Matrix Protein Tenascin-C Promotes Kidney Fibrosis via STAT3 activation in Response to Tubular Injury

Chuanming Hao (chuanminghao@fudan.edu.cn)
Department of Nephrology Huashan Hospital, Fudan University

Qionghong Xie

Min Zhang
Huashan Hospital, Fudan University

Xiaoyi Mao
Huashan Hospital,Fudan University

Mingyue Xu
Huashan Hospital,Fudan University

Shaojun Liu
Huashan Hospital of Fudan university

Da Shang
Huashan Hospital,Fudan University

Yunyu Xu
Huashan Hospital,Fudan University

Ruiying Chen
Huashan Hospital,Fudan University

Yi Guan
Huashan Hospital,Fudan University

Xinzhong Huang
Affiliated Hospital of Nantong University

Roy Zent
Vanderbilt University Medical Center

Ambra Pozzi
Vanderbilt University Medical Center

Article

Keywords: Tenascin C, kidney fibrosis, extracellular matrix, STAT3

Posted Date: October 10th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2002214/v1

Matrix Protein Tenascin-C Promotes Kidney Fibrosis via STAT3 activation in Response to Tubular Injury

Qionghong Xie*[1], Min Zhang*[1], Xiaoyi Mao*[1], Mingyue Xu[1], Shaojun Liu[1], Da Shang[1], Yunyu Xu[1], Ruiying Chen[1], Yi Guan[1], Xinzhong Huang[2], Roy Zent[3,4], Ambra Pozzi[3,4] and Chuan-Ming Hao[1]

[1]Division of Nephrology, Huashan Hospital, Fudan University, Shanghai China

[2]Division of Nephrology, Affiliated Hospital of Nantong University, Nantong China

[3]Division of Nephrology, Vanderbilt University Medical Center, Nashville, Tennessee

[4]Department of Veterans Affairs, Nashville, Tennessee

*These authors contributed equally to the study

Corresponding author:

Chuan-Ming Hao, MD, PhD

12 Wulumuqi Road (middle)

Division of Nephrology, Huashan Hospital, Fudan University

Shanghai 200040, China

Tel: 86-21-52888303

Fax: 86-21-52888304

E-mail: chuanminghao@fudan.edu.cn

Abstract

Accumulating evidence indicates that the extracellular matrix (ECM) is not only a consequence of fibrosis, but also contributes to the progression of fibrosis, by creating a

pro-fibrotic microenvironment. Tenascin-C (TNC) is an ECM glycoprotein that contains multiple functional domains. We showed that following kidney injury, TNC was markedly induced in fibrotic areas in the kidney from both mouse models and humans with kidney diseases. Genetically deletion of TNC in mice significantly attenuated unilateral ureteral obstruction-induced kidney fibrosis. Further studies showed that TNC promoted the proliferation of kidney interstitial cells via STAT3 activation. To identify and characterize TNC expressing cells, we generated a TNC promoter driven CreER2-IRES-eGFP knock-in mouse line and found that the TNC reporter eGFP was markedly induced in cells around injured tubules that had lost epithelial markers, suggesting TNC was induced in response to epithelium injury. Most of the eGFP positive cells were both NG2 and PDGFRβ positive, only half of which were αSMA positive. These cells did not carry markers of progenitor cells or macrophages. In normal kidney, TNC is constitutively expressed by the renal medullary interstitial cells (RMICs) in renal papilla that has been reported to contain stem cells. Cell lineage tracing study revealed that the cells expressing TNC in renal cortex after fibrosis were not originated from these RMICs. In conclusion, this study provides strong evidence that matrix protein TNC contributes to kidney fibrosis. TNC pathway may serve as a potential therapeutic target for interstitial fibrosis and progression of chronic kidney disease.

Key words: Tenascin C, kidney fibrosis, extracellular matrix, STAT3

Background

Kidney fibrosis is the final common pathway to end stage renal disease and characterized by extracellular matrix (ECM) accumulation and destruction of normal structure [1, 2]. Accumulating evidence indicates that the ECM is not only a consequence of fibrosis, but also contributes to the progression of fibrosis, by creating a pro-fibrotic microenvironment or a fibrogenic niche [3, 4].

It has been documented that the ECM not only provides physical scaffolds to cells by

forming a three-dimensional network, but also regulates many biologic processes including proliferation, migration, differentiation, survival and morphogenesis during development or in certain physiological/pathophysiological conditions [5-7]. Matricellular proteins (or non-structural matrix proteins) are a group of ECM proteins that are characterized by dynamical expressions and regulatory roles[8]. Rather than serving as stable structural elements, they are usually transiently expressed during development or after injury [9]. They contain multiple functional domains, such as binding sites for other ECM proteins, ligands for cell surface receptors, and sites that can sequester specific growth factors, playing important roles in regulating cellular processes [10].

Tenascin-C (TNC), a hexametric glycoprotein, is a member of the matricellular proteins that is expressed during development and is at low levels in most of the adult tissues, but re-induced following injury and associated with the severity of diseases and prognosis[11, 12]. The monomer of TNC contains an N-terminal assembly domain, epidermal growth factor like (EGF-L) repeats, fibronectin type-III (FNIII) like repeats and a C-terminal fibrinogen-like globe [13, 14]. These functional domains have been suggested to interact with specific cell-surface receptors, such as epidermal growth factor receptor (EGFR), integrins and toll like receptor 4 (TLR4) [13]. They can also recruit cytokines and growth factors, and present them to or prevent them from cell-surface receptors depending on their relative locations [14]. Mice lacking TNC develop normally, suggesting that TNC is not indispensable for the development. However, accumulating evidence showed that TNC was markedly increased in the fibrotic tissues and associated with organ fibrosis[15-17]. Recently, TNC was also found to promote renal interstitial fibroblast proliferation via

integrin/focal adhesion kinase/mitogen-activated protein kinase pathway [18].

In the present study, we characterized the cells that expressed TNC and examined their role in renal fibrosis. This study provided novel information about the role of TNC in renal fibrosis.

Methods

Patients

To characterize the distribution of TNC expression in normal human kidney, specimens from healthy parts of human carcinoma nephrectomy were obtained. TNC mRNA was measured by qPCR in fresh tissue, and the distribution of TNC protein was investigated in paraffin-embedded tissue by immunohistochemistry (IHC). To examine the induction of TNC in diseased kidneys, TNC protein was detected in paraffin-embedded sections from patients with biopsy-proven IgA nephropathy (IgAN, n=15) and diabetic nephropathy (DM, n=6) by IHC. This study was approved by the ethics committee of Huashan Hospital, Fudan University.

Animals and Models

Mice. $TNC^{CreER2\text{-}eGFP/+}$ mouse line was generated as described previously [19]. In brief, an inducible CreER2 gene with an eGFP reporter was knocked into the 2^{nd} exon of TNC at the site of starting codon ATG. The insertion of this cassette also resulted in TNC deletion (Figure 2A). The TNC deletion ($TNC^{-/-}$) mice were fertile, and developed normally. Male $TNC^{-/-}$ mice and their wild-type littermates, aged 8-12 weeks, were used to examine the role of TNC in fibrosis. $R26^{tdTomato}$ reporter mice were purchased from the Jackson

Laboratory (stock number, 007909). The bi-transgenic $TNC^{CreER2\text{-}eGFP/+}$;$R26^{tdTomato/+}$ reporter mice were generated by crossing $TNC^{CreER2\text{-}eGFP/+}$ with $R26^{tdTomato}$ mice. All of the mice were maintained in the animal facility of Fudan University and allowed free access to standard rodent chow and water. All of the animal experiments were approved by the Animal Care and Use Committees of Fudan University.

TNC reporter mice. In the $TNC^{CreER2\text{-}eGFP/+}$ mice, eGFP is driven by endogenous TNC promoter (TNCp-CreER2-IRES-eGFP), and is thus used as a TNC reporter. In the bi-transgenic $TNC^{CreER2\text{-}eGFP/+}$;$R26^{tdTomato/+}$ reporter mice, the recombination is induced by TNC promoter driven CreER2 in the presence of tamoxifen, and then tdTomato (red fluorescence protein) will label the TNC expressing cells and their daughter cells permanently.

Cell lineage experiments. To determine whether the constitutive TNC expressing cells in renal medulla were precursors of the interstitial cells or TNC producing cells in the cortex after fibrosis, a cell lineage tracing experiment was conducted, as shown in Figure 8A and supplement Figure 3A. The bi-transgenic mice $TNC^{CreER2\text{-}eGFP/+}$;$R26^{tdTomato/+}$ were given tamoxifen 1.5mg/d for 5 days to induce recombination at 8 weeks of age. After 12 weeks' washout time, these mice were used to perform UUO or unilateral kidney ischemia reperfusion (UIR) operation. When they were sacrificed at the end of the experiments, the tdTomato positive cells we observed were originated from the cells that constitutively expressed TNC in the renal medulla. In the other set of animals, if these mice were given a second series of tamoxifen at the time of UUO or UIR operation, tdTomato would be also expressed in the cells where TNC was newly induced after operation. The mice with sham

operation but similar tamoxifen exposure was used as controls.

Models. UUO induced kidney fibrosis model was used in our study. The mice were anesthetized with chloral hydrate (400 mg/kg body weight) by intra-peritoneal injection and body temperatures were maintained at 36.5-37.5℃ throughout the procedure. The left ureter was exposed via a flank incision and ligated with two 3.0 silk ties at the level of the lower renal pole. The mice were sacrificed at day 7, 10 or 14 after the operation. The EdU cooperation assay was used to assess cell proliferation of the kidney: 0.1mg of EdU was injected (i.p.) at day 3 and 5 after UUO operation and the mice were sacrificed at day 7.

Cell Experiments

Cell lines. The TNC expressing cells (TNC-Cell) were obtained by sorting the tdTomato positive cells in the UUO kidneys of $TNC^{CreER2\text{-}eGFP/+}$;$R26^{tdTomato/+}$ mice using flow cytometry, and immortalized using SV40 T lentivirus (Figure 9B). This cell line was cultured in polylysine (20μg/mL, Sigma) coated dish with DMEM/F12 media containing 10% FBS, 1ng/mL basic-FGF (Peprotech) and 5ng/mL insulin (Sigma). The normal rat kidney fibroblast NRK49F was purchased from American Type Culture Collection (ATCC). Both NRK49F and mouse embryo fibroblast NIH3T3 were cultured in DMEM media (GIBCO, Invitrogen Co, CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS, GIBCO, USA), 100U/ml penicillin and 100μg/ml streptomycin. Cells were incubated at 37℃ in a 5% CO2, humidified atmosphere.

Intervention. The cultured cells at 60% confluence were incubated with 1% FBS containing media for 24 hours followed by treatment of human TNC (Millipore, CAT. CC605, 5μg/mL or 10μg/mL) or TGFβ (Sigma, 2ng/mL to 10ng/mL). The STAT3 inhibitor Stattic (Selleck,

2μM) was added to the media 30 minutes before TNC treatment.

Cell Proliferation. After TNC treatment for 24 hours, the number and viability of the cultured cells were determined using the Cell Counting Kit-8 (CCK-8, Dojindo Laboratories) following the manufacturer's instructions. Briefly, water-soluble tetrazolium salt WST-8 is reduced by dehydrogenase in the cells and turns into an orange formazan dye which is soluble in the culture media, and the amount of the formazan dye is proportional to the number of living cells. The cell proliferation was determined by detecting the proportion of EdU positive cells using the Click-iT® EdU Imaging Kits (Invitrogen) according to the manufacturer's instructions. EdU is detected based on a copper-catalyzed covalent reaction between an alkyne (contained in the EdU) and azide (contained in the Alexa Fluor dye).

Histology and Pathology

Tissue Preparation. The mice were anesthetized with chloral hydrate (500 mg/kg body weight) intra-peritoneal injection before sacrifice and immediately perfused with 60 ml ice-cold PBS via the left ventricle. The kidneys were hemi-sectioned horizontally and fixed in 4% paraformaldehyde (PFA) on ice for 1 hour. Then half of each kidney was incubated in 30% sucrose-PBS at 4℃ overnight and embedded in OCT, and the other half was processed and embedded in paraffin.

Immunohistochemistry. Paraffin-embedded 2μm-thick sections of human kidney were deparaffinized and rehydrated in water. After incubation with 3% H_2O_2 for 30 minutes at room temperature (RT), the tissues were given microwave antigen retrieval in citrate buffer pH 6.0 (100% power for 10 minutes), blocked with 5% BSA in PBS for 1 hour at RT, and

followed by the incubation with anti-human TNC antibody (Sigma rabbit polyclonal, 1:500) at 4℃ overnight. After 3 washes with PBS, the samples were incubated in a horseradish peroxidase (HRP) conjugated secondary antibody for 45 minutes at RT, followed by coloration with 3,3- diaminobenzidine solution (DAB).

Immunofluorescence. Frozen tissue embedded in OCT was cut in 5μm thick section. For IF analysis, the sections were fixed in cold acetone for 3 minutes, washed in PBS, blocked in 5% bovine serum albumin (BSA) for 30 minutes and then incubated with primary antibodies in blocking buffer overnight at 4℃. The primary antibodies included: anti-GFP (Abcam rabbit polyclonal, 1:200; Aves Labs chicken antibodies, 1:500;), anti-AQP2 (Santa Cruz goat polyclonal, 1:100), anti-CD31 (Abcam rat monoclonal, 1:100), anti-PDGFRβ (eBioscience rat monoclonal, 1:100), anti-CD34 (Abcam rat monoclonal, 1:200), anti-CD44 (Abcam rat monoclonal, 1:200), anti-α-SMA (Sigma mouse monoclonal, 1:100), anti-FSP-1 (Abcam rabbit polyclonal, 1:200), anti-NG2 (Millipore rabbit polyclonal, 1:100), anti-TNC (Sigma rat monoclonal, 1:100), anti-F4/80 (AbD serotec rat monoclonal, 1:200), and anti-CD68 (AbD serotec rat monoclonal, 1:200), anti-THP (Santa Cruz rabbit polyclonal, 1:200) antibody. After 3 washes in PBS, the tissue was incubated with FITC- or Cy3- conjugated anti-IgG/IgY secondary antibody (Millipore, 1:200) for 1 hour at room temperature (RT), followed by 3 washes in PBS and then covered by Vectashield Mounting Medium with DAPI (Vector Labs). Fluorescein lotus tetragonolobus lectin (LTL, Vector Labs, 1:100) was used to label proximal tubules.

EdU detection. Paraffin-embedded renal tissue from the mice which had received EdU injection after operation was used for EdU detection according to the manufacturer's

instructions. EdU positive cells were calculated per high power field to compare the proliferation between wildtype and TNC$^{-/-}$ mice.

Quantitative RT-PCRs

Renal cortex and papilla isolated from mice kidneys were used to measure TNC mRNA and TNC reporter eGFP mRNA to determine the expression of TNC in different regions of kidney. The entire kidney from UUO model was homogenized to exam the TNC expression and evaluate the severity of fibrosis by detecting the mRNA of TNC, collagen Ⅰα, fibronectin and PAI-1. Total mRNA was extracted using TRIzol Reagent (Invitrogen), reversely transcribed using RevertAid RT reagent Kit (Takara) according to the manufacturer's protocol. Levels of mRNA were determined by real time qPCR using SYBR Premix Ex Taq (Takara) and normalized to the eukaryotic 18s rRNA or β-actin. The primers used were TNC: F 5'-CAA CTG TGC CCT GTC CTA C-3', R 5'-AAC GCC CTG ACT GTG GTT A-3'; eGFP: F 5'-CCT CAA GGA CGA CGG CAA C-3', R 5'-CTC GAT GCG GTT CAC CAG-3'; collagen Ⅰα: F 5'-TGA CTG GAA GAG CGG AGA G-3', R 5'-GAC GGC TGA GTA GGG AAC A-3'; fibronectin: F 5'-TGG GAG CAT TGT TGT GTC-3', R 5'-AGC GGT GTC ACT ACT CTG T-3'; PAI-1: F 5'-ACT TTA CCC CTC CGA GAA-3', R 5'-CCT GCT GAA ACA CTT TTA C-3'.

Western Blot and Immunoprecipitation

Total protein was extracted from the whole kidney or cultured cells using RIPA buffer (0.05M Tris, 0.15M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, pH7.4) with PMSF, protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche). The concentration of the protein was determined using the BCA protein assay kit (Beyotime).

For Western blot, equal amount of protein (20-50μg per lane) was loaded in a 7.5% or 10% SDS-PAGE mini-gel and transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% BSA in PBST buffer (100mM PBS, pH 7.5, 0.1% Tween-20) and then incubated in primary antibody diluted with blocking buffer overnight at 4℃. The primary antibodies included: anti-TNC (IBL), anti-collagen Iα (Novus), anti-αSMA (Sigma), anti-STAT3 (Abcam), anti-phospho-STAT3 (Tyr 705, Abcam), anti-GAPDH (CST) and anti-β-actin (Sigma) antibody. Membranes were then incubated with appropriate secondary antibodies and subjected to chemiluminescence detection using ECL Reagent (Millipore).

Statistical Analysis

Statistical analysis was performed using Prism 5.0 (GraphPad Software Inc.). Comparison of two factors was performed by two-tailed t test and comparison of two factors with multiple levels was performed by two-way ANOVA test. A *P* value <0.05 was considered significant. The results were presented as means and error bars indicate ±SEM.

Results

TNC was constitutively expressed by the renal medullary stromal cells in normal kidney

To determine the distribution of TNC in non-fibrosis kidneys, we examined TNC expression using immunohistochemistry (IHC) in non-fibrosis human kidneys and found that TNC immuno-protein was primarily located in the interstitium of renal medulla, but rarely detected in the renal cortex (Figure 1A, Supplement Figure 1A&B). This result was supported by TNC mRNA measurement in human kidneys which were resected because

of renal carcinoma (Supplement Figure 1C) and mouse kidneys (Figure 2B) [19].

Since TNC is an extracellular matrix protein, IHC will not be able to identify cells that express TNC. Therefore, we developed a TNC promoter driven eGFP reporter mice (Figure 2A) [19]. Co-staining for TNC reporter eGFP and AQP2, a marker of collecting ducts, showed that eGFP positive cells were not epithelial cells. They were primarily expressed by the renal medullary interstitial cells (RMICs) in normal mouse kidney (Figure 2C&D and Supplement Figure 2C&D) [20, 21]. To characterize the cell types that expressed TNC, we co-stained eGFP with specific cell markers in normal kidney and found that eGFP positive cells expressed platelet-derived growth factor receptor beta (PDGFRβ), a marker of stromal cells of mesenchymal origin [22] (Figure 2E and Supplement Figure 2E). They did not express CD31 (a marker of endothelial cells [23]), CD34 (a marker of endothelial cells and early hematopoietic stem cells and progenitors [24]), or CD44 (a marker of progenitors [25, 26]), suggesting that TNC was neither expressed in endothelial cells nor progenitor cells (Figure 2F&G&H and Supplement Figure 2F&G&H). In the normal renal medulla, there were only a few cells positive for αSMA (a marker of myofibroblasts[27]), FSP1 (recognized as a specific marker of fibroblasts [28], but subsequently identified as a marker of macrophages[29]), or NG2 (a transmembrane glycoprotein used to label pericytes [30]). We did not find eGFP was colocalized with these three markers (Figure 2I&J&K and Supplement Figure 2I&J&K).

TNC was markedly induced in the fibrotic area of diseased kidney

We previously reported that TNC was significantly increased in injured glomeruli of IgA nephropathy and expressed by PDGFRβ positive mesangial cells[31]. TNC was also

markedly induced in the fibrotic interstitial area of renal cortex of biopsy specimen from patients with IgA nephropathy and from patients with diabetic nephropathy (Figure 1B-F). To further examine the expression of TNC in kidney fibrosis, we generated a unilateral ureteral obstruction (UUO) mouse model and found that TNC mRNA and protein were significantly increased in the fibrotic kidneys (Figure 3A-C). Transgenic TNC reporter mice also showed a remarkable increase of eGFP mRNA expression and eGFP positive cells in the UUO kidneys (Figure 3B&D). Similar results were also found in the ischemia reperfusion (IR) induced kidney fibrosis model (Supplement Figure 3).

TNC deficiency reduced kidney fibrosis in animal models

To investigate the role of TNC induction in kidney fibrosis, we generated a TNC homozygous knockout mice. Deletion of TNC ($TNC^{-/-}$) significantly attenuated the induction of collagen Ⅰ expression in the kidneys assessed by IF following UUO by approximate 30% at day 7 and 10 compared with their wild type littermates (Figure 4A, $p<0.05$). Consistently, mRNAs of fibrosis markers, such as collagen Ⅰα, fibronectin and plasminogen activator inhibitor-1 (PAI-1), were significantly lower in the $TNC^{-/-}$ mice after UUO at day 7 and 10 (Figure 4B, $p<0.05$). Western blot showed that the proteins of collagen Ⅰα and α-SMA were also significantly reduced in $TNC^{-/-}$ mice after UUO at day 7 (Figure 4C, $n=7$, $p<0.05$).

TNC expressing cells in the fibrotic kidneys were not epithelial cells

To confirm the above results that TNC was significantly induced in fibrosis, we generated another TNC mouse reporter $TNC^{CreER2\text{-}eGFP/+}$;$R26^{tdTomato/+}$, in which the cells that express TNC will be labeled with red fluorescence protein tdTomato permanently following

tamoxifen (Figure 5A). There are some differences between tdTomato reporter mice and eGFP reporter mice. First, the tdTomato reporter not only labels the cells which are expressing TNC but also the cells once expressed TNC, while the eGFP reporter only labels the cells which are expressing TNC. Second, tdTomato is only expressed in the presence of tamoxifen while eGFP not. Third, the expression of tdTomato is driven by the promoter Rosa26 and the expression level is high with strong autofluorescence. The tdTomato expression does not reflect TNC expression, while eGFP does. Fourth, the downstream IRES-eGFP protein may be less efficiently translated than the coding sequence of Cre which is directly adjacent to the promoter. However, tamoxifen induction efficiency as well as excision rate vary among tissue types.

Using this bi-transgenic reporter mice $TNC^{CreER2\text{-}eGFP/+}$;$R26^{tdTomato/+}$, we confirmed that TNC expressing cells were primarily expressed in the medulla of normal kidney (Figure 5B&D). Then we generated UUO model in this mice and administered tamoxifen right after operation, and also found that the tdTomato positive cells were significantly increased in the cortex of fibrotic kidneys (Figure 5B-E). These tdTomato positive cells did not stained for Lotus tetragonolobus lectin (LTL, proximal tubule) nor expressed AQP2 (collecting duct), suggesting that they were not epithelial cells (Figure 5F&G and Supplement Figure 4).

TNC expressing cells in the fibrotic kidneys were mostly $PDGFR\beta^{+}NG2^{+}$ stromal cells

To characterize the cells that express TNC in the fibrotic kidneys, we co-labeled TNC reporter eGFP with specific markers in UUO model. We did not use tdTomato as reporter because its autofluorescence is too strong that it can be detected in the green channel.

We found that the eGFP positive cells were positive for PDGFRβ, consistent with stromal cells (Figure 6A and Supplement Figure 5A). Some of the eGFP positive cells were αSMA positive, and not all αSMA positive myofibroblasts expressed TNC (Figure 6B&I and Supplement Figure 5B&I). In contrast, most of the eGFP positive cells were NG2 (a marker of pericyte) positive (Figure 6C&J and Supplement Figure 5C&J). None of the eGFP positive cells were positive for FSP1 (Figure 6D and Supplement Figure 5D). In addition, the eGFP positive cells were neither CD34 nor CD44 positive (Figure 6E&F and Supplement Figure 5E&F), and were neither CD68 nor F4/80 (markers of macrophages) positive (Figure 6G&H and Supplement Figure 5G&H).

Interestingly, the TNC reporter eGFP or tdTomato positive cells in UUO models were predominantly located adjacent to the tubular structures that were dilated and negative for LTL, THP (Tamm-Horsfall protein, a marker of the thick ascending limb), AQP2, and CD31 (Figure 7A-C and Supplement Figure 6A-C). To examine whether these tubular structures surrounded by TNC reporter positive cells were injured renal tubules, we co-labeled TNC reporter with LTL or antibodies against THP or AQP2 on serial sections. This serial section study showed that the tubules surrounded by TNC expressing cells had continuation to the tubules with positive AQP2 staining, supporting that TNC expressing cells were localized surrounding the injured tubular epithelial cells in UUO kidneys (Figure 7C and Supplement Figure 6C).

The expanded TNC expressing cells in the fibrotic renal cortex were not originated from the constitutive TNC expressing cells in renal papilla

It has been reported that renal papilla contains stem cells[32], and TNC has also been

reported to be expressed in stem cells[33]. To determine whether the TNC expressing cells in renal medulla were progenitors or source of the expanded stromal cells in the renal cortex after fibrosis, we conducted a cell linage tracing study in $TNC^{CreER2\text{-}eGFP/+}$;$R26^{tdTomato/+}$ mice. If not exposed to tamoxifen, there were only a few tdTomato positive cells in normal or fibrotic kidneys, suggesting that the reporter mice had little leakage and could be used for lineage tracing analysis (Supplement Figure 7). As detailed in Figure 8A, the bi-transgenic mice were treated with tamoxifen (1.5mg/d for 5 days) at 8 weeks old and subjected to UUO operation after 12 weeks' washout (Figure 8A). The cells that were labeled by tdTomato in the kidney were examined 7 days after UUO: the tdTomato positive cells were limited to the renal medulla, although they were significantly increased (Figure 8B&C). However, if a second series of tamoxifen was given after UUO operation, the tdTomato positive cells were found throughout the kidney (Figure 8D). These results suggested that following UUO, the original TNC expressing cells in renal medulla proliferated, but they were neither the source of TNC nor the progenitors of expanded interstitial cells in the cortex. Similar results were found in IR induced fibrosis model (Supplement Figure 8).

TNC enhanced fibrosis by promoting interstitial cells proliferation via STAT3 pathway

To explore the mechanism by which TNC enhanced fibrosis, we examined the effect of TNC on interstitial cell proliferation and the potential signaling mechanism. Firstly, we evaluated the cell proliferation using EdU incorporation study. In vivo, EdU positive cells were markedly increased and predominantly located in the renal interstitium after UUO,

and TNC deletion significantly reduced the number of EdU positive cells in the obstructed kidney compared with wild-type mouse (Figure 9A). To further examine the effect of TNC on cell proliferation, we cultured TNC-Cell (an interstitial cell line obtained from fibrotic kidney, Figure 9B), NIH3T3 and NRK49F cells. Exogenous TNC dose dependently increased the number of these cultured cells as assessed by CCK8 kit (Figure 9C-E). Exogenous TNC also markedly increased EdU incorporation in NRK49F cells, consistent with increasing cell proliferation (Figure 9F).

To determine the signaling mechanism by which TNC promoted cell proliferation, we screened 45 phospho-kinases in cultured fibroblast treated with or without TNC, and identified STAT3 as one of the candidates that responded to TNC treatment. The potential significance of STAT3 signaling in mediating the effect of TNC was further validated in UUO kidney and cultured cells. In vivo, the STAT3 and phosphor-STAT3 levels were markedly increased after UUO, and TNC deletion significantly reduced the phospho-STAT3 levels (Figure 10A&B). In cultured cells (TNC-Cell), TNC markedly increased the phosphorylation of STAT3, peaking at 45 minutes (Figure 10C). The effect of TNC on cell proliferation was blocked by the STAT3 inhibitor Stattic (Figure 10D).

Discussion

Fibrosis is a common event that leads to irreversible loss of organ function and organ failure, and so far has no effectively therapy [34]. The excessive deposition of ECM is a key feature of fibrosis. Accumulating evidence has revealed that ECM is not only the product of fibrosis, but also actively drives the persistence of fibrosis [4, 6, 35]. TNC is an

ECM glycoprotein that is under tight temporal and spatial regulation [36, 37]. A recent study suggests that TNC played an important role in kidney fibrosis [18]. The present studies showed that (1) the TNC expression levels and the number of TNC expressing cells dramatically increased during fibrosis in the kidney; (2) deletion of TNC attenuated kidney fibrosis; (3) TNC facilitated fibrosis, at least in part, by promoting the phosphorylation of STAT3; (4) the cells expressing TNC were mainly localized surrounding the injured tubules, suggesting stromal cells in response to epithelium damage; (5) the cells that produced TNC were a special population of stromal cells, 80% of which were positive for NG2 and 50% positive for αSMA.

Our study provided strong evidence that TNC plays an important role in promoting kidney fibrosis using a TNC knock-out mouse line, consistent with a previous study using siRNA-TNC injection approach [18]. In chronic kidney disease progression, tubular epithelia have been reported to sense and response to damage [38-41], followed by the interstitial stroma activation [42]. Our study showed that the TNC expressing cells (eGFP positive cells) in the fibrotic cortex were primarily localized around the injured tubules that had lost their epithelial markers. This specific localization may indicate that TNC plays an important role in linking tubule injury and interstitial cell activation and fibrosis. Interestingly, TNC was reported to be expressed only in the mesenchyme surrounding the epithelia undergoing differentiation during embryonic development [43]. TNC was induced by epithelial-mesenchymal interactions where epithelia are undergoing differentiation or restoration [44]. TGFβ, a well-established driver of fibrosis, was found to be upregulated in the injured tubular epithelium [45, 46]. Our study showed that TNC was, at least partially,

induced by TGFβ (Supplement Figure 9). Taken together, these data highlight a potential role of TNC in linking tubule damage to persistence of interstitial fibrosis.

TNC is a matricellular protein with multiple domains including EGF-like repeats, FNⅢ repeats and fibrinogen-like globe [13]. These domains can serve as ligands of cell-surface receptors and activate intracellular signals. Receptors for TNC include EGFR, integrins and toll-like receptors [47-50]. These receptors have an enzymatic activity in intracellular domain and usually regulate downstream kinase cascade. Therefore, we screened the kinases and identified STAT3 as a potential signaling pathway mediating the effect of TNC. In UUO induced kidney fibrosis models, deficiency of TNC gene significantly reduced the phosphorylation of STAT3. In cultured cells, TNC promoted fibroblast proliferation by upregulating the phosphorylation of STAT3, because STAT3 inhibitor blocked the cells proliferation induced by TNC. STAT3 is a transcription factor and has been reported as a downstream target of EGFR [51, 52]. Mounting evidence has indicated that STAT3 activation is associated with cell proliferation [53] and the development of fibrosis [54, 55].

Myofibroblasts, characterized by positive staining for αSMA, are the key contributor of kidney fibrosis [56]. It is well known that myofibroblasts are the major source of ECM [57]. Our study showed some of the αSMA positive cells expressed TNC, but not all TNC was expressed by αSMA positive myofibroblasts. This result was consistent with an early study in myocardial infarction which suggests that TNC was induced in the very early stage after injury even before the appearance of myofibroblasts [58]. NG2 is a transmembrane proteoglycan and usually used to label pericytes ($NG2^+PDGFR\beta^+$) which had been suggested to be a precursor of myofibroblasts [59]. However, NG2 expression has also

been identified in several types of immature cells (such as progenitor and tumor cells) and is downregulated when cells become mature and quiescent [30, 60, 61]. Similarly, TNC expression has also been found in developing cells, such as progenitors during development, tumor stroma and mesenchymal stem cells [33, 44, 62]. In our study, most of the TNC expressing cells were positive for both NG2 and PDGFRβ. These studies suggest TNC is expressed in early stages of activated interstitial cells that will be transdifferentiated to collagen producing myofibroblast in response to injury.

It has been reported that renal papilla contains stem cells or progenitors which repopulate after kidney injury[32]. TNC is constitutively expressed in the renal medullary interstitial cells[19]. To examine whether these TNC expressing cells in renal medulla were progenitors that migrated to renal cortex after injury, we used a genetic mouse model that specifically tracked and fate-mapped renal medullary interstitial TNC expressing cells. The results demonstrated that the cortical interstitial cells that express TNC during fibrosis were not originated from papilla.

Conclusions

In conclusion, our study provides strong evidence that non-structural matrix protein TNC that contains multiple functional domains plays an important role in kidney fibrosis. TNC is mostly expressed by $NG2^{+}PDGFR\beta^{+}$ cells around the injured tubules and STAT3 is a signaling pathway at least partially mediating the profibrotic effect of TNC. TNC pathway may serve as a potential therapeutic target to treat interstitial fibrosis and progression of kidney injury.

List of abbreviations

ECM, extracellular matrix

TNC, tenascin-C

STAT3, signal transducer and activator of transcription 3

CreER2, a Cre recombinase fused to a human estrogen receptor ligand binding domain

IRES, internal ribosome entry site

eGFP/GFP, enhanced green fluorescent protein/ green fluorescent protein

NG2, neuron glial antigen

PDGFRβ, platelet derived growth factor receptor β

αSMA, smooth muscle actin

RMICs, renal medullary interstitial cells

EGF-L, epidermal growth factor like

EGFR, epidermal growth factor receptor

TLR, toll like receptors

IHC, immunohistochemistry

IF, immunofluorescence

AQP2, aquaporin 2

FSP1, fibroblast-specific protein 1

UUO, unilateral ureteral obstruction

IR, ischemia reperfusion

LTL, lotus tetragonolobus lectin

THP, Tamm-Horsfall protein

CCK8, cell counting kit-8

TGFβ, transforming growth factor β

Declarations

Ethics approval and consent to participate

Human study was approved by the ethics committee of Huashan Hospital, Fudan University, approval number KY2016-394. Informed consent was signed for each patient. Animal study was approved by the Animal Care and Use Committees of Fudan University, approval number 20150588A274.

Competing interests

Investigators declare no other conflicts of financial interest for participation in this research.

Funding

This work was supported by Natural Science Foundation of China grants NSFC: 81520108006 and 81930120 (C. M. H.), and 82170735 (Q. X.), and 82170750, and VA Merit Awards I01 BX002196 (R. Z.) and I01 BX002025 (A. P.) as well as R01 DK069921 (R. Z.) and R01 DK119212 (A. P.), and P30-DK114809 (A. P. and R. Z.). A. P. is the recipient of a Senior Research Career Scientist award from the Veteran Affairs. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors' contributions

Conceptualization, C.M.H., Q.H., M.Z., and X.M;

Methodology, Q.H., C.M.H., X.M., and S.L;

Investigation, Q.H., M.Z., X.M., and S.L;

Writing – Original Draft, C.M.H., Q.H., R.Z. and A.P;

Funding Acquisition, C.M.H, R.Z. and A.P;

Resources, D.S., Y.X., R.C., Y.G., and X.H.

Acknowledgements

We acknowledge that all authors had access to the data and played a role in writing this article.

Reference

1. Kuncio GS, Neilson EG, Haverty T: **Mechanisms of tubulointerstitial fibrosis**. *Kidney Int* 1991, **39**(3):550-556.
2. Ben Salem C, Slim R, Fathallah N: **Fibrosis--A Common Pathway to Organ Injury and Failure**. *N Engl J Med* 2015, **373**(1):95.
3. Herrera J, Henke CA, Bitterman PB: **Extracellular matrix as a driver of progressive fibrosis**. *J Clin Invest* 2018, **128**(1):45-53.
4. Wight TN, Potter-Perigo S: **The extracellular matrix: an active or passive player in fibrosis?** *American Journal of Physiology-Gastrointestinal and Liver Physiology* 2011, **301**(6):G950-G955.
5. Frangogiannis NG: **The extracellular matrix in myocardial injury, repair, and remodeling**. *J Clin Invest* 2017, **127**(5):1600-1612.
6. Bonnans C, Chou J, Werb Z: **Remodelling the extracellular matrix in development and disease**. *Nature Reviews Molecular Cell Biology* 2014, **15**(12):786-801.
7. Clause KC, Barker TH: **Extracellular matrix signaling in morphogenesis and repair**. *Curr Opin Biotechnol* 2013, **24**(5):830-833.
8. Bornstein P, Sage EH: **Matricellular proteins: extracellular modulators of cell function**. *Curr Opin Cell Biol* 2002, **14**(5):608-616.
9. Murphy-Ullrich JE, Sage EH: **Revisiting the matricellular concept**. *Matrix Biol* 2014, **37**:1-14.
10. Theocharis AD, Skandalis SS, Gialeli C, Karamanos NK: **Extracellular matrix structure**. *Adv Drug Deliv Rev* 2016, **97**:4-27.
11. Xu Y, Li N, Gao J, Shang D, Zhang M, Mao X, Chen R, Zheng J, Shan Y, Chen M *et al*: **Elevated Serum Tenascin-C Predicts Mortality in Critically Ill Patients With Multiple Organ Dysfunction**. *Front Med (Lausanne)* 2021, **8**:759273.
12. Gao W, Li J, Ni H, Shi H, Qi Z, Zhu S, Hao C, Xie Q, Luo X, Xie K: **Tenascin C: A Potential Biomarker for Predicting the Severity of Coronary Atherosclerosis**. *J Atheroscler Thromb* 2019, **26**(1):31-38.
13. Midwood KS, Hussenet T, Langlois B, Orend G: **Advances in tenascin-C biology**. *Cell Mol Life Sci*

2011, **68**(19):3175-3199.

14. De Laporte L, Rice JJ, Tortelli F, Hubbell JA: **Tenascin C promiscuously binds growth factors via its fifth fibronectin type III-like domain**. *PLoS One* 2013, **8**(4):e62076.
15. Shimojo N, Hashizume R, Kanayama K, Hara M, Suzuki Y, Nishioka T, Hiroe M, Yoshida T, Imanaka-Yoshida K: **Tenascin-C may accelerate cardiac fibrosis by activating macrophages via the integrin alphaVbeta3/nuclear factor-kappaB/interleukin-6 axis**. *Hypertension* 2015, **66**(4):757-766.
16. El-Karef A, Yoshida T, Gabazza EC, Nishioka T, Inada H, Sakakura T, Imanaka-Yoshida K: **Deficiency of tenascin-C attenuates liver fibrosis in immune-mediated chronic hepatitis in mice**. *The Journal of Pathology* 2007, **211**(1):86-94.
17. Bhattacharyya S, Wang W, Morales-Nebreda L, Feng G, Wu M, Zhou X, Lafyatis R, Lee J, Hinchcliff M, Feghali-Bostwick C *et al*: **Tenascin-C drives persistence of organ fibrosis**. *Nat Commun* 2016, **7**:11703.
18. Fu H, Tian Y, Zhou L, Zhou D, Tan RJ, Stolz DB, Liu Y: **Tenascin-C Is a Major Component of the Fibrogenic Niche in Kidney Fibrosis**. *J Am Soc Nephrol* 2017, **28**(3):785-801.
19. He W, Xie Q, Wang Y, Chen J, Zhao M, Davis LS, Breyer MD, Gu G, Hao CM: **Generation of a tenascin-C-CreER2 knockin mouse line for conditional DNA recombination in renal medullary interstitial cells**. *PLoS One* 2013, **8**(11):e79839.
20. Fushimi K, Uchida S, Hara Y, Hirata Y, Marumo F, Sasaki S: **Cloning and expression of apical membrane water channel of rat kidney collecting tubule**. *Nature* 1993, **361**(6412):549-552.
21. Nielsen S, DiGiovanni SR, Christensen EI, Knepper MA, Harris HW: **Cellular and subcellular immunolocalization of vasopressin-regulated water channel in rat kidney**. *Proc Natl Acad Sci U S A* 1993, **90**(24):11663-11667.
22. Heldin CH, Westermark B: **Mechanism of action and in vivo role of platelet-derived growth factor**. *Physiol Rev* 1999, **79**(4):1283-1316.
23. Favaloro EJ, Moraitis N, Koutts J, Exner T, Bradstock KF: **Endothelial cells and normal circulating haemopoietic cells share a number of surface antigens**. *Thromb Haemost* 1989, **61**(2):217-224.
24. Andrews RG, Singer JW, Bernstein ID: **Precursors of colony-forming cells in humans can be distinguished from colony-forming cells by expression of the CD33 and CD34 antigens and light scatter properties**. *J Exp Med* 1989, **169**(5):1721-1731.
25. Hostettler KE, Gazdhar A, Khan P, Savic S, Tamo L, Lardinois D, Roth M, Tamm M, Geiser T: **Multipotent mesenchymal stem cells in lung fibrosis**. *PLoS One* 2017, **12**(8):e0181946.
26. Morath I, Hartmann TN, Orian-Rousseau V: **CD44: More than a mere stem cell marker**. *Int J Biochem Cell Biol* 2016, **81**(Pt A):166-173.
27. Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G: **The myofibroblast: one function, multiple origins**. *Am J Pathol* 2007, **170**(6):1807-1816.
28. Strutz F, Okada H, Lo CW, Danoff T, Carone RL, Tomaszewski JE, Neilson EG: **Identification and characterization of a fibroblast marker: FSP1**. *J Cell Biol* 1995, **130**(2):393-405.
29. Osterreicher CH, Penz-Osterreicher M, Grivennikov SI, Guma M, Koltsova EK, Datz C, Sasik R, Hardiman G, Karin M, Brenner DA: **Fibroblast-specific protein 1 identifies an inflammatory subpopulation of macrophages in the liver**. *Proc Natl Acad Sci U S A* 2011, **108**(1):308-313.
30. Stallcup WB: **The NG2 Proteoglycan in Pericyte Biology**. *Adv Exp Med Biol* 2018, **1109**:5-19.
31. Yan M, Liu S, Zhang M, Lai L, Xie Q, Hao CM: **Mesangial cell-derived tenascin-C contributes to**

mesangial cell proliferation and matrix protein production in IgA nephropathy. *Nephrology (Carlton)* 2022, **27**(5):458-466.

32. Oliver JA, Maarouf O, Cheema FH, Martens TP, Al-Awqati Q: **The renal papilla is a niche for adult kidney stem cells**. *J Clin Invest* 2004, **114**(6):795-804.
33. Chiquet-Ehrismann R, Orend G, Chiquet M, Tucker RP, Midwood KS: **Tenascins in stem cell niches**. *Matrix Biol* 2014, **37**:112-123.
34. Rockey DC, Bell PD, Hill JA: **Fibrosis--a common pathway to organ injury and failure**. *N Engl J Med* 2015, **372**(12):1138-1149.
35. Herrera J, Henke CA, Bitterman PB: **Extracellular matrix as a driver of progressive fibrosis**. *Journal of Clinical Investigation* 2018, **128**(1):45-53.
36. Jones FS, Jones PL: **The tenascin family of ECM glycoproteins: structure, function, and regulation during embryonic development and tissue remodeling**. *Dev Dyn* 2000, **218**(2):235-259.
37. Chiquet-Ehrismann R, Chiquet M: **Tenascins: regulation and putative functions during pathological stress**. *J Pathol* 2003, **200**(4):488-499.
38. Kang HM, Ahn SH, Choi P, Ko YA, Han SH, Chinga F, Park AS, Tao J, Sharma K, Pullman J *et al*: **Defective fatty acid oxidation in renal tubular epithelial cells has a key role in kidney fibrosis development**. *Nat Med* 2015, **21**(1):37-46.
39. Wu H, Lai CF, Chang-Panesso M, Humphreys BD: **Proximal Tubule Translational Profiling during Kidney Fibrosis Reveals Proinflammatory and Long Noncoding RNA Expression Patterns with Sexual Dimorphism**. *J Am Soc Nephrol* 2020, **31**(1):23-38.
40. Gewin LS: **Renal fibrosis: Primacy of the proximal tubule**. *Matrix Biol* 2018, **68-69**:248-262.
41. Qi R, Yang C: **Renal tubular epithelial cells: the neglected mediator of tubulointerstitial fibrosis after injury**. *Cell Death Dis* 2018, **9**(11):1126.
42. Humphreys BD: **Mechanisms of Renal Fibrosis**. *Annu Rev Physiol* 2018, **80**:309-326.
43. Aufderheide E, Chiquet-Ehrismann R, Ekblom P: **Epithelial-mesenchymal interactions in the developing kidney lead to expression of tenascin in the mesenchyme**. *J Cell Biol* 1987, **105**(1):599-608.
44. Midwood KS, Orend G: **The role of tenascin-C in tissue injury and tumorigenesis**. *Journal of Cell Communication and Signaling* 2009, **3**(3-4):287-310.
45. Meng XM, Nikolic-Paterson DJ, Lan HY: **TGF-beta: the master regulator of fibrosis**. *Nat Rev Nephrol* 2016, **12**(6):325-338.
46. Yamamoto T, Noble NA, Cohen AH, Nast CC, Hishida A, Gold LI, Border WA: **Expression of transforming growth factor-beta isoforms in human glomerular diseases**. *Kidney Int* 1996, **49**(2):461-469.
47. Iyer AK, Tran KT, Borysenko CW, Cascio M, Camacho CJ, Blair HC, Bahar I, Wells A: **Tenascin cytotactin epidermal growth factor-like repeat binds epidermal growth factor receptor with low affinity**. *J Cell Physiol* 2007, **211**(3):748-758.
48. Swindle CS, Tran KT, Johnson TD, Banerjee P, Mayes AM, Griffith L, Wells A: **Epidermal growth factor (EGF)-like repeats of human tenascin-C as ligands for EGF receptor**. *J Cell Biol* 2001, **154**(2):459-468.
49. Tucker RP, Chiquet-Ehrismann R: **Tenascin-C: Its functions as an integrin ligand**. *Int J Biochem Cell Biol* 2015, **65**:165-168.
50. Midwood K, Sacre S, Piccinini AM, Inglis J, Trebaul A, Chan E, Drexler S, Sofat N, Kashiwagi M,

Orend G *et al*: **Tenascin-C is an endogenous activator of Toll-like receptor 4 that is essential for maintaining inflammation in arthritic joint disease**. *Nat Med* 2009, **15**(7):774-780.

51. Abdelhamed S, Ogura K, Yokoyama S, Saiki I, Hayakawa Y: **AKT-STAT3 Pathway as a Downstream Target of EGFR Signaling to Regulate PD-L1 Expression on NSCLC cells**. *J Cancer* 2016, **7**(12):1579-1586.
52. Wu J, Patmore DM, Jousma E, Eaves DW, Breving K, Patel AV, Schwartz EB, Fuchs JR, Cripe TP, Stemmer-Rachamimov AO *et al*: **EGFR-STAT3 signaling promotes formation of malignant peripheral nerve sheath tumors**. *Oncogene* 2014, **33**(2):173-180.
53. Daniel JM, Dutzmann J, Bielenberg W, Widmer-Teske R, Gunduz D, Hamm CW, Sedding DG: **Inhibition of STAT3 signaling prevents vascular smooth muscle cell proliferation and neointima formation**. *Basic Res Cardiol* 2012, **107**(3):261.
54. Seo HY, Jeon JH, Jung YA, Jung GS, Lee EJ, Choi YK, Park KG, Choe MS, Jang BK, Kim MK *et al*: **Fyn deficiency attenuates renal fibrosis by inhibition of phospho-STAT3**. *Kidney Int* 2016, **90**(6):1285-1297.
55. Chakraborty D, Sumova B, Mallano T, Chen CW, Distler A, Bergmann C, Ludolph I, Horch RE, Gelse K, Ramming A *et al*: **Activation of STAT3 integrates common profibrotic pathways to promote fibroblast activation and tissue fibrosis**. *Nat Commun* 2017, **8**(1):1130.
56. Meran S, Steadman R: **Fibroblasts and myofibroblasts in renal fibrosis**. *Int J Exp Pathol* 2011, **92**(3):158-167.
57. Klingberg F, Hinz B, White ES: **The myofibroblast matrix: implications for tissue repair and fibrosis**. *J Pathol* 2013, **229**(2):298-309.
58. Imanaka-Yoshida K, Hiroe M, Nishikawa T, Ishiyama S, Shimojo T, Ohta Y, Sakakura T, Yoshida T: **Tenascin-C modulates adhesion of cardiomyocytes to extracellular matrix during tissue remodeling after myocardial infarction**. *Lab Invest* 2001, **81**(7):1015-1024.
59. Humphreys BD, Lin SL, Kobayashi A, Hudson TE, Nowlin BT, Bonventre JV, Valerius MT, McMahon AP, Duffield JS: **Fate tracing reveals the pericyte and not epithelial origin of myofibroblasts in kidney fibrosis**. *Am J Pathol* 2010, **176**(1):85-97.
60. Stallcup WB: **The NG2 proteoglycan: past insights and future prospects**. *J Neurocytol* 2002, **31**(6-7):423-435.
61. Stallcup WB, Huang FJ: **A role for the NG2 proteoglycan in glioma progression**. *Cell Adh Migr* 2008, **2**(3):192-201.
62. Chiquet-Ehrismann R, Mackie EJ, Pearson CA, Sakakura T: **Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis**. *Cell* 1986, **47**(1):131-139.

Figures

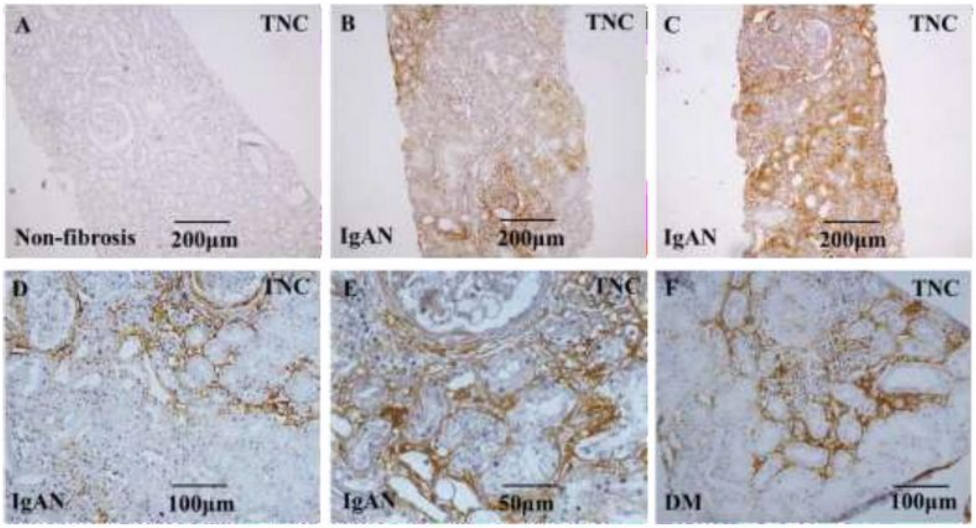


Figure 1. TNC expression in normal and fibrotic kidneys. IHC staining showed that TNC level was low in the renal cortex of non-fibrotic kidney from patients with minimal change disease (A, n=6), and significantly increased in fibrotic kidneys from patients with IgA nephropathy (B-E, n=6) and diabetic nephropathy (F, n=6).

Figure 1

See image above for figure legend.

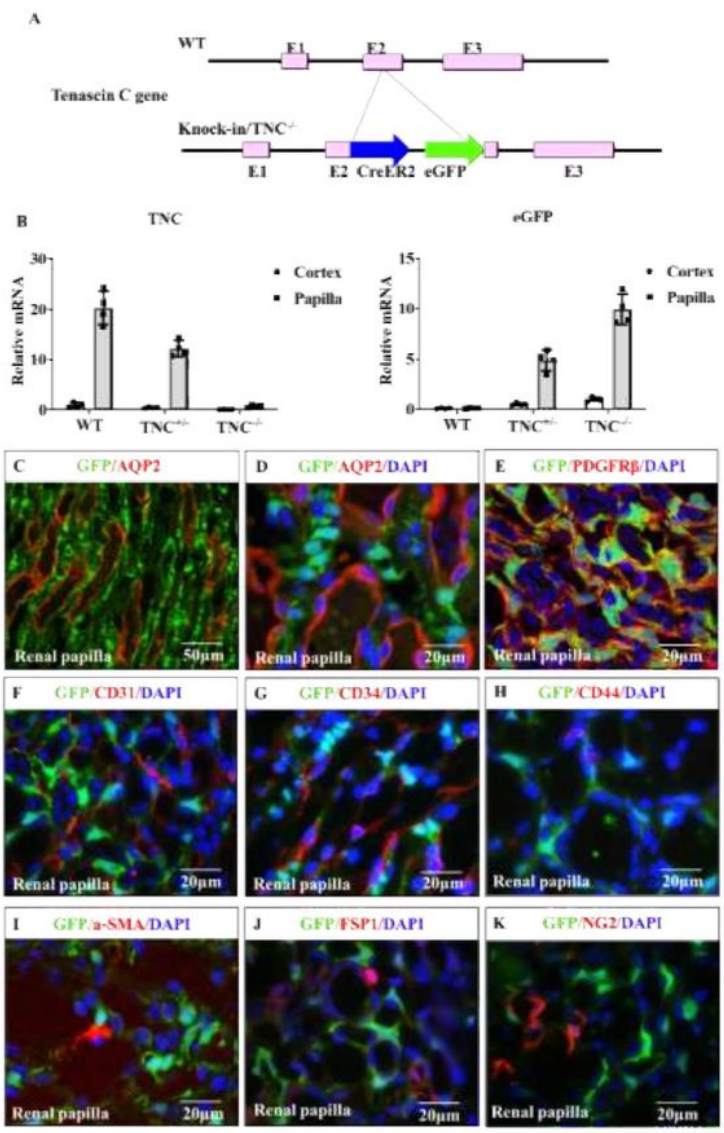


Figure 2. TNC was constitutively expressed by renal medullary stromal cells in normal kidney. A TNC promoter driven inducible CreER2 knock-in mouse line with an eGFP reporter was generated, and the insertion of CreER2-eGFP lead to TNC deletion (A). Messenger RNA of TNC and TNC reporter eGFP was significantly higher in the renal papilla than in the renal cortex (B, n=4 for each, $p<0.001$). Co-staining for eGFP and AQP2 showed that eGFP positive cells were located in the renal medullary interstitium (C&D). These eGFP positive cells were PDGFRβ positive, consistent with stromal cells (E), but negative for CD31 (a marker of endothelial cells, F), CD34 (a marker of endothelial cells and early hematopoietic stem cells and progenitors, G) and CD44 (a marker of progenitors, H). They were also negative for αSMA (a marker of myofibroblasts, I), FSP1 (a marker of fibroblasts, J) and NG2 (a marker of pericytes, K). (n=4, 3 slides for each)

Figure 2

See image above for figure legend.

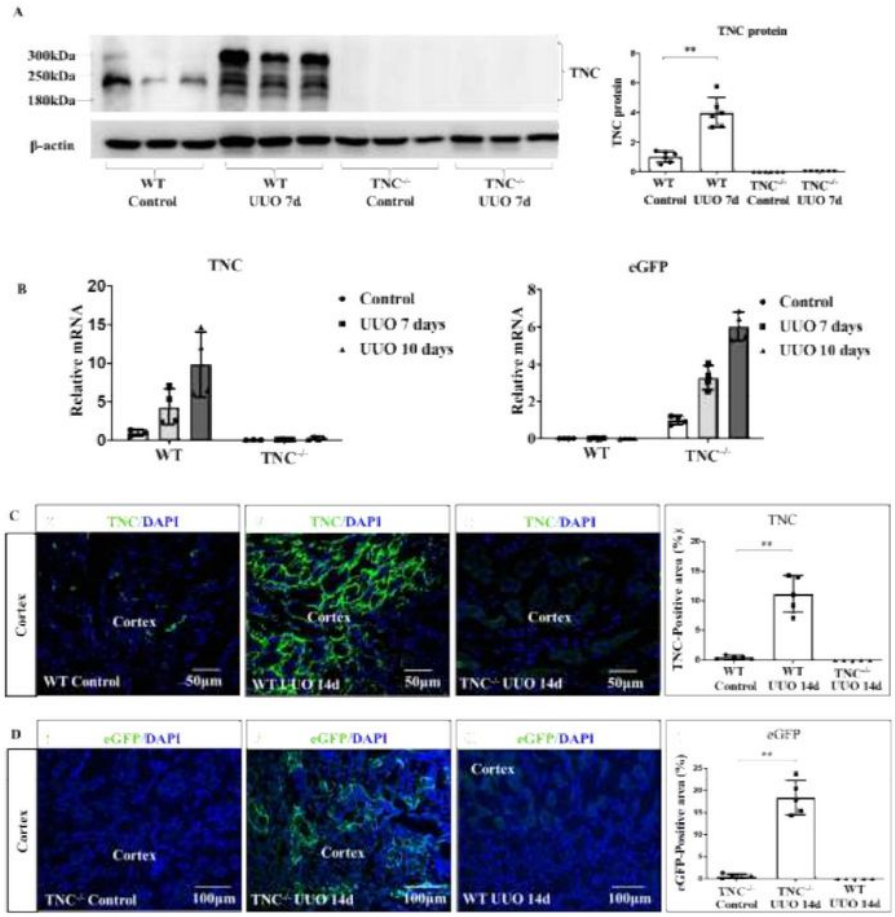


Figure 3. TNC was significantly increased in the fibrotic kidneys. Western blot showed that TNC protein was dramatically increased with different splicing variants in UUO kidneys (A; n=6 for each, $p<0.01$). qPCR showed that TNC mRNA in wild-type mice and eGFP mRNA in the reporter mice were also significantly elevated (B ; n=4 for each, $p<0.01$). IF showed that TNC was markedly increased in renal cortex, and eGFP staining in the reporter mice confirmed this result (C&D; n=5 for each, $p<0.01$).

Figure 3

See image above for figure legend.

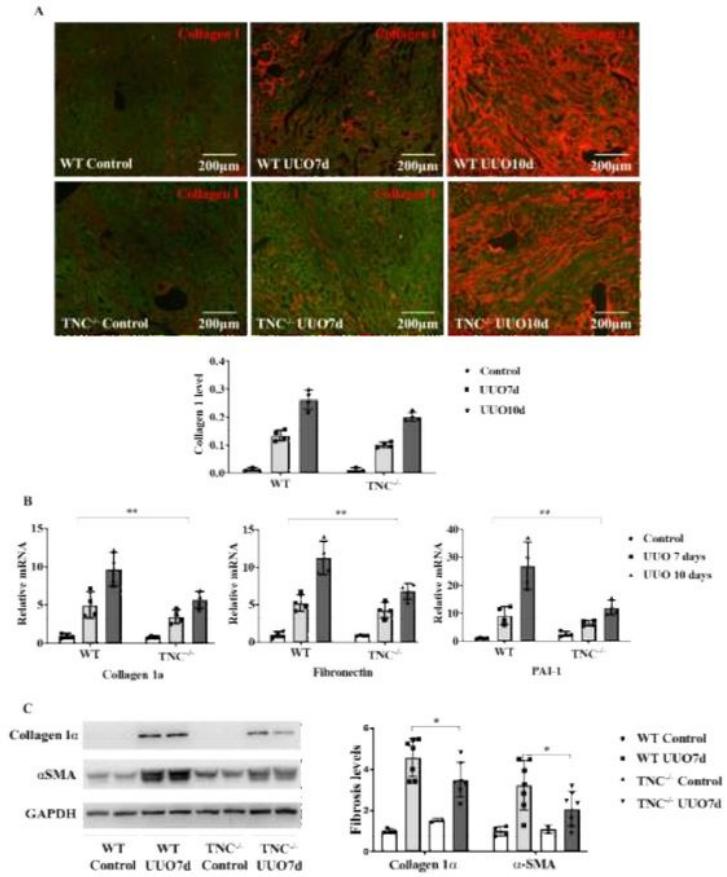
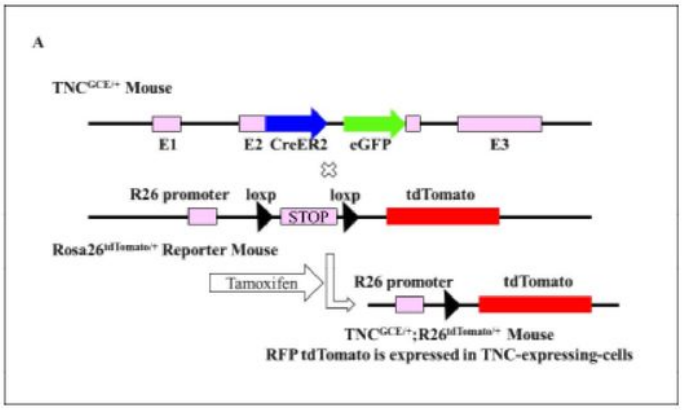


Figure 4. TNC deficiency reduced kidney fibrosis in animal models. Deletion of TNC ($TNC^{-/-}$) significantly attenuated the induction of collagen Ⅰ assessed by IF following UUO by approximate 30% at day 7 and 10 compared with their wild type littermates (A, n=4 for each time point, two-way ANOVA $p<0.05$). Consistently, the expression of fibrosis markers, such as collagen Ⅰα, fibronectin and plasminogen activator inhibitor-1 (PAI-1), were significantly lower in $TNC^{-/-}$ mice at UUO day 7 and 10 (B, n=4 for each time point, two-way ANOVA, $p<0.05$). Western blot showed that the proteins of collagen Ⅰα and α-SMA were also significantly reduced in $TNC^{-/-}$ mice at UUO day 7 (Figure 4C, n=7, $p<0.05$).

Figure 4

See image above for figure legend.



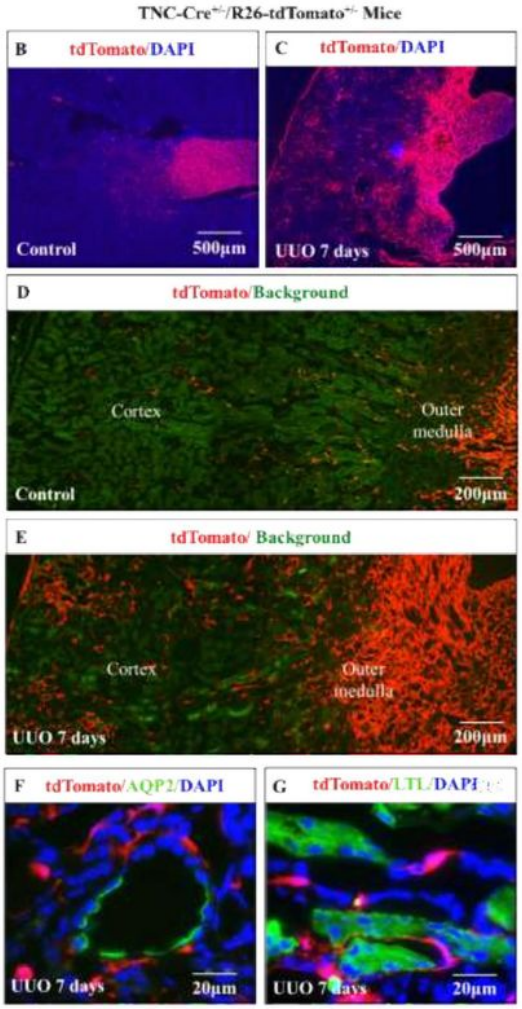


Figure 5. TNC expressing cells were significantly increased in fibrotic kidneys. In bi-transgenic TNC$^{CreER2\text{-}eGFP/+}$;R26$^{tdTomato/+}$ mice, the recombination happens in the cells that express TNC in the presence of tamoxifen, and then these TNC expressing cells and their daughter cells will be labeled with red fluorescence protein tdTomato permanently (A). In normal kidneys, tdTomato also identified TNC expressing cells rich in the papilla, scattered in the outer medulla, and rare in the cortex (B&D). After UUO (tamoxifen was given for 5 days after operation), the tdTomato positive cells were significantly increased in both the renal cortex and medulla (C&E). These tdTomato positive cells were neither AQP2 nor Lotus Tetragonolobus Lectin (LTL) positive tubular epithelial cells in fibrotic kidneys (F&G). (n=4, 3 slides for each)

Figure 5

See image above for figure legend.

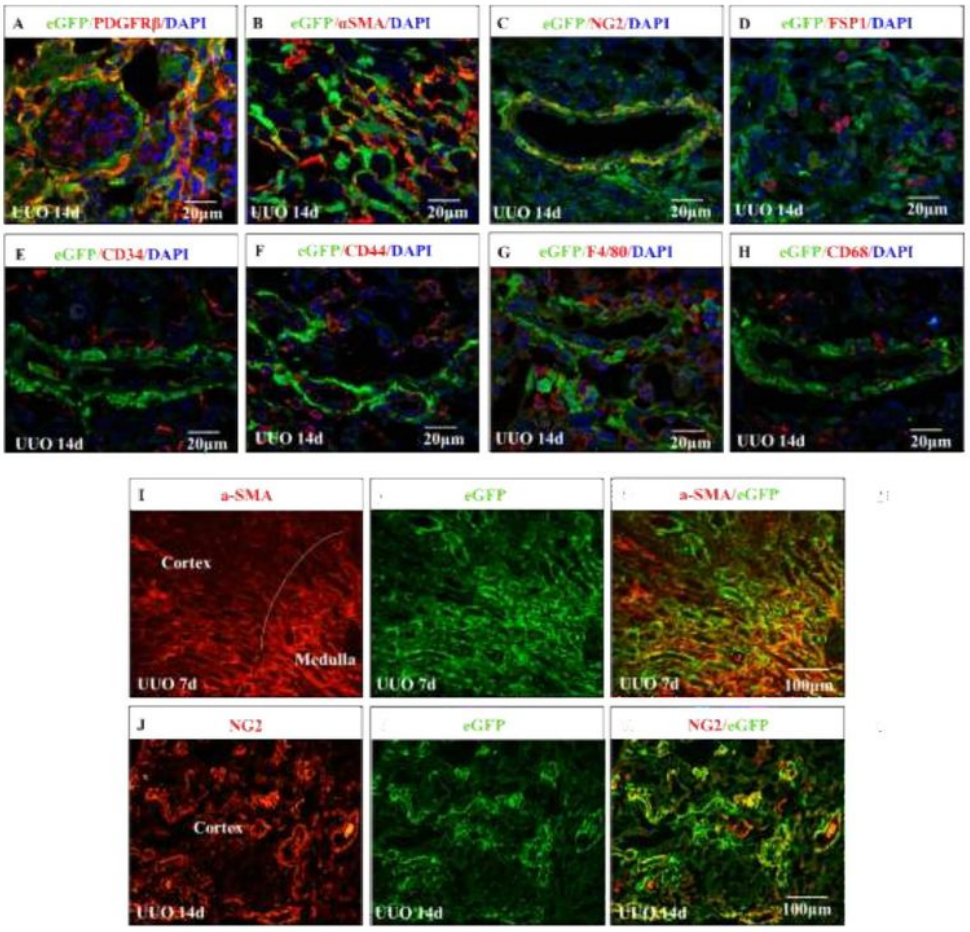


Figure 6. TNC expressing cells in fibrotic kidneys were mostly PDGFRβ^{+}NG2^{+} stromal cells. In TNC$^{CreER2\text{-}eGFP/+}$ reporter mice, eGFP positive cells were all PDGFRβ positive, consistent with stromal cells (A). Some of the eGFP positive cells were αSMA positive, and not all αSMA positive myofibroblasts were eGFP positive (B&I). Most of the eGFP positive cells were NG2 (a marker of pericyte) positive, while these eGFP^{+}NG2^{+} cells accounted for about half of the total NG2 positive cells (C&J). These eGFP positive cells were not positive for FSP1 (D). They were neither CD34 nor CD44 positive progenitors (E&F), and were neither CD68 nor F4/80 positive macrophages (G&H). (n=4, 3 sections for each)

Figure 6

See image above for figure legend.

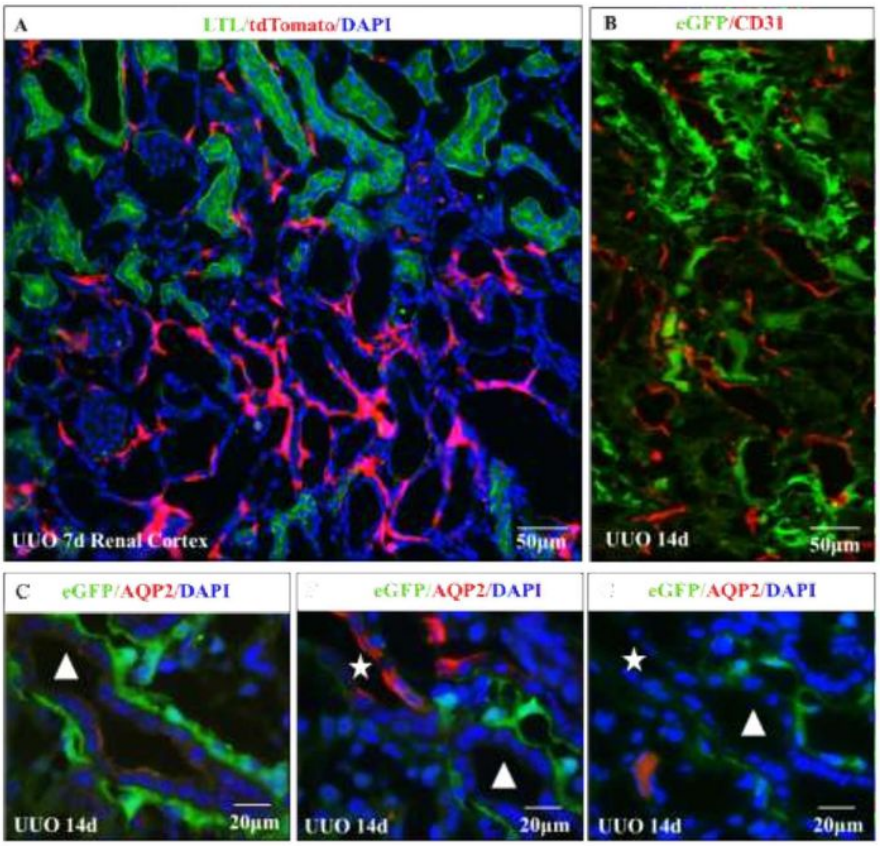


Figure 7. TNC expressing cells surrounded the injured renal tubules in fibrotic kidneys. TNC reporter eGFP or tdTomato positive cells were predominantly located adjacent to the tubular structures in UUO kidneys (A&B). These tubules were negative for CD31, a maker of endothelial cells, suggesting not vessels (B). They were dilated and negative for epithelial markers including LTL and AQP2, presumably injured tubules which lost their markers (A-C). Serial section experiments showed that these tubules (△) had continuation to structures with positive AQP2 staining(☆), further supporting that TNC expressing cells were localized surrounding the injured renal tubules in UUO kidneys (C). (n=4, 3 sections for each)

Figure 7

See image above for figure legend.

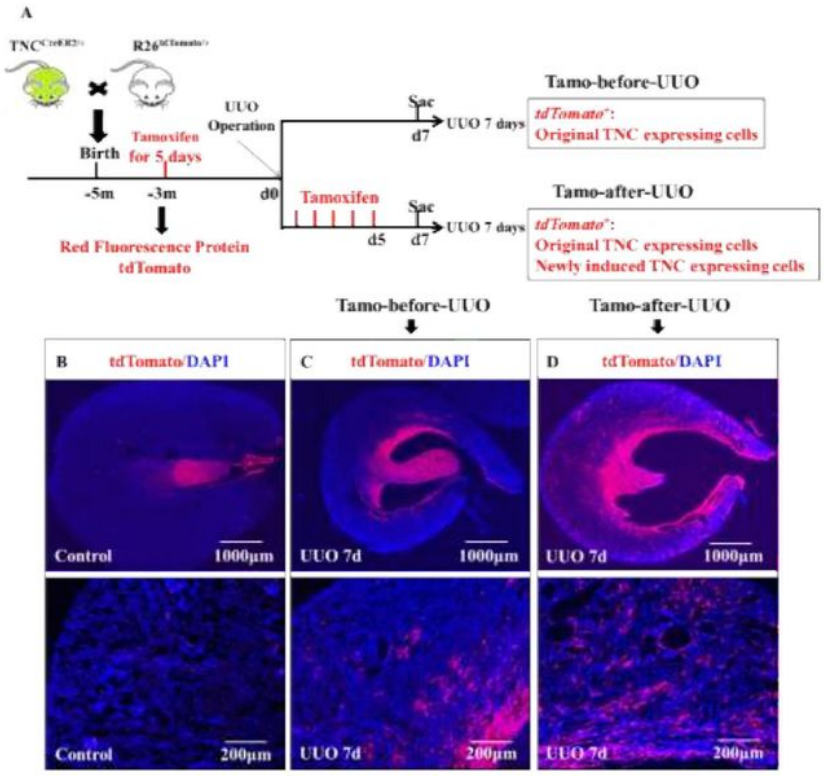


Figure 8. Cell lineage study in UUO induced renal fibrosis model. To determine whether TNC expressing cells in renal papilla or medulla were progenitors of the expanded interstitial cells in renal cortex after fibrosis, a cell linage tracing study was conducted. As detailed in Figure A, the bi-transgenic mice $TNC^{CreER2\text{-}eGFP/+}$; $R26^{tdTomato/+}$ were treated with tamoxifen (1.5mg/d for 5 days) at 8 weeks old and subjected to UUO operation after 12 weeks' washout. Seven days later, tdTomato positive cells were limited to the renal medulla, although they were significantly increased (B&C). However, if a second series of tamoxifen was given after UUO operation, the tdTomato positive cells were found throughout the kidney (D). (n=4 mice for each group, 3 slides for each mouse, $p<0.05$) These results suggested that following UUO, the original TNC expressing cells in renal medulla proliferated, but not progenitors of the expanded TNC expressing cells in renal cortex.

Figure 8

See image above for figure legend.

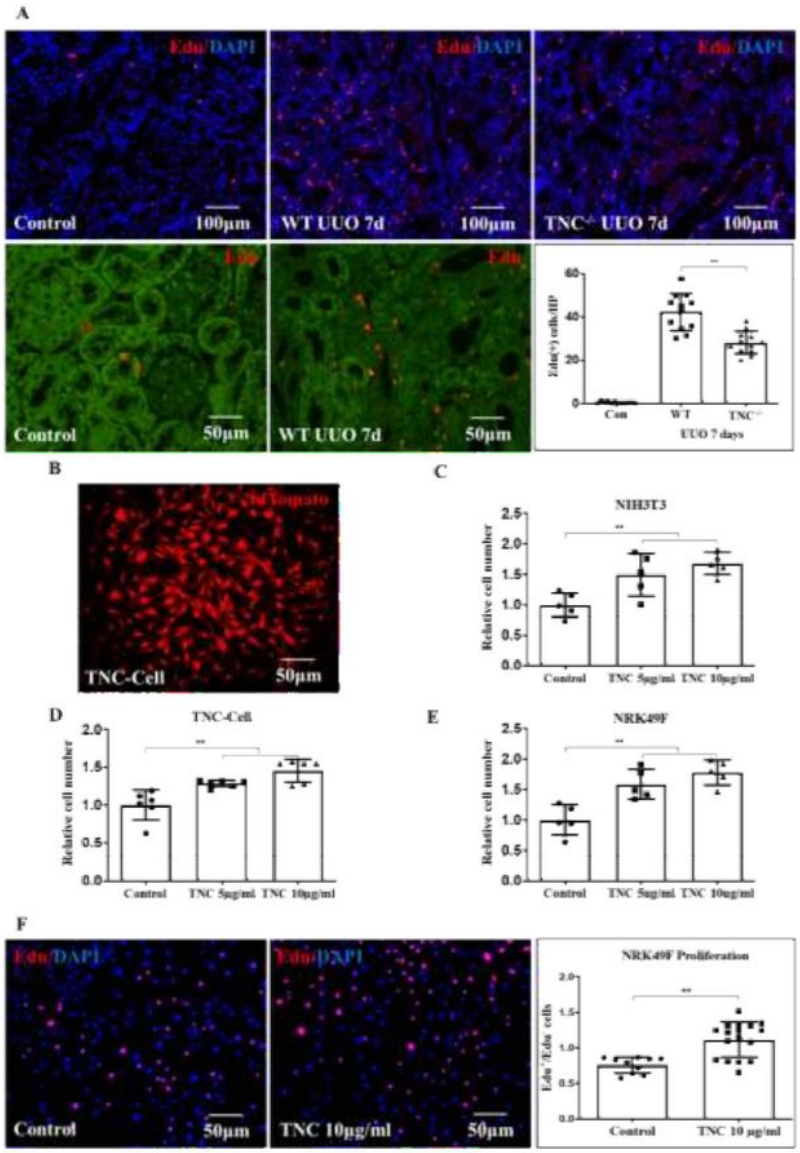


Figure 9. TNC promoted interstitial cells proliferation. In vivo, EdU positive cells were markedly increased and predominantly located in the renal interstitium after UUO, and TNC deletion significantly reduced the number of EdU positive cells in the obstructed kidney compared with wild-type mouse (A, n=4 mice, 3 slides for each mouse, $p<0.05$). To further examine the effect of TNC on cell proliferation, TNC expressing cells (TNC-Cell) were obtained by sorting the tdTomato positive cells in UUO kidneys, and then immortalized by transfecting SV40 T lentivirus (B). Exogenous TNC dose dependently increased the cell number of TNC-Cell, NIH3T3 and NRK49F in vitro, assessed by CCK8 kit (C-E, $p<0.01$). Exogenous TNC also markedly increased EdU incorporation in NRK49F cells, consistent with promoting cell proliferation (F, $p<0.01$).

Figure 9

See image above for figure legend.

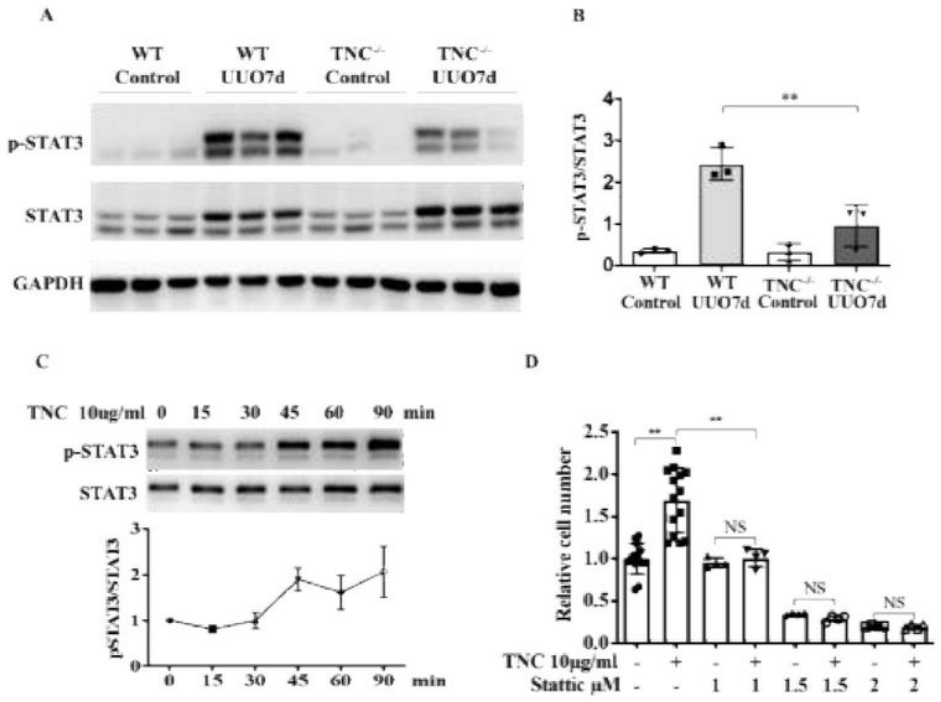


Figure 10. TNC promoted stromal cells proliferation via STAT3 pathway. In vivo, STAT3 and phosphor-STAT3 were markedly increased after UUO, and TNC deletion significantly reduced the phospho-STAT3 levels (A&B). In cultured cells (TNC-Cell), exogenous TNC markedly increased the phosphorylation of STAT3, peaking at 45 minutes (C). The effect of TNC on cell proliferation was blocked by the STAT3 inhibitor Stattic (D).

Figure 10

See image above for figure legend.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementalfigures.pdf