Glycogen Synthase Kinase-3 Inhibition Attenuates Fibroblast Activation And Development Of Fibrosis Following Renal Ischemia/Reperfusion In Mice

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ABSTRACT
Glycogen synthase kinase-3β (GSK3β) is a serine/threonine protein kinase that plays an important role in renal tubular injury and regeneration in acute kidney injury. However, its role in the development of renal fibrosis, often a long-term consequence of acute kidney injury, is unknown. Using a mouse model of renal fibrosis induced by ischemia/reperfusion injury, we demonstrate increased GSK3β expression and activity in fibrotic kidneys and its presence in myofibroblasts in addition to tubular epithelial cells. Pharmacological inhibition of GSK3 using TDZD-8 starting before or after ischemia/reperfusion significantly suppressed renal fibrosis by reducing myofibroblast population, collagen-1 and fibronectin deposition, inflammatory cytokines and macrophage infiltration. GSK3 inhibition in vivo reduced TGF-β1, SMAD3 activation and plasminogen activator inhibitor-1 levels. Consistently in vitro, TGF-β1 treatment increased GSK3β expression and GSK3 inhibition abolished TGF-β1 induced SMAD3 activation and α-smooth muscle actin expression in cultured renal fibroblasts. Importantly, overexpression of constitutively active GSK3β stimulated α-smooth muscle actin expression even in the absence of TGF-β1 treatment. These results suggest that TGF-β regulates GSK3β, which in turn is important for TGF-β/SMAD3 signaling and fibroblast-to-myofibroblast differentiation. Overall, these studies demonstrate that GSK3 could promote renal fibrosis by activation of TGF-β signaling and the use of GSK3 inhibitors might represent a novel therapeutic approach for progressive renal fibrosis that develops as a consequence of acute kidney injury.
Introduction

Renal fibrosis is a classic outcome of multiple forms of chronic kidney disease. It is characterized by excessive extracellular matrix (ECM) remodeling, which results in progressive loss of renal function and often leads to end stage renal disease (Chuang et al., 2013). Renal fibrosis can also develop as a consequence of maladaptive repair after acute kidney injury (AKI) (Bonventre and Yang, 2011; Venkatachalam et al., 2010). For instance, AKI caused by ischemia/reperfusion (I/R) can result in inadequate tubular regeneration, chronic inflammation, macrophage infiltration and fibroblast activation, leading to excessive ECM deposition and fibrosis (Bonventre and Yang, 2011; Jang et al., 2014; Kim and Padanilam, 2014; Yang et al., 2010; Zhou et al., 2014).

The glycogen synthase kinase-3 (GSK3) family of protein kinases consists of GSK3α and GSK3β isoforms and plays an important role in injury and repair of renal tubular epithelial cells in AKI. A pro-apoptotic role for the GSK3β isoform was demonstrated in experimental AKI using gene silencing in vitro (Wang et al., 2010), or gene deletion in vivo (Howard et al., 2012). Moreover, pharmacological inhibition using isoform non-selective GSK3 inhibitors reduced apoptosis and renal tubular injury in AKI induced by lipopolysaccharides, I/R and nephrotoxins (Bao et al., 2012; Howard et al., 2012; Plotnikov et al., 2013; Wang et al., 2009; Wang et al., 2010). In previous studies we demonstrated that renal proximal tubule-specific gene deletion of GSK3β could accelerate renal tubular repair after HgCl₂ induced AKI in mice (Howard et al., 2012). We also showed that GSK3 inhibition using TDZD-8, 48 hours after a nephrotoxic insult can significantly improve renal tubular repair by increasing pro-proliferative cyclin-D1, c-myc and β-catenin (Howard et al., 2012). These results were subsequently affirmed by studies using LiCl in cisplatin and I/R injury models of AKI (Bao et al., 2014). Thus, inhibition of GSK3 could be a viable strategy for the treatment of AKI. However, it is unclear whether GSK3β is expressed in renal myofibroblasts, the major producers of ECM, or if GSK3β is involved in the development of renal fibrosis.

GSK3 regulates multiple cell signaling pathways by suppressing accumulation or transcriptional activity of key mediators of these pathways in the absence of ligands or activators (Beurel et al., 2014). Some of these cell-signaling pathways, including TGF-β, Wnt, sonic hedgehog, EGFR and BMP signaling are important for fibrosis (Chuang et al., 2013; LeBleu et al., 2013). Hence, it could be hypothesized that inhibition of GSK3 would mimic activation of these pro-fibrotic signaling pathways, leading to fibrosis. However, the role of GSK3β in fibrosis seems to be cell and context dependent. For instance, in vitro,
GSK3 inhibition can cause epithelial to mesenchymal transition (EMT) in renal tubular epithelial cells by activation of TGF-β1 and increase in Snail levels, (Lan et al., 2014; Noh et al., 2012) while in cultured glomerular mesangial cells, GSK3 inhibition reduces TGF-β1 signaling and fibronectin accumulation (Ho et al., 2012). Moreover, GSK3β inhibition can reduce inflammation, an essential prelude to renal fibrosis (Martin et al., 2005; Wang et al., 2011). Given this complexity and our previous observations that inhibition of GSK3 reduces injury and accelerates repair in the acute setting of AKI, its effect on renal fibrosis, a long-term outcome of AKI, remains to be defined.

In the current studies we examined the role of GSK3β in the development of renal fibrosis and tested the effect of pharmacological inhibition of GSK3 in an I/R induced mouse model of renal fibrosis. The results of these studies are presented.
Results:
Renal GSK3β expression increases following I/R

To determine the role of GSK3β in the development of renal fibrosis, we first examined its expression and activation in the kidneys of mice subjected to bilateral renal I/R. A time course analysis of renal GSK3β expression following I/R showed significant increase in total GSK3β levels by day-2, which at day-12 remained 2 fold higher than day-0 (Fig-1A,B). The Serine-9 phosphorylated (inactive) form of GSK3β (pGSK3β), increased significantly by day-2, following which it returned to baseline levels. The ratio of pGSK3β to GSK3β did not change significantly on day-2 and was further reduced on days-3 and -12, suggesting an increase in GSK3β activity (Fig-1A,B). Expression levels of renal α-smooth muscle actin (α-SMA), a marker of myofibroblasts also increased, starting on day 2 following I/R (Fig-1A). Immunofluorescence staining demonstrated that GSK3β co-localizes with α-SMA in day-2 as well as day-12 I/R kidneys (Fig-1C). The day-12 I/R kidneys were fibrotic as determined by Masson’s-trichrome staining and Sirius red staining (Supplemental-1A). GSK3β expression was detected in proximal tubules, and to a lower extent in collecting ducts, but not thick ascending limbs (Supplemental-1B). Unlike proximal tubules and myofibroblasts, the F4/80 staining macrophages in day-12 I/R kidneys rarely stained for GSK3β (Supplemental-1C).

To determine if TGF-β1, a known stimulator of fibroblast-to-myofibroblasts differentiation can stimulate GSK3β expression, immortalized rat fibroblasts (NRK-49F cells) were treated with TGF-β1. Treatment with TGF-β1 dose-dependently increased GSK3β as well as α-SMA. GSK3β expression doubled at a dose of 2 ng/mL of TGF-β1 and co-localized with α-SMA in myofibroblasts (Fig-1E,F,G), while pGSK3β to GSK3β ratio remained unchanged (Fig-1E,F). These results demonstrate for the first time increased and sustained GSK3β expression in fibrotic kidneys following I/R or following TGF-β1 treatment in vitro, and its association with myofibroblasts.

Inhibition of GSK3 attenuates development of renal fibrosis after I/R

To determine the role of GSK3 activity in I/R induced renal fibrosis, we examined the effect of TDZD-8 (TDZD) (Martinez et al., 2002) a highly specific ATP non-competitive inhibitor of GSK3 that we and others have effectively used in mice (Howard et al., 2012; Tao et al., 2015; Wang et al., 2010). Since GSK3β expression increased by day-2 (Fig-1A), TDZD treatment (1mg/Kg BWt.) was started on day-2 after I/R in one group of mice (TDZD-Post), and in a second group, one hour before I/R (TDZD-Pre) (Fig-2A). All mice
were sacrificed on day-12 after I/R. TDZD treatment did not affect renal GSK3β levels, but significantly increased pGSK3β and pGSK3β/GSK3β levels when compared to vehicle treated I/R kidneys suggesting GSK3β inhibition (Fig-2B,C). As illustrated by trichrome and Sirius red staining, the TDZD-Pre and TDZD-Post, I/R groups showed a striking reduction in fibrosis compared to vehicle treated I/R group (Fig-D,E).

Dilated tubules and tubular atrophy were observed in vehicle treated I/R group by H&E staining (Fig-2D), while the TDZD-Pre and TDZD-Post treatment groups showed minimal injury. Following I/R, blood urea nitrogen (BUN) levels in TDZD-Pre treatment group never increased to the high levels as the vehicle treated group (Fig-2F). Similarly in the TDZD-Post treatment group, BUN levels reduced significantly as early as 24h after initiation of treatment (day-3) and decreased to TDZD-Pre treatment levels by day-12. These results indicate that GSK3 inhibition not only preserves renal function in AKI, but TDZD treatment, either pre- or post- I/R, can reduce fibrosis.

**TDZD treatment inhibits renal ECM deposition and reduces myofibroblast population**

To determine the effect of GSK3 inhibition on ECM deposition, we measured expression levels of collagen-1 and fibronectin, major matrix components. Since active myofibroblasts are a major source of collagen and fibronectin, we also determined myofibroblast population in the kidneys by measuring α-SMA expression. Immunostaining for fibronectin, collagen-1 and α-SMA was higher in vehicle treated I/R kidneys compared to sham kidneys and TDZD treatment reduced their expression (Fig-3A). Western blot analysis confirmed these results and showed that compared to TDZD-Pre, the TDZD-Post treatment group had significantly lower levels of collagen-1, fibronectin and α-SMA (Fig-3B, supplemental-2). Fibronectin, collagen-a1, collagen-3a1 and α-SMA mRNA levels were also increased in vehicle treated I/R kidneys compared to sham and significantly reduced in TDZD treatment groups (Fig-3C,D,E,F), although no significant difference was observed in mRNA levels between TDZD-Pre and TDZD-Post treatment groups. These results suggest that inhibition of GSK3 activity can reduce myofibroblast population and ECM deposition following I/R injury-induced fibrosis.

**GSK3 inhibition reduced proinflammatory factors and macrophage infiltration**

Fibroblast activation can be stimulated by pro-inflammatory cytokines, chemokines and growth factors secreted by damaged epithelial cells, myofibroblasts and infiltrating
cells that further enhance tissue fibrosis. We found significant up regulation of mRNA encoding the cytokines, TNF-α, IL-6 and IL-1β, in vehicle treated I/R kidneys, compared to sham (Fig-4A,B,C). Messenger RNA levels of ICAM-1, a myofibroblast-expressed mediator for interaction with infiltrating leukocytes were also increased in vehicle treated I/R kidneys (Fig-4D). Similarly, monocyte chemo-attractants CCL-2 (Furuichi et al., 2009) and CCL-3 (Correa-Costa et al., 2014) were significantly increased in vehicle treated I/R kidneys (Fig-4E,F), accompanied by increased macrophage infiltration determined by immunostaining for F4/80 (Fig-4G). These proinflammatory factors and macrophage infiltration were significantly reduced in TDZD-Pre and TDZD-Post treatment groups. These results suggest that GSK3 activity could contribute to macrophage infiltration and the production of proinflammatory cytokines in I/R induced fibrosis.

**GSK3 inhibition blocks TGF-β signaling but increases β-catenin in epithelial cells.**

TGF-β signaling plays a crucial role in development of renal fibrosis. Hence we examined the effect of TDZD treatment on canonical TGF-β signaling. TGF-β1 mRNA levels were 60% higher in vehicle treated I/R group compared to sham (Fig-5A), with a corresponding increase in TGF-β signaling, suggested by increased pERK and pSMAD3 levels (Fig-5B,C). Immunofluorescence staining demonstrated pSMAD3 expression in GSK3β expressing cells (Fig-5D). In TDZD-Pre and TDZD-Post I/R kidneys, TGF-β1 mRNA, pERK and pSMAD levels were significantly reduced compared to vehicle treated I/R kidneys (Fig-5B,C). TGF-β receptor 1 and 2, and CTGF mRNA levels were unchanged in the TDZD treatment groups compared to vehicle treatment group (Data not shown). PAI-1, an inhibitor of collagen degradation is an important gene up regulated by TGF-β signaling (Ma and Fogo, 2009; Samarakoon et al., 2012). In vehicle treated I/R kidneys, PAI-1 mRNA levels were 3 fold higher compared to sham, and TDZD treatment significantly reduced PAI-1 mRNA levels (Fig-5E).

Canonical Wnt signaling regulates expression of multiple pro-inflammatory and profibrotic factors (DiRocco et al., 2013). We examined β-catenin, an important component of Wnt signaling to determine if TDZD treatment increased its stabilization. Indeed, in TDZD-Pre and TDZD-Post I/R kidneys, β-catenin protein levels were significantly higher than in sham or vehicle treated I/R kidneys (Fig-5F, Supplemental-3). However, β-catenin expression in TDZD-Pre and TDZD-Post I/R kidneys could be detected only in renal tubules, including LTA- staining proximal tubules (Fig-5G) but not in the few α-SMA expressing myofibroblasts (Fig-5G). These results suggest that inhibition of GSK3
suppressed renal fibrosis following I/R by inhibiting TGF-β signaling and not β-catenin-dependent mechanisms.

**GSK3 is crucial for TGF-β induced fibroblast-to-myofibroblast differentiation in vitro.**

To further determine the role of GSK3 in myofibroblasts, we tested the effect of GSK3 inhibitors, TDZD and SB216763, a small molecule inhibitor of GSK3 on TGF-β1 induced α-SMA expression in NRK-49F cells. Pretreatment with SB216763 (Fig-6A,C) or TDZD (Fig-6B,D) significantly reduced α-SMA expression in a dose dependent fashion. The GSK3 inhibitors also reduced TGF-β1 induced pSMAD3 levels (Fig-6A,B). To further determine if increase in GSK3β in renal fibroblasts can stimulate α-SMA expression, we overexpressed constitutively active GSK3β (AdGSK3β-CA, serine 9-alanine mutant) or control GFP (AdGFP) in NRK-49F cells. After 48 hours, GSK3β expression increased in the AdGSK3β-CA transduced cells, accompanied by increase in α-SMA expression and decrease in β-catenin levels (Fig-6E,F). These studies demonstrate that GSK3β plays a crucial role in TGF-β signaling and fibroblast activation.
Discussion:
Renal fibrosis is characterized by activation and proliferation of fibroblasts, which continually produce and deposit extracellular matrix proteins leading to progressive fibrosis. Herein we demonstrate that GSK3β is expressed in myofibroblasts and GSK3β expression and activity are increased in mouse kidneys following I/R and in cultured fibroblasts following TGF-β1 treatment. Pharmacological inhibition of GSK3 using TDZD significantly reduced pro-inflammatory and pro-fibrotic cytokines, macrophage infiltration and ECM deposition, thereby reducing fibrosis. GSK3 inhibition reduced myofibroblast population in vivo, and fibroblast-to-myofibroblast differentiation in vitro by a TGF-β/SMAD signaling dependent mechanism. Thus, GSK3β plays a pro-fibrotic role in the kidney following I/R, and its inhibition, even after the injury has occurred, could prevent the future development of fibrosis.

GSK3β is expressed in proximal tubules (Norregaard et al., 2015) and increased GSK3β has been detected in renal tubules of human chronic renal allograft dysfunction tissue by immunohistochemical staining (Gong et al., 2008; Yan et al., 2012). In the current study we found a time dependent and sustained increase in GSK3β expression and activity following I/R in mouse kidneys. Importantly, and unlike the previous studies, GSK3β expression was detected in α-SMA-expressing myofibroblasts, as early as day-2 following I/R. Moreover, increased GSK3β expression could be linked to fibroblast activation because overexpression of constitutively active GSK3β (Serine 21 to alanine mutation) by itself led to increase in α-SMA expression similar to the effect of TGF-β1 treatment in NRK-49F cells.

Fibroblast activation /differentiation into myofibroblasts which produce large amounts of ECM components is a key step in the development of fibrosis (Kalluri and Zeisberg, 2006; LeBleu et al., 2013; Strutz and Zeisberg, 2006). In the vehicle treated I/R kidneys we found a large population of myofibroblasts, which was significantly reduced by TDZD treatment. Fibroblasts can be activated by paracrine and autocrine factors including pro-inflammatory and pro-fibrotic cytokines and chemokines produced by injured tubular epithelial cells, macrophages, and myofibroblasts themselves (Grande and Lopez-Novoa, 2009; Kendall and Feghali-Bostwick, 2014). In I/R kidneys, a significant increase in IL-6, IL-1β, TNF-α, TGF-β1, macrophage chemo-attractants and infiltration of macrophages was found, which were abolished by TDZD treatment. These results are consistent with a pro-inflammatory role for GSK3 (Dugo et al., 2007; Gong et al., 2008; Martin et al., 2005) and
suggest that suppression of one or more of these cytokine/chemokine production could have reduced fibroblast activation in the TDZD treated mice.

The chemokine TGF-β, which promotes fibroblast activation, proliferation, migration and ECM synthesis, is a critical mediator of fibrosis (Kramann et al., 2013; Zarjou and Agarwal, 2012). Canonical TGF-β signaling involves binding of TGF-β to its receptors and activation of SMAD3, which together with SMAD4 regulates the expression of pro-fibrogenic genes (Farris and Colvin, 2012). The role of GSK3 in TGF-β signaling is controversial. In in vitro studies, GSK3 inhibition by pharmacological inhibitors or activation of Wnt signaling attenuated TGF-β1 mediated ECM accumulation in cultured renal glomerular mesenchymal cells, lung, gingival and skin fibroblasts (Bahammam et al., 2013; Ho et al., 2012; Liu et al., 2012). Similarly, in cultured human renal tubular epithelial cells and corneal fibroblasts, GSK3 inhibition reduced TGF-β1 induced SMAD3 activity (Choi et al., 2011; Zhang et al., 2007). On the other hand, TGF-β inhibits GSK3β via ERK/MAPK in hepatocellular carcinoma (Ding et al., 2005) and cultured peritoneal mesothelial cells (Jang et al., 2013), and GSK3 inhibition can lead to SMAD3 activation and fibrosis in cultured cardiac myocytes and fibroblasts (Hua et al., 2010; Lal et al., 2014). In the kidney, GSK3 inhibition has been associated with EMT in unilateral ureteral obstruction (UUO) in vivo and renal epithelial cells in vitro by increasing TGF-β1 induced β-catenin and Snail accumulation (Lan et al., 2014; Noh et al., 2012; Yoshino et al., 2007; Zhou et al., 2004). Moreover, in a transgenic mouse expressing constitutively active GSK3α and GSKβ, α-SMA levels did not increase following three days of UUO (Voelkl et al., 2013). The results of the current studies in I/R model of AKI are consistent with a pro-fibrotic role for GSK3β by its involvement in TGF-β signaling in the kidney. We demonstrate that pSMAD3 expression co-localized with GSK3β in fibrotic kidney following I/R and systemic GSK3 inhibition significantly reduced TGF-β1, pSMAD3 and PAI-1, a SMAD-regulated profibrotic gene. This suggests that TGF-β/ SMAD3 signaling is active in vehicle treated I/R kidneys and GSK3 inhibition suppresses it. Moreover GSK3 inhibition using TDZD or SB216763 abolished TGF-β1 induced SMAD3 activation and α-SMA expression in cultured renal fibroblasts. Since TGF-β1 treatment also increased GSK3β expression and activity, GSK3β could play an essential role in TGF-β1 signaling mediated fibroblast activation and development of fibrosis.

In addition to SMAD, the Wnt signaling pathway also mediates the effects of TGF-β1 (Akhmetshina et al., 2012; Zhou, 2011). Wnt signaling has been implicated in the pathogenesis of renal fibrosis based on the findings that expression of Wnt ligands are up
regulated in fibrotic kidneys, and inhibition of Wnt signaling using DKK-1, sFRP4 or paricalcitol can reduce renal fibrosis in mice (DiRocco et al., 2013; Hao et al., 2011; He et al., 2009; Ren et al., 2013; Surendran et al., 2005). GSK3 is linked to canonical Wnt signaling by virtue of its ability to prevent cytoplasmic accumulation of β-catenin in the absence of Wnt ligands (Kaidanovich-Beilin and Woodgett, 2011). In the absence of Wnt ligands, GSK3β phosphorylates β-catenin, which prevents its cytoplasmic accumulation (Yost et al., 1996). In the presence of Wnt ligands, GSK3β is unable to phosphorylate β-catenin, leading to its accumulation and increased activity. While the role of Wnt ligands in the development of renal fibrosis is clear, the role of β-catenin, an important component of canonical Wnt signaling has been inconclusive. Renal β-catenin is increased in experimental models of renal fibrosis (He et al., 2009; Ren et al., 2013; Surendran et al., 2005) and its systemic inhibition can reduce fibrosis (Hao et al., 2011). However, while DKK-1, a Wnt antagonist significantly reduced renal β-catenin abundance (He et al., 2009), the anti-fibrotic effects of DKK-1 were found to be independent of β-catenin in UUO and I/R models of fibrosis (Ren et al., 2013). Moreover, gene deletion of β-catenin in tubular epithelium did not reduce renal fibrosis (Zhou et al., 2013). In the current studies, renal β-catenin abundance increased significantly in TDZD treated mice consistent with our previous studies (Howard et al., 2012). However, β-catenin expression in TDZD treated mice was localized to tubules, and consistent with Zhou et al's observation (Zhou et al., 2013), increased renal tubular β-catenin levels did not result in increased α-SMA, ECM or fibrosis. Regardless of the role of β-catenin in the development of renal fibrosis, the involvement of GSK3 in fibroblast activation or renal fibrosis does not seem to be linked to β-catenin in the post-I/R kidney. This is further supported by the findings that both increased β-catenin (von Toerne et al., 2009) and increased GSK3β (Gong et al., 2008; Yan et al., 2012) occur in renal tubules of human chronic renal allograft nephropathy.

Injured tubules are known to recruit macrophages, secrete cytokines and chemokines and generally induce fibrosis (Bonventre and Yang, 2011). Since GSK3β expression was observed in both renal tubular epithelial cells and interstitial fibroblasts, it cannot be excluded at this point that GSK3 inhibition could have led to complete and proper repair of the renal tubules after I/R and thereby resulted in reduced inflammation, TGF-β signaling and fibrosis. However, the in vitro and in vivo findings that (a) GSK3β is expressed in myofibroblasts, (b) GSK3β activity is crucial for TGF-β signaling and α-SMA expression and (c) constitutively active GSK3β can increase α-SMA expression in vitro,
suggest that GSK3β could play an important role in fibroblast differentiation and renal fibrosis.

In summary, our study is the first to demonstrate that GSK3 is a key pathogenic determinant in the development of renal fibrosis. The pro-fibrogenic role of GSK3 in the post I/R kidney could be coupled to TGF-β signaling, although additional studies are needed to address the mechanism. As such, the results show that pharmacological inhibition of GSK3, even after the detection of AKI in patients could suppress fibroblast activation and development of renal fibrosis.

**Materials and Methods**

**Bilateral I/R Surgery and Experimental Protocol:**

Bilateral I/R was carried out essentially as described earlier (Wei and Dong, 2012) on male C57/BL6J mice (Jackson Laboratory, Bar Harbor, MN) weighing approximately 25g. Briefly, both renal pedicles were exposed by flank incisions and clamped using micro aneurysm clamps for 30 min under pentobarbital anesthesia (60 mg/Kg BWt. i.p.). At the end of the ischemic period, the clamps were released for reperfusion.

Study Groups: 1. Sham- Mice underwent surgery to expose renal pedicle, without clamping. 2. Vehicle treated I/R- Mice underwent surgery for I/R and received vehicle injection (10% DMSO) 1h before clamping. 3. TDZD-Pre I/R- Mice underwent surgery for I/R and received TDZD daily, starting 1h before I/R. 4. TDZD-Post I/R Mice underwent surgery for I/R and received TDZD daily, starting 48 hours after I/R. n=6 each group.

TDZD (TDZD-8) was dissolved in 10% DMSO and administered by daily IP injection at 1mg/Kg BWt. dose. All experiments were approved by the IACUC committee of University of Kansas Medical Center. Blood was collected from tail vein and plasma used to measure BUN using a QuantiChrom Urea Assay Kit from BioAssay Systems (Hayward, CA, USA) following manufacturer’s instructions.

**Quantitative Real-Time PCR (RT-PCR)**

Total RNA was extracted using TRlzo1 (Sigma-Aldrich, MO, USA) and first-strand cDNA synthesized using a reverse transcription system kit (Applied Biosystems, NY, USA). RT-PCR was performed on an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). Primer sequences are provided in supplemental data Table-1. The mRNA levels were calculated relative to β-actin levels for each sample.
In vitro studies: NRK-49F cells (ATCC, VA, USA) were cultured in DMEM medium containing 5% fetal bovine serum, 0.5% penicillin and streptomycin. Cells were serum starved for 16h followed by TGF-β1 (Sigma-Aldrich, MO, USA) treatment for 48 hours. Adenovirus, AdGSK3β-CA, carrying a Serine to Alanine substitution at Ser-9 in the NH2-terminal region of GSK3β was a gift from Dr. Thomas Force (Haq et al., 2000). AdGSK3β-CA and control, AdGFP have been described before (Rao et al., 2004). The recombinant viruses were propagated in HEK 293 cells, and high titer stocks (2 x 10^10 particles/mL) were purified by CsCl density gradient centrifugation. For infection of NRK-49F cells, virus of 5 or 10 multiplicity of infection (MOI) was added to each culture dish.

Western Blot analysis

Kidney tissues were lysed in RIPA buffer and loaded onto SDS-PAGE gels, transferred to nitrocellulose membranes and blocked with 5% milk in TBST. Membranes were probed with primary antibody followed by TBST washes and horseradish peroxidase secondary antibody application. Secondary antibodies were purchased from Dako (CA, USA).

Antibodies Used

For Western blot and Immunostaining of tissue sections, antibodies for GSK3β, pGSK3β, β-Catenin, fibronectin, α-SMA (Cell Signaling Technology, Inc., MA, USA), Collagen I (MD Bioproducts, MN, USA), GAPDH, pERK and ERK (Santa Cruz Biotechnology, Inc. TX, USA), β-actin (Sigma Aldrich, MO, USA) were used. Secondary antibodies were purchased from Dako (CA, USA). Antibodies used only for Immunohistochemistry/Immunofluorescence staining were, anti-α-SMA, F4/80 (Serotec, NC, USA), PCNA (Dako, CA, USA), DBA and LTA (Vector Laboratories, CA, USA) and BrdU (Cell Signaling Technology, Inc., MA, USA), (pSMAD3 (Rockland, PA).

Histology/ Immunohistochemistry/ Immunofluorescence:

Kidney sections were fixed in 4% paraformaldehyde and blocked in paraffin. Picro sirius red staining (Polysciences, PA, USA) was carried out and fibrillar collagen was visualized under polarized light. Masson’s trichrome staining was carried out using (Polysciences, PA, USA). For both IHC and IF, paraffin sections were de-paraffinised, washed in PBS containing 0.1% Tween 20 (PBST) and blocked in 10% normal goat serum. Primary antibodies were applied to sections and incubated at 4°C overnight. For IHC, slides were blocked with Avidin/Biotin (Invitrogen, NY, USA), and then biotinylated goat anti-rabbit IgG or anti-mouse IgG (Invitrogen, NY, USA) secondary antibodies were
applied, followed by incubation with Streptavidin HRP conjugate (Invitrogen, NY, USA). Finally slides were developed with DAB (Vector Laboratories, CA, USA) and counterstained with Harris Haematoxylin, dehydrated, and mounted with Permount (Fisher Scientific). For IF, after incubation with primary antibody, goat anti-Rabbit IgG fluor, Goat anti-mouse IgG Texas red, or Goat anti-chicken IgG Alexa 555 secondary antibodies were applied, and following incubation, washed, stained with DAPI and mounted with Flour-G (Invitrogen, NY, USA). All images were captured using a Nikon 80i microscope in KUMC imaging center.

**Statistics:** Values are expressed as mean ± standard error for all bar charts, except for band density measurements of western blots, which is expressed as mean ± standard deviation. Data was analyzed using Graphpad Prism software (Version 5.0d). Two-tailed unpaired t-test with Welch’s correction and F test to compare variances and One-Way Analysis of Variance followed by Tukey’s multiple comparison test and Bartlett’s test for equal variance were used. A probability level of 0.05 (P ≤ 0.05) was considered significant.

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**References:**


Figures:

Fig 1. Increased GSK3β expression after renal I/R. (A) Western blot analysis and (B) quantitation of band density show increased GSK3β protein levels and reduced inactive pGSK3β-serine-9/ GSK3β ratio and increased α-SMA in kidneys after I/R. *P<0.05, **P<0.01 compared to day 0. (C) GSK3β (green) and α-SMA (red) co-staining shows that GSK3β is expressed in renal myofibroblasts on day-2 and (D) day-12 after I/R. (Scale bar=25µm). (E) In rat renal fibroblast-NRK-49F cells, TGF-β1 treatment for 48 hours, dose dependently increased GSK3β and pGSK3β levels. (F) Relative band densities of total GSK3β and pGSK3β to GSK3β ratio. (G) GSK3β co localizes with α-SMA in NRK-49F cells (Scale bar=25µm). **P<0.01, compared to 0 ng/mL TGF-β1 treatment. n=6 mice/group for immunoblotting and n=4 mice/group for immunostaining.
Fig 2. Treatment with TDZD, a GSK3 inhibitor reduced fibrosis following I/R. (A) Scheme of experimental design. Treatment with TDZD (1mg/Kg BWt.), starting one hour before I/R (TDZD-Pre) and 2 days after I/R (TDZD-Post). (B) Western blot analysis and (C) densitometry shows increased pGSK3β levels in TDZD treated kidneys (D) Masson’s Trichrome, Picro-sirius red and H&E stained kidney sections, show reduced injury and fibrosis in TDZD-Pre and TDZD-Post treatment groups 12 days after I/R. (E) Fibrosis score based on Masson’s Trichrome staining. (F) Blood urea nitrogen levels. *P<0.05, **P<0.01, ***P<0.001 compared to vehicle treated I/R group, n=6 mice/group. Scale bar=50µm. All tissue samples were from day 12 after I/R.
Fig 3. GSK3 inhibition reduced ECM deposition following I/R. (A) Immunostaining and (B) Western blot for fibronectin, collagen-1 and α-SMA show reduced levels in TDZD-Pre and TDZD-Post treatment groups. (C) Quantitative RT-PCR to determine mRNA levels of fibronectin, (D) collagen-a1 and (E) collagen 3a1 and (F) α-SMA (fold change, relative to β-actin). **P<0.01, ***P<0.001 compared to vehicle treated I/R group, n=6 mice/group. Scale bar=50µm
Fig 4. GSK3 Inhibition reduced pro-inflammatory cytokines and macrophage infiltration following I/R.

Messenger RNA levels of (A) TNF-α, (B) IL-6, (C) IL-1β, (D) ICAM-1, (E) CCL-2, and (F) CCL-3 (fold change, relative to β-actin) are lower in TDZD treated than vehicle treated I/R kidneys. (G) F4/80 staining shows reduced macrophage infiltration in TDZD treated compared to vehicle treated I/R kidneys. **P<0.01, ***P<0.001 compared to vehicle treated I/R group, n=6 mice/group. Scale bar=50µm
Fig 5. GSK3 Inhibition reduced TGF-β signaling after I/R.

(A) Quantitative RT-PCR shows reduced TGF-β1 mRNA in TDZD treated compared vehicle treated I/R kidneys (fold change, relative to β-actin). (B) Western blot analysis for pSMAD3, SMAD3, pERK1/2 and ERK1/2 and (C) pSMAD3 to SMAD3 ratio based on relative band density. (D) Immunostaining for pSMAD3 (red) and GSK3β (green). (Scale bar=25µm). (E) Quantitative RT-PCR for PAI-1. (F) Western blot analysis shows increased β-catenin levels in TDZD treated mouse kidney compared to sham or vehicle treated kidneys. (G) Immunostaining for β-catenin (red), LTA (green) (Scale bar=25µm) or α-SMA (green). *P<0.5, **P<0.01, ***P<0.001 compared to vehicle treated I/R group, n=6 mice/group.
Fig 6. GSK3 activity is critical for TGF-β1 induced fibroblast activation.

Inhibition of GSK3 using (A) SB216763 (SB) or (B) TDZD, dose-dependently reduced α-SMA expression and pSMAD3 levels in NRK-49F cells treated with TGF-β1 for 48 hours. (C,D) Quantitation of relative band density for α-SMA. (E) Overexpression of constitutively active GSK3β-serine 9-alanine (GSK3β-CA) in NRK-49F cells increases α-SMA but reduces β-catenin compared to cells overexpressing GFP (control). (F) Quantitation of relative band density. *P<0.5, **P<0.01, ***P<0.001, compared to control, n=4.
Translational Impact:

Clinical issue: Chronic kidney disease affects about 10% of the population and is a major cause of death in the United States. Renal fibrosis is a classic outcome of multiple forms of chronic kidney disease. It is characterized by excessive extracellular matrix (ECM) remodeling, which results in progressive loss of renal function and often leads to end stage renal disease. Renal fibrosis can also develop as a consequence of maladaptive repair after acute kidney injury (AKI). For instance, AKI caused by ischemia/reperfusion (I/R) can result in inadequate regeneration, chronic inflammation, macrophage infiltration and fibroblast activation, leading to excessive ECM deposition and fibrosis. Glycogen synthase kinase-3β (GSK3β) is a serine/threonine protein kinase that we and others have found to play an important role in renal tubular injury and regeneration in acute kidney injury. While inhibition of GSK3β can reduce injury and accelerate repair, its role in the development of renal fibrosis, often a long-term consequence of acute kidney injury is currently unknown.

Results: Using a mouse model of renal fibrosis induced by ischemia/reperfusion injury, we demonstrate increased GSK3β expression and activity in fibrotic kidneys and its presence in myofibroblasts, the major producers of ECM. Pharmacological inhibition of GSK3 using TDZD-8 starting before or after ischemia/reperfusion significantly suppressed renal fibrosis by reducing myofibroblast population, collagen-1 and fibronectin deposition, inflammatory cytokines and macrophage infiltration. GSK3 inhibition in vivo reduced TGF-β1, SMAD3 activation and plasminogen activator inhibitor-1 levels. Consistently in vitro, TGF-β1 treatment increased GSK3β expression and GSK3 inhibition abolished TGF-β1 induced SMAD3 activation and α-SMA expression in cultured renal fibroblasts. Importantly, overexpression of constitutively active GSK3β stimulated α-SMA expression even in the absence of TGF-β1 treatment. These results suggest that TGF-β regulates GSK3β, which in turn is important for TGF-β/SMAD3 signaling and fibroblast-to-myofibroblast differentiation.

Implications and future directions: Overall these studies demonstrate that GSK3 could promote renal fibrosis by activation of TGF-β signaling. The finding that GSK3 inhibition, starting even after AKI has occurred can reduce fibrosis is important, because a large percentage of AKI cases are detected after the injury has occurred. The use of GSK3 inhibitors might represent a novel therapeutic approach for progressive renal fibrosis that develops as a consequence of AKI.