Syndecan-4 Knockout Leads to Reduced Extracellular Transglutaminase-2 and Protects against Tubulointerstitial Fibrosis

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ABSTRACT

Transglutaminase type 2 (TG2) is an extracellular matrix crosslinking enzyme with a pivotal role in kidney fibrosis. The interaction of TG2 with the heparan sulfate proteoglycan syndecan-4 (Sdc4) regulates the cell surface trafficking, localization, and activity of TG2 in vitro but remains unstudied in vivo. We tested the hypothesis that Sdc4 is required for cell surface targeting of TG2 and the development of kidney fibrosis in CKD. Wild-type and Sdc4-null mice were subjected to unilateral ureteric obstruction and aristolochic acid nephropathy (AAN) as experimental models of kidney fibrosis. Analysis of renal scarring by Masson trichrome staining, kidney hydroxyproline levels, and collagen immunofluorescence demonstrated progressive fibrosis associated with increases in extracellular TG2 and TG activity in the tubulointerstitium in both models. Knockout of Sdc-4 reduced these effects and prevented AAN-induced increases in total and active TGF-β1. In wild-type mice subjected to AAN, extracellular TG2 colocalized with Sdc4 in the tubular interstitium and basement membrane, where TG2 also colocalized with heparan sulfate chains. Heparitinase I, which selectively cleaves heparan sulfate, completely abolished extracellular TG2 in normal and diseased kidney sections. In conclusion, the lack of Sdc4 heparan sulfate chains in the kidneys of Sdc4-null mice abrogates injury-induced externalization of TG2, thereby preventing profibrotic crosslinking of extracellular matrix and recruitment of large latent TGF-β1. This finding suggests that targeting the TG2-Sdc4 interaction may provide a specific interventional strategy for the treatment of CKD.


CKD is characterized by glomerulosclerosis and tubulointerstitial fibrosis that result from excessive extracellular matrix (ECM) accumulation.1–3 In recent years, the role of transglutaminase type 2 (TG2) has been shown to be crucial to both the ECM expansion1,4,5 and TGF-β1 activation6–9 that underlies this fibrotic remodeling.

TG2 belongs to the eight-member transglutaminase family that catalyzes a calcium-dependent acyl-transfer reaction (EC 2.3.2.13) between the γ-carboxamide group of peptide-bound glutamine and the ε-amino group of peptide-bound lysine,10 generating stable ε-(γ-glutamyl)-lysine isopeptide crosslinks. In fibrotic diseases (e.g., renal, liver, and pulmonary fibrosis), increased TG2 externalization and/or expression results in abundant crosslink formation, contributing to ECM accumulation.5,7,11–16 In early CKD, ε-(γ-glutamyl)-lysine crosslinking in the ECM

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Figure 1. Sdc4-KO protects against the development of renal fibrosis in the UUO model of CKD. Paraffin sections from WT and Sdc4 KO kidneys (control and 21 days after UUO) were stained with MT (A–D), collagen I (E–H), collagen III (I–J), and collagen IV (M–P). Collagen staining (red) and nuclei staining (blue). Representative images at ×200 magnification are shown. Detail of collagen IV staining at ×400 magnification is shown (M).
results predominantly from cell externalization of existing TG2 as the renal TG2 level remains constant. The externalized TG2 is known to exert a profibrotic function also through a nonenzymatic “structural” activity, by enhancing arginine-glycine-aspartic acid-independent cell adhesion and, consequently, contraction of the ECM. In experimental CKD, pan TG inhibition preserved

**Figure 2.** Sdc4-KO protects against the development of renal fibrosis in the AAN model of CKD. Paraffin sections from WT and Sdc4 KO kidneys (control and AAN at 12 weeks) were stained with MT (A–D), collagen I (E–H), collagen III (I–L), and collagen IV (M–P). Collagens staining (green) and nuclei staining (blue). Representative images at ×200 magnification are shown.
kidney function because of a reduction in kidney fibrosis in both diabetic and nondiabetic disease.\textsuperscript{2,16} Mice deficient in TG2 were protected against the development of fibrosis in obstructive nephropathy resulting from impaired collagen I synthesis related to decreased TGF-\textbeta 1 activation.\textsuperscript{8}

However, clinical application of anti-TG2 therapy has been hampered by the complexity to develop TG2-specific inhibitors due to a highly conserved catalytic core across the TG family,\textsuperscript{10} with inhibition of factor XIIIa and the keratinocyte transglutaminase causing particular concern.\textsuperscript{20} Consequently, elucidation of the mechanism whereby TG2 is released from cells has been an object of intense scrutiny, because TG2 is unconventionally secreted via a potentially unique non-Golgi route,\textsuperscript{21,22} which may offer a specific interventional strategy to decrease extracellular TG2.

Recently, we have shown that heparan sulfate proteoglycans (HSPG), such as syndecan-4 (Sdc4), may have a key role in the cell surface trafficking of TG2 \textit{in vitro}.\textsuperscript{23} Sdc4 and TG2 coassociated in cell membranes \textit{via} the HS chains of Sdc4, for which TG2 has high affinity.\textsuperscript{18,23,24} Lack of Sdc4/HS or functional inhibition of HS led to a lower level of cell-surface TG2 antigen and crosslinking activity \textit{in vitro}, causing a parallel accumulation of cytosolic TG2 with no changes in the total level of TG2 expression.\textsuperscript{23} Membrane-proximal Sdc4/HS may, therefore, affect the unconventional secretion of TG2, as described for fibroblast growth factor-2, by acting as a cell-surface “molecular trap.”\textsuperscript{25} Thus, Sdc4/HS may modulate TG2 profibrotic function by controlling its cell-surface trafficking. Sdc4/HS has also been implicated in kidney fibrosis, being upregulated in progressive proliferative kidney diseases (IgA nephropathy) and diabetic nephropathy, but not in nonproliferative diseases.\textsuperscript{26–28}

To investigate the possible role of Sdc4 in regulating cell-surface trafficking of TG2 \textit{in vivo}, we induced kidney fibrosis in Sdc4-knockout (KO) mice\textsuperscript{29} and assessed whether or not Sdc4 deletion affected TG2 externalization/extracellular activity and tubulointerstitial fibrosis development. We used two distinct experimental models of kidney fibrosis: unilateral ureteric obstruction (UUO)\textsuperscript{30} and aristolochic acid nephropathy (AAN).\textsuperscript{31,32} Sdc4-KO ameliorated tubulointerstitial fibrosis in both models, and deletion of Sdc4 led to a lowering of extracellular TG2 in the ECM. Binding of TG2 to the tubular interstitium depended on the HS chains of proteoglycans, with which TG2 was found to be strongly associated in normal and diseased kidney. These data suggest for the first time that Sdc4 plays a critical role in the pathogenesis of

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\caption{Sdc4-KO prevents ECM protein increases in the UUO and AAN model of CKD. Graphical presentation of kidney fibrosis in the UUO (A–D) and AAN (E–H) fibrotic lesions. The fibrosis score was calculated by multiphase analysis of MT-stained kidney sections as the ratio blue/green (collagen)/pink/red (tissue area) (A and E). Deposition of collagen I (B and F), collagen III (C and G), and collagen IV (D and H) was measured by multiphase analysis of immunofluorescence-stained kidney sections (collagens/DAPI). All data were normalized by the WT control at the lower time point (day 7 for UUO, week 9 for AAN). Raw data at these time points were the following: MT UUO, 0.02; MT AAN, 0.001; collagen I UUO, 0.01; collagen I AAN, 0.03; collagen III UUO, 0.04; collagen III AAN, 0.06; collagen IV UUO, 0.03; collagen IV AAN, 0.08. *P<0.05 versus control; †P<0.05 versus Sdc4-KO UUO or AAN; ‡P<0.05 versus WT day 7 UUO or week 9 AAN; \( ^{\circ} \)P<0.05 versus Sdc4-KO day 7 UUO or week 9 AAN; \( ^{\circ} \)P<0.05 versus WT day 14 UUO; \( ^{\circ} \)P<0.05 versus Sdc-KO day 14 UUO.}
\end{figure}
kidney fibrosis by regulating TG2 trafficking and localization via HS chain-binding.

RESULTS

Kidney Fibrosis Is Reduced in Sdc4-Null Mice

The phenotype of Sdc4-KO mice was investigated in two models of CKD: UUO and AAN. Both models led to the progressive development of interstitial fibrosis in wild-type (WT) kidneys as assessed by Masson trichrome (MT) staining (Figures 1, A and B, and 2, A and B). Fibrotic areas caused by Aristolochic acid I (AAI) were mainly located in the outer cortex with areas of scarred tissue tracking down the medullary ray, whereas in the UUO, there was a more diffuse fibrosis through the cortex. Sdc4 deletion resulted into a reduced collagen staining in both models compared with WT (Figures 1D and 2D). There were no differences between WT and Sdc4-KO kidneys at baseline (Figures 1, A and C, and 2, A and C).

Multiphase image analysis of collagen-positive staining on MT sections revealed that in the UUO model the increase in collagen was significant compared with the change in controls at all the time points (P<0.05) (Figure 3A), while differences between WT and Sdc4-KO fibrotic kidneys were significant at days 7 and 21 after UUO (Figure 3A). In the AAN model, MT staining was significantly higher than the control at 12 weeks (Figure 3F), with a reduction of MT staining in the Sdc4-KO compared with WT fibrotic kidneys (Figure 3F). In both models, fibrosis development was slower in Sdc4-KO mice than in the WT (Figure 3, A and F). Whole-kidney hydroxyproline analysis confirmed the progressive increase of collagen in both models of fibrosis, with lower collagen accumulation in Sdc4-KO at day 21 after UUO and at 12 weeks in the AAN model (Figure 3, B and G).

Individual changes in the levels of collagen I, III, and IV were analyzed by immunofluorescence on paraffin sections from WT and Sdc4-KO kidneys. There were no significant differences in any collagen staining in control kidneys between WT and Sdc4-KO mice (Figures 1 and 2, E, G, I, K, M, O). All collagens were increased in diseased kidneys, particularly so in WT kidneys (Figures 1 and 2, F, J, N) compared with Sdc4-KO kidneys (Figures 1 and 2, H, L, P). These differences were confirmed by multiphase image analysis of the staining for collagen I (Figure 3, C and H), collagen III (Figure 3, D and I), and collagen IV (Figure 3, E and J) from both CKD models. In diseased animal, kidneys from Sdc4-KO mice showed reduced levels of collagen I deposition compared with WT at days 7 and 21 in the UUO model (Figure 3C) and at 12 weeks in the AAN model (Figure 3H). Lower levels of collagen III accumulation were also detected at day 21 in Sdc4-KO obstructed kidneys (UUO) compared with WT (Figure 3D), but not in response to AAI (Figure 3I). Changes in collagen IV, typically abundant in the tubular and glomerular basement membranes, were greater in the WT than in the Sdc4-KO obstructed kidneys at 21 days after UUO (Figure 3E) and at 12 weeks in the AAN model (Figure 3J). Evaluation of collagen gene expression by quantitative RT-PCR analysis revealed significant elevation of interstitial collagen α1(I) and collagen III transcripts and the basement membrane collagen IV transcript in the UUO mice and AAI-treated mice compared with the controls. There was a lower expression level in the Sdc4-KO mice, which was significant for collagen α1.
II, III, and IV in the AAN model but reached significance only for collagen $\alpha_1$ (I) in the UUO model (Supplemental Figure 1). Therefore, the knockout of Sdc4 caused a reduction in the development of kidney fibrosis in both models, and this was principally due to less deposited collagen.

The kidney weight to body weight ratio was lower in AAN but was preserved in the Sdc4-KO mice (Supplemental Figure 2A). Serum creatinine and creatinine clearance were respectively increased and decreased ($P<0.05$) in response to AAI but with no difference between the two genotypes. However, in this model, loss in function was higher at 9 weeks when only an early level of fibrosis was seen (Figure 3F), than after 12 weeks when fibrosis was more advanced (Supplemental Figure 2, B and C).

Sdc4-KO Lowers Extracellular TG2 in Two Mouse Models of CKD

The link between TG2 externalization and tubulointerstitial fibrosis is well known, being associated with post-translational modification of the ECM and the recruitment of large latent TGF-$\beta_1$ that facilitates its activation. Therefore, we examined the consequences of Sdc4-KO on extracellular TG2 in normal and diseased kidneys. Extracellular TG2 antigen was specifically detected on unfixed cryosections after washout of the intracellular enzyme. Extracellular TG2 was mainly localized in the tubulointerstitial space and within the glomerular tuft (most likely within the mesangial matrix and glomerular basement membrane) in normal kidneys from WT and Sdc4-KO mice, with no quantitative difference in the level of extracellular TG2 between WT and Sdc4-KO control mice (Figures 4 and 5).

In the UUO model, the levels of TG2 increased with the progression of kidney fibrosis in the tubulointerstitium, with the WT kidneys having significantly more extracellular TG2 than the Sdc4-KO kidneys at 14 and 21 days after UUO (Figure 4, A and C). Again, the level of externalized TG2 was significantly higher in the WT animals than in the Sdc4-null mice in the AAI-treated mice at 12 weeks (Figure 5A). Extracellular TG activity (Figures 4B and 5B) mirrored the distribution of TG2 antigen, with no quantitative difference in level between WT and Sdc4-KO control kidneys. UUO and AAN kidneys had a significantly higher level of TG activity at all the time points compared with the relative controls (Figures 4D and 5D). In the UUO, although mean extracellular TG activity was always higher in WT than in Sdc4-KO kidneys, this difference reached significance from day 14 (Figure 4D). In the AAN, extracellular TG activity was higher in WT than in Sdc4-KO kidneys at 12 weeks (Figure 5D).

Western blot analysis of total homogenates revealed no differences in TG2 expression between the two genotypes (WT and Sdc4-KO) in control and diseased kidneys (Figure 6). This rules out the possibility that the lower extracellular TG2 detected in the Sdc4-KO kidneys that underwent experimental fibrosis was due to any difference in TG2 production between the two genotypes. Therefore, TG2 was elevated outside the cell in both models (Figures 4 and 5) through an increase in cellular trafficking, rather than an upregulation per se, which was deficient in Sdc4-KO diseased kidneys.

As TG2 was not found in the ECM of Sdc4-KO fibrotic kidney sections to the same extent as in the ECM of WT diseased kidneys, we analyzed whether it was retained intracellularly or in the cytosol. WT and Sdc4-KO fibrotic kidneys

Figure 5. Sdc4-KO decreases extracellular TG2 and TG activity in the AAN model of CKD. Extracellular TG2 was detected using cryostat sections of WT and Sdc4-KO kidneys from the AAN model through immunofluorescence (A). TG in situ activity was measured by incorporation of biotinylated cadaverine and revealed by TexasRed-labeled streptavidin (B). Quantification of levels of TG2 (C) and TG in situ activity (D) were performed using multiphase image analysis by dividing the TG2 fluorescence or the incorporated cadaverine fluorescence by tissue area (green autofluorescence). All data were normalized by the WT control at week 9; the original values were 0.04 for TG2 and 0.03 for TG in situ activity. *$P<0.05$ compared to control; †$P<0.05$ compared to Sdc4-KO AAN; *changes from 9 and 12 weeks in the WT AAN groups.
were fractionated into cytosol and membrane fractions, and equal loading of protein samples was analyzed by Western blotting for TG2 expression. Sdc4-KO membrane fractions contained significantly less TG2 antigen than did the WT membrane preparations (Figure 7, A and D). Conversely, the Sdc4-KO cytosolic fractions had a higher amount of TG2 antigen compared with the WT (Figure 7, B and D). The combined level of TG2 was not significantly different in the two genotypes (Figure 7, C and D). We next visualized intracellular TG2 in paraffin sections, where ECM TG2 cannot be detected.\textsuperscript{1,12} Compared with the WT fibrotic kidneys, staining of Sdc4-KO sections revealed an increase in cytosolic TG2 that was paralleled by a lower level of externalized TG2 after UUO, as specifically detected in cryostat sections (Figure 7E).\textsuperscript{12} This finding is consistent with our prior data that Sdc4-null cells had an increased retention of TG2 inside the cell.\textsuperscript{22} Therefore, Sdc4 influences the externalization of profibrotic TG2.

Extracellular TG2 Availability in the Renal Interstitium Depends on Binding to HSPG

To investigate the mechanism of Sdc4 modulation of TG2 externalization, we began with visualizing extracellular TG2 and Sdc4 in normal and fibrotic kidneys after AAI. Immunofluorescence analysis of unfixed cryostat sections revealed, in normal tissues, that Sdc4 and TG2 had mainly a tubular basement membrane localization; however, Sdc4 staining was more diffuse and clearly outlined the perimeter of all cells of the renal interstitium, in keeping with its matrix cell-receptor role (Figure 8). In AAN kidneys, extracellular TG2 staining was more intense and filled the widened interstitial space, decorating the thickened basement membrane. Extracellular TG2 and Sdc4 clearly showed a parallel alignment with areas of intense extracellular TG2 staining lying adjacent to Sdc4 staining in AAN kidneys (Figure 8). Quantitative colocalization analysis according to Manders\textsuperscript{35} indicated a good overlap coefficient (mean±SEM, 0.67±0.07 [1 is total colocalization]). The Pearson correlation coefficient was 0.64±0.04. The partial overlap and location in immediately adjacent regions, especially in the basement membrane, suggested that TG2 association with Sdc-4 could be mediated by the sulfated domains within HS chains, rather than the HSPG core protein. Syndecans are characterized by three to five GAG chains,\textsuperscript{36} which typically vary in length and size, extending extensively in the extracellular space. Dual staining of extracellular TG2 and HS in sections from control and AAN kidneys (Figure 9) showed that HS were strongly expressed in the tubulointerstitium and were prominent in the basement membranes of the diseased kidney, where HS largely colocalized with TG2. Quantitative colocalization indicated an improved overlap coefficient (0.85±0.09) and Pearson correlation coefficient (0.77±0.08). To confirm that TG2-association with the interstitial matrix depended on binding to HS chains of proteoglycans, sections were preincubated with heparitinase I to selectively cleave HS (Figure 10). Following heparitinase I pretreatment, both the interstitial space and the basement membrane were almost completely devoid of TG2 binding, indicating that HS

Figure 6. The expression of TG2 in kidney is similar in the WT and Sdc4-KO genotype. TG2 expression was evaluated by Western blot in control kidneys and fibrotic kidneys (total homogenates) from WT and Sdc4 KO mice. (A) AAN model. (B) UUO model. Cyclophilin A (Cyp A) was used as loading control. Twenty-five micrograms of kidney proteins was used, and 100 ng guinea pig liver TG2 was loaded as a control (M). TG2 protein level is expressed as mean±SEM of TG2/Cyp A (TG2 level); data are normalized for WT control. Differences in TG2 between the two genotypes and treatments were nonsignificant (P>0.05).
plays a critical role in the cell-surface availability of TG2 (Figure 10).

Because we showed that Sdc-4 is critical to the availability of cell-surface TG2 in the diseased kidney (Figures 4 and 5), these findings suggest a role for the Sdc4-HS chains in recruiting TG2 at the basolateral membrane and the renal interstitium in progressive kidney fibrosis. HS not only characterizes membrane HSPG but also secreted proteoglycans. It would be reasonable to think that once TG2 is externalized in the renal interstitium, through the critical involvement of transmembrane Sdc4, it may be trafficked in the matrix by binding to basement membrane HSPG.

**Sdc4-KO Reduces TGF-β1 Activation**

Given that TG2 is involved in the activation of TGF-β1 by recruiting large latent TGF-β complex, and failure to localize/recruit latent TGF-β1 results in altered TGF-β1 activity, the level of active TGF-β1 was evaluated through the mink lung bioassay (Figure 11). Active TGF-β1 was similar in WT and Sdc-4 KO control kidneys. Active TGF-β1 was increased in the fibrotic kidneys of WT mice at 12 weeks of AAI treatment compared with controls but was not elevated to the same extent in the Sdc4-KO AAI-treated kidneys (Figure 11A). Total TGF-β1 was also significantly elevated in the fibrotic WT kidneys and not in the Sdc4-KO kidneys (Figure 11B). Overall, the percentage of active TGF-β1 in the WT AAI-treated kidneys was significantly higher than in the Sdc4-KO AAI-treated kidneys (Figure 11C). Syndecan-2 (Sdc2), the main syndecan protein found to directly interact with TGF-β1, was not differently expressed in the Sdc4-KO normal and diseased kidneys compared with WT counterparts (Supplemental Figure 2). Therefore, we have confirmed a reduction in TGF-β1 activity associated with Sdc4 deletion.

**DISCUSSION**

All types of CKD progress to kidney failure through the common pathway of kidney fibrosis and scarring. Therefore, understanding the mechanisms involved in this is essential for the development of antifibrotic therapies. Studies have repeatedly highlighted the benefit of TG2 modulation on the progression of kidney fibrosis, but the tightly conserved catalytic triad within the TG family has challenged the design of specific small-molecule TG2 inhibitors suitable to clinics. In this paper, we have exploited recent in vitro observations that Sdc4 may...
have an important role in TG2 export and retention of TG2 at the cell surface,\textsuperscript{23,40,41} to see whether or not targeting the TG2-Sdc4 interaction may provide such an interventional step.

We have chosen to do these studies in two models of CKD in parallel in order to prevent concerns over any model-specific responses. UUO is a well-established model for rodents that allows the fibrotic state to be developed in a relatively short time without the use of exogenous toxins and the development of uremia.\textsuperscript{42} Although obstruction of the urinary tract has clinical relevance,\textsuperscript{43} the interstitial fibrosis of this model is not typical of most types of CKD. Therefore, we have also used the relatively new AAN model, which does provide a typical tubulointerstitial fibrosis.\textsuperscript{31,32,44}

The results in both models were highly consistent with lower levels of fibrosis detected in the Sdc4-KO animals, suggesting a protective role for Sdc4-KO in kidney fibrosis. As fibrosis is an abnormal tissue repair process, this result fits with the involvement of Sdc4 in wound healing and the reported delay in wound repair in Sdc4-KO mice.\textsuperscript{45,46}

Although, to our knowledge, this is the first study in CKD using Sdc4-KO mice, Sdc4-KO was investigated in mice with unilateral nephrectomy.\textsuperscript{47} Glomerulosclerosis was reported in Sdc4-KO male mice after and was attributed to the compensatory expression of Sdc2 and activation of TGF-\(\beta\).\textsuperscript{47} However, unilateral nephrectomy typically causes renal hypertrophy rather than overt fibrosis and, subsequently, is not similar to the two CKD models used here. Moreover, we did not see Sdc2 compensation in Sdc4-KO mice after UUO.

Extracellular TG activity positively correlated with fibrosis development in the two experimental models of renal fibrosis. To establish whether or not the protective effect of Sdc4-KO was related to its previously reported role in TG2 trafficking/cell-surface localization,\textsuperscript{23,41} tissues were subjected to assays for both extracellular TG2 antigen and \textit{in situ} TG activity. In both models, increased extracellular TG2 and TG activity was found to be in the same interstitial and periglomerular regions where increased collagen deposition was detected. Importantly, the increase in extracellular TG2 occurred in parallel with fibrosis, being significantly lower in the Sdc4-KO diseased animals. Due to the total TG2 level being similar in both WT and Sdc4-null mice, the difference in TG2 antigen outside the cell could only be due to Sdc4 affecting the cell-surface availability of TG2. This conclusion was corroborated by the finding of reduced distribution of TG2 to the membrane and a higher proportion of cytosolic TG2 following Sdc4 silencing in kidney, consistent with similar findings in dermal fibroblasts from Sdc-4 KO mice.\textsuperscript{23} Taken together, the \textit{in vivo} and \textit{ex vivo} data strongly support the hypothesis that Sdc4 plays a significant role in TG2 export and localization and that interference with TG2-Sdc4 interaction may have therapeutic value.

In the tubular epithelial cell line NRK-52E, we previously reported that TG2 is transported basolaterally into the tubular basement membrane through an unconventional pathway, most likely linked to direct molecular trap involving transmembrane transport.\textsuperscript{21} Here we have shown for the first time that HS are critical for extracellular TG2 association with the tubular basement membrane. Therefore, TG2 is likely to be “trapped” by HS as soon as secreted from the plasma...
TG2 largely colocalizes with HS chains in tubulointerstitial fibrotic lesions. HS and TG2 immunostainings of cryostat sections were performed using mouse monoclonal anti-HS antibody and rabbit polyclonal anti-TG2 antibody, respectively, followed by goat anti-mouse (IgG) FITC and donkey anti-rabbit IgG AlexaFluor 568. Representative pictures of HS, TG2, and DAPI-stained sections are shown separately and merged (with and without DAPI staining) for control and AAI-treated kidneys (two fibrotic lesions are shown). Basolateral membrane and interstitial localization of HS and TG2 after AAI are shown at higher magnification (details a, b, and c). Dual staining controls were carried out in TG2 KO kidney sections (Supplemental Figure 3). Scale bars are shown under each column of images.

Figure 9. TG2 largely colocalizes with HS chains in tubulointerstitial fibrotic lesions. HS and TG2 immunostainings of cryostat sections were performed using mouse monoclonal anti-HS antibody and rabbit polyclonal anti-TG2 antibody, respectively, followed by goat anti-mouse (IgG) FITC and donkey anti-rabbit IgG AlexaFluor 568. Representative pictures of HS, TG2, and DAPI-stained sections are shown separately and merged (with and without DAPI staining) for control and AAI-treated kidneys (two fibrotic lesions are shown). Basolateral membrane and interstitial localization of HS and TG2 after AAI are shown at higher magnification (details a, b, and c). Dual staining controls were carried out in TG2 KO kidney sections (Supplemental Figure 3). Scale bars are shown under each column of images.

The components required for TG2-membrane translocation are unknown, but the membrane-proximal HS chains of Sdc4 are a key factor. TG2 is a high-affinity ligand for heparin/HS with a dissociation constant in the low nanomolar range,22 and folding of TG2 forms a functional heparin-binding domain.24 Upon injury-induced tissue damage, the externalized TG2 is thought to be activated by the increased calcium ions and low guanine nucleotides,10 leading to matrix stabilization by crosslinking of fibronectin and collagen. Although not tested, it is conceivable to speculate that further diffusion of TG2 from the cell surface may be regulated by shedding of TG2-Sdc4 from the cell surface by matrix metalloproteases, which are abundant, into a wound healing/fibrotic context. Furthermore, the HS chains could facilitate diffusion of TG2 through dissociation/reassociation via adjacent binding sites, thus allowing TG2 to “slide” in the matrix, depending on the specificity of the HS interaction. The significant but partial attenuation of extracellular TG2 activity and export in two Sdc4-KO mice models of CKD, and its complete abolishment by digestion of the HS chains, suggest that additional HSGP may be implicated in TG2 localization in the renal interstitium. Secreted proteoglycans, such as perlecan, agrin, and collagen VIII (typical glomerular basement membrane proteoglycans), are also expressed in the tubule interstitium.37

Taken together, our finding suggests that TG2 cell-surface availability may depend on trapping of TG2 by the HS chains of transmembrane HSPG Sdc4. Further engagement of TG2 with basement membrane-secreted HSPG may facilitate the distribution of extracellular TG2 into the matrix.

Our findings support the idea that Sdc4 and TG2 cooperate in the fibrotic process and Sdc4-KO reduces fibrosis through this route. In addition to controlling the cell-surface availability and function of extracellular TG2, Sdc4 regulates the export of other fibrogenic factors, such as fibroblast growth factor-2.23 Sdc4/HS have also been shown to influence and integrate the procontractile signals from TGF-β1, leading to increased cell adhesion during scarring,48 and trapping of exogenously provided TGF-β.49 TG2 participates in the TGF-β1 recruitment/activation cascade by crosslinking the amino-terminal region of LTBP-1 to ECM proteins (diagram c in Figure 12).6,33,50 This, together with matrix accumulation and stabilization by direct post-translational modification of ECM proteins (diagram b in Figure 12), is accountable for the fibrotic role of TG2 in vivo.8,34 Of note, we found the protective role of Sdc4-KO on fibrosis to be associated with lower activation of TGF-β1. This is predictable given the lower TG2 activity in the ECM. The TG2-KO mouse is protected against kidney UUO-induced fibrosis and pulmonary fibrosis,7 and this is due partly to lower levels of profibrotic active TGF-β1 in the damaged organ or bronchoalveolar lavage, respectively.7 Given that Sdc4-KO leads to lower extracellular TG2, which is needed for one of the three known mechanisms of TGF-β1 recruitment and activation51 then it is reasonable to suggest that the lower active TGF-β1 is affected by impaired TG2 recruitment of the LTBP. Although a direct role of Sdc4 in TGF-β1 activation has not been reported, the ability of Sdc4 to act as a reservoir of growth factors leaves open the possibility of a synergistic action between TG2 and Sdc4 in TGF-β1 activation (diagram d in Figure 12). Therefore, because Sdc4 and TG2 affect multiple pathways in the fibrotic program, targeting the TG2-Sdc4 interaction.
interaction, would probably form part of a multifaceted interventional strategy against fibrosis.

A surprising finding in this study is that although Sdc4-KO is clearly protective, with strong reduction in fibrosis in both models of kidney fibrosis, this did not have a knock-on effect on kidney function in the AAN model. The most obvious explanation is that the AAN is primarily a model of tubulointerstitial fibrosis. At 12 weeks, there was only minimal glomerulosclerosis because the disease had not gained glomerular involvement at this stage. It is therefore not surprising that there was no effect on glomerular filtration. This raises the question as to why both WT and Sdc4-KO animals treated with AAI had elevated serum creatinine and lower clearance at both time points. In rodents and mice especially, creatinine is secreted from the tubules and because AAI is cytotoxic to the tubular epithelial cells; this would clearly interfere with creatinine excretion through this route. Thus, because serum creatinine decreased from 9 to 12 weeks, this simply reflected the epithelial cell recovery to AAI toxicity, which undoubtedly masked any early fibrotic changes on kidney function due to fibrosis.

In conclusion, Sdc4-KO is protective in two models of tubulointerstitial fibrosis. In both cases this is related to a reduction of extracellular TG2 antigen and activity, which has previously been shown to be a primary cause of scar tissue formation in renal fibrosis by both direct post-translational modification and indirect TGF-β1 recruitment. Therefore, Sdc4 is involved in extracellular trafficking and cell-surface targeting of profibrogenic TG2 in vivo. These data may ultimately be useful in designing interventional strategies for CKD.

**CONCISE METHODS**

**Experimental Models**

Experimental UUO was performed on Sdc4-KO29 and control C57BL/6J mice. Anesthesia was induced with 5% fluorothane and maintained with 2% fluorothane during the surgical process, wherein the left ureter of each animal was blocked with a legating clip (Hemoclip Plus; Weck Closure Systems). The peritoneum was flooded with ADEPT (4% icodextrin solution) to prevent postsurgical adhesions before closing. The muscle wall was closed with single crossover stitching using dissolvable stitches. After UUO, mice were provided with buprenorphine (0.1 mg/kg) for 40 hours for analgesic purposes. Mice were allowed to recover and had free access to food and water. Kidneys were harvested at days 7, 14, and 21 (Supplemental Table 1).

AAN was induced in 8-week-old male C57BL/6J and Sdc4-KO mice by intraperitoneal injection of AAI (Sigma-Aldrich).52 Administration of AAI, 3 mg/kg, once every 3 days for 3 or 6 weeks, as described in Supplemental Table 2, established progressive fibrosis.32,50 Control animals received intraperitoneal injection of vehicle control DMSO (Sigma-Aldrich). Mice were maintained at 20°C and 45% humidity on a 12-hour light/dark cycle and allowed free access to standard rodent chow and water.

All procedures were carried out under license according to regulations laid down by Her Majesty’s Government, United Kingdom (Animals Scientific Procedures Act, 1986).

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**Figure 10.** TG2 extracellular location depends on the HS chains of HSPGs. Kidney cryosections were treated with 50 mU/ml protease-free heparitinase I (Hep-I) for 2 hours at 37°C. Extracellular TG2 was immunolabeled using mouse anti-TG2 IA12 antibody followed by goat anti-mouse DyLight 594. Images were obtained by confocal microscopy (A) and the level of extracellular TG2 was quantified by ImageJ intensity analysis in four kidneys per treatment (B). Three fields are shown per treatment, representative of control and AAN fibrotic kidneys (A).
Kidney Function
Terminal blood samples were taken. Twenty-four-hour urine samples were collected using metabolic cages immediately before termination. Serum and urine creatinine was measured as previously described, with modifications detailed in the Supplemental Methods.

Fibrosis Measurement
Kidney fibrosis was assessed on kidney sections stained with MT as previously described. To assess the scarring index in the UUO study, 10 images per kidney were acquired at 3.200 magnification. For the AAN model, the measurement was performed using images acquired at 3.100 magnification along the whole cortical area of each kidney.

Immunofluorescence
Immunodetection of collagens was performed on 4-μm paraffin sections. After antigen retrieval and blocking, sections were incubated with primary antibodies against collagen I (rabbit anticollagen I; Abcam; 1:250) collagen III (goat anticollagen III; Southern Biotech; 1:10) or collagen IV (rabbit antihuman collagen IV; MP Biomedicals; 1:35). AlexaFluor 568 (UUO model) or AlexaFluor 488 (AAN model) secondary antibodies (Invitrogen; 1:200) were used for the immunofluorescent staining, and coverslips were mounted with 4',6-diamidino-2-phenylindole (DAPI)-containing Vectashield (Vectorlab). Ten nonoverlapping images of the cortical regions were acquired at ×200 magnification and analyzed using multiphase image analysis with correction to DAPI staining.

Immunodetection of extracellular TG2 was performed on 14-μm-thick cryostat sections, as previously described by Johnson et al. in 1999 and 2003 and reported in the Supplemental Methods. We used mouse monoclonal anti-TG2 (clone ID10; Abcam; 1:100) followed by goat anti-mouse AlexaFluor 568 (Invitrogen; 1:200), or mouse anti-TG2 monoclonal IA12 (University of Sheffield; 1:100), followed by goat anti-mouse IgG DyLight 594 (Abcam; 1:200). Coverslips were mounted with DAPI-containing Vectashield. For TG2 quantification, ten ×200 pictures of the cortical region were acquired for each kidney and analyzed as described above.

TG2 and Sdc4 double staining was performed using rabbit polyclonal anti-Sdc4 (Zymed; 1:50) and mouse anti-TG2 IgG monoclonal IA12 (University of Sheffield; 1:100) followed by donkey anti-rabbit IgG AlexaFluor 568 (Invitrogen; 1:200) and goat anti-mouse IgG DyLight 594 (Abcam; 1:200). Coverslips were mounted with DAPI-containing Vectashield. For TG2 quantification, ten ×200 pictures of the cortical region were acquired for each kidney and analyzed as described above.

Enzymatic Pretreatment
Proteinase-free heparitinase I (EC 4.2.2.8) (Sigma-Aldrich) was used to digest the side chains of HSPG. Enzymatic pretreatments of cryosections were performed with heparitinase I (0.05 U/ml) dissolved according to the manufacturer’s instructions and incubated for 30 minutes. HS and TG2 dual staining was performed using mouse IgM anti-HS antibody (Amsbio, 1:50) and rabbit polyclonal anti-TG2 (Abcam; 1:50) followed by goat anti-mouse IgM FITC (Sigma-Aldrich; 1:100) and donkey anti-rabbit IgG AlexaFluor 568 (Invitrogen; 1:200) in Tris-buffered saline with Tween-20 containing 1% vol/vol serum (donkey and goat). The dual staining protocol is described in the Supplemental Methods. Images were captured using a Leica SP5 confocal microscope scanning system coupled to a ×40 and ×63 oil immersion objective-inverted microscope. Successive serial optical sections (0.5–1 μm) were recorded over a range of specimen planes (3–7 μm). Colocalization was estimated as described in Supplemental Methods.

Enzymatic Pretreatment
Proteinase-free heparitinase I (EC 4.2.2.8) (Sigma-Aldrich) was used to digest the side chains of HSPG. Enzymatic pretreatments of cryosections were performed with heparitinase I (0.05 U/ml) dissolved according to the manufacturer’s instructions and incubated for 30 minutes.

Figure 11. Sdc4-KO lowers TGF-β1 in the AAN model of CKD. Active TGF-β (A), total TGF-β (B), and percentage of activated TGF-β (C) were evaluated in WT and Sdc4-KO kidneys using the mink lung TGF-β bioassay, as described in the Concise Methods. Total TGF-β was converted to a biologically active form for analysis by acid activation. The percentage of activation was calculated by expressing the level of active TGF-β as a percentage of total TGF-β. (D) Recombinant TGF-β standard curve. Values are the mean of four kidneys ± SEM, each assessed in triplicate. RLU, relative light unit. *P<0.05; **P<0.01; ***P<0.001 compared with the control or compared with Sdc4 KO AAN.
Detection of TG In Situ Activity

In situ activity was detected as previously described by Huang and colleagues using 0.1 mM Texas red cadaverine instead of biotinylated cadaverine.

TGF-β1 Activity

Active and total TGF-β1 were determined using the MLEC luciferase TGF-β quantitative bioassay as previously described. Details are provided in the Supplemental Methods.

Statistical Analyses

Data are shown as mean ± SEM. Data analyses were performed using two-way ANOVA followed by a Bonferroni post hoc test or a t test. A probability of 95% (P < 0.05) was taken as indicating a statistically significant result.

ACKNOWLEDGMENTS

We would like to thank Mabrouka Maamra (University of Sheffield) for kindly providing the mouse monoclonal anti-TG2 (IA12) antibody. We would also like to thank Anne Schneider (JvGRC, Nottingham Trent University) for the cryostat sectioning advice. We are grateful to Daniel Aeschlimann (Cardiff University) for useful comments on this study. We are indebted to Martin Griffin (Aston University) and Takashi Muramatsu (Aichi Gakuin University) for generously providing cell lines and animals in this study.

This work has been supported by Wellcome Trust project (087163) to T.S.J. and E.M.M. Verderio, the Sheffield Kidney Research Foundation, and Kidney Research UK.

DISCLOSURES

None.

REFERENCES


Figure 12. Interplay between TG2 and Sdc4/HS in TG2 externalization and matrix crosslinking during the development of fibrosis. Transmembrane Sdc4 traps TG2 at the cell surface through its HS chains, facilitating TG2 externalization. It is unclear how TG2 crosses the plasma membrane. The HS chains of secreted HSPG could facilitate diffusion of TG2 via adjacent binding sites, thus allowing TG2 to “slide” in the matrix (a). Engagement with protein substrates (collagen, fibronectin) leads to activation of TG2 transamidation, resulting in matrix stabilization by crosslinking (b). Furthermore, TG2 promotes TGF-β1 large latent complex (LLC) deposition into the ECM by covalently linking the latent TGF-β1–binding protein (LTBP) to matrix components (c), leading to its activation in the fibrotic kidney. TGF-β1 and the latency-associated peptide (LAP) are proteolytically separated at the site indicated by the arrowhead. Sdc4 could trap LLC via HS chains and/or contribute to TGF-β1 activation directly or indirectly through other Sdc4-linked pathways (d).


38. Abe M, Harpel JG, Metz CN, Nunes I, Loskutoff DJ, Rifkin DB: An assay for transforming growth factor-beta using cells transfected with a
plasminogen activator inhibitor-1 promoter-luciferase construct.  

Anal Biochem 216: 276–284, 1994


50. Huang L, Johnson TS: Development of a chronic kidney disease model in C57BL/6 mice that is relevant to human disease. Presented at the BRS/RA Conference, Birmingham, United Kingdom, June 6, 2011.


This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2013050563/-/DCSupplemental.
SUPPLEMENTARY METHODS

Serum and urine creatinine quantification

Serum and urine creatinine was measured as previously described. 1 100ul of serum was mixed with 1ml of acetonitrile or 10ul of urine mixed with 0.5ml of acetonitrile. Samples were then centrifuged at 13,000g for 15 minutes at 4°C. Supernatant was transferred, air-dried and resuspended in 50ul or 100ul of 5mM sodium acetate (pH5.1) for serum or urine samples respectively. 25ul of processed sample was injected into an Agilent 1100 HPLC system with a 10µm 4.1x100mm PRP-X200 cation exchange column (Hamilton). Isocratic HPLC was performed at a flow rate of 1.5 ml/min for 12 minutes after each injection using UV absorbance at 234nm. A creatinine standard curve between 0 to 60ng was used for serum and between 0 to 750ng for urine.

Kidney homogenates and western blotting

For kidney homogenate, 100-200mg of snap-frozen kidney tissue was weighed and homogenised in 9 times the volume of STE buffer (0.32M sucrose, 5mM Tris, 2mM EDTA, pH 7.4) containing protease inhibitors (1mM leupeptin, 5mM benzamidine and 1mM phenylmethylsulphonylfluoride (Sigma, UK) by using an Ultra Turrax T25 Homogeniser (Merck, UK). One hundred ng of tissue proteins were resolved by 10% SDS-PAGE under reducing conditions, and TG2 was detected by Western blotting using anti-TG2 monoclonal antibody (IA12, University of Sheffield) in blocking buffer (5% non-fat milk in TBST). TG2 was revealed by enhanced chemiluminescence, after incubation with rabbit anti-goat-HRP (Dako). Cyclophilin was revealed as loading control using a polyclonal rabbit anti cyclophilin (Abcam) followed by rabbit anti goat IgG. Quantitative comparison of protein
Kidney fractionation

Control or AAN kidneys were homogenised in homogenisation buffer (0.25M sucrose, 1mM MgCl₂, 2mM EDTA, 10mM Tris-HCl pH 7.4) containing protease inhibitors (1mM leupeptin, 5mM benzamidine and 1mM phenylmethylsulphonylfluoride) (Sigma, UK) to prepare a 10% (w/v) kidney homogenate using Ultra Turrax T25 Homogeniser (Merck, UK). The kidney homogenate was centrifuged at 1000g for 5 min at 4°C to remove larger particulates and the supernatant transferred to a new tube. This was then centrifuged at 20,000g at 4°C for 30 min to precipitate membranes and retrieve the cytosolic fraction in the supernatant. 40 µg of tissue proteins were resolved by 12% SDS-PAGE under reducing conditions. TG2 was detected by western blotting using anti-TG2 monoclonal antibody (IA12, University of Sheffield), Cyclophilin A using the anti-Cyclophilin A polyclonal antibody (ab41684, Abcam), β-Tubulin with the anti-Beta Tubulin polyclonal antibody (ab6046, Abcam) and Sodium Potassium ATPase with the anti-Sodium Potassium ATPase monoclonal antibody (ab7671, Abcam) in blocking buffer (5% non-fat milk in TBST). The western blots were revealed by chemiluminescence, after incubation with HRP-conjugated goat anti-mouse or goat anti-rabbit antibodies (Dako, Glostrup/Denmark). Quantitative comparison of protein band intensity was obtained by Aida Image Analyser v.4.03, according to the manufacturer’s guidelines.

Mink lung epithelial cell (MLEC) luciferase TGF-β1 bioassay

TGF-β1 activity is determined using MLEC permanently transfected with the construct p800neoLUC containing a truncated plasminogen activator inhibitor-1 promoter (PAI-1),
with the TGF-β1 response element fused to the firefly luciferase reporter gene.² Kidney samples were prepared based on a protocol previously described.³,⁴ Briefly, control or AAN kidneys were homogenised in homogenisation buffer (0.25M sucrose, 1mM MgCl₂, 2mM EDTA, 10mM Tris-HCl pH 7.4) containing protease inhibitors (1mM leupeptin, 5mM benzamidine and 1mM phenylmethylsulphonylfluoride) (Sigma, UK) to prepare a 10% (w/v) kidney homogenate using Ultra Turrax T25 Homogeniser (Merck, UK). The kidney homogenate was centrifuged at 1000g for 5 min at 4°C, the supernatant diluted 1:25 in serum-free DMEM containing 0.1% (w/v) BSA and sterile-filtered (2µm, Sartorius Stedim, Goettingen/Germany). Total TGF-β1 was determined by activating latent TGF-β1 using the acid treatment described previously⁵ and applied to 5x10⁴ MLEC in a 96-well plate. After 22 h, the cells were washed twice with PBS and lysed in 1x Reporter Lysis Buffer (Promega, Madison, WI). A total of 50 µl of cell lysate was mixed with 50 µl of luciferase substrate (Promega) and loaded onto a Polarstar Optima (BMG Labtech, Ortenberg/Germany) luminometer.

Immunolabelling of kidney cryostat sections

Cryostat sections (16 μm thick) from AAI-treated or control kidneys were washed twice with PBS containing 0.1% Tween-20, and then incubated with PBS containing 1% Triton X-100 for 30 minutes at room temperature to clean the sections. Sections were blocked for 30 minutes at room temperature in blocking buffer containing normal serum from the same species of which the secondary antibody was raised (TBS containing 0.1% v/v Tween-20 (TBST) and 10% v/v serum (donkey and goat). Sections were incubated with primary antibodies for 15 h at +4°C diluted in TBST containing 1% v/v serum (donkey and goat). Following three washes in TBST, sections were fixed with ice cold acetone for 5 minutes at -20°C and allowed to dry. Sections were then incubated for 2 hour at room temperature with secondary antibodies diluted in TBST containing 1% v/v serum (donkey and goat). Slides
were washed three times with TBST and coverslips mounted using DAPI-containing vectashield.

**Immunolabelling of kidney paraffin sections**

Paraffin sections (4µm thick) from UUO or control kidneys de-waxed in xylene for 10 min and rehydrated sequentially in 100%, 95%, 75% and 50% Ethanol for 5 min each. After washing with ddH₂O for 10 min and PBS for 10 min, the sections were heated at 92°C for 10 min in Unmasker Solution (ID-Labs Technology) and allowed to cool down gradually. After washing twice for 10 min with PBS, sections were trypsinised with Digest all-2 (Invitrogen, Camarillo/USA) at 37°C for 15 min according to the manufacturer's directions. The sections were washed twice with PBS for 10 min and blocked with PBS containing 10% v/v blocking serum (donkey) for 30 min at RT. Sections were incubated with the primary antibody against TG2 (rabbit polyclonal, ab421, Abcam, Cambridge/UK), diluted 1:50 in PBS containing 1% donkey serum (v/v) and protease inhibitors, for 16 h at 4°C. Following three washes in PBS, sections were incubated with the secondary antibody donkey anti-rabbit conjugated with Alexafluor-568 (Invitrogen) diluted 1:200 in in PBS containing 1% donkey serum (v/v) and protease inhibitors for 1 hour at RT. Sections were washed three times with PBS and coverslip mounted using DAPI-containing Vectashield (Vector Laboratories, Burlingame/USA).

**Quantification of co-localisation in confocal images**

Following dual immune fluorescence staining, the degree of co-localisation was estimated using WCIF ImageJ intensity correlation analysis plugins (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2004), consisting of intensity correlation quotient (ICQ with minimum value -0.5 and max value 0.5
indicating that the overlap is not random) and Mander’s colocalisation coefficient (0-1, with maximum value of 1 indicating total overlap). Four kidneys were analysed per treatment (approx. 3 sections per kidney; 4-6 non overlapping images per section). The ICQ values ranged from 0.25 to 0.35.

**Hydroxyproline measurement**

Kidney tissue was homogenised in 4 volumes of homogenation buffer (50 mM Tris HCl, pH 7.4 containing 0.25 M sucrose, 10 mM EDTA and proteases inhibitors) using a motor-driven pellet pestle (Sigma). Total protein content were assessed by diluting 2 µl homogenate in 18 µl homogenation buffer containing 1% SDS. 1.5 mg proteins from the homogenate were hydrolysed in 1.5 ml 6 M HCl 110°C for 18 h. The samples were freeze-dried for 8 h and resuspended in 200 µl Lithium citrate loading buffer (0.2 M, pH 2.2) for amino acid analysis. 40 µl of the sample was loaded onto a Biochrom 30 Amino Acid Analyser. The fractionation was performed using 96361 LiHP control program and analysis package, and the readings of Ninhydrin-derived peaks at 440nm were recorded. The estimated hydroxyproline concentration in the volume loaded was calculated by reference to the 10 nmol/20 µl Biochrom calibration standard.

**Real time reverse transcription (RT) PCR assay**

A two-step RT-PCR was performed. Total RNA (2µg) was reverse transcribed with random hexamers (Promega) in a total volume of 20 µl, using Superscript reverse transcriptase (Invitrogen), according to manufacturer’s instructions. The cDNA, corresponding to 100ng of reverse-transcribed total RNA, was amplified by real-time PCR using iQ SYBR green supermix (Biorad), using oligonucleotide primers specific for the Syndecan family (Sdc1, Sdc2 and Sdc4), the collagens (Col1a1, Col1a2, Col3a1, Col4a1) and Cyclophilin-A (Cyp A)
(Supplementary Table 3). The primers were validated by melting point analysis and end-point analysis of PCR products (gel electrophoresis) showing that all fragments were of the anticipated size. To rule out PCR amplification of contaminating genomic DNA, not reverse transcribed RNA samples were included in each PCR reaction. Primers pairs (Sigma Genosys) were designed manually or using the Primer3 software (available at SDSC Biology workbench 3.2; http://workbench.sdsc.edu/), and their level of secondary structures evaluated by the DNA calculator software (available at http://www.sigmagenosys.com/calc/DNACalc.asp).

Real-time PCR reactions were performed in a Corbett Rotor-GeneTM 6000 rotary analyzer (initial denaturation: 95°C, 3 min; followed by 40 cycles: 95°C, 30 sec; 58-63°C, 30-45 sec; 72°C, 20-45 sec; depending on the transcript to be amplified). For all transcripts studied, a ramp temperature from 72°C to 95°C was used to generate the melt curves, which were used to check the homogeneity of the amplified transcripts. Each sample was run in triplicate and a no-template control was included to rule out contamination. Quantifications were conducted relatively, using the $2^{-\Delta\Delta C_T}$ method, by comparing gene expression between control and UUO samples. Each primer set was validated by melting point analysis and end-point analysis of PCR products, and data calculated after normalizing to Cyp A.
**References**


Supplementary Table 1

Experimental UUO design.

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<td>21 days</td>
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Supplementary Table 2

Experimental AAN design. Animal received intraperitoneal injection of 3mg/kg AAI once every 3 days; control animal received vehicle control DMSO.

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## Supplementary Table 3

Real-time RT-PCR oligonucleotide primer sequences

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Supplementary Figure 1

Collagens I(α1), III (α1), and IV expression in the UUO and AAN models of CKD. Col1a1, Col3a1 and Col4a1 RNA transcripts were quantified by real-time RT-PCR in control, UUO (A-C), control and AAN (D-F) kidneys from WT and Sdc4 KO mice by using oligonucleotide primers specific for Col1a1 (A and D), Col3a1 (B and E) and Col4a1 (C and F) (Supplementary Table 3). Quantifications were conducted relatively, by comparing gene expression to the WT control as described in the Supplementary Methods.
**Supplementary Figure 2**

Ratio of kidney weight to body weight and kidney function in AAN model. Kidneys were removed at termination. Ratio of kidney weight to body weight was calculated using kidney weight divided by terminal body weight (A). Kidney function was measured by serum creatinine (B) and creatinine clearance (C). * indicates p<0.05 compared to the control. † and ‡ indicate p<0.05 compared to Sdc4 KO AAN and control respectively. a, b and c indicate changes from 9 and 12 weeks in the same groups respectively.

### A
**Kidney Weight to Body Weight**
- WT control
- Sdc4 KO control
- WT AAN
- Sdc4 KO AAN

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### B
**Serum Creatinine**
- WT control
- Sdc4 KO control
- WT AAN
- Sdc4 KO AAN

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### C
**Creatinine Clearance**
- WT control
- Sdc4 KO control
- WT AAN
- Sdc4 KO AAN

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Supplementary Figure 3

Extracellular Transglutaminase-2 levels in UUO. Extracellular TG2 was detected using cryosections from WT and Sdc4KO kidneys through immunofluorescence (a-d and e-h). Representative images of TG2 staining overlapped to phase contrast pictures are shown (magnification 200X). Details of extracellular TG2 staining at day 21 post UUO are shown in Figure 7E. Scale, 50 µm.
Supplementary Figure 4

TG2 and Sdc4 staining controls in TG2 KO and Sdc4 KO kidney sections. Background immunostainings of cryostat kidney sections were tested in sections from TG2 KO mice (kindly donated by Prof G Melino, University of Rome “Tor Vergata”) and Sdc4 KO mice. (A) As in Figure 7, sections were stained with rabbit polyclonal anti-Sdc4 and mouse monoclonal anti-TG2 IA12, followed by, respectively, donkey anti-rabbit AlexaFluor®488 and goat anti-mouse DyLight®594. (B) As in Figure 8, sections were stained with mouse monoclonal IgM anti-HS antibody and rabbit polyclonal IgG anti-TG2 antibody, followed by respectively goat anti-mouse IgM-FITC and donkey anti-rabbit IgG AlexaFluor®568. No or negligible background staining was observed.
Supplementary Figure 5

Syndecan 2 expression is not enhanced in fibrotic UUO kidneys upon Sdc4 KO. Sdc2 RNA transcripts were quantified by real-time RT-PCR in control and UUO kidneys from WT and Sdc4 KO mice by using oligonucleotide primers specific for Sdc2 (Supplementary Table 3). Quantifications were conducted relatively, by comparing gene expression to the WT control as described in the Supplementary Methods.