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Susceptibility to kidney fibrosis in mice is associated with early growth response-2 protein and tissue inhibitor of metaloproteinase-1 expression

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Abstract

Patients with chronic kidney disease and experimental animal models of kidney fibrosis manifest diverse progression rates. Genetic susceptibility may contribute to this diversity, but the causes remain largely unknown. We have previously described kidney fibrosis with a mild or severe phenotype in mice expressing transforming growth factor-beta1 (TGF-β1) under the control of a mouse albumin promoter (Alb/TGF1β1), on a mixed genetic background with CBAxC57Bl6 mice. Here, we aimed to examine how genetic background may influence kidney fibrosis in TGF-β1 transgenic mice, and in the unilateral ureteral obstruction (UUO) and subtotal nephrectomy (SNX) mouse models. Congenic C57Bl6(B6)-TGFβ and CBAxB6-TGFβ (F1) transgenic mice

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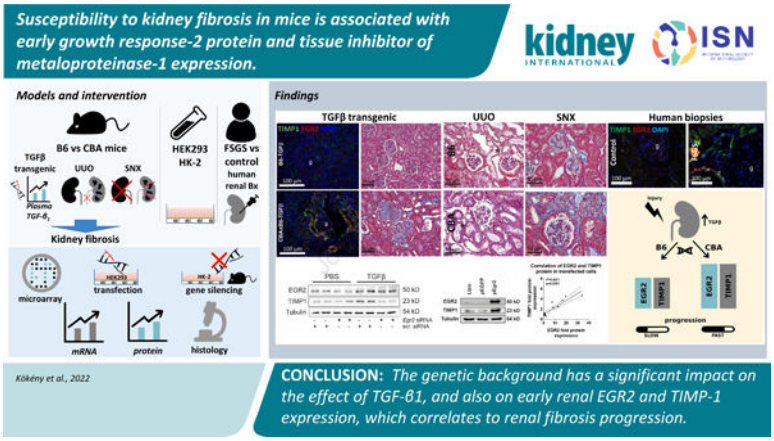
were generated and survival, proteinuria, kidney histology, transcriptome and protein expressions were analyzed. We investigated the kidneys of B6 and CBA mice subjected to UUO and SNX, and the effects of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) neutralization on the fibrotic process. CBAxB6-TGFβ mice developed severe kidney fibrosis and premature death, while B6-TGF-β mice had mild fibrosis and prolonged survival. Kidney early growth response factor-2 (EGR2) and TIMP-1 expression were induced only in CBAxB6-TGFβ mice. Similar strain-dependent early changes in EGR2 and TIMP-1 of mice subjected to UUO or SNX were observed. TIMP-1 neutralization *in vivo* hindered fibrosis both in transgenic mice and the SNX model. EGR2 over-expression in cultured HEK293 cells induced TIMP-1 while EGR2 silencing hindered TGF-β induced TIMP-1 production in HK-2 cells and ureteral obstructed kidneys. Finally, EGR2 and TIMP1 was increased in human kidneys manifesting focal segmental glomerulosclerosis suggesting a correlation between animal studies and patient clinical settings. Thus, our observations demonstrate a strong relationship between genetic background and the progression of kidney fibrosis, which might involve early altered EGR2 and TIMP-1 response, but the relationship to patient genetics remains to be explored.

Graphical Abstract



Keywords

chronic kidney disease; proteinuria; focal segmental glomerulosclerosis; fibrosis; gene expression; transcription regulation

Introduction

Progressive renal fibrosis, which often eventuates in end-stage kidney disease, is the final common pathway of chronic renal diseases of different etiologies, and represents one of the major challenges in nephrology. Patients with one particular renal disease, such as diabetic nephropathy and arterionephrosclerosis (in the case of African-Americans, often misattributed in the past to hypertension) show different progression rates, presumably due to genetic susceptibility[1].

Transforming growth factor-beta1 (TGF-β1) is a multifunctional cytokine that regulates the dynamic balance of extracellular matrix (ECM) components, as well have effects on cell growth and differentiation. TGF-β1 plays a pivotal role in the pathogenesis of fibrosis, but also in development, wound healing, immune processes and carcinogenesis. Experimental overexpression of TGF-β1 is associated with renal[2], myocardial[3], pulmonary[4], and hepatic fibrosis.

Several lines of evidence suggest a role for strain-dependent differences in the development and progression of experimental renal diseases. Rat models of puromycin aminonucleoside nephrosis and subtotal nephrectomy[5–7] have demonstrated the importance of strain differences. Studies involving albumin overload[8, 9], subtotal nephrectomy[10, 11] and diabetic nephropathy[12] mouse models show that the C57Bl6/J (B6) strain is resistant to renal fibrosis compared to other mouse strains, including 129Sv, Balb/C or DBA/2. Furthermore, despite early renal TGF-β1 overexpression after injury, B6 mice are resistant to fibrosis induced by renal ablation[10, 13]. Importantly, the molecular mechanism underlying strain-dependent renal fibrosis remain largely unknown.

We examined the role of genetic background on the progression of TGF-β1-induced renal fibrosis in transgenic mice overexpressing TGF-β1[14]. Originally, TGF-β1 transgenic mice maintained on mixed (CBAxB6) genetic background developed progressive renal fibrosis with phenotypic variability, characterized as either mild or severe[15]. Therefore, we generated congenic B6-TGFβ transgenic mice which manifested only a mild renal phenotype and CBAxB6-TGFβ F1 mice that had severe proteinuria and glomerulosclerosis, in order to analyze the expression of various fibrosis related molecules in these transgenic strains. Further, we made similar inter-strain comparisons in the unilateral ureter obstruction (UUO) and subtotal renal ablation (SNX) models, complemented with cell culture studies and analysis of human FSGS kidney biopsies.

Our experimental data show that genetic background and progression of TGFβ1-induced renal fibrosis strongly correlate with altered TIMP-1 expression, associated with early EGR2 response. We suggest that the underlying mechanism of resistance to renal fibrosis in B6 mice might involve the lack of early EGR2 and TIMP-1 response.

Methods

Concise methods are provided as Supplementary Methods.

Results

Characterization of novel congenic B6-TGFβ1 transgenic mice

In order to confirm the role of genetic background on renal fibrosis progression in this model, male CBA.B6-Alb/TGF-β_1 transgenic mice were backcrossed to both C57Bl6 (B6) and CBA inbred strains, as a CBAxB6 F1 hybrid mouse was the founder of this transgenic line.

Backcross of Alb/TGF-β_1 transgenic mice to CBA strain failed, as 70% of the CBAxB6-TGFβ F1 males died before six weeks of age (Figure 1A, Supplementary Figure S1A).

In contrast, backcross of Alb/TGF-β_1 transgenic mice for 22 generations to the B6 strain greatly increased survival as compared to the original transgenic strain, which exhibited 100% mortality by the age of 52 weeks. We found that 72% of B6-TGFβ mice survived to age 15 weeks (Figure 1A) and at 52 weeks, 39% were alive, compared to 100% survival of B6 wild type controls (n=33, log-rank test, $p<0.001$).

At the age of 4 and 9 months, plasma TGF-β1 levels were 2-fold higher in B6-TGFβ transgenic mice as compared to age-matched B6 controls (Supplementary Table S1). Body weights of both B6 and B6-TGFβ mice increased comparably with age (Supplementary Table S1). There were no differences in kidney weights normalized to body weight, and urinary protein to creatinine ratio among the groups. We observed mild but significant glomerulosclerosis in B6-TGFβ kidneys at the age of 4 and 9 months, as compared to age-matched B6 controls (Supplementary Table S1, Supplementary Figure S1B). We did not observe tubulointerstitial damage in B6-TGF-β1 mice at any investigated age.

Proteinuria and shortened survival of novel CBAxB6-TGFβ F1 transgenic mice despite comparable plasma TGF-β1 levels

Plasma levels of TGF-β1 at the age of 14 days in B6-TGFβ and CBAxB6-TGFβ F1 mice were similar and were 11–14 fold higher as compared to controls (Table 1). Body weights of transgenic mice were similar to their wild-type control (Table 1). In contrast to B6-TGFβ mice, the survival of CBAxB6-TGFβ F1 mice was dramatically shorter, as only 60% of these F1 mice survived to age 2 weeks, and all mice died by age 12 weeks (Figure 1A). Despite comparable plasma TGF-β1 levels in B6-TGFβ and CBAxB6-TGFβ strains, only CBAxB6-TGFβ mice had significantly elevated urine protein/creatinine ratio (Figure 1B). Similarly, kidney weights and levels of serum urea were significantly higher only in CBAxB6-TGFβ F1 mice (Figure 1C). Due to the early uremic death of most CBAxB6-TGFβ F1 mice, we were not able to continue the backcross to CBA strain and all experimental samples were obtained at the age of 14 days.

Onset and progression of renal fibrosis in TGF-β_1 transgenic mice is strain-dependent

At 14 days of age, B6-TGFβ mice exhibited renal histology similar to wild-type controls. However, CBAxB6-TGFβ mice manifested glomerular hypertrophy with mesangial expansion and capillary obliteration affecting 60% of glomeruli, together with mild tubulointerstitial fibrosis and tubular hyaline deposits (Figure 1D,E and Figure 1F, right panel). Significant glomerular and tubulointerstitial collagen-4 (Figure 2A,B) and fibronectin accumulation were present (Figure 2C,D). Glomerular expression of TGFB1 protein was only significant in CBAxB6-TGFβ kidneys (Figure 2E,F), while mild tubular expression was present in B6-TGFβ as well.

The evaluation of renal cortical gene expression at age 14 days showed that collagen-1 (*Cola1*) and collagen-3 *(Col3a1)* mRNA expression in B6-TGFβ kidneys were mildly elevated as compared to controls, while these were significantly increased (5- and 4-fold, respectively) in kidneys of CBAxB6-TGFβ mice (Figure 3A,B). This increase was accompanied by renal *Lcn2* (Figure 3C), *Tgfb1* and connective tissue growth factor (*Ctgf*) mRNA over-expression in CBAxB6-TGFβ mice (Table 2), in parallel with increased

expression of TGF-β1 inhibitors, biglycan *(Bgn)* and decorin (*Dcn)* (Table 2). At the age of 14 days, expression of matrix metalloproteinease-2 *(Mmp2)* was similar in wild-type and transgenic kidneys. There was no significant alteration in the expression of TGF-β_1 receptor-II (*Tgfbr2*) or any of the Smad mRNAs (*Smad2, 3, 4, 6, 7*) or SMAD3 phosphorylation (Supplementary Figure S2A). Interestingly, *Mmp9* levels in B6 and B6-TGF-β kidneys were comparable while CBAxB6 and CBAxB6-TGF-β kidneys had 3- to 4-fold increases in *Mmp9* mRNA expression, respectively. Expression of *Timp2* mRNA was comparable in wild type and in B6-TGFβ kidneys but was elevated in CBAxB6-TGF-β mice (Table 2).

Strikingly, there was a 100-fold increase in *Timp1* mRNA expression in CBAxB6-TGFβ kidneys as compared to B6-TGF-β and wild type mice (Figure 3D). Immunoblot analysis revealed 10-fold increase in TIMP1 protein expression (Figure 3E), mainly localized to tubules in CBAxB6-TGFβ kidneys (Figure 3G). This TIMP1 overexpression in CBAxB6-TGFβ kidneys resulted in reduced renal MMP9 activity as shown by gelatin zymography (Figure 3F).

We wished to elucidate whether the hepatic expression of the TGF-β transgene exerts local fibrotic effects in the liver or the elevated plasma TGF-β_1 levels distantly affect the heart that could substantially contribute to the short survival of CBAxB6-TGFβ mice. However, the extent of fibrosis in the liver and the myocardial expression of *Col1a1* mRNA were similar in both transgenic strains (Supplementary Figure S2B,C).

In order to investigate whether alterations in other kidney disease related genes could be responsible for this phenotype, we performed cDNA microarray analysis of B6-TGFβ and CBAxB6-TGFβ kidneys. The microarray showed 311 significant gene alterations (Supplementary Figure S3,S4 and Supplementary Table S4,S5) and confirmed *Timp1* being the most upregulated gene related to matrix remodeling. Significant changes in RNA expression were seen for 27 transcription factors (Supplementary Table S6). Among them, *Egr2* was most strikingly induced in CBAxB6-TGFβ kidneys (Figure 4A-C). Early growth response factor-2 (EGR2), drew our attention, as it has been implicated in the pathogenesis of skin and lung fibrosis[18]. Interestingly, renal expression of *Egr2* mRNA was similar in B6-TGFβ and wild-type mice (Figure 4A-C). We observed nuclear EGR2 protein expression mostly in some interstitial cells in control kidneys and B6-TGFβ mice that increased significantly in CBAxB6-TGFβ kidneys accompanied by tubular overproduction that co-expressed with TIMP1 (Figure 4C).

EGR2 and TIMP-1 overexpression are hallmarks of fibrosis initiation in TGFβ-transgenic mice

As histology revealed nearly end-stage glomerulosclerosis in CBAxB6-TGFβ (F1) kidneys at the age of 14 days, we investigated the initiation and early development of renal fibrosis in TGF-β1-transgenic mice at birth and at 5 days. Glomerular size and mesangial matrix content increased significantly in CBAxB6-TGFβ mice at 5 days (Glomerulosclerosis index (GSI) B6-TGFβ: 0.05±0.01 vs. CBAxB6-TGFβ: 0.45±0.21, $p<0.05$; Figure 1F, left). Renal *Tgfb1* mRNA expression in 5 day-old CBAxB6-TGFβ mice was also significantly higher compared to B6-TGF-β or controls (Table 3), although the expression of *Ctgf*, and type-I *(Col1a1)* and type III *(Col3a1)* collagens were similar in all groups at this age.

However, *Timp1* mRNA expression at this early age was 50% higher in CBAxB6-TGFβ kidneys, accompanied by elevated *Egr2* expression (Table 3). In B6-TGFβ kidneys, EGR2 immunostaining was observed in interstitial cells similar to controls, despite a mild tubular TIMP1 immunostaining (Supplementary Figure S5). However, EGR2 and TIMP1 were already co-expressed in tubules of CBAxB6-TGFβ mice at this early age although to a less extent as in 14 days-old mice (Figure 4C). In contrast, kidneys of newborn B6-TGFβ and CBAxB6-TGFβ mice depicted no renal histological alterations (data not shown) and had comparable *Timp1* and *Egr2* mRNA expression levels. The kinetics of *Egr2* and *Timp1* mRNA expression from 0 to 14 days of age, while only correlative, could indicate a mechanistic role for EGR2 and TIMP1 in the pathophysiological process of renal fibrosis in this model (Figure 4D, E).

TIMP-1 neutralization reduced fibrosis in CBAxB6-TGFβ F1 transgenic mice

As these data suggested the central role of TIMP1 in TGF-β1 induced renal fibrosis, we injected male CBAxB6-TGFβ mice intraperitoneally with anti-TIMP1 neutralizing antibody or isotype IgG for 5 consecutive days beginning at 8 days of age, and the animals were euthanized at 14 days of age. The early systemic TIMP1 inhibition was associated with 20% reduction in kidney weight in fibrosis prone CBAxB6-TGFβ mice as compared to isotype IgG-treated transgenic littermates (Figure 5A), accompanied by 50% reduction in proteinuria (Figure 5B) and serum urea levels (Figure 5C) and less glomerular and tubulointerstitial damage than in IgG treated littermates (Figure 5D-F).

Early EGR2 and TIMP1 over-expression are associated with progression after UUO and SNX

In order to test whether strain dependent early onset of renal fibrosis is associated with EGR2 or TIMP1 protein over-expression on other fibrosis models, we investigated B6 and CBA wild type mice after UUO and subtotal renal ablation (SNX). Overt renal fibrosis develops usually 3–5 days after UUO[19]. We first examined young wild-type B6 and CBA male mouse kidneys 24 hours after UUO. Kidney histology at this early stage showed only a few dilated tubules, otherwise normal structure and no signs of extracellular matrix accumulation (Figure 6A). Renal mRNA expression of *Lcn2*, as a marker of tubular damage (Figure 6B) and *Tgfb1* (Figure 6C), increased comparably in both B6 and CBA UUO. By contrast, *Col1a1* mRNA expression was 2-fold higher in CBA UUO as compared to B6 UUO kidneys (Figure 6B), accompanied by significant over-expression of *Egr2* and *Timp1* mRNA (Figure 6C, D) and EGR2 protein (Supplementary Figure S6A,B). The higher TIMP1 expression resulted in lower renal MMP9 activity in CBA UUO kidneys (Figure 6E). We also analyzed kidneys at 7 days after UUO when significant interstitial fibrosis developed in B6 but slightly more severe fibrosis in CBA kidneys accompanied by higher *Timp1* mRNA expression and reduced renal MMP9 activity, but *Egr2* mRNA only tended to be higher in CBA at this late time point (Supplementary Figure S7A-D).

In the second set of experiments, we observed marked interstitial fibrosis and glomerulosclerosis in male CBA mice at 6 weeks after SNX, as compared to B6 (Figure 7A,B). The significant renal fibrosis of CBA SNX mice was associated with elevated urinary protein/creatinine ratio (Figure 7C) although *Tgfb1* mRNA was comparable in B6 and CBA

SNX (Figure 7D). However, CBA SNX kidneys over-expressed *Col1a1* and *Lcn2* mRNA (Figure 7E, F), as well as TIMP1 (Figure 7G, H) and EGR2 mRNA and protein (Figure 7I, Supplementary Figure S8), the latter co-localized with TIMP1 by immunostaining in most of the CBA SNX tubules (Figure 7H).

TIMP1 deficiency inhibits the development of fibrosis after SNX in CBAxB6 mice

In order to confirm the central role of TIMP1 in the initiation and progression of renal fibrosis, we compared TIMP1-deficient (Timp1-null) male mice on fibrosis prone CBAxB6 background and wild-type CBAxB6 F1 male mice (Timp1+) subjected to SNX. Six weeks after nephrectomy, Timp1+ SNX kidneys manifested glomerulosclerosis, tubular atrophy and interstitial matrix accumulation, whereas Timp1-null SNX kidneys depicted only mild, non-significant glomerulosclerosis and interstitial fibrosis (Figure 8A,B). This was reflected by marked difference in proteinuria (Figure 8C). Interestingly, expression of type I and type III collagen mRNA was slightly but significantly elevated in Timp1-null SNX kidneys (Figure 8D,E), despite their histology was similar to control kidneys. Notably, Timp1+ SNX kidneys had 10-fold increased expression of *Col1a1* and *Col3a1*, accompanied by 2-fold increase in *Tgfb1* mRNA (Figure 8F). In contrast, Timp1-null SNX kidneys had *Tgfb1* expression similar to controls. The lack of TIMP1 resulted in higher MMP9 gelatinase activity in Timp1-null SNX kidneys (Figure 8G).

In vitro over-expression of EGR2 upregulates TIMP1 while EGR2 silencing reduces TIMP1

For *in vitro* evaluation of a possible EGR2-TIMP1 regulation, we transiently transfected HEK293 cells with pCMV plasmid containing full length *Egr2* gene (pEGR2). Controls were either transfected with EGFP coding plasmid (pEGFP) or received only transfection reagent Lipofectamine2000 (Lipo). Transfection with pEGR2 for 24h significantly increased EGR2 protein expression, which plausibly was responsible for an over-expression of TIMP-1 mRNA and protein by 2.5-fold and 4-fold, respectively (Figure 9A,B). Further, EGR2 levels correlated with TIMP-1 (Figure 9C). In another set of experiments, we transfected HK-2 cells with *EGR2* siRNA or scrambled siRNA 24h prior to TGF-β treatment. Silencing *EGR2* inhibited the TGF-β induced EGR2 and TIMP1 production, as well as increased MMP9 gelatinase activity of HK-2 cells (Figure 9D,E). Interestingly, TGF-β induced *TIMP1* and *EGR2* mRNA over-expression also in human fibroblasts (Supplementary Figure S9). *In vivo*, EGR2 siRNA administration prior to UUO significantly lowered renal EGR2 accompanied by reduced TIMP1 expression (Supplementary Figure S10A, B).

Increased EGR2 and TIMP1 expression in human FSGS kidney biopsies

In order to test whether upregulation of EGR2 and TIMP-1 in mice and the co-regulation in HEK293 and HK-2 cells has human clinical relevance, we investigated human kidney cortex biopsies obtained from FSGS patients and controls (the latter samples were taken from healthy cortex area of kidneys removed due to renal cancer (Supplementary Table S7). We observed more than 2-fold over-expression of both *EGR2* and *TIMP1* mRNA in FSGS samples compared to control tissues (Figure 9F) despite non-significant differences in *TGFB1* mRNA. Immunostaining revealed increased tubular EGR2 and TIMP1 expression in FSGS biopsies with notable co-expression in several tubules (Figure 9G).

Discussion

Experimental animal and human studies have demonstrated that TGF-β1 plays a central role in the initiation and progression of renal fibrosis. CBA.B6-Alb/TGF-β1 transgenic mice, which were maintained on mixed (CBAxB6) genetic background, develop renal fibrosis characterized as either mild or severe[15], presumably as a consequence of the inhomogeneous genetic background. In the present study, we generated congenic B6-TGFβ transgenic mice manifesting only a mild renal phenotype and CBAxB6-TGFβ F1 mice having shortened survival, proteinuria and glomerulosclerosis. We identified the transcription factor EGR2 (Krox-20) as a novel participant in the development of progressive renal fibrosis in mice and FSGS patients. We also demonstrate that genetic background of mice (B6 vs CBA) is strongly associated with altered early EGR2 and down-stream TIMP-1 response, which contributes to the progression of fibrosis in various models, including unilateral ureter obstruction, subtotal renal ablation and TGF-β1-transgenic mice.

We have addressed the hypothesis that genetic differences among inbred mouse strains contribute to the variability in TGF-β1 induced progressive fibrosis and we sought evidence for specific differences in expression of relevant molecules. Although the backcross of TGF-β1 transgene to the inbred B6 strain (congenic B6-TGFβ mice) did not reduce the elevated plasma TGF-β1 levels, B6-TGFβ mice developed only mild glomerulosclerosis and lacked proteinuria and their survival improved significantly (Figure 1). Our finding corroborates reports of B6 mice as a resistant strain to renal fibrosis[10, 12, 13].

We also wished to examine the effects of CBA genetic background on the progression. The CBAxB6-TGFβ transgenic mice had elevated plasma TGF-β1 levels similar to those of the B6-TGFβ strain, although their renal disease was markedly accelerated and survival was greatly shortened as compared to B6-TGFβ mice. CBAxB6-TGFβ mice manifested glomerular matrix accumulation as soon as by 5 days of age (Figure 1F) and 50% of these mice succumbed to uremic death by 21 days of age. Our study provides evidence that genetic background strongly influences the pro-fibrotic effect of TGF-β1 in this model. We observed similar genetic differences in the progression of kidney disease using two classical models of renal fibrosis, the unilateral ureter obstruction (Figure 6) and subtotal renal ablation (Figure 7).

The analysis of fibrosis-related molecules in CBAxB6-TGFβ mouse kidneys revealed several-fold increases in mRNA expression of *Tgfb1* and interstitial collagens as early as at 14 days of age, compared to B6-TGFβ mice. The observed strain differences were not related to altered expression of the natural inhibitors of TGF-β_1[20, 21] or to TGF-β_1 signaling molecules[22].

TGF-β1 influences the extracellular matrix turnover by repressing matrix metalloproteinases (MMPs) and inducing their tissue inhibitors (TIMPs)[23]. MMP2 cleaves fibronectin and collagen-1 and −3[24], while MMP9 cleaves collagen-4 with higher affinity[25]. Among their inhibitors, TIMP-1, -2 and -3 are expressed in the kidney[25]. Analysis of TIMPs in TGFβ1-transgenic mice revealed a striking TIMP-1 over-expression in CBAxB6-TGFβ kidneys as compared to B6-TGFβ, mainly localized in tubules, that resulted in reduced MMP9

gelatinase activity. Additionally, the transcription factor EGR2 was significantly induced and correlated to TIMP-1 in CBAxB6-TGFβ kidneys. EGR2 has been described as a key mediator of TGF-β action in mouse models of scleroderma and lung fibrosis and in human scleroderma[18]. Of note, Vollmann and colleagues found that *in vivo EGR2* gene silencing reduces liver fibrosis in mice[26]. To the best of our knowledge, the present study is the first to link EGR2 to renal fibrosis progression. As several pathways (including inflammation) were upregulated in the microarray study, we might also consider them as possible contributors in the pathogenesis. We focused on EGR2 as the most upregulated transcription factor and TIMP-1 as the most over-expressed participant of ECM remodeling. The importance of TIMP-1 in kidney fibrosis has been postulated, as elevated TIMP-1 levels have been reported in human diabetic nephropathy[27] or glomerulonephritis[28] and in experimental murine lupus glomerulonephritis[29], protein overload proteinuria[30] and obstructive uropathy[31]. However, this raises the question of whether the TIMP-1 over-expression in CBAxB6-TGFβ mice is a component of the excessive scarring, or the upregulated TIMP-1 has a causal role in the development of fibrosis in this TGF-β1-transgenic model.

Therefore, we analyzed mice at the age of 5 days, when CBAxB6-TGFβ1 kidneys exhibited only mild glomerular hypertrophy and in approximately 30% of glomeruli, slight mesangial expansion. Even at this early age, EGR2 and TIMP-1 were significantly upregulated in CBAxB6-TGFβ mice, as compared to B6-TGFβ (Figure 4D,E, Supplementary Figure S5), accompanied by elevated renal *Tgfb1* mRNA, but not collagen overexpression. The comparable renal *Egr2* and *Timp1* mRNA expression in newborn TGFβ1-transgenic mice indicates that the differences in renal fibrosis progression were not a consequence of increased perinatal EGR2 or TIMP-1 expression. Taken together, these results demonstrate that early induction of renal EGR2 and TIMP-1 in CBAxB6-TGFβ mice precedes the over-production of ECM components, and the TIMP-1 driven inhibition of MMP9 activity contributes to ECM accumulation. Administration of neutralizing TIMP-1 antibody ameliorated the potent profibrotic effect of TGFβ1 in CBAxB6-TGFβ kidneys, and reduced fibrosis, proteinuria and serum urea levels (Figure 5), supporting the important role of TIMP-1 in promoting renal scarring.

Transgenic mouse models often raise the question whether the observations are unique consequences of the transgene, acting in mouse context itself, or might lead to broader understanding of human pathophysiology. Unilateral ureter obstruction (UUO) and subtotal renal ablation (SNX) are widely used experimental models of kidney fibrosis. UUO causes tubulointerstitial damage that usually leads to fibrosis within 7 days[19], while SNX causes hyperfiltration in the remnant glomeruli and leads to glomerulosclerosis with concomitant interstitial fibrosis within usually 15–20 weeks[32]. Based on the results of our TGF-β1-transgenic mice, we investigated how genetic background influences the early events of fibrosis within 24 hours and 6 weeks in UUO and SNX models, respectively. Both UUO and SNX performed on wild type B6 and CBA mice lead to early renal EGR2 and TIMP-1 induction in the susceptible CBA strain (Figures 6 and 7). Thus, genetic susceptibility to kidney fibrosis was strongly associated with EGR2 and TIMP-1 not only in the TGF-β1-transgenic model, but also after UUO or SNX. Our comparison of Timp1-null and Timp1+ control male mice subjected to SNX further supports the central role of TIMP-1 in the

pathogenesis of renal fibrosis (Figure 8). Interestingly, similar fibrosis rate was previously reported in TIMP-1 deficient and control mice 14 days after UUO[33], but that study used a different, 129Sv mouse strain. Moreover, the very late (14 days) evaluation time point in that study is partly in line with our observation that despite the early differences at 24h after UUO in B6 and CBA males, basically similar fibrosis developed at later stage (7 days), although the MMP9 gelatinase activity was still higher in B6 UUO than CBA UUO even at 7 days. To overcome the limitations of UUO model due to hydronephrosis we performed the SNX models as well, that resembles human kidney fibrosis development more closely than UUO.

Additionally, over-expressing EGR2 *in vitro* induced TIMP1 protein of HEK293 cells, and TIMP1 was correlated to EGR2 (Figure 9), while *EGR2* silencing in HK-2 cells inhibited the TGF-β1 induced EGR2 and TIMP1 over-production, resulting in higher MMP9 activity. Moreover, *Egr2* silencing *in vivo* markedly reduced the UUO induced EGR2 and TIMP1 overexpression in mice. Of note, expression in kidney biopsies from FSGS patients supported our *in vitro* and *in vivo* results. These data suggest a novel role for the transcription factor EGR2 in renal fibrogenesis.

In conclusion, our studies demonstrate that genetic background has a significant impact on the effect of TGF-β1, a potent pro-fibrotic cytokine. Genetic background influences early renal EGR2 and TIMP-1 expression that regulates MMP9 activity, which associates with resistance (B6 background) or susceptibility (CBA background) to fibrosis progression in mice, suggesting an important role for EGR2 and TIMP-1 in the initiation of fibrosis (Figure 10). Still, further studies are needed to clarify how genetic background of mice influences the early renal EGR2 response to injury (eg. via altered miRNA or lncRNA) and whether EGR2 has direct or indirect effect on TIMP1.

Data availability statement.

Microarray data of this study are openly available in repository (NCBI Gene Expression Omnibus, GSE54441) at URL: https://www.ncbi.nlm.nih.gov/geo.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Keith DS, Nichols GA, Gullion CM, et al. Longitudinal follow-up and outcomes among a population with chronic kidney disease in a large managed care organization. Arch Intern Med 2004; 164: 659–663. [PubMed: 15037495]
2. August P, Suthanthiran M. Transforming growth factor beta and progression of renal disease. Kidney Int Suppl 2003: S99–104. [PubMed: 14531781]
3. Khan R Examining potential therapies targeting myocardial fibrosis through the inhibition of transforming growth factor-beta 1. Cardiology 2007; 108: 368–380. [PubMed: 17308385]
4. Murray LA, Chen Q, Kramer MS, et al. TGF-beta driven lung fibrosis is macrophage dependent and blocked by Serum amyloid P. Int J Biochem Cell Biol 43: 154–162. [PubMed: 21044893]
5. Kokeny G, Nemeth Z, Godo M, et al. The Rowett rat strain is resistant to renal fibrosis. Nephrol Dial Transplant 25: 1458–1462.
6. Grond J, Beukers JY, Schilthuis MS, et al. Analysis of renal structural and functional features in two rat strains with a different susceptibility to glomerular sclerosis. Lab Invest 1986; 54: 77–83. [PubMed: 3941543]
7. Grond J, Muller EW, van Goor H, et al. Differences in puromycin aminonucleoside nephrosis in two rat strains. Kidney Int 1988; 33: 524–529. [PubMed: 3361753]
8. Chen JS, Chen A, Chang LC, et al. Mouse model of membranous nephropathy induced by cationic bovine serum albumin: antigen dose-response relations and strain differences. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association 2004; 19: 2721–2728. [PubMed: 15385633]
9. Ishola DA, Jr., van der Giezen DM, Hahnel B, et al. In mice, proteinuria and renal inflammatory responses to albumin overload are strain-dependent. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association 2006; 21: 591–597. [PubMed: 16326737]
10. Ma LJ, Fogo AB. Model of robust induction of glomerulosclerosis in mice: importance of genetic background. Kidney Int 2003; 64: 350–355. [PubMed: 12787428]
11. Salzler HR, Griffiths R, Ruiz P, et al. Hypertension and albuminuria in chronic kidney disease mapped to a mouse chromosome 11 locus. Kidney Int 2007; 72: 1226–1232. [PubMed: 17851470]
12. Gurley SB, Clare SE, Snow KP, et al. Impact of genetic background on nephropathy in diabetic mice. Am J Physiol Renal Physiol 2006; 290: F214–222. [PubMed: 16118394]
13. Rumberger B, Vonend O, Kreutz C, et al. cDNA microarray analysis of adaptive changes after renal ablation in a sclerosis-resistant mouse strain. Kidney Blood Press Res 2007; 30: 377–387. [PubMed: 17890868]
14. Sanderson N, Factor V, Nagy P, et al. Hepatic expression of mature transforming growth factor beta 1 in transgenic mice results in multiple tissue lesions. Proc Natl Acad Sci U S A 1995; 92: 2572–2576. [PubMed: 7708687]
15. Mozes MM, Bottinger EP, Jacot TA, et al. Renal expression of fibrotic matrix proteins and of transforming growth factor-beta (TGF-beta) isoforms in TGF-beta transgenic mice. J Am Soc Nephrol 1999; 10: 271–280. [PubMed: 10215326]
16. Truett GE, Heeger P, Mynatt RL, et al. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). BioTechniques 2000; 29: 52, 54. [PubMed: 10907076]
17. Piecha G, Kokeny G, Nakagawa K, et al. Calcimimetic R-568 or calcitriol: equally beneficial on progression of renal damage in subtotally nephrectomized rats. Am J Physiol Renal Physiol 2008; 294: F748–757. [PubMed: 18199601]
18. Fang F, Ooka K, Bhattacharyya S, et al. The early growth response gene Egr2 (Alias Krox20) is a novel transcriptional target of transforming growth factor-beta that is upregulated in systemic sclerosis and mediates profibrotic responses. Am J Pathol 2011; 178: 2077–2090. [PubMed: 21514423]

19. Klahr S, Morrissey J. Obstructive nephropathy and renal fibrosis. Am J Physiol Renal Physiol 2002; 283: F861–875. [PubMed: 12372761]
20. Border WA, Noble NA, Yamamoto T, et al. Natural inhibitor of transforming growth factor-beta protects against scarring in experimental kidney disease. Nature 1992; 360: 361–364. [PubMed: 1280332]
21. Hildebrand A, Romaris M, Rasmussen LM, et al. Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta. Biochem J 1994; 302 (Pt 2): 527–534. [PubMed: 8093006]
22. Poncelet AC, de Caestecker MP, Schnaper HW. The transforming growth factor-beta/SMAD signaling pathway is present and functional in human mesangial cells. Kidney Int 1999; 56: 1354–1365. [PubMed: 10504488]
23. Seeland U, Haeuseler C, Hinrichs R, et al. Myocardial fibrosis in transforming growth factor-beta(1) (TGF-beta(1)) transgenic mice is associated with inhibition of interstitial collagenase. Eur J Clin Invest 2002; 32: 295–303. [PubMed: 12027867]
24. Allan JA, Docherty AJ, Barker PJ, et al. Binding of gelatinases A and B to type-I collagen and other matrix components. Biochem J 1995; 309 (Pt 1): 299–306. [PubMed: 7619071]
25. Olson MW, Toth M, Gervasi DC, et al. High affinity binding of latent matrix metalloproteinase-9 to the alpha2(IV) chain of collagen IV. The Journal of biological chemistry 1998; 273: 10672–10681. [PubMed: 9553130]
26. Vollmann EH, Cao L, Amatucci A, et al. Identification of Novel Fibrosis Modifiers by In Vivo siRNA Silencing. Molecular therapy Nucleic acids 2017; 7: 314–323. [PubMed: 28624207]
27. Suzuki D, Miyazaki M, Jinde K, et al. In situ hybridization studies of matrix metalloproteinase-3, tissue inhibitor of metalloproteinase-1 and type IV collagen in diabetic nephropathy. Kidney Int 1997; 52: 111–119. [PubMed: 9211353]
28. Sanders JS, van Goor H, Hanemaaijer R, et al. Renal expression of matrix metalloproteinases in human ANCA-associated glomerulonephritis. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association 2004; 19: 1412–1419. [PubMed: 15034162]
29. Nakamura T, Ebihara I, Tomino Y, et al. Effect of a specific endothelin A receptor antagonist on murine lupus nephritis. Kidney Int 1995; 47: 481–489. [PubMed: 7723234]
30. Eddy AA. Molecular insights into renal interstitial fibrosis. J Am Soc Nephrol 1996; 7: 2495–2508. [PubMed: 8989727]
31. Sharma AK, Mauer SM, Kim Y, et al. Altered expression of matrix metalloproteinase-2, TIMP, and TIMP-2 in obstructive nephropathy. J Lab Clin Med 1995; 125: 754–761. [PubMed: 7769370]
32. Shimamura T, Morrison AB. A progressive glomerulosclerosis occurring in partial five-sixths nephrectomized rats. Am J Pathol 1975; 79: 95–106. [PubMed: 164779]
33. Kim H, Oda T, Lopez-Guisa J, et al. TIMP-1 deficiency does not attenuate interstitial fibrosis in obstructive nephropathy. J Am Soc Nephrol 2001; 12: 736–748. [PubMed: 11274235]

Translational Statement

Patients with chronic kidney disease, regardless of etiology, develop renal fibrosis, but they manifest varying rates of progressive loss of kidney function. Moreover, ongoing kidney fibrosis is a major therapeutic challenge in nephrology. Differences in genetic susceptibility may contribute to differences in progression rates, driven by as yet unknown molecular mechanisms. In the present study, we addressed the question whether genetic background affects the development and progression of kidney disease in several mouse models of renal fibrosis. We found that renal fibrosis progression is strongly associated with mouse genetic background. This genetic susceptibility involves very early and marked overproduction of TIMP-1 that associated with EGR2 *in vivo* and *in vitro*. Further, we observed similar expression patterns in human renal biopsies manifesting FSGS. These results may help to clarify the initial molecular mechanisms of kidney fibrosis and might lead to identification of early diagnostic markers and the development of pharmacological inhibitors to slow, halt, or even reverse the fibrotic process.

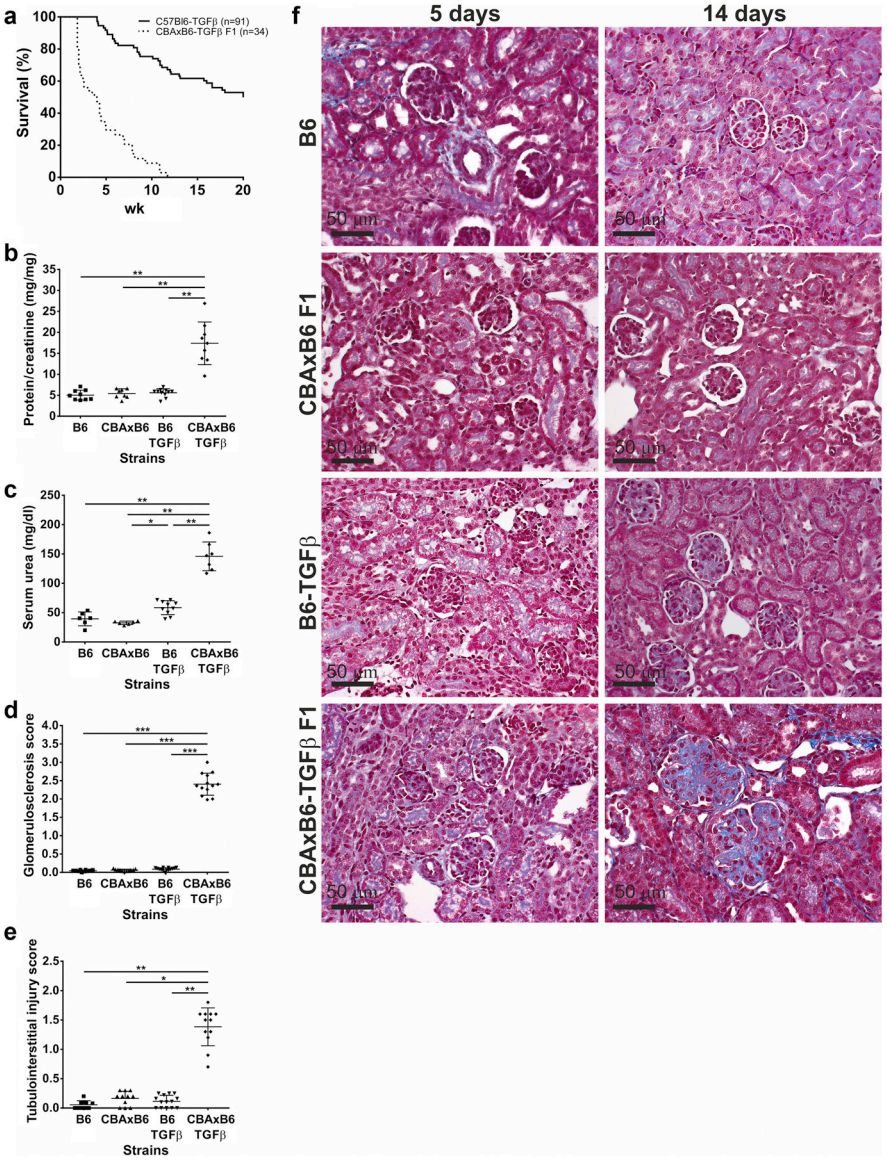

Figure 1. Characterization of TGF-β_1 transgenic mice and controls.

Survival (a) of CBAxB6-TGFβ mice (n=34) (…) was dramatically shorter as compared to B6-TGFβ mice (n=91) (▬) (Kaplan-Meier analysis, p<0.0001). Urine protein/creatinine ratio (b, n=8–11/group) and serum urea levels (c, n=6–10/group) were only elevated in CBAxB6-TGFβ F1 mice. Histology scores (n=10–14/group) for glomerulosclerosis (d) and tubulointerstitial damage (e) show significant fibrosis with mesangial expansion in CBAxB6-TGFβ F1 mice, as compared to B6-TGFβ and wild type mice. The histopathology (f) is shown on representative photomicrographs of paraffin embedded kidney sections comparing TGF-β_1 transgenic and wild type control mice at the ages of 5 days (left panel) and 14 days (right panel). B6-TGFβ kidneys had no histological alterations in any time points as compared to wild type controls. CBAxB6-TGFβ F1 kidneys depicted mild enlargement and sclerosis in some glomeruli at the age of 5 days, but severe glomerular hypertrophy and sclerosis at the age of 14 days (Masson's trichrome stain. 400x

magnification; bar represents 50 μm). Data are presented as mean ± SD. Kruskal-Wallis test, * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

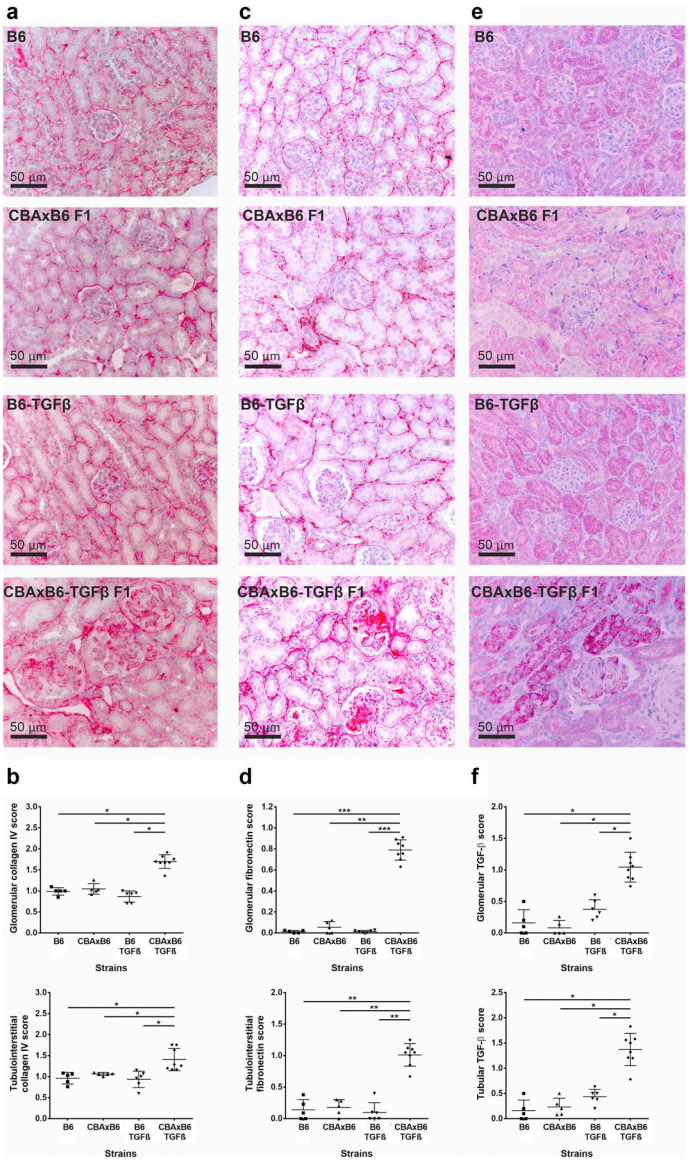

Figure 2. Renal expression of TGF-β and extracellular matrix proteins.

Evaluation of renal Type-IV collagen, fibronectin and TGF-β_1 immunostaining in TGF-β transgenic mice and wild type controls at the age of 14 days. (a) Representative photomicrographs of Type IV collagen expression depict marked increase in CBAxB6-TGFβ kidneys, both in glomeruli and tubulointerstitium (b). The similar pattern in renal fibronectin (c, d) and TGF-β_1 (e, f) immunostaining scores further underscore the marked fibrosis in CBAxB6-TGFβ kidneys. (a, c, e) 400x magnification; bar represents 50 μm. Data are presented as mean ± SD (n=6–8/group). Kruskal-Wallis test, * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

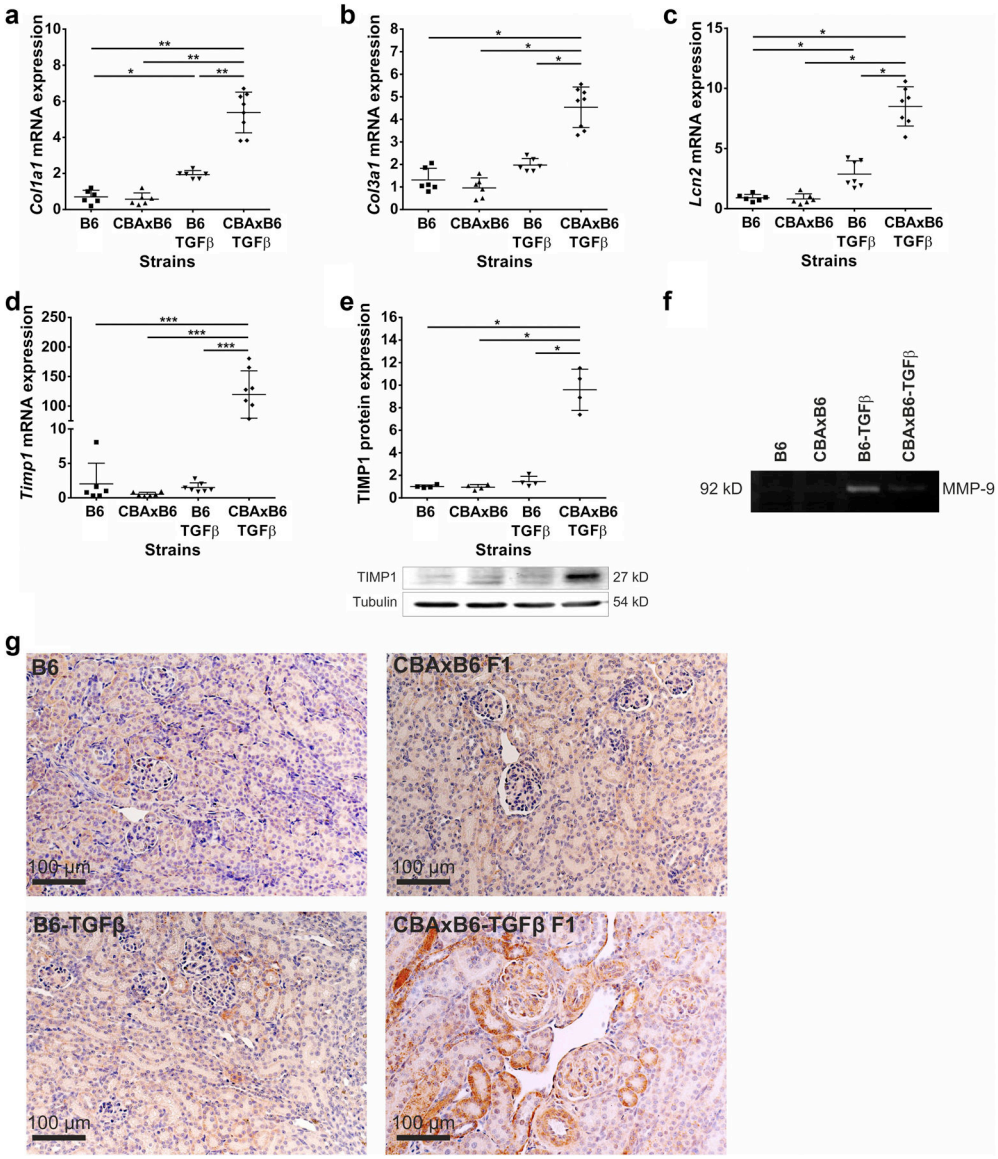

Figure 3. Renal expression of mRNA encoding extracellular matrix and related proteins.

Renal cortical expression of type I (a) and type III (b) collagen, Lipocalin-2 (*Lcn2*, c) and TIMP-1 mRNA (d) in wild type and TGF-β_1 transgenic mouse strains at the age of 14 days (n=6–8/ group). (e) TIMP1 immunoblot analysis (n=4/group) depicted 10-fold increase in CBAxB6-TGFβ kidneys which was mainly localized to tubules (g) by immunostaining (200x magnification, bar represents 100 μm) and resulted in reduced renal MMP9 gelatinase activity as shown by representative zymography (f). All data are presented as fold expression to control sample (mean ± SD). Kruskal-Wallis test, * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

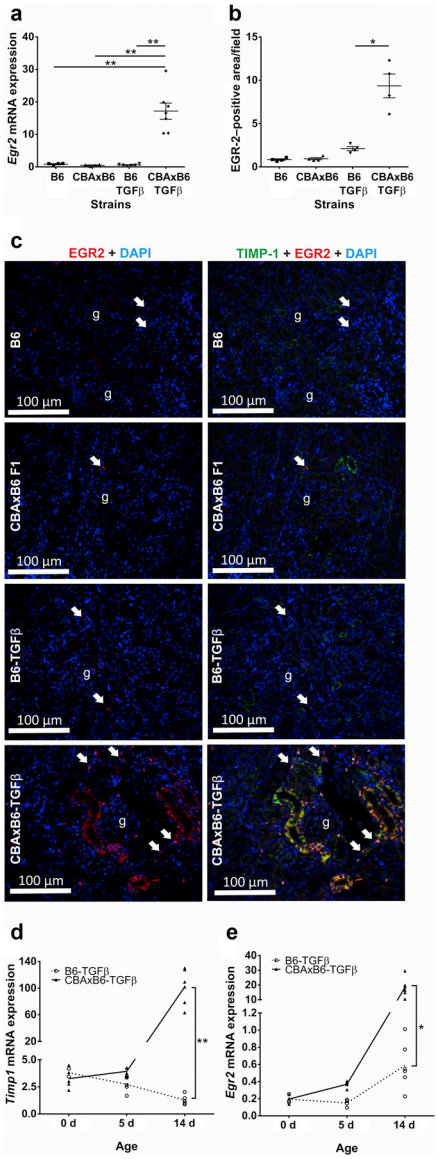

Figure 4. Renal EGR2 expression in wild type and TGF-β_1 transgenic mouse strains.

At 14 days of age, EGR2 increased markedly only in CBAxB6-TGFβ kidneys both at mRNA (a) and protein level (b). Representative photomicrographs of immunostaining (c) depicted EGR2 (red) mostly in the tubulointerstitium of control and B6-TGFβ kidneys (see arrows) with minimal tubular TIMP1 positivity (green). Apart of increased interstitial and to less extent, glomerular staining, EGR2 appeared also in tubules of CBAxB6-TGFβ mice where it mostly co-localized with TIMP1 (yellow;400x magnification, bar represents 50 µm). The kinetics of both *Timp1* (d) and *Egr2* (e) in kidneys of B6-TGFβ and CBAxB6-TGFβ F1 transgenic mice at the age of 0 (n=3/group), 5 (n=5/group) and 14 days (n=6/group) show early overexpression only in CBAxB6-TGFβ F1 mice. Data are presented as fold expression to control sample (mean ± SD, n=4–7/group). Kruskal-Wallis test, * $p<0.05$; ** $p<0.01$.

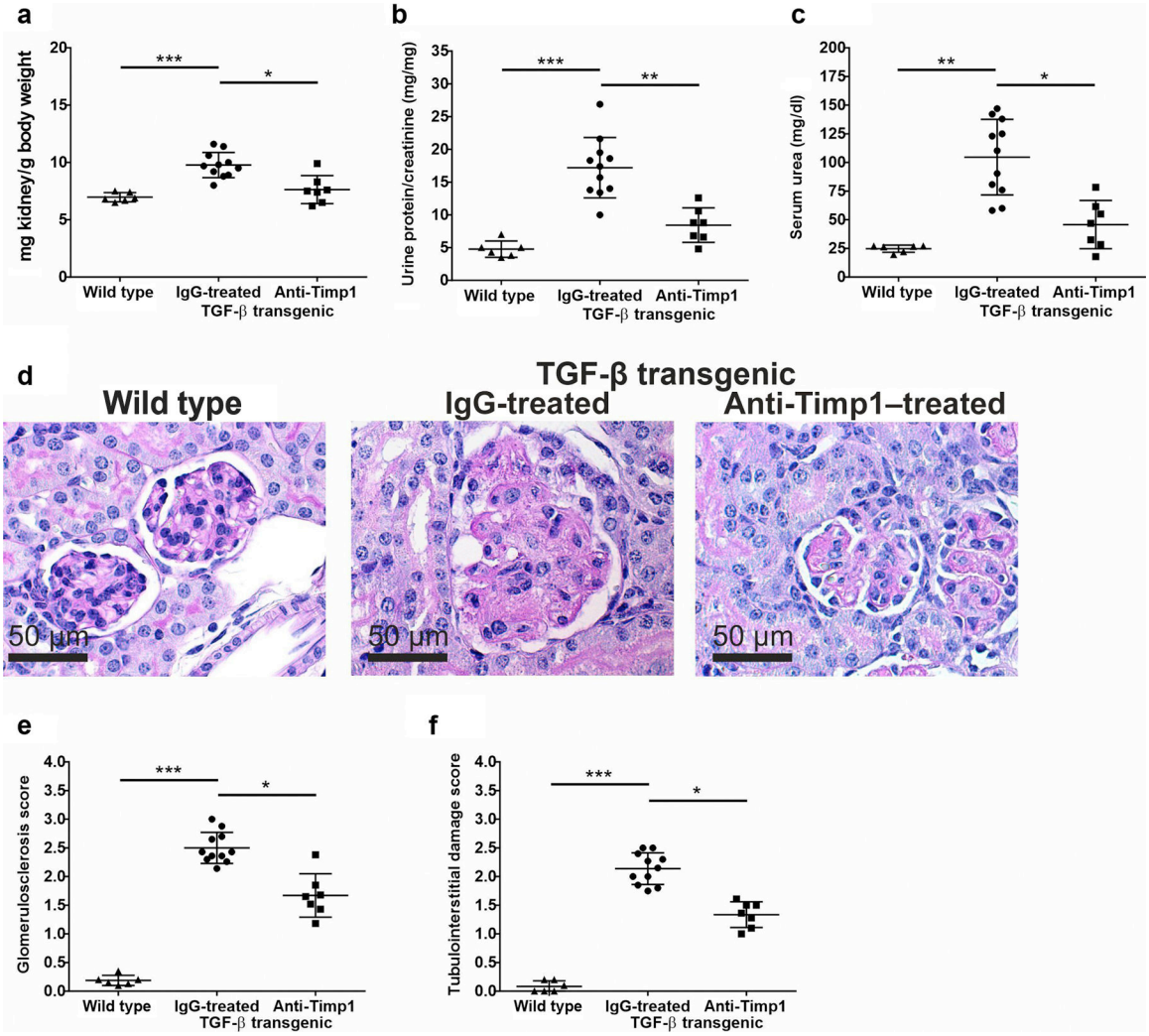

Figure 5. Effects of TIMP-1 neutralization in fibrotic CBAxB6-TGFβ mice.

TIMP-1 neutralization for 5 consecutive days significantly reduced renal hypertrophy (a), proteinuria (b), and serum urea levels (c) in CBAxB6-TGFβ F1 mice. Administration of anti-TIMP-1 antibody ameliorated glomerulosclerosis (d, e) and tubulointerstitial fibrosis (d, f), as compared to isotype IgG treated CBAxB6-TGFβ F1 mice. PAS staining, 400x magnification. Data are presented as mean ± SD for CBAxB6 F1 (wild type, n=5), IgG treated CBAxB6-TGFβ F1 (IgG treated TGF-β transgenic, n=12) and anti-TIMP-1 treated CBAxB6-TGFβ F1 mice (anti-Timp1 TGF-β transgenic, n=7). *p<0.05, ** p<0.01, *** p<0.001 (Kruskal-Wallis test and ANOVA).

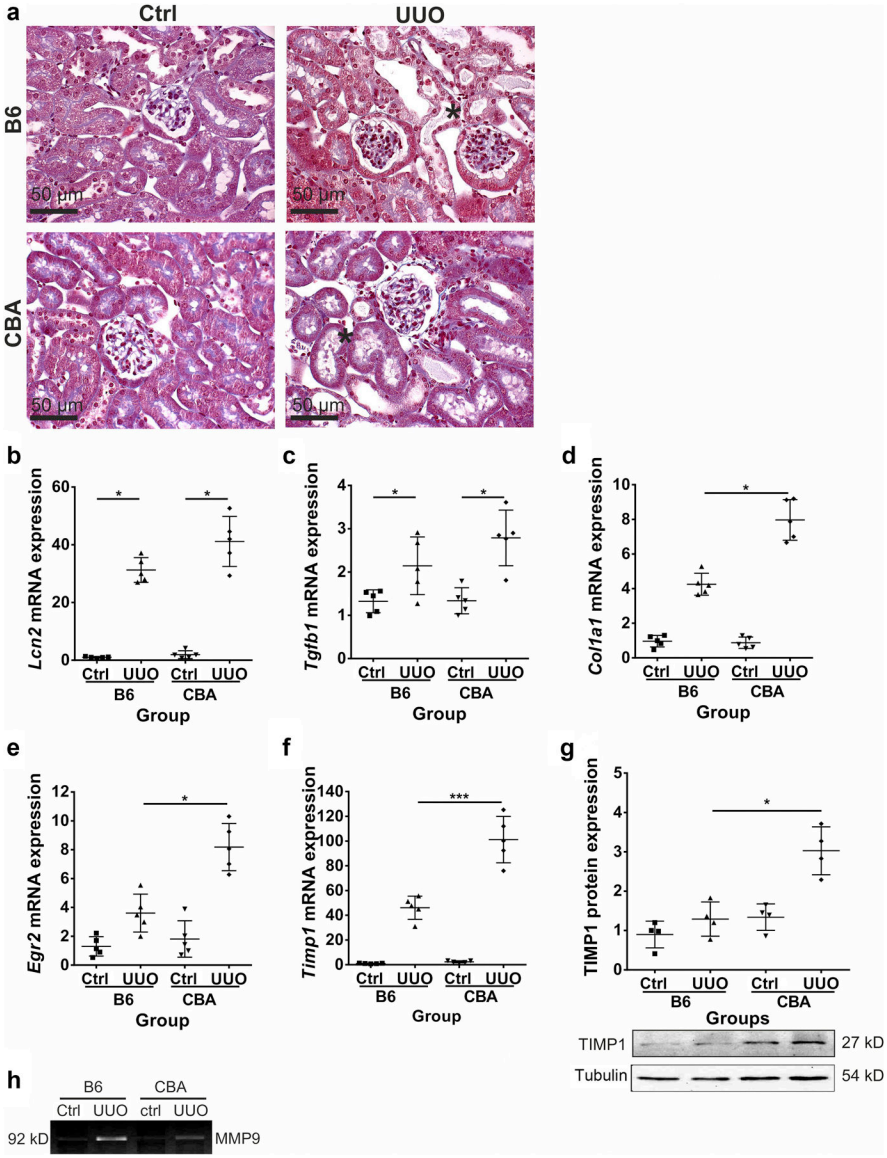

Figure 6. Strain-related early differences in *Egr2* and *Timp1* after unilateral ureter obstruction.

After unilateral ureter obstruction (UUO) in both B6 and CBA mice, we observed similar extent of tubular dilation within 24 hours (a, asterisks show dilated tubules). Renal mRNA expression of lipocalin-2 *(Lcn2)* (b) and TGF-β *(Tgfb1)* (c) were similarly elevated in both B6 and CBA UUO mice. However, both renal Type-I collagen *(Col1a1)* (d), *Egr2* (e) and *Timp1* (f) mRNA expressions (n=5/group) were significantly higher in CBA UUO as compared to B6 UUO kidneys, accompanied by already 2-fold TIMP1 protein overexpression (n=4/group) in CBA UUO (g) at this early stage. The increased TIMP1 reduced MMP9 activity in CBA UUO as compared to B6 UUO as shown by representative gelatin zymography (h). All data are presented as fold expression to control sample (mean ± SD). Kruskal-Wallis test, * $p<0.05$; *** $p<0.001$.

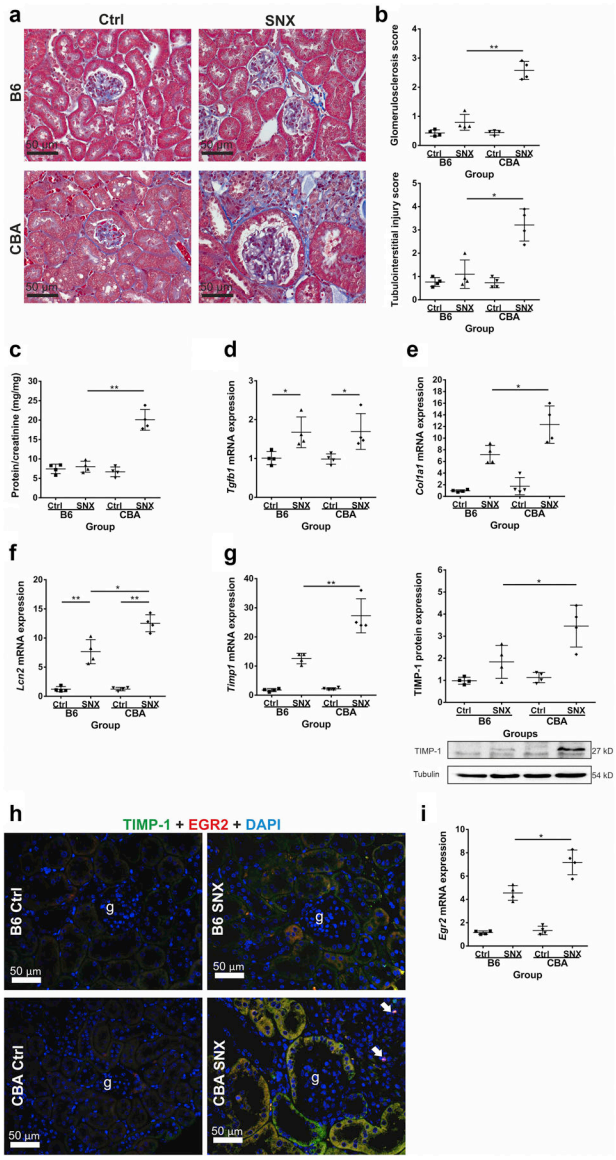

Figure 7. Strain-related differences in *Egr2* and *Timp1* after subtotal renal ablation.

Six weeks after subtotal renal ablation (SNX) we observed only mild glomerular scarring and tubulointerstitial fibrosis in B6 mice (a, b), but severe glomerulosclerosis and interstitial fibrosis in CBA mice (a, b). CBA SNX mice developed marked proteinuria (c) despite the TGF-β mRNA *(Tgfb1)* expression was comparable to B6 SNX (d). Kidneys of CBA SNX mice depicted overexpression of type-I collagen mRNA *(Col1a1)* (e) and lipocalin-2 *(Lcn2)* mRNA (f) as well as significant TIMP-1 mRNA *(Timp1)* and protein overexpression (g) accompanied by *Egr2* mRNA (i) as compared to B6 SNX or controls. Immunostaining (h) depicted significant co-localization of tubular TIMP1 and EGR2 in CBA SNX kidneys and increased number of interstitial EGR2 positive cells (arrows; EGR2: red, TIMP1: green, EGR2+TIMP1: yellow, DAPI:blue, nuclear stain; scale bar: 50 µm). All data are presented as fold expression to control sample (mean ± SD, n=4/group). Kruskal-Wallis test, * $p<0.05$; ** $p<0.01$.

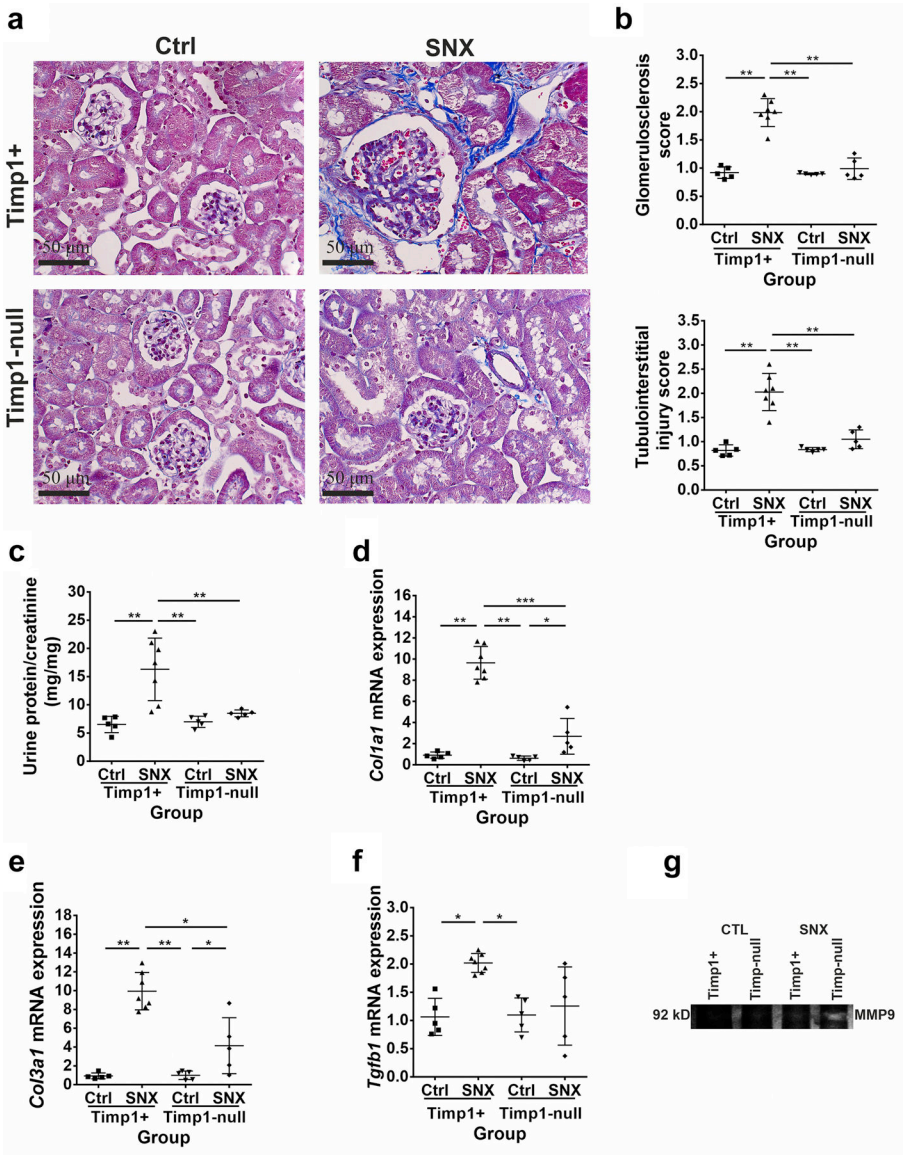

Figure 8. Subtotal renal ablation (SNX) in Timp1-null mice.

Subtotal renal ablation (SNX) in Timp1-null male mice on the fibrosis-prone CBAxB6 F1 genetic background induced only minor glomerular or tubulointerstitial changes (a, b), as compared to significant glomerular scarring and interstitial fibrosis in Timp1+ control CBAxB6 F1 mice (a, b). Timp1-null SNX mice did not develop proteinuria (c) and only mild overexpression of type-I *(Col1a1)* and type-III collagen *(Col3a1)* mRNA overexpression (d, e) as compared to the Timp1+ SNX control mice with 2-fold higher proteinuria and 3-fold higher renal collagen overexpression. Interestingly, renal TGF-β_1 mRNA *(Tgfb1)* expression was similar to controls in Timp1-null SNX kidneys (f), despite the 2-fold overexpression in wild type SNX kidneys. Representative photo of MMP9 gelatinase activity shows only visible band in Timp1-null SNX (g). All data are presented as fold expression to control sample (mean ± SD, n=5–7/group). Masson's trichrome stain, scale bar=50 μm. Kruskal-Wallis test, * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

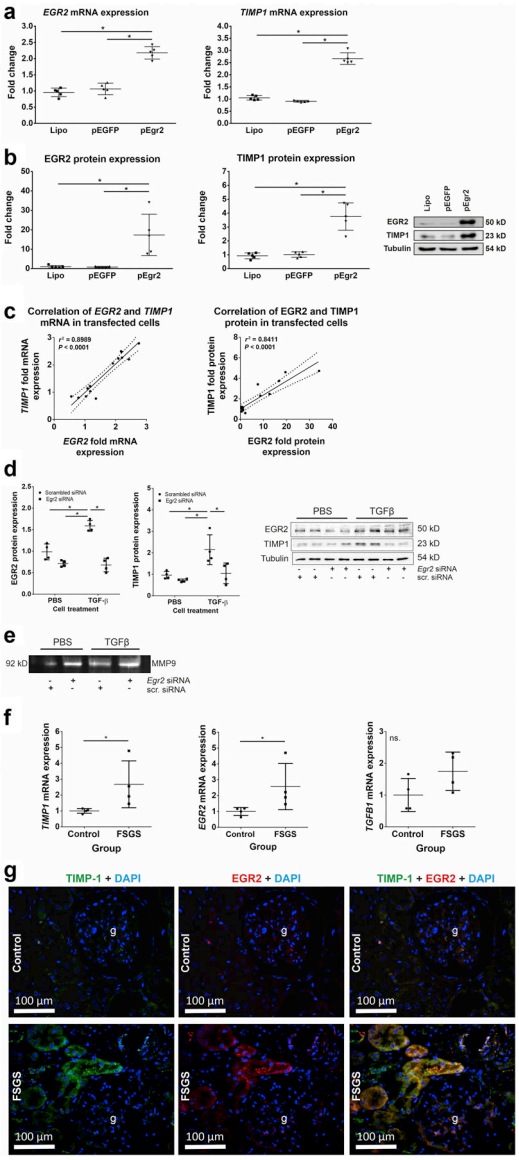

Figure 9. The effect of EGR2 overexpression in cultured HEK293 cells and EGR2 expression in human FSGS.

HEK293 cells were transiently transfected with pCMV plasmid containing full length *Egr2* gene (pEGR2, n=5) or *EGFP* (pEGFP, n=5), while non-transfected negative controls were treated with Lipofectamine (Lipo, n=5). Transfection for 24h with pEGR2 increased the expression of *EGR2* mRNA and *TIMP1* mRNA (a), also EGR2 and TIMP1 protein (b). Representative immunoblots for EGR2 and TIMP1 are also shown. Furthermore, EGR2 correlated tightly with TIMP-1 (c) both at mRNA level (n=15) and protein level (n=12). In HK-2 cells, transfection with *EGR2* siRNA 1 days prior to TGF-β treatment basically inhibited the TGF-β induced EGR2 and TIMP1 production as compared to scrambled siRNA-treated cells (d). This reduced TIMP1 resulted in elevetad MMP9 gelatinase activiy as shown by representative zymogram performed from conditioned cell media (e). Additionally, kidney cortex biopsies of FSGS patients (n=4) were compared to control kidney samples (extracted from healthy cortex area of nephrectomized tissues,

due to renal cancer, n=4). FSGS samples showed significantly higher *EGR2* and *TIMP1* mRNA expression as compared to controls but only tendentially increased *TGFB1* mRNA (f). Immunostaining of FSGS biopsies (g) revealed a marked tubular TIMP1 expression (green) as compared to control samples, accompanied by increased interstitial and tubular EGR2 expression (red) that largely co-localized with TIMP1 (yellow). (g=glomerulus, scale bar=100 μm). All data are presented as fold expression to control sample (mean ± SD), Kruskal-Wallis or Mann-Whitney test were performed, and two-way ANOVA for gene silencing study, * $p<0.05$).

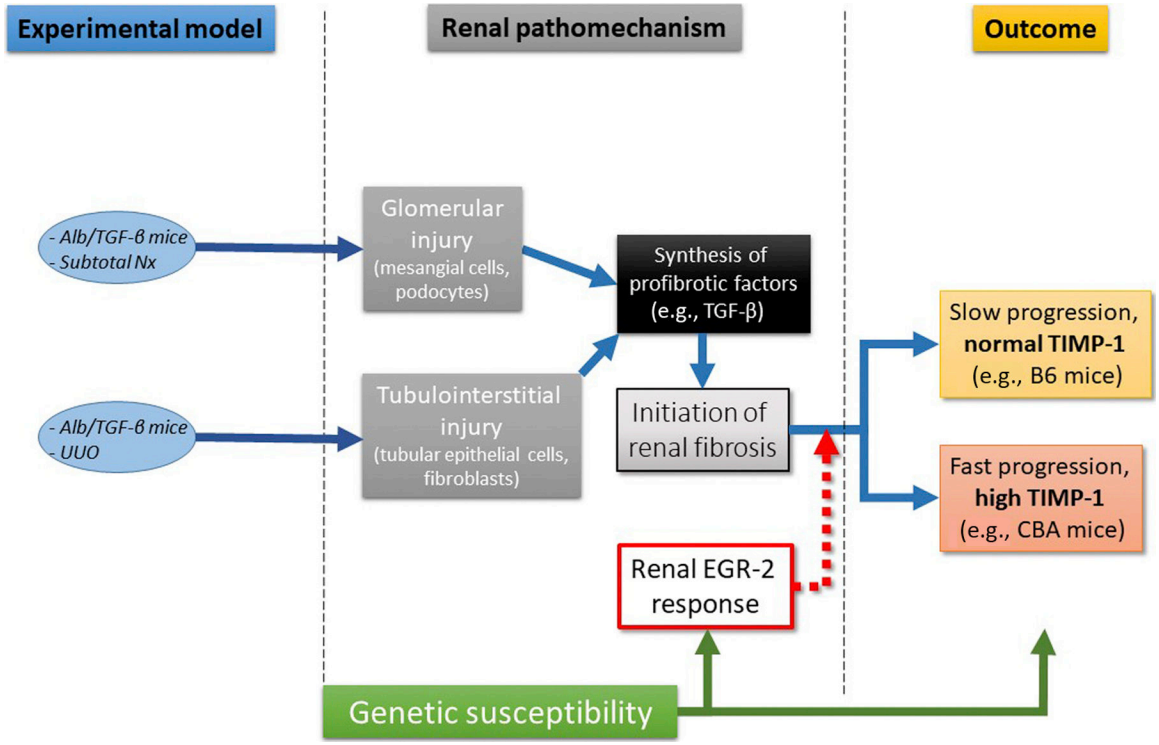

Figure 10. Proposed effect of genetically determined renal fibrosis progression in mice.

In our experiments, we confirmed the importance of genetically determined renal TIMP-1 response in experimental models of both glomerular and tubulointerstitial injury. The elevated circulating TGF-β_1 levels of Alb/TGF-β_1 transgenic mice induce glomerulosclerosis and tubulointerstitial fibrosis. Subtotal nephrectomy (Nx) leads to glomerular hyperfiltration in the remnant nephrons, inducing glomerulosclerosis. Unilateral ureter obstruction (UUO) causes tubular injury and dilatation, mainly leading to interstitial fibrosis. Regardless of glomerular or tubulointerstitial injury, the consecutive renal synthesis of TGF-β_1 and other profibrotic factors initiate the kidney scarring processes. We propose that the genetically determined early EGR2 response in the kidneys influences the progression rate of the initiated fibrosis. The outcome will be either slow progression due to normal or mildly elevated EGR2 and TIMP-1 levels (*e.g.* in fibrosis resistant B6 mice) or alternatively, fast fibrosis progression due to high EGR2 and TIMP-1 expression in the kidneys (*e.g.* in fibrosis prone CBA mice) influenced by genetic susceptibility. Therefore, this renal EGR2-TIMP-1 response might play a major role in the outcome of fibrosis.

Table 1.

Pathology and laboratory data of transgenic B6-TGFβ and CBAxB6-TGFβ F1 mice and wild-type controls, at the age of 14 days.

	Body weight (g)	Kidney weight / body weight (mg / g)	Plasma TGF-β_1 (ng / mL)	Urea nitrogen (mg / dl)	Proteinuria (mg protein / mg creatinine)
B6 (n=10)	7.25 ± 0.62	7.60 ± 1.03	4.04 ± 2.01	36.5 ± 10.6	5.03 ± 1.18
CBAxB6 F1 (n=8)	8.45 ± 0.95^{a}	7.40 ± 0.79	3.10 ± 1.76	34.8 ± 6.4	5.41 ± 1.11
B6-TGFβ (n=14)	7.21 ± 0.87^{c}	7.14 ± 0.55	57.61 ± 16.77^{ac}	58.5 ± 11.9^{ac}	5.58 ± 0.97
CBAxB6-TGFβ F1 (n=15)	8.54 ± 1.06^{ab}	9.94 ± 1.03^{abc}	44.49 ± 19.15^{ac}	152.0 ± 31.8^{abc}	15.20 ± 6.66^{abc}
ANOVA / Kruskal-Wallis	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.005

Legend. Data from male TGF-β1 transgenic mice and male wild type controls at the age of 14 days are shown. Despite similar plasma TGFβ$_1$ levels to B6-TGFβ mice, CBAxB6-TGFβ mice had significant renal hypertrophy, accompanied by elevated serum urea nitrogen levels and proteinuria. Values are expressed as mean ± SD. ANOVA or Kruskal-Wallis test,

^{a}p<0.05 vs B6;

^{b}p<0.05 vs B6-TGFβ;

^{c}p<0.05 vs CBAxB6 F1.

Table 2.

Renal expression of fibrosis pathway genes in transgenic B6-TGFβ and CBAxB6-TGFβ F1 mice at 14 days of age.

Gene symbol	B6 (n=5)	CBAxB6 F1 (n=5)	B6-TGFβ (n=7)	CBAxB6-TGFβ F1 (n=7)	Kruskal-Wallis test
Tgfb1	1.00 ± 0.18	1.05 ± 0.12	0.80 ± 0.17	1.61 ± 0.42^{abc}	p<0.0001
Ctgf	1.00 ± 0.19	0.69 ± 0.13	0.29 ± 0.06	1.88 ± 0.56^{abc}	p<0.0001
Bgn (biglycan)	1.00 ± 0.39	0.37 ± 0.06	1.01 ± 0.23	1.78 ± 0.46^{abc}	p<0.0001
Dcn (decorin)	1.00 ± 0.28	0.43 ± 0.13	1.09 ± 0.27^{c}	1.84 ± 0.43^{abc}	p<0.0001
Mmp2	1.00 ± 0.29	0.40 ± 0.21	0.97 ± 0.19	0.91 ± 0.38	n.s.
Mmp9	1.00 ± 0.33	3.24 ± 0.57^{a}	0.88 ± 0.30^{c}	4.06 ± 1.04^{ab}	p<0.0001
Timp1	1.00 ± 0.53	0.52 ± 0.28	1.39 ± 0.45	108.48 ± 28.57^{abc}	p<0.0001
Timp2	1.00 ± 0.20	0.61 ± 0.08	0.98 ± 0.09	1.53± 0.11^{c}	p<0.05
Timp3	1.00 ± 0.21	1.31 ± 0.24	1.16 ± 0.07	1.35 ± 0.39	n.s.
Tgbr2	1.00 ± 0.13	0.91 ± 0.15	1.03 ± 0.34	1.15 ± 0.16	n.s.
Smad2	1.00 ± 0.34	0.72 ± 0.18	0.86 ± 0.14	0.95 ± 0.39	n.s.
Smad3	1.00 ± 0.08	0.81 ± 0.19	0.89 ± 0.12	1.03 ± 0.23	n.s.
Smad4	1.00 ± 0.28	0.85 ± 0.22	0.97 ± 0.12	0.91 ± 0.24	n.s.
Smad6	1.00 ± 0.47	0.95 ± 0.39	1.12 ± 0.19	1.10 ± 0.60	n.s.
Smad7	1.00 ± 0.10	0.70 ± 0.17	0.86 ± 0.11	1.07 ± 0.24	n.s.

Legend. Renal mRNA expression values of male TGF-β1 transgenic mice and male wild type controls at the age of 14 days. Expression of each gene was normalized to 18S rRNA *(Rn18s)* using the $2^{-\Delta\Delta Ct}$ formula. Data are presented as fold expression to a pooled control sample (mean ± SD).

^{a}p<0.05 vs B6;

^{b}p<0.05 vs B6-TGFβ;

^{c}p<0.05 vs CBAxB6 F1.

Table 3.

Renal expression of fibrosis-related genes in transgenic B6-TGFβ and CBAxB6-TGFβ F1 mice at 5 days of age.

Gene	B6 (n=4)	CBAxB6 F1 (n=4)	B6-TGFβ (n=6)	CBAxB6-TGFβ F1 (n=6)	Kruskal-Wallis test
Tgfb1	1.00±0.25	1.09±0.06	0.83±0.15	1.34±0.32^{abc}	p<0.05
Ctgf	1.00±0.06	1.14±0.23	1.01±0.32	1.18±0.07	n.s.
Col1a	1.00±0.36	0.85±0.14	1.08±0.30	1.01±0.37	n.s.
Col3a1	1.00±0.25	0.78±0.14	1.07±0.45	1.13±0.29	n.s.
Mmp2	1.00±0.11	0.89±0.14	0.90±0.10	1.75±0.90^{abc}	p<0.05
Mmp9	1.00±0.35	0.92±0.21	1.06±0.69	1.27±0.51	n.s.
Timp1	1.00± 0.53	1.25±0.50	1.02±0.44	1.94±0.62^{abc}	p<0.05
Egr2	1.00± 0.26	0.94±0.31	0.99±0.27	2.20±0.42^{abc}	p<0.001

Legend. Renal mRNA expression values of fibrosis-related genes of male TGF-β$_1$ transgenic mice and male wild type controls at the age 5 days. Of note, *Timp1* and *Egr2* expression were already elevated in CBAxB6-TGFβ mice at this very early age. Gene expression was normalized to 18S rRNA *(Rn18s)* using the $2^{-\Delta\Delta Ct}$ formula. Data are presented as fold expression to a pooled control sample (mean ± SD).

^{a}p<0.05 vs B6;

^{b}p<0.05 vs B6-TGFβ;

^{c}p<0.05 vs CBAxB6 F1.