Stromal-cell deletion of STAT3 protects mice from kidney fibrosis by inhibiting pericytes trans-differentiation and migration

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SUMMARY:

Signal transducer and activator of transcription 3 (STAT3) is a key transcription factor implicated in the pathogenesis of kidney fibrosis. Although tubular *Stat3* deletion in tubular epithelial cells is known to protect mice from kidney fibrosis, the exact function of STAT3 in stromal cells remains unknown. We utilized stromal-cell *Stat3* knock-out (KO) mice, CRISPR and pharmacologic activators and inhibitors of STAT3 to investigate its function in pericyte-like cells. STAT3 is phosphorylated in tubular epithelial cells in acute kidney injury whereas its activation expanded to interstitial cells in chronic kidney disease in mice and humans. Stromal cell-specific deletion of *Stat3* protects mice from folic acid- and aristolochic acid-induced kidney fibrosis. Mechanistically, STAT3 directly regulates the inflammatory pathway, differentiation of pericytes into myofibroblasts. Specifically, STAT3 activation leads to an increase in migration and profibrotic signaling in genome-edited pericyte-like cells, 10T1/2. Conversely, *Stat3* KO or blocking STAT3 function inhibits detachment, spreading, migration, and profibrotic signaling. Furthermore, STAT3 binds to *Collagen1a1* promoter of fibrotic mouse kidneys and in pericyte-like cells. Together, our study identifies a previously unknown function of STAT3 in stromal cells that promotes kidney fibrosis and may have therapeutic value in fibrotic kidney disease.

KEYWORDS: STAT3, fibrosis, inflammation, stromal cells, pericytes, myofibroblasts transformation

INTRODUCTION:

Diverse cell types play crucial functions in the cellular processes of tissue fibrosis, (1-4) including kidney fibrosis(5-8). Although many cell types in the tubulo-interstitium of the kidneys produce extracellular matrix (ECM), fibroblasts being the principal cells, the contribution of other cell types are not well understood. During the fibrotic process of kidneys, fibroblasts undergo transformation into myofibroblasts, which are identified as α -Smooth Muscle Actin (α -SMA) positive cells. One of the central questions is to delineate the origin, activation, and regulation of these matrix-producing myofibroblasts (9-14). Many reports have suggested that activation of interstitial fibroblasts, differentiation of pericytes, phenotypic conversion of tubular epithelial cells and endothelial cells and recruitment of circulating fibrocytes are involved in this process (6, 15-19). Proliferation of interstitial cells and activation of myofibroblast are the two main phenotypical changes acquired by myofibroblasts to induce fibrosis (20), which is caused by inflammation and release of cytokines in the diseased kidneys (21, 22).

Previously, ECM-producing myofibroblasts were thought to be derived from resident fibroblasts after kidney injury (23). More recently, due to the availability of common markers for different cell types, there has been immense amount of effort towards identifying the “weight” of contribution of specific cell types to the fibrosis process and evidence that myofibroblasts can originate from many different cell types (10, 24) is starting to develop. Recent reports indicate that pericytes are a critical source of myofibroblasts in fibrotic kidneys (11, 16, 25, 26). Pericytes are a subset of the stromal cells that partially cover capillary walls, thereby stabilizing the endothelium. Following

kidney injury, pericytes get detached from the endothelium, undergo proliferation and migration, and transform into myofibroblasts (16, 25).

Multiple mechanisms could account for the loss of peritubular capillaries in the fibrotic kidneys, one of them being the detachment of pericytes causing pericyte deficiency and destabilization of the endothelium (11). A recent study showed that perivascular CD73 positive cells attenuate inflammation and interstitial fibrosis in the kidney (27). Thus, investigating pericytes detachment and its associated cellular signaling may provide mechanistic insights into the initiation and progression of kidney fibrosis.

Secretion of inflammatory cytokines, (TGF- β (28, 29), CTGF (30), IL-1b, TNF- α (31)) including IL-6 (32), after cellular injury triggers inflammatory pathways in the kidney causing deterioration of kidney function (33). In the inflammatory cells, IL-6 binds to growth receptors to phosphorylate Signal Transduction and Activator of Transcription 3 (STAT3), resulting in increased inflammation (34, 35). We and others have shown that STAT3 regulates the expression of Kidney Injury Molecule-1 (KIM-1) following acute kidney injury (36, 37) and fibrinogen gamma chain in fibrotic kidneys (38). Furthermore, we found that IL-6 mediates STAT3 phosphorylation in human proximal tubular epithelial cells in acute kidney injury (AKI) (36) and IL-6-mediated STAT3 is observed in fibroblasts *in vitro* (38). Genetic depletion of STAT3 from tubular epithelial cells, using *KSP Cre*, protects mice from nephrectomy induced kidney fibrosis by inhibiting tubulo-
interstitial crosstalk (39).

However, the role of STAT3 in the stromal cells particularly in pericytes in the kidney fibrosis remains unknown. Previous studies show that *Foxd1 Cre* driver can be used to investigate the function of pericytes (16, 27, 40-42). In the present study we found that

Stat3 KO mice in the stromal cells are protected from folic acid and aristolochic acid induced kidney fibrosis. Our gain of function and loss of function studies revealed that STAT3 directly regulates the inflammatory pathway, DNA damage, proliferation, migration, and differentiation of pericytes into myofibroblasts. Furthermore, we found that STAT3 binds to the promoter of *Collagen1a1* gene to execute the fibrotic process. Together, our study defines a previously unknown function by which STAT3 in stromal cells promotes kidney fibrosis.

RESULTS:

Increased phosphorylation of STAT3 in AKI and CKD in mouse and human

kidneys. We obtained human kidney specimens from the Brigham and Women's Hospital pathology core from healthy individuals, those with Acute Tubular Necrosis (ATN) as AKI, and Diabetic Nephropathy (DN) as CKD. We found that phosphorylation of STAT3 is increased in AKI and CKD kidneys when compared to healthy kidney. In humans, pSTAT3 expression is limited to tubular epithelial cells of patients with ATN, while pSTAT3 was found in both tubular epithelial and the interstitial cells of the patients with CKD (**Figure 1A, B, C**). Of Note, the total number of tubular epithelial cells in both AKI and CKD were not significantly altered but the number of interstitial cells were significantly increased in CKD (**Supplemental Figure 1A, B**). Analogous to humans, the phosphorylation of STAT3 was also increased in mouse kidneys with FA or AA-induced injury. Specifically, we found increased pSTAT3 in tubular epithelial cells in AKI (2 days post FA or 4 days post AA treatments) and in interstitial cells in fibrotic kidneys (7 days post FA or AA treatment) when compared to those without FA treatment (**Figure 1D, E, F, H, and I**).

In addition, we found that interstitial myofibroblasts, which are known to play important roles in fibrosis, were positive for pSTAT3 as demonstrated by co-expression of a myofibroblast marker, α -SMA following 7 days post FA treatment (**Figure 1D and 1G**). Furthermore, we investigated the pSTAT3 positive cell types within the interstitium and found that in addition to myofibroblasts (**Figure 1J**), pericytes also have increased pSTAT3 as shown by co-immunostaining for either NG2 or PDGFR β in AA induced fibrotic kidneys (**Figure 1K and 1L**) at 14 days post AA treatment.

Stromal cell-specific deletion of *Stat3* protects mice from kidney fibrosis

A previous study showed that *Stat3* deletion from tubular epithelial cells protects mice from kidney fibrosis (39). We aimed to investigate the functions of STAT3 activation in the stromal cells, a cell population known to differentiate into myofibroblasts. To this aim, we developed a stromal cell specific *Stat3* deficient mice by crossing *Stat3*^{flox/flox} mice and *Foxd1*^{GC} mice (**Supplemental Figure 2A, 2B & 2C**). We did not find any significant difference in BUN and sCr in AKI (post FA treatment **Figure 2A, B** nor 4 days post AA treatment **Figure 2C, D**) between control (*Stat3*^{fl/fl}) and *Stat3* KO (*Foxd1*^{GC}*Stat3*^{fl/fl}) mice. Furthermore, our results show similar tubular injury in the AKI phase for both control and *Stat3* KO mice (**Supplemental Figure 3A, D**); these observations were confirmed by mRNA levels of *Tnfa* and proximal tubular epithelial cell injury marker, *Hacvr1* suggesting that *Stat3* deletion in stromal cells did not affect the intensity of AKI (**Supplemental Figure 3B, C, E and F**).

We then assessed the functional roles of STAT3 in the fibrotic models using FA or AA treated mice for 7 days and 14 days. While our results revealed no significant difference in kidney function as assessed by BUN and sCr between control and *Stat3* KO mice in FA treated groups, we found that *Stat3* KO group had better kidney function in AA treated mice (**Figure 2A, B, C and D**). We then evaluated the extent of kidney injury by assessing the degree of fibrosis, myofibroblast activation and tubular damage in both FA and AA treatment groups. Using MTS staining, we found a significant decrease in fibrosis in *Stat3* KO mice as compared to control mice (**Figure 2E, F, G and H**). Myofibroblast activation, assessed by α -SMA staining, was also decreased in *Stat3* KO mice (**Figure 2I, J, K and L**). We further assessed the tubular function, by brush border

using LTL staining, and found an increase in LTL staining in *Stat3* KO mice, indicating the preservation of tubular function (**Figure 2I, K**). In addition, we found a decrease in the expression of *Collagen1a1*, *Fibronectin1* and *Acta2* in *Stat3* KO mice when compared with control mice (**Figure 2M, N, O, Q, R and S**) in both FA- and AA-induced fibrosis. Furthermore, we found decreased gene expression of *Havcr1* in *Stat3* KO mice as compared to control mice (**Figure 2P, T**). Together, our results suggest that *Stat3* depletion from stromal cells has protective effects on kidney fibrosis in FA and AA treated models.

Stromal cell-specific *Stat3* deletion results in reduced inflammation, DNA damage and cell proliferation in the fibrotic kidneys, *in vivo*. To understand the mechanisms by which stromal cell-specific deletion of *Stat3* protects mice from kidney fibrosis, we investigated the involvement of inflammatory pathways in the pericytes and macrophages in AA induced kidney fibrosis model. We found that infiltration of macrophages (stained by F4/80) in the kidney was significantly reduced in *Stat3* KO as compared to control mice (**Figure 3A, B**). We then isolated the macrophages from control and *Stat3* KO fibrotic mice kidneys using F4/80 and Cd11b markers and found decreased number of macrophages in *Stat3* KO mice kidneys as compared to control kidneys (**Figure 3C, D**). Furthermore, gene expression of inflammatory mediators including *Il1b*, *Il6*, *Il12*, *Il23*, *Ifng*, *Nos2* and *Tnfa*, was decreased in the macrophages of *Stat3* KO mice as compared to control mice (**Figure 3E**). On the other hand, expression levels of *Il10* and *Tgfb*, markers of M2 macrophages, a reparative phenotype, were increased in the fibrotic kidneys of *Stat3* KO mice (**Figure 3F**).

Furthermore, to assess the involvement of STAT3 in DNA damage signaling as well as proliferation of tubular and interstitial cells, we stained for pH2AX and Ki67, respectively. We found that *Stat3* KO mice show reduced DNA damage of interstitial as well as tubular epithelial cells (**Figure 3G, H and I**). We also found decreased numbers of interstitial and tubular Ki67 positive cells in *Stat3* KO mice (**Figure 3J, K and L**). Taken together, our results indicated that the depletion of *Stat3* from stromal cells protects mice from kidney fibrosis by reducing inflammation, DNA damage, proliferation of the interstitial cells and increasing the polarization of M2 macrophages.

IL-6 mediated STAT3 phosphorylation leads to increased proliferation, migration and profibrotic signaling in pericyte-like 10T1/2 cells, *in vitro*. It has been shown that stromal cells including pericytes can differentiate into myofibroblasts (16, 43-46). Due to the lack of a pericytes cell line, we utilized 10T1/2 cells of mesenchymal stem cell origin, which are widely used for studying pericyte biology in various organs including kidney (47-49). As an increase in IL-6 has been reported in the pathogenesis of kidney disease, (50-52) we explored its function in STAT3 mediated proliferation, migration, and induction of fibrotic signaling in 10T1/2 cells. We found that pSTAT3 is increased and translocated to the nucleus after IL-6 treatment, and this was inhibited by stattic, a specific inhibitor of STAT3, indicating that these cells responded to IL-6 (**Figure 4A, B, C and D**). We also found increased proliferation of 10T1/2 cells after IL-6 induced STAT3 activation, which was also inhibited by stattic as shown by BrdU incorporation and Ki67 staining (**Figure 4E, F, G and H**). Migration of pericytes to the site of injury is one of the critical steps for the development of fibrosis (12, 25). We

found that an increase in the migration of 10T1/2 cells was associated with the phosphorylation of STAT3, as shown by scratch assay and trans well migration assay, which can be inhibited by stattic (**Figure 4I, J, K and L; Supplemental Figure 4**). We also report here that the phosphorylation of STAT3 increased the phosphorylation and nuclear translocation of profibrotic transcription factor SMAD2, which was also inhibited by stattic (**Figure 4M, N**). In addition, increased STAT3 phosphorylation by IL6 resulted in higher levels of the fibrotic markers, Collagen1, Fibronectin and α -SMA (**Figure 4O, P, Q, R, S and T**). Interestingly, static effectively inhibited IL-6-induced upregulation of these fibrotic markers. Thus, these data indicate that IL-6 mediated-STAT3 phosphorylation increases proliferation, migration and profibrotic signaling in 10T1/2 cells.

STAT3 directly regulates proliferation, migration and profibrotic signaling in pericytes, *in vitro*. To investigate the function of STAT3 phosphorylation in pericytes, we established cellular models for gain and loss of STAT3 function. We generated CRISPR mediated STAT3 Synergistic Activation Mediator (SAM), that transcriptionally activates STAT3 expression as well as STAT3-C by mutating Alanine 662 and Asparagine 664 to Cystines that leads to dimerization and constitutive activation of STAT3 (**Figure 5A**). As expected, *Stat3* mRNA was significantly increased in SAM STAT3 with no change in STAT3-C (**Supplemental Figure 5**). SAM STAT3 and STAT3-C cells showed abundance of pSTAT3 as compared to control cells (**Figure 5B, C**). Furthermore, we found that the expression of profibrotic transcription factor pSMAD2 and fibrotic markers Collagen1, Fibronectin and α -SMA was increased in

STAT3 activated cells as compared to control cells (**Figure 5D, E, F, G, H, I, J and K**).

In addition, we tested the function of STAT3 in migration of 10T1/2 cells. STAT3 activated cells (SAM STAT3 and STAT3-C) showed increased trans well migration with increased cell area as compared to control cells (**Figure 5L, M and N**).

We then investigated whether STAT3 played direct roles in cell proliferation, cell detachment, cell spreading and cell migration. To investigate these phenotypes, we knocked out *Stat3* in 10T1/2 using CRISPR as a loss of function model. *Stat3* KO cells showed genomic deletion and absence of STAT3 protein as compared to *Stat3* WT cells (**Supplemental Figure 6**). Our results demonstrated that *Stat3* KO cells exhibited decreased proliferation, surface attachment, spreading and migration as compared to *Stat3* WT cells (**Figure 5O, P, Q, R, S, T and U**). We next examined pericytes from fibrotic kidneys of control and *Stat3* KO mice. For this, we isolated pericytes via FACS-sorting using NG2, CD140b positive and CD31 negative markers as shown in **Figure 5V**. Interestingly, we found that the gene expressions of the fibrotic factors including *Acta2*, *Col1a1*, *Fn1*, *Tgfb1*, and *Tgfb1* was decreased in *Stat3* KO pericytes (**Figure 5V, W**). Taken together, these data indicate that STAT3 plays a direct role in regulating proliferation, cell detachment, cell spreading, cell migration and profibrotic signaling of pericytes leading to the development of fibrosis.

STAT3 transcriptionally promotes *Collagen1a1* gene expression to execute fibrotic signaling *in vivo* and *in vitro*. To further probe how STAT3 regulates profibrotic signaling, we performed in silico analysis of promoter binding on *Collagen1a1* promoter. It was predicted that human and mouse *Collagen1a1* promoters contain

consensus sequence for STAT3 binding (**Supplemental Figure 7**). Further, we used STAT3 ChIP-seq data from mouse kidney tissues (53). Re-analysis of ChIP sequencing data from WT kidneys shows that STAT3 binds on the *Collagen1a1* promoter but not on the *Fibronectin1* promoter (**Figure 6A, B**). To confirm the binding of STAT3 on the promoter of *Collagen1a1* gene, we performed ChIP assay in mouse kidneys and found that STAT3 binding was increased at day 14 post AA as compared to mice without AA treatment (**Figure 6C**). Moreover, we found a significant increase in the binding of STAT3 on the *Collagen1a1* promoter in our STAT3 gain of function pericytes cells as compared to control cells (**Figure 6D**). We then assessed the STAT3 transactivation in gain and loss of function pericytes cell models, using luciferase assay. We found a significant increase in luciferase activity in STAT3 gain of function models, while a significant decrease in loss of function models as compared to the respective control cells (**Figure 6E, F**). Thus, these data indicate that STAT3 directly regulates a key pro-fibrotic factor, *Collagen1a1*, by binding to its promoter *in vivo* and *in vitro*.

DISCUSSION:

Kidney fibrosis affects 10-15% of the population in the USA. While the functions of various cell types and their involvement has been documented but till now, the function of pericytes and its signaling pathway in tissue fibrosis remains poorly studied. Recent studies using mouse models of kidney, heart, and liver fibrosis by Kramann *et. al.* showed that sonic hedgehog mediated activation of Gli pathways controls fibrosis development (5, 54).

The origin of myofibroblasts in kidney fibrosis has been controversial. Whereas one group support the hypothesis that neither PDGFR β positive nor NG2-positive cells contribute to the myofibroblast pool in the development of kidney fibrosis (17). Another group demonstrated that PDGFR β positive cells do indeed contribute to the myofibroblast pool in kidney, lung, and heart fibrosis (55). Our data supports the hypothesis that PDGFR β positive and NG2 positive pericytes detach from the endothelium and acquire myofibroblastic phenotype. The PDGFR β positive myofibroblasts have been repeatedly described (16, 56, 57) but recent studies using *in vivo* genetic lineage tracing methods indicate that all myofibroblasts express PDGFR β and that Gli1 positive cells do not acquire significant NG2 expression in fibrosis models (5).

Understanding the cellular and molecular signaling of the cell types involved in progression of kidney fibrosis is an important step towards the development of anti-fibrotic therapeutic strategies. STAT3 signaling has been implicated in organ fibrosis including kidney fibrosis (39, 58), where genetic ablation of *Stat3* from tubular epithelial cells protects mice from 5/6 nephrectomy induced kidney fibrosis by disrupting

tubulointerstitial communication (39). In this previous report tubular cell function of STAT3 was well documented but the function of stromal cells remains unknown. Our data show that STAT3 in the stromal cells is crucial for the development of kidney fibrosis and genetic deletion of *Stat3* from Foxd1 positive cells protects mice from kidney fibrosis. Although delineating the quantitative contribution of specific cell subtypes from the stromal cells is needed to confirm the extent of protection, our results indicate 50% protection after *Stat3* deletion using the fibrotic assays such as IFTA scoring, semi-quantitative analyses of *Fibronectin1*, *Collagen1a1* and *Acta2* in two mechanistically different mouse models of kidney fibrosis. In addition to the mouse data, we found that STAT3 phosphorylation is significantly increased in interstitial cells of CKD patients. These findings are particularly important for the translational potential of STAT3 inhibition in patients with CKD.

Initiation of fibrosis is orchestrated by sequential activation of various cell types and one of the most common prominent pathways is inflammation. We and others have shown that DNA damage in AKI generates Reactive Oxygen Species (ROS), which triggers DNA damage signaling and activation of STAT3 pathways. In fact, STAT3 upregulation is associated with AKI (32, 59) and fibrosis development (38, 60-62). Persistent inflammation of tubules or interstitium initiates the progression of fibrosis. However, function of STAT3 in pericytes and its function/role in DNA damage and inflammation remains unclear. Our data for the first time show that *Stat3* depletion from stromal cells limits macrophage infiltration, restricts its inflammatory capabilities, and promotes M2 polarization, which helps to resolve inflammation. In accordance with these data, we

found that *Stat3* depletion reduced DNA damage signaling and decreased proliferation of tubular epithelial cells and interstitial cells in the fibrotic kidneys of *Stat3* KO mice.

Myofibroblasts, a cell type that is well studied to lay matrix leading to fibrosis in chronic kidney disease, are differentiated from different cell types, pericytes being one of them (17, 57, 63). How pericytes differentiate into myofibroblasts is not well understood. We found that STAT3 is phosphorylated in tubular epithelial cells as well as in interstitial cells in human CKD kidneys and experimental mouse models of kidney fibrosis. Our data show that *Stat3* KO in stromal cells protects mice from FA or AA induced kidney fibrosis. Multiple models used in our study to increase STAT3 activation, including IL-6-mediated increase, CRISPR mediated transcriptional as well as mutation induced gain of function, all show that STAT3 is involved in proliferation, migration and increase in profibrotic signaling in pericyte-like cells. We found a significant increase in profibrotic signaling in STAT-C cells as compared to SAM STAT3 cells, which positively correlates with the amount of nuclear STAT3. Thus, confirming that STAT3 phosphorylation and its nuclear translocation directly regulate profibrotic signaling in pericyte-like cells. Stattic, a specific small molecular inhibitor of STAT3, inhibited IL-6 mediated proliferation, migration and profibrotic signaling and *Stat3* KO from the pericyte-like cells reduced the detachment, spreading proliferation and migration of these cells. Furthermore, pericytes isolated from *Stat3* KO fibrotic mice kidneys showed decreased expression of profibrotic signaling molecules, *Fibronectin1*, *Collagen1a1* and *Acta2*. Together, these data confirm a crucial function of STAT3 in the pericytes.

Binding of STAT3 on specific gene promoters decide the outcome of the cellular phenotype. For instance, STAT3 binding on *OCT4* and *NANOG* gene promoters

regulates the stem cell proliferation (64), STAT3 binding on promoters of *Cyclin D1* and *c-MYC* cause cancer cell proliferation, controls cell cycle and deposits extracellular matrix (65), while binding of STAT3 on *Havcr1* promoter leads to tubular cell damage though activation of DNA damage induced inflammation (37, 60). Previously, we have shown that STAT3 binds on promoters of *Havcr1* and *fibrinogen α , β* as well as γ genes in AKI and fibrotic kidneys respectively (38, 60). Here, we found that STAT3 binds on the promoter of *Collagen1a1* gene in the mouse kidneys and pericyte-like cells and induces profibrotic signaling in the pericytes. The binding of STAT3 on specific DNA sequences during fibrosis progression needs further investigation to understand the dynamics of the whole genome occupancy. Thus, in this study we have identified a crucial role of STAT3 phosphorylation to increase profibrotic signaling in the stromal cells. These findings provide potential therapeutic interventions of pericytes using STAT3 as a therapeutic target (**Graphical Abstract**).

STAR METHODS:

Key Resources table

Primary and secondary antibodies:

Antibodies	Vendor	Clone	Cat. number
Anti-CD3e-PerCP	Biolegend	145-2C11	100326
Anti-NK1.1-PerCP	Biolegend	PK136	108726
Anti-CD19-PerCP	Biolegend	6D5	115532
Anti-CD45-FITC	Biolegend	30-F11	103108
Anti-CD11b-PE	Biolegend	M1/70	101208
Anti-MHC-II-APC-Cy7	Biolegend	M5/114.152	107628
Anti-F4/80-PE-Cy7	Biolegend	BM8	123114
Anti-CD45-PE-Cy7	Biolegend	30-F11	103114
Anti-CD31-PE	Biolegend	390	102407
Anti-CD140b-APC	Biolegend	APB5	136007
Anti-NG2-AF488	EMD Millipore	N/A	AB5320A4
7AAD viability solution	Biolegend	N/A	420404
Anti-pSTAT3	Cell Signaling	D3A7	9145
Anti-STAT3	Cell Signaling	124H6	9139
Anti-Ki67	Cell Signaling	8D5	9449
Anti-BrdU	Cell Signaling	Bu20a	5292
Anti-Fibronectin	Abcam	N/A	ab2413
Anti-Collagen1	Abcam	N/A	ab34710
Anti-alpha Smooth Muscle	Millipore	1A4	A5228

Actin	Sigma		
Anti-SMAD2	Cell Signaling	D43B4	5339
Anti-Na ⁺ K ⁺ ATPase	Abcam	EP1845Y	ab76020
Anti-PDGFR beta	Abcam	Y92	ab32570
Anti-F4/80	Cell Signaling	D4C8V	30325
Anti-pH2AX	Cell Signaling	20E3	9718
Anti-NG2	Millipore Sigma	132.38	MAB5384-I-100UG
Anti-GAPDH	Millipore Sigma	N/A	SAB2108668- 100UL
Anti-Rabbit conjugated HRP	Cell Signaling	N/A	7074
Anti-Mouse conjugated HRP	Cell Signaling	N/A	7076
Alexa Flour 488 conjugated Goat anti- Rabbit IgG	Thermo Fisher Scientific	N/A	A32731
Alexa Flour 488 conjugated Goat anti-Rat IgG	Invitrogen	N/A	A-11006
Alexa Flour 488 conjugated Goat anti- Mouse IgG	Invitrogen	N/A	A-32723

Alexa Flour 594 conjugated Goat anti- Rabbit IgG	Invitrogen	N/A	A-11037
Alexa Flour 647 conjugated Goat anti- Rabbit IgG	Invitrogen	N/A	A-32733
Alexa Flour 594 conjugated Goat anti- Mouse IgG	Invitrogen	N/A	A-11005

Chemicals and recombinant proteins:

Chemicals and proteins	Vendor	Cat. number
Stattic	Millipore Sigma	R415
IL-6	R&D Systems	7270-IL-025/CF
DAPI	Vector laboratories	H-1200-10
F-Actin	Invitrogen	A-12381
Cell mask	Thermo Fisher Scientific	C2925
Eagle's Basal medium	Thermo Fisher Scientific	21010-046
BrdU	Thermo Fisher	B5002-5G

	Scientific	
Heparin	Fisher Scientific	H19
LTL	Vector Laboratories	NC0127927
Dual luciferase assay kit	Promega Corporation	E1910
Glutamine	ATCC	30-2214
Lightning-Link® Rapid Alexa Fluor 488 Antibody Labeling Kit	Novus Biologicals	ab236553

Mouse:

Mouse	Source	Stock number
<i>Stat3^{tm1Xyfu/J}</i> (<i>Stat3^{fllox}</i>)	The Jackson Laboratory	016923
<i>Foxd1^{GFP-Cre}</i> (<i>Foxd1^{GC}</i>)	(66)	N/A

Software:

Name	Vendor	Identification number
GraphPad Prism 8	GraphPad Software	RRID:SCR_002798

FIJI v1.0	(67)	RRID:SCR_003070
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Human subjects: Human kidney biopsy sections were obtained through Brigham and Women's Hospital's pathology service core, and they were classified as patients without evidence of ATN (healthy, n=5), patients with evidence of Acute Tubular Necrosis (ATN) as AKI (n=5) and patients with Diabetic Nephropathy (DN) as CKD (n=5). The Institutional Review Board approved the protocol for extracting paraffin-embedded, formalin-fixed sections from patients with and without ATN and DN.

Animals: Male C57BL/6J mice (age 8-10 weeks) were purchased from the Jackson Laboratory (Bar Harbor, ME). *Stat3^{fllox}* (*Stat3^{fl}*) mice were a kind gift from Dr. Kevin Haigis (Dana Farber Cancer Institute, Boston, MA) and *Foxd1^{GC}* mice were a kind gift from Dr. Benjamin D. Humphreys (Washington University in St. Louis, MO). *Stat3* homozygous and *Foxd1* heterozygous mice were bred to generate *Stat3^{fl/fl} Foxd1^{GC}* (*Stat3* KO) mice in *Foxd1* positive cell population. *Stat3* homozygous mice without *Foxd1 cre* allele named as *Stat3^{fl/fl}* (control) mice were used as littermate controls. Mice were treated with single dose of either 250 mg/kg body weight of folic acid or 5 mg/kg body weight of aristolochic acid intraperitoneally and blood was collected at acute and chronic phases of injuries. Mice were injected with 50 mg/kg body weight of BrdU, 3 hrs prior to sacrifice as previously described (68). Mice were sacrificed and kidneys were harvested at day 7 and 14 post treatments. All the experiments were conducted with the approval and guidelines of IACUC and Center for Comparative Medicine of Brigham

and Women's Hospital and were housed in a facility equipped with a 12-hour light/dark cycle.

Estimation of BUN and sCr:

BUN and sCr analysis were performed at O'Brien Centre AKI core at University of Alabama, AL and University of California, San Diego, CA, respectively.

Fibrosis characterization:

Histology: Periodic Acid Schiff (PAS) stain and Mason's Trichrome staining (MTS) was performed at Harvard rodent pathology core. Images were captured by a Nikon DS-Qi1Mc camera attached to a Nikon Eclipse 90i fluorescence microscope using an 40X or 60X objective using Nikon NIS elements AR version 3.2 software.

Interstitial Fibrosis and tubular atrophy (IFTA) scoring: IFTA scores were performed by two pathologist and average of the scores were plotted.

Chemical and antibodies:

Chemicals: IL-6 was purchased from R&D Systems (Minneapolis, MN) and resuspended as per manufacturer's instructions. Stattic was purchased from Millipore Sigma (Burlington, MA) and dissolved in DMSO. Phalloidin (cat. no. R415) and Cell Tracker Green Dye (cat. no. C2925) was purchased from Thermo Fisher Scientific (Waltham, MA). Lotus Tetragonolobus Lectin (LTL) was purchased from Vector laboratories (cat. no. NC0127927).

Antibodies: Anti-pSTAT3 Tyrosine 705 (cat. no. 9145) and anti-STAT3 (cat. no. 9139), anti-Ki67 (cat. no. 9449), anti-F4/80 (cat. no. 30325), pH2AX (cat. no. 9718), pSMAD2 (cat. no. 18338) were purchased from Cell Signaling Technology (Danvers, MA). Anti-Fibronectin (cat. no. ab2413), anti-Collagen1 (cat. no. ab34710) and anti-Na⁺K⁺ATPase (cat. no. ab76020) were purchased from Abcam (Cambridge, MA). Anti- α -SMA (cat. no. A5228) and anti-GAPDH (cat. no. SAB2108668-100UL) were purchased from Thermo Fisher Scientific. Horse Radish Peroxidase (HRP) conjugated anti-rabbit (cat. no. 7074) and anti-mouse (cat. no. 7076) antibodies were purchased from Cell Signaling Technology. Anti-Rabbit, anti-rat, or anti-mouse Alexa488, Alexa 594 and Alexa 647 conjugated antibodies were purchased from Thermo Fisher Scientific. For co-immunostaining using the same species antibodies, primary antibodies were conjugated using Lightning-Link® Rapid Alexa Fluor 488 Antibody Labeling Kit (Novus Biologicals, Centennial, CO 80112).

Cell culture: 10T1/2 a pericyte-like cells was purchased from ATCC (Manassas, VA) and maintained in BME medium with 10% FBS and 2 mg/ml sodium pyruvate. Cells were grown in a 37°C incubator with 5%CO₂ and 37% relative humidity.

CRISPR mediated gain and loss of function:

CRISPR mediated *Stat3* KO: sgRNAs were designed using Broad Institute/MIT CRISPR design tool (<http://crispr.mit.edu>) for the genomic location around transcription start site in the exon 1. sgRNAs were cloned into pSpCas9(BB)-2A-GFP plasmid (Addgene, Watertown, MA). Genomic DNA was extracted using the PureLink® Genomic DNA Mini Kit (cat. no. K182002, Thermo Fisher Scientific). PCR was performed using *Stat3* KO primers described in Table 1. The deletion was confirmed by DNA sequencing followed by western blotting of STAT3 (**Supplemental Figure 6A, B, C**).

Cells were transfected with the designed plasmid and incubated for 48 hrs with plasmids. Single cells positive for GFP were then sorted into a 96 well plate and were grown for 14 days with changing media every 4-5 days. Cells were then stained with pSTAT3 antibodies to confirm the expression levels of phosphorylated STAT3. Positive clones were selected for further studies.

CRISPR mediated Synergistic Activation Mediators (SAM): For CRISPR mediated SAM, sgRNAs were designed for promoter region of *Stat3* (<https://epd.epfl.ch//index.php>) as discussed in the previous section and 4 residues were removed from 5' end to make it a 16 nucleotides sgRNA. The 16-base pair sgRNA loses its capability to break the genomic DNA but retains the binding ability to specific DNA sequence (69). Two sgRNAs from proximal, central and distal promoter regions were selected and were cloned into the lenti-CRISPRV2 plasmid (Addgene). Lentivirus was produced in HEK293 cells by transfecting lenti-CRISPRV2, VSV-G, REV and GAG plasmids. The

supernatant after incubation for 48 hrs was collected and centrifuged to get the clear supernatant containing viral particles.

Pericyte-like cells, 10T1/2 was transduced with the lentiviral particles. SAM activation plasmid containing MS2-p65-HSF1-VP64 was co-transfected in the cells along with the lentiviral particles. *Stat3* gene expression was confirmed by PCR amplification using TaqMan probes described in Table 1 and immunostaining with STAT3 Tyrosine 705 was performed at 72 hrs post transductions.

CRISPR mediated mutation to generate STAT3-C: sgRNAs were designed by selecting the genomic location of Alanine662 and Asparagine664. sgRNAs were cloned into the pSpCas9(BB)-2A-GFP plasmid. Ultramers containing these two mutations to Cysteines were synthesized and co-transfected with the plasmid. GFP positive cells were sorted and were grown as single cell clones as described above. Immunostaining for pSTAT3 was performed to identify the cells with increased pSTAT3. List of sgRNAs and ultramers is shown in Table 1.

Isolation of kidney pericytes and kidney resident macrophage using FACS:

Control and *Stat3* KO mice were sacrificed at day 14 post AA treatment and perfused with ice cold PBS. Immediately, kidneys were harvested and digested with Iscove's Modified Dulbecco's Medium (IMDM) (Gibco) containing Collagenase D (2.5 mg/ml, Roche Diagnostics) and DNase I (1 mg/ml, Sigma-Aldrich) at 37 °C for 45 min. Digested tissue was passed through a 70 µm cell strainer and centrifuged at 1600 rpm for 5 min at 4 °C. Pellets were resuspend in 37% Percoll (GE Healthcare) and overlaid on top of 70% Percoll and centrifuged at 2000 rpm for 25 min at RT with no brake for gradient

centrifugation. Mononuclear cells at the interphase were collected and washed twice with cold Hank's Buffered Saline Solution (HBSS, Gibco). Samples were split into two, one for macrophage sorting and the other for pericyte sorting. Cells at the bottom of the tubes were also collected, washed twice with cold HBSS and included in the pericyte sorting. For pericyte sorting, cells were stained with 7-AAD, anti-CD45-PerCP, anti-CD3-PerCP, anti-NK1.1-PerCP, anti-CD19-PerCP, anti-CD31-PE, anti-CD140b-APC, anti-NG2-Alexa Fluor 488. For macrophage sort, cells were stained with 7-AAD, anti-CD45-PE-Cy7, anti-MHC II-APC-Cy7, anti-CD11b-PE, anti-F4/80-APC. Cells were then sorted using FACS Aria IIu Cell Sorter (Becton Dickinson) using 100 μ m nozzle. Collected cells were lysed with RLT buffer containing 1% β -mercaptoethanol.

Scratch assay: 10T1/2 cells were plated in a 6-well plate at a confluency of 80% and next day a scratch was created with a 200 μ l microtip. Cells were washed three times with PBS and treated with IL-6 and stattic for 12 hrs. Cells were fixed and images were taken using Nikon Eclipse 90i microscope using 10X objective.

Trans well migration assay: 10T1/2 cells were plated 6-well plates and serum starved for 16 hrs. Ten thousand cells were then plated onto the upper well of 8 μ m pore trans well inserts (BD Bioscience). The lower chamber contained either control medium, medium with IL-6 (100 ng/ml) or medium with IL-6 (100 ng/ml) and stattic (2.5 μ M). Cells were incubated for additional 12 hrs. Using cotton swab the cells from the upper wells were removed and cells were fixed with 4% paraformaldehyde. Cells were then stained

with cell mask green (1:10,000 dilution, Thermo Fisher Scientific) and were imaged after removing from the insert and fixing them on a glass slide using Nikon Eclipse 90i microscope using 10X objective.

Western blotting: Western blotting was performed as previously described (36). Briefly, kidney tissues or cells were lysed using RIPA buffer containing protease and phosphatase inhibitors. Tissue was homogenized using homogenizer. Cell lysates were cleared using centrifugation and equal protein was loaded onto the gel. Protein was transferred onto the PVDF membrane and probed with specific antibodies. GAPDH or β -Actin were used as a loading control. Blots were developed using Luminata Forte HRP reagent using a Bio-Rad (Hercules, CA) or Syngene (Fredrick, MD) imaging systems. Quantification of blots was performed using Image lab for Bio-Rad or GeneSys for Syngene software and the data was normalized to the loading controls to calculate the fold change.

RNA isolation, cDNA synthesis, and quantitative Real-Time PCR: Total RNA was isolated from cell pellets using the RNeasy Micro or Mini Kit (Qiagen, Germantown, MD) and first-strand cDNA synthesis was performed for each RNA sample from 0.5 to 1 μ g of total RNA using TaqMan reverse transcription reagents.

TaqMan RT-PCR: RNA from kidney tissues or cells was isolated using miRNeasy Mini Kit (Qiagen, Germantown, MD) as per manufacturer's instruction. Five hundred nanograms of total RNA was converted into cDNA using High-Capacity cDNA Reverse

Transcription Kits (Applied Biosystems, ThermoFisher Scientific) according to manufacturer's protocol. Two microliters of five times diluted cDNA were used for PCR using TaqMan™ Universal PCR Master Mix with FAM labeled gene specific probes (ThermoFisher Scientific) in duplicate on a QuantStudio 7 thermal cycler (ThermoFisher Scientific). Fold change was calculated by delta Ct methods with *Gapdh* as reference gene and presented as fold change with respect to experimental controls.

RT-PCR: For genotyping, DNA was extracted from mouse tail samples using Kappa Genotyping Kit (Roche, Basel, Switzerland) as per manufacturer's instructions and RT-PCR was performed using specific primers described in Table 1. PCR products were resolved onto agarose gel and imaged using Bio-Rad gel imaging system ChemiDoc MP.

Immunofluorescence staining: Immunofluorescence on OCT or paraffin tissue sections or cells were performed as previously described (36, 38, 68). Briefly, formalin fixed paraffin tissue sections were deparaffinized using xylene followed by antigen retrieval using citrate buffer. For the OCT block from PFA fixed tissue sections, PBS was added to the slides and antigen retrieval was performed using proteinase K treatment. For staining the cells, 4% PFA was used for fixation. Samples were permeabilized using 1% Triton X-100 for 15 min. Two percent normal sheep serum containing 5% BSA was used for blocking for 1 hr. Primary antibodies were incubated overnight at 4 °C in five times diluted blocking buffer containing 0.1% Tween 20. After three washes of PBST (0.1% Tween 20), fluorescence labeled secondary antibodies (Thermo Fisher Scientific) were added and incubated for 1 h at room temperature.

Slides were washed and mounted with medium containing DAPI (Vector laboratories). Images were captured on a Nikon Confocal Imaging system (Nikon C1 Eclipse, Nikon) using 60 X objective.

BrdU incorporation assay: BrdU incorporation assay was performed as per manufacturer's protocol (Abcam). Briefly, two thousand cells were plated in a 96 well plate and 2 hrs before endpoints, BrdU was added. Cells were fixed and anti-BrdU antibody was added and incubated at room temperature for 30 min. After washing, anti-mouse peroxidase IgG was added, and washing was repeated. TMB substrate was added and incubated in dark for 30 min at room temperature. Reaction was stopped by stop solution and plate was read at 450 nm. Control cells were referenced as 100% proliferation to plot the graphs.

Cell detachment assay: Cells were plated on chambered slides and incubated for 1 h. Cells were then rotated on shaker for 15 min and washed thrice with PBS. Cells were fixed with 4% PFA and stained with phalloidin. Cells were then mounted with mounting medium containing DAPI. Images were captured using a Nikon confocal imaging system at 60x magnification. The number of cells present were counted, and graph were plotted.

Cell spreading assay: Cells were plated on chambered slides and incubated for 3 h. Cells were fixed with 4% PFA and stained with phalloidin. Cells were then mounted with

mounting medium containing DAPI. Images were captured using a Nikon confocal imaging system at 60x magnification. The area of cells was calculated using ImageJ software and graph were plotted with respect to control cells.

In silico promoter analysis and ChIP sequencing analysis:

In silico STAT3 binding prediction: Promoter region of *Collagen1a1* and *Fibronectin1* gene were extracted from (<https://epd.epfl.ch/index.php>) and search was performed for STAT3 binding sites using (<http://jaspar.genereg.net>).

ChIP sequencing analysis: ChIP data was analyzed from previously performed STAT3 ChIP sequencing in kidneys for promoter binding of *Collagen1a1* and *Fibronectin1* gene ([GSM3176738](#), [GSM3176739](#)). Peaks on the log scale were shown as DNA binding sites for STAT3.

Chromatin Immunoprecipitation (ChIP) assay: ChIP assay was performed as previously described (36). Briefly, 3-mm diameter of snap frozen kidney tissues or cells were treated with 1% formaldehyde/protease inhibitor DNA to crosslink proteins. Sonication was performed to get DNA fragments ranging from 200 to 1000 bp. Fifteen hundred µg protein was immunoprecipitated with 10 µg STAT3, normal rabbit IgG (negative control), or RNA polymerase (positive control) antibodies. Fifteen micrograms (1%) protein from each sample was frozen in 80°C as input. Protein G was used to pull down bound immune complex and DNA was isolated. Five-microliter samples were used for real time PCR using promoter-specific primers of *Collagen1a1* gene promoter (Table 1).

Luciferase assay: Cells were plated in a 12 well plate and were co-transfected with Firefly *STAT3* luciferase and Renilla *Gapdh* luciferase (gift from Dr. Matthias Brock, Center of Experimental Rheumatology, University Hospital Zurich, Zurich, Switzerland) plasmids. After 24 hours cells were treated with IL-6 and stattic. Cells were harvested at 72 hrs post transfection and lysed using passive lysis buffer using Dual-Luciferase Reporter assay purchased from Promega (Madison, WI). Luciferase assay was performed as previously described (36). Ratio of Firefly and Renilla luciferase readings were plotted with respect to control cells.

STATISTICAL ANALYSES:

Statistical analyses were performed using an unpaired 2-tailed Student's *t* test, or one- or two-way ANOVA as indicated. A *P* value of less than 0.05 was considered statistically significant. Data are generally presented as the mean $\pm$ SD. Please see individual figure captions for more details. GraphPad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA) was used for all statistical analyses.

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COMPETING INTERESTS STATEMENT:

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS:

Conceptualization, AA, VS, AW, GM, DF, JB and LLH; Data curation, AA, LZ, SV, MF, KR, PC, AM, AB, MK, ST; Investigation, AA, LZ, SV; Visualization, AA, SV, MF, GM, ST; Methodology, AA, LZ, SV, GM, SJ, I-JC, KR, ST, VS; Writing, AA and LLH, Review and editing, all authors; Funding acquisition, AA, VS, JB, LLH.

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FIGURE LEGENDS:

Figure 1. STAT3 is expressed in tubular epithelial cells in AKI and also in interstitial cells in CKD of human and mouse kidneys. (A) pSTAT3 staining (red) in healthy, AKI and CKD human kidneys. Na⁺K⁺ ATPase (green) for tubular staining. DAPI (blue) for nuclei. (B) Quantitation of number of pSTAT3 positive tubular epithelial cells. (C) Quantitation of number of pSTAT3 positive interstitial cells. * represents p<0.05 as compared to healthy. Scale bar=10 μm. (D) pSTAT3 (red) and α-SMA (green) in normal (day 0) and folic acid induced AKI (day 2) and fibrotic (day 14) kidneys. (E) Quantitation of percentage of tubular pSTAT3 positive cells. (F) Quantitation of percentage of interstitial pSTAT3 positive cells. (G) Quantitation of percentage of pSTAT3 and α-SMA positive cells. * represents p<0.05 as compared to normal. Scale bar=10 μm. (H) Western blotting for pSTAT3 and STAT3 in mouse normal (day 0) kidneys and aristolochic acid induced AKI (day 4) and fibrotic (day 7) kidneys. GAPDH served as loading control. (I) Quantitation of fold change in pSTAT3 compared to normal kidneys. * represents p<0.05 as compared to day 0. (J, K, L) Co-immunofluorescence staining for pSTAT3 and α-SMA or NG2 or PDGFRβ positive cells respectively in mouse kidneys 14 days post AA treatment. White arrow indicates double positive cells. Scale bar=10 μm.

Figure 2. Stromal cell depletion of *Stat3* protects mice from folic acid and aristolochic acid induced kidney fibrosis. (A-D) BUN and SCr estimation for kidney dysfunction in FA- (A, B) or AA- (C, D) induced kidney injury. (E-H) Masson Trichrome Staining (MTS) and IFTA scoring of kidneys following 14 days post FA- (E, F) or AA- (G,

H) induced kidney fibrosis. **(I-L)** Co-immunostaining for LTL (green) and α -SMA (red) and its quantitation in kidneys 14 days post FA- **(I, J)** or AA- **(K, L)** induced kidney fibrosis. **(M-T)** Gene expression analysis for *Col1a1*, *Fn1*, *Acta2* and *Havcr1* in FA- **(M, N, O** and **P)** or AA- **(Q, R, S** and **T)** induced kidney fibrosis. Scale bar=10 μ m. * represents $p<0.05$ as compared to wildtype kidneys. \$ represents $p<0.05$ as compared to 14 days FA or AA wildtype kidneys.

Figure 3. Stromal cell depletion of *Stat3* shows decreased macrophage infiltration, inflammation, decreased DNA damage signaling and reduced cell proliferation in fibrotic kidneys. **(A)** Co-immunostaining for F4/80 (green) and Na⁺ K⁺ ATPase in the mouse kidneys 14 days post AA injection. **(B)** Quantitation of F4/80 intensity. Scale bar=10 μ m. **(C** and **D)** FACS sorting strategy for macrophages from fibrotic kidneys. Quantitative RT-PCR for **(E)** inflammatory mediators and **(F)** M2 markers from the macrophages isolated from kidney 14 days post AA-induced fibrosis. **(G)** Immunostaining for pH2AX (green) in the mouse kidneys 14 days post AA injection. Quantitation of **(H)** interstitial and **(I)** tubular pH2AX positive cells. **(J)** Immunostaining for Ki67 (Cyan) in the mouse kidneys 14 days post AA injection. Quantitation of **(K)** interstitial and **(L)** tubular Ki67 positive cells. Scale bar=10 μ m. * represents $p<0.05$ as compared to wildtype kidneys.

Figure 4. IL-6 mediated STAT3 phosphorylation regulates proliferation, migration and profibrotic signaling in pericyte-like 10T1/2 cells. **(A** and **B)** Western blotting for STAT3 phosphorylation following treatment with IL-6 or IL-6 in combination with static

in 10T1/2 cells and its quantitation. **(C and D)** Nuclear translocation of STAT3 in 10T1/2 cells following IL-6 and or stattic treatment and its quantitation. **(E and F)** Proliferation of 10T1/2 cells following IL-6 and stattic treatments using BrdU assay in a **(E)** time and **(F)** STAT3 dependent manner. **(G and H)** Immunofluorescence staining for Ki67 with or without IL-6 and stattic treatments and its quantitation. **(I and J)** Migration of 10T1/2 cells following IL-6 and stattic treatment using scratch assay and its quantitation. **(K and L)** Trans well migration of 10T1/2 cells treated with IL-6 and stattic and its quantitation for cell area normalized to cell number. **(M and N)** Immunostaining for pSMAD2 (green) and its quantitation. **(O and P)** Immunostaining for Collagen 1 (Col1, green) and its quantitation. **(Q and R)** Immunostaining for Fibronectin (Fn, green) and its quantitation. **(S and T)** Immunostaining for α -SMA (green) and its quantitation. Scale bar=10 μ m. * represents $p < 0.05$ as compared to control cells. \$ represents $p < 0.05$ as compared to IL-6 treated cells.

Figure 5. STAT3 directly regulates attachment, spreading, migration, proliferation and profibrotic signaling in 10T1/2 cells. **(A)** Scheme for STAT3 activation using synergistic activation mediators (SAM) and activation mutants (STAT3-C). **(B and C)** Immunostaining for pSTAT3 (green) and its quantitation. **(D and E)** Immunostaining for pSMAD2 (green) and its quantitation. **(F and G)** Immunostaining for Collagen 1 (Col1, green) and its quantitation. **(H and I)** Immunostaining for Fibronectin (Fn, green) and its quantitation. **(J and K)** Immunostaining for α -SMA (green) and its quantitation. **(L)** Trans well migration of 10T1/2 cells with STAT3 activation. Quantitation of **(M)** cell area normalized to cell number and **(N)** total number of migrated cells. Scale bar=10 μ m. *

Represents $p < 0.05$ as compared to control cells. **(O)** Proliferation of *Stat3* WT and KO 10T1/2 cells as assayed by BrdU incorporation assay. **(P and Q)** Adherence assay (F-actin, red) and quantitation of intensity of cells normalized to total number of cells. **(R and S)** Spreading assay (F-actin, red) and quantitation of cell area normalized to total number of cells. **(T and U)** Trans well migration assay (F-actin, red) and quantitation of total number of migrated cells. **(V)** FACS sorting strategy for isolating pericytes from fibrotic mice kidneys and its quantitation. **(W)** Quantitative RT-PCR for pro-fibrotic factors in the pericytes isolated from fibrotic control and *Stat3* KO mice. Scale bar=10 μm . *represents $p < 0.05$ as compared to wildtype 10T1/2 cells or control mice pericytes.

Figure 6. STAT3 binds on *Collagen1a1* promoter in mouse kidneys and in pericytes cell line to induce profibrotic signaling. **(A and B)** Analysis of previously published STAT3 ChIP sequencing data showing STAT3 binding on mouse *Collagen1a1* and Fibronectin 1 promoter region. **(C)** ChIP assay using anti-STAT3 antibody in normal and 14 days post AA injection in the mice kidneys to detect STAT3 binding on *Collagen1a1* promoter. Rabbit IgG and RNA polymerase (RP) antibodies are negative and positive controls. **(D)** ChIP assay using anti-STAT3 antibody in 10T1/2 cells with activated STAT3 to detect STAT3 binding on *Collagen1a1* promoter. Luciferase assay to detect STAT3 binding on STAT3 consensus sequence in **(E)** STAT3 activated and **(F)** *Stat3* KO 10T1/2 cells. * represents $p < 0.05$ as compared to control cells.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 6

Figure 7

Graphical abstract

Table 1

List of TaqMan Assays primers (Thermo Fisher Scientific)

Gene name	Assay ID
<i>Havcr1</i>	Mm01291075_m1
<i>Col1a1</i>	Mm00801657_gH
<i>Fn1</i>	Mm01256729_m1
<i>Acta2</i>	Mm01546133_m1
<i>Tnfa</i>	Mm01161290_g1
<i>Stat3</i>	Mm00456961_m1
<i>Spry1</i>	Mm00839969_g1
<i>Socs3</i>	Mm05810749_s1
<i>Gapdh</i>	Mm99999915_g1
<i>Il6</i>	Mm00446190_m1
<i>Il12</i>	Mm00434169_m1
<i>Il23</i>	Mm00518984_m1
<i>Nos2</i>	Mm00440502_m1
<i>Il10</i>	Mm01288386_m1
<i>Tgfb</i>	Mm01178820_m1
<i>Il6st</i>	Mm00439665_m1
<i>Il1b</i>	Mm00434228_m1
<i>Ifng</i>	Mm01168134_m1

Stat3 genotyping primers

Name	Sequence (5'-3')	Annealing temp	Product size (bp)
<i>Stat3 F</i>	CACCCTGTCCCCAAGAGTAA	56	Flox = 250
<i>Stat3 R</i>	AGGAAGGAAACAAGCAAGCA	55	KO = 350
<i>Stat3 genex</i>	AAATGCCAAGTCCACATTCC	53	Wt = 150
<i>Cre F</i>	GAACCTGATGGACATGTTTCAGG	55	Cre positive = 150
<i>Cre R</i>	AGTGCGTTTCAACGCTAGAGCCTGT	55	

Collagen 1a1 ChIP primers

Name	Sequence (5'-3')
<i>Coll1a1</i> promo F	AAGGTGTGTCTGGCATGGCA
<i>Coll1a1</i> promo R	CACCTCATGGTGTGGACCCA

STAT3 KO sgRNAs

Name	Sequence (5'-3')
<i>Stat3 KOsg1 F</i>	TCGAGACCCCTGACTGCAGC
<i>Stat3 KOsg2 F</i>	GACCCCTGACTGCAGCAGGA

STAT3 KO primers

Name	Sequence (5'-3')
<i>Stat3 KO1 F</i>	GTATAGTGTGTTGATCCCCAGCAT
<i>Stat3 KO2 F</i>	ACCTCTGTTCTCCCAATCCT

STAT-C sgRNA

Name	Sequence (5'-3')
<i>Stat3C-1F</i>	CATCATGGGCTATAAGATCA
<i>Stat3C-2F</i>	AAGTGGAGACACCAGGATGT

Ultramer for STAT3C (5'-3')

ACATGTCATTTGCTGAAATCATCATGGGCTATAAGATCATGGATTGTACCTGCATCCTGGTGTCTCCACTTGTCTACCTC
TACCCCGACATTCCCAAGGA

SAM STAT3 sgRNA

Primer Name	Sequence (5'-3')
<i>Stat3</i> SAM-1F (distal)	CAGCTGAGTTTTTCAGC
<i>Stat3</i> SAM-2F (distal)	GTCTCCTCCCTCCCCC
<i>Stat3</i> SAM-3F (central)	TTAGTGGGCGGGGCTT
<i>Stat3</i> SAM-4F (central)	ATTCCTTAATTATGCA
<i>Stat3</i> SAM-5F (proximal)	GGAACGAGCCCTAAC
<i>Stat3</i> SAM-6F (proximal)	TCGGCTAGGAGAGGCC