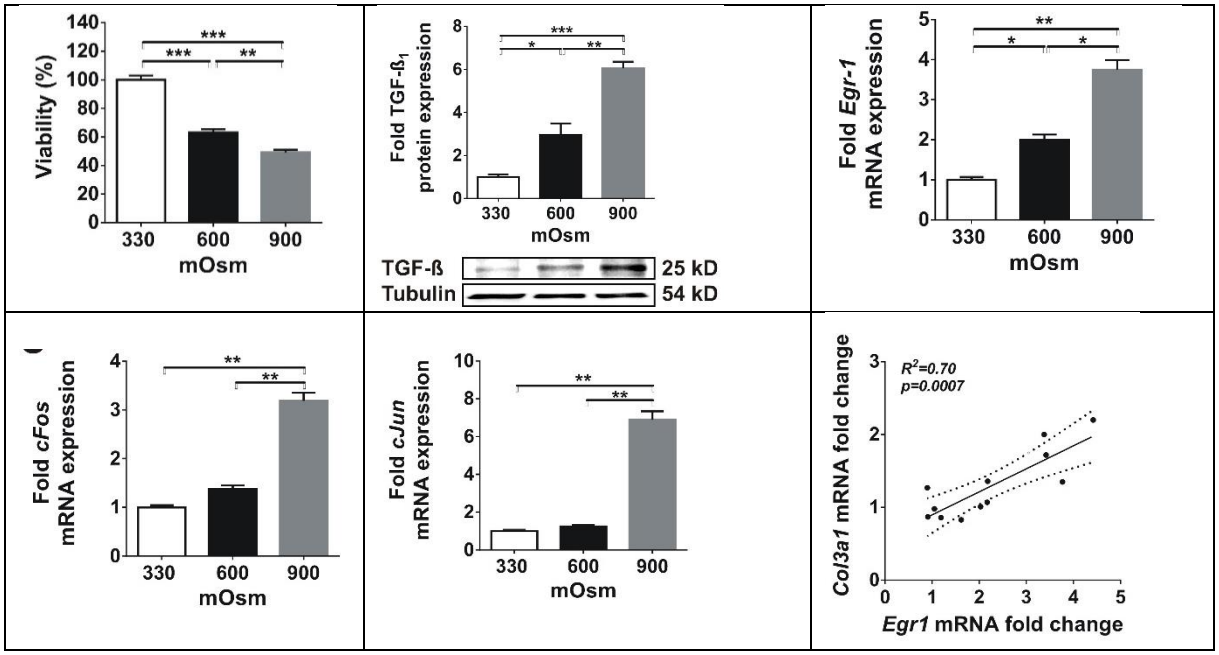
MicroRNA regulation of TIMP-1 expression in progressive renal fibrosis

Final report

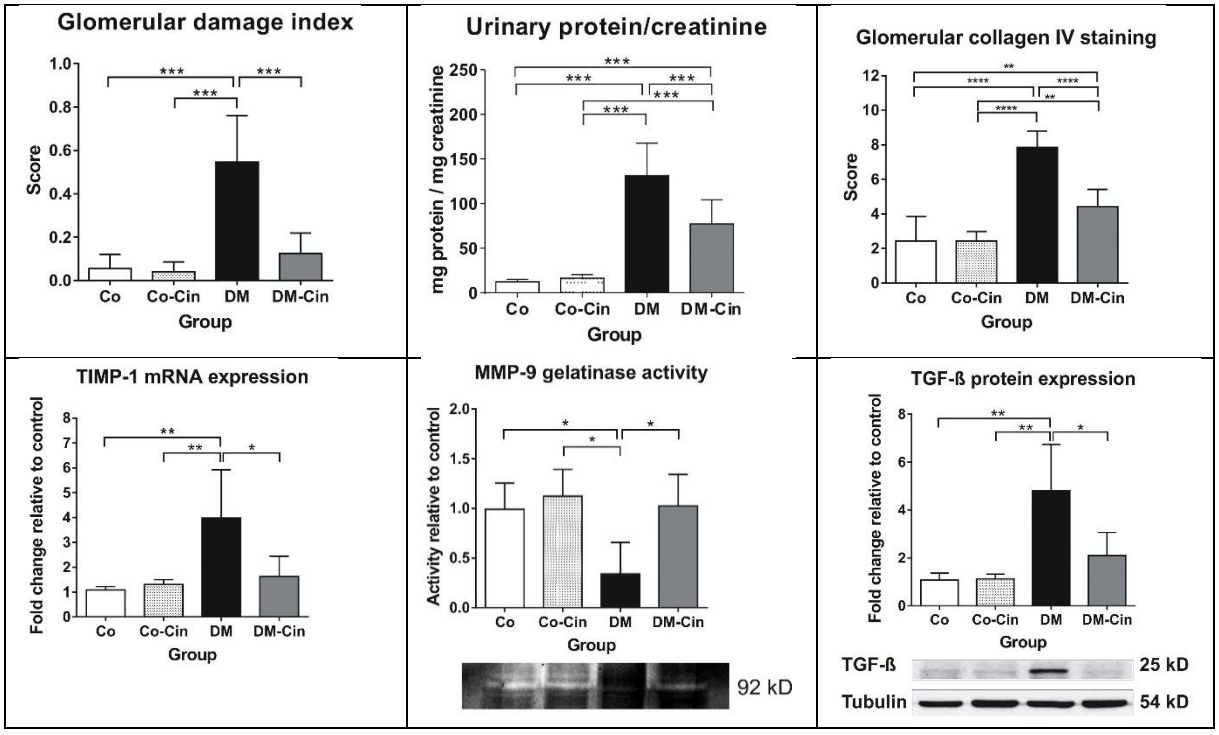
Transforming growth factor-beta (TGF-ß) is one of the major profibrotic cytokines, involved in many diseases with extracellular matrix overproduction and cell proliferation. During fibrosis, the dynamic balance of extracellular matrix protein (ECM) synthesis and degradation is disrupted. TGF-β can affect the extracellular matrix homeostasis directly at the gene expression level, or via inducible transcription factors. The balance between matrix metalloproteinases (MMPs) and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), is definitive in the remodeling of extracellular matrix. TGF-ß is known to induce TIMP-1 transcription through the AP-1 binding site of the TIMP-1 promoter. AP-1 (activating protein-1) is a group of transcription factor subunits (cJun, cFos) that bind to the AP-1 binding site on target DNA. Among the several transcription factors identified to play important role in tissue fibrosis, EGR-1 and EGR-2 are early response factors that can directly induce collagen production, thus enhancing the effect of TGF-ß.

Moreover, both TGF-ß and EGR-1 play an important role in response to acute changes of renal medullary osmolarity, but their role under sustained hypo- or hyperosmolar conditions has not been elucidated. Using immortalized murine inner medullary collecting duct cell culture (mIMCD), we gradually increased the osmolarity from 330 mOsm to 900 mOsm over 6 days, and analyzed gene and protein expressions at 330, 600 and 900 mOsm. In this study, hyperosmolarity led to gradually decreased cell viability and overexpression of both TGF-ß and EGR-1 mRNA (2.5-fold and 3.5-fold increase from 330 to 900 mOsm, respectively ($p < 0.05$)), which was accompanied by significant cFos, cJun ($p < 0.01$), and type III and type IV collagen overexpression. These results indicate that chronic medullary hyperosmolarity might induce or worsen ongoing fibrotic remodeling in the kidneys. We published these results in the Open Access journal *BMC Nephrology* ***18:****(1) Paper 209. 11 p. (2017).*



Renal TGF-ß and TIMP-1 expressions are known to increase in diabetic kidneys as well. The altered production of endothelial nitric oxide (NO) and decreased glomerular cyclic 3′,5′ guanosine monophosphate (cGMP) levels are suggested to account for the expansive glomerular pathology in diabetes. The intact NO-cGMP axis has antifibrotic properties, as reduces TGF-ß and extracellular matrix production. NO activates the soluble guanylate cyclase (sGC), which converts guanosine triphosphate (GTP) to cGMP, and then cGMP mediates the biological functions of NO. Therefore, we investigated the renal molecular actions of the sGC activator cinaciguat in streptozotocin-

induced type-1 diabetes. In our study, cinaciguat attenuated the diabetes induced glomerulosclerosis and proteinuria, and renal collagen-IV expression. This was accompanied by 50% reduction of TIMP-1 expression and increased MMP-9 activity, with significantly attenuated TGF-ß overexpression. Our data indicate that sGC activators could be possible adjuvants in the treatment of diabetic nephropathy. These results were published as Open Access in *SCIENTIFIC REPORTS 7:(1) Paper 11218. 15 p. (2017).*

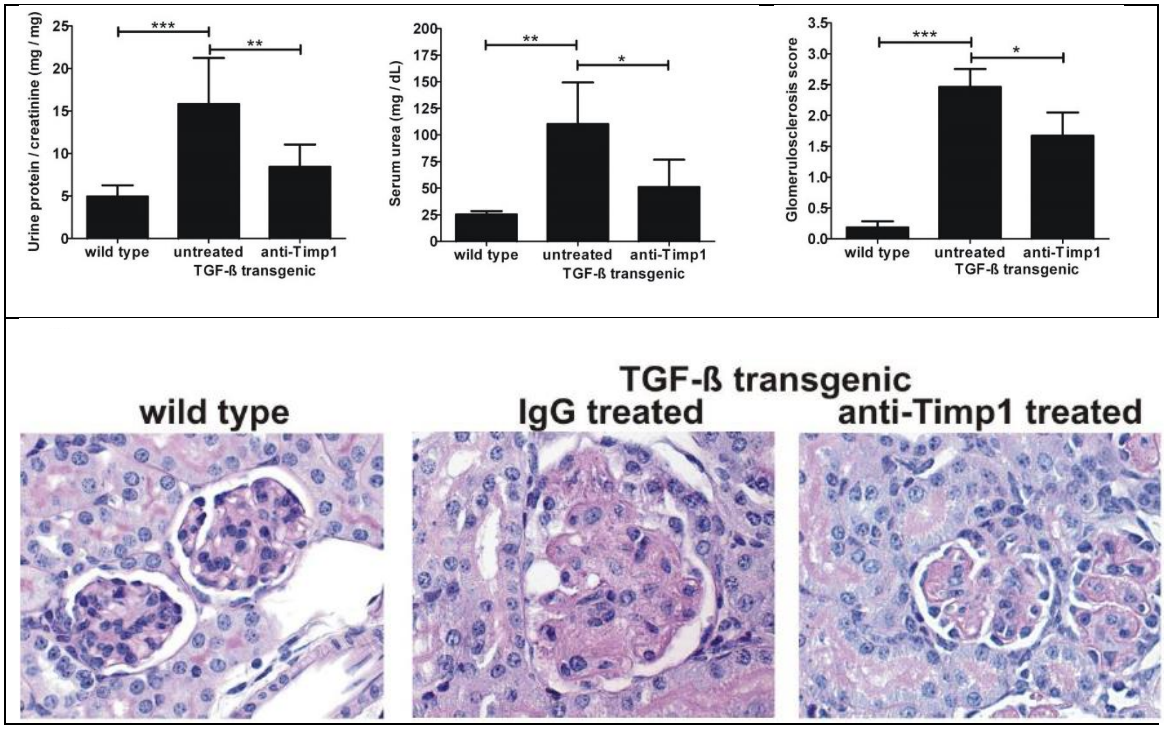


Patients with renal diseases such as diabetic or hypertensive nephropathy show different progression rate, presumably due to genetic susceptibility. Several lines of evidence suggest strain-dependent differences in the development and progression of experimental renal diseases. Mouse experiments indicate that C57Bl/6 (B6) strain is resistant to renal fibrosis compared to other inbred mouse strains. Yet, the molecular mechanism has not been elucidated.

In our mouse studies we have first showed that renal TIMP-1 overexpression is associated with genetic susceptibility to renal fibrosis. First, we used the TGF-ß transgenic mouse model, and generated TGF-ß transgenic mice on B6 and CBAxB6 genetic background. At the beginning of mouse experiments and according to the workplan, we first had to establish successful breeding colonies for B6 and CBA mice. Unfortunately, as they are bad breeders, the CBA colony establishment took 4 more months as compared to the B6 colony, what caused a significant delay in our mouse studies. To overcome the delay, in the meantime we were able to finish the above mentioned experiments on TGF-β and hyperosmolarity, as well as the investigation of renal sGC and TIMP-1 association in diabetes.

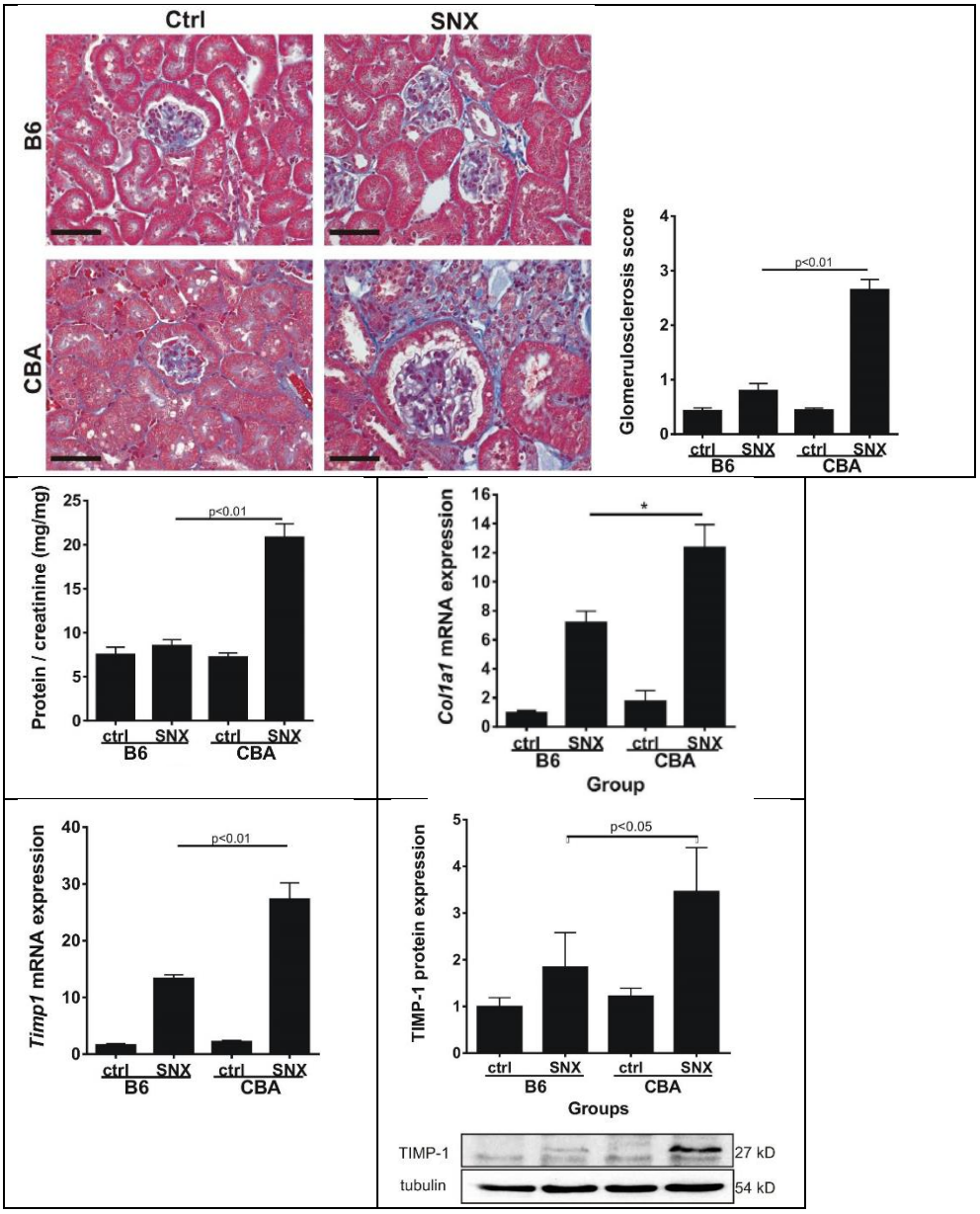
CBAxB6-TGFß mice succumb to uremic death within 21 days post partum, showing dramatic renal TIMP-1 overexpression and grade-IV glomerulosclerosis with massive proteinuria at the age of 14 days. Therefore, we treated CBAxB6-TGFß mice daily with intraperitoneal injections of 300 ug/kg goat anti-mouse TIMP-1 neutralizing antibody dissolved in PBS (anti-Timp group) for 5 consecutive days, starting at the age of 8 days. Control pups were the same breed, injected with goat isotype IgG only (untreated group, 300ug/kg IgG in PBS). TIMP-1 neutralization resulted in proteinuria levels reduced by 50% accompanied by reduction of serum urea levels. Accordingly, we observed less glomerulosclerosis in TIMP-1 neutralized mice than in controls. Among the investigated matrix

molecules, collagen-4 mRNA expression was reduced in anti-Timp kidneys (untreated: 1.3±0.1, anti-Timp: 0.8±0.3, $p<0.05$).

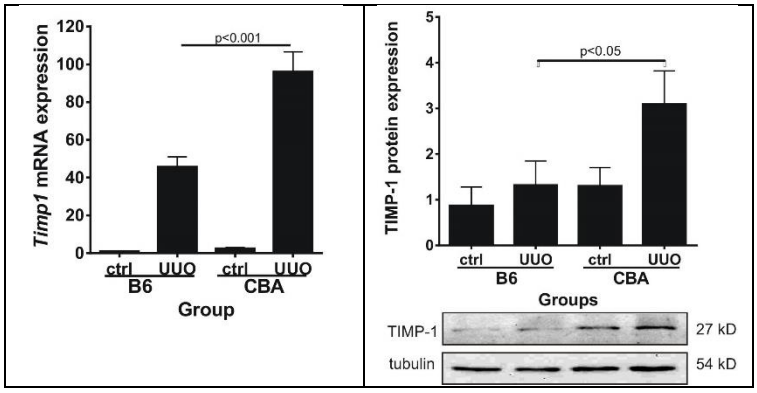


In order to further confirm the central role of TIMP-1 in the progression of TGF-ß induced renal fibrosis, we injected TIMP-1 knockout mice (8 days old) on fibrosis prone CBAxB6 background (TIMP-KO) with human recombinant hTGF-ß dissolved in 0.1% BSA at 2 ug/day or 0.1% BSA and as a positive control, we also injected CBA wild type mice for 5 consecutive days. TGF-ß treated TIMP-1 KO mice had significantly reduced glomerulosclerosis as compared to the wild type mice (TIMP-KO+BSA: 0.1±0.1; TIMP-KO+TGFß: 0.2±0.1; CBA+TGFß: 2.2±0.3), accompanied by reduced type-I collagen (TIMP-KO+BSA: 1.0±0.1; TIMP-KO+TGFß: 2.7±0.4; CBA+TGFß: 3.3±0.5) and lipocalin-2 expressions (Lcn2 or NGAL, marker or tubular damage: TIMP-KO+BSA: 1.0±0.1; TIMP-KO+TGFß: 0.8±0.2; CBA+TGFß: 1.5±0.4)) in the absence of TIMP-1.

We also wanted to confirm the central role of early TIMP-1 overexpression in genetic susceptibility to renal fibrosis, therefore we investigated early renal changes after renal ablation (SNX) and unilateral ureter obstruction (UUO). SNX induces glomerular hyperfiltration and glomerulosclerosis usually within 12 weeks after surgery. We performed SNX on wild type B6 and CBA mice (n=5/group), and kidneys were analyzed 6 weeks after surgery, to investigate earlier changes. Both proteinuria and glomerulosclerosis were significantly higher in CBA mice as compared to B6, accompanied by dramatically elevated TIMP-1 and type I collagen expression (see figures below). Similarly, the mRNA expression of EGR-2 was also significantly higher in CBA mice (B6 SNX: 0.9±0.3 vs CBA SNX: 4.8±1.3, $p<0.05$).

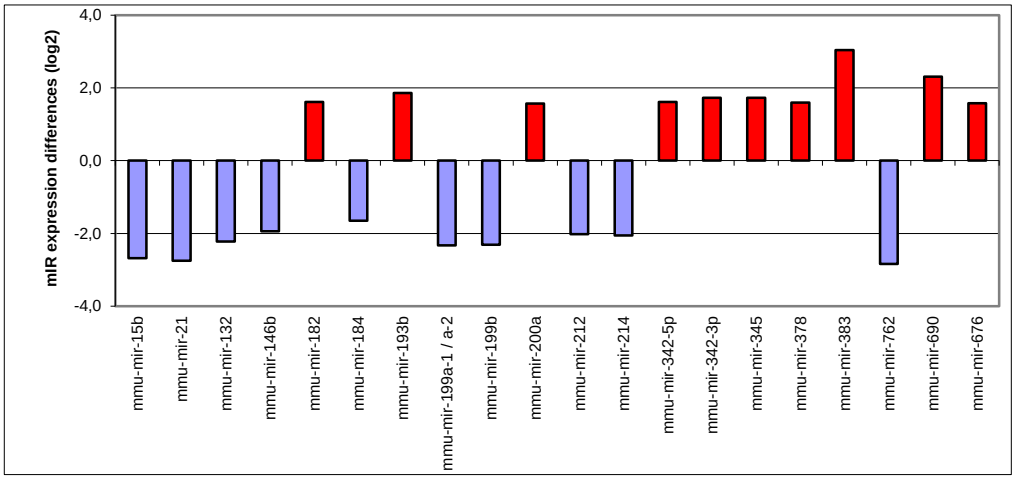


Tubulointerstitial fibrosis usually develop 3-5 days after unilateral ureter obstruction (UUO). Therefore, we investigated early (at 24 hours) and very early changes (at 6 hours) after UUO performed on male B6 and CBA mice. Both at 6 hours and 24 hours after surgery TIMP-1 expression was significantly higher in CBA mice. This was accompanied by EGR-2 overexpression at both 6h (B6 CTL 6h: 1.0±0.3; CBA CTL 6h: 2.1±1.2; B6 UUO 6h: 8.9±2.6; CBA UUO 6h: 30.1±9.2) and 24h time-points (EGR-2 B6 CTL 24h: 1.0±0.3; CBA CTL 24h: 8.3±1.2; B6 UUO 24h: 14.1±3.1; CBA UUO 24h: 18.1±4.9). We have also observed marked and very early increase in renal Lcn2 mRNA expression in both strains, but it was 5-fold higher in CBA UUO (B6 UUO 6h: 9.0±4.9; CBA UUO 6h: 45.7±19.2). Interestingly, B6 UUO kidneys at 6h did not show CTGF overexpression, but CBA had almost 2-fold increase (B6 CTL 6h: 1.0±0.7; CBA CTL 6h: 1.2±0.1; B6 UUO 6h: 1.1±0.3; CBA UUO 6h: 1.9±0.8).



Apart of EGR-2, we have observed a dramatic early increase in gene expression of transcription factors EGR-1 and RUNX1. At 6 hours post occlusion, EGR-1 expression in CBA UUO kidneys was 3-fold higher than in B6 UUO kidneys (B6 UUO 6h: 9.4±0.3; CBA UUO 6h: 27.6±2.5), showing augmented difference than at 24 hours (B6 UUO 24h: 7.3±2.1; CBA UUO 24h: 11.2±3.2). As compared to B6, the expression of RUNX1 was 4-fold higher in CBA UUO kidneys at 6h (B6 UUO 6h: 2.2±0.9; CBA UUO 6h: 8.9±6.7) and 2-fold increased at 24h (B6 UUO 24h: 6.4±3.3; CBA UUO 24h: 12.9±2.1). We found a strong correlation between the renal expression of EGR-2 and TIMP-1 in both SNX (R-square: 0.73, p=0.0007) and UUO (R-square: 0.79, p<0.0001) experiments.

We have also postulated that genetic differences might lead to altered microRNA (miRNA) regulation of profibrotic genes. Therefore, we also performed miRNA microarray experiment using Affymetrix miRNA 1.0 array on B6-TGFß and CBAxB6-TGFß kidneys (n=3/group). In our samples, 19 miRNAs were statistically significant and the expression difference exceeded 1.5-fold change (log2). The significantly upregulated miRNAs (showing log2 fold-change (FC)) in B6-TGFß kidneys were: miR-383 (FC: 3.04), miR-690 (FC: 2.3), miR-193b (FC: 1.86), miR-345 (FC: 1.72), miR-342-5p (FC: 1.6), miR-182 (FC: 1.6), miR-378 (FC: 1.6), mIR-676 (FC: 1.58) and miR-200a (FC: 1.57). The significantly downregulated miRNAs were: miR-762 (FC: 2.8), miR-21 (FC: 2.7), miR-15b (FC: 2.6), miR-199a (FC: 2.3), miR-199b (FC: 2.3), miR-132 (FC: 2.2), miR-214 (FC: 2.1), miR-212 (FC: 2.0), miR-146 (FC: 1.9) and miR-184 (FC: 1.6) (see figure below).

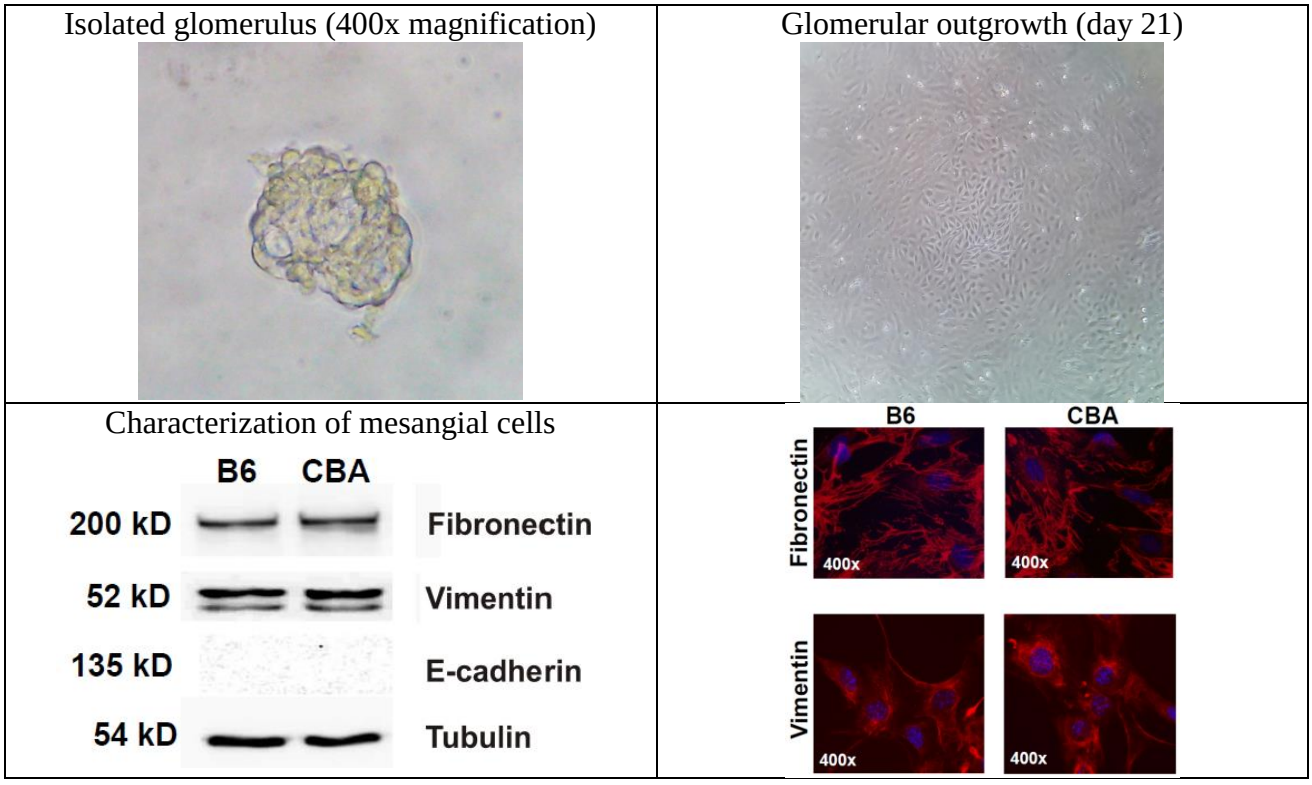


Among these miRNAs, only miR-21 has been associated with renal fibrosis, and it has been also shown that miR-21 might influence TIMP-1 expression. Based on miRNA database searches (miRDB, ebi.ca.uk, cm.jefferson.edu), and Ingenuity Pathway Analysis of our previous cDNA microarray results with the miRNA microarray by our collaborator Andrew Oler at NIH, Bethesda, mIR-200a and miR-199 has been predicted to inhibit TIMP-1. Therefore, we verified the array data by qPCR focusing on miR-200, mIR-199 using Exiqon mIRCURY LNA assays. According to the array, miR-199a expression was lower in B6-TGFß kidneys (B6-TGFß: 1.2±0.2, CBAxB6-TGFß: 2.1±0.3). In contrast

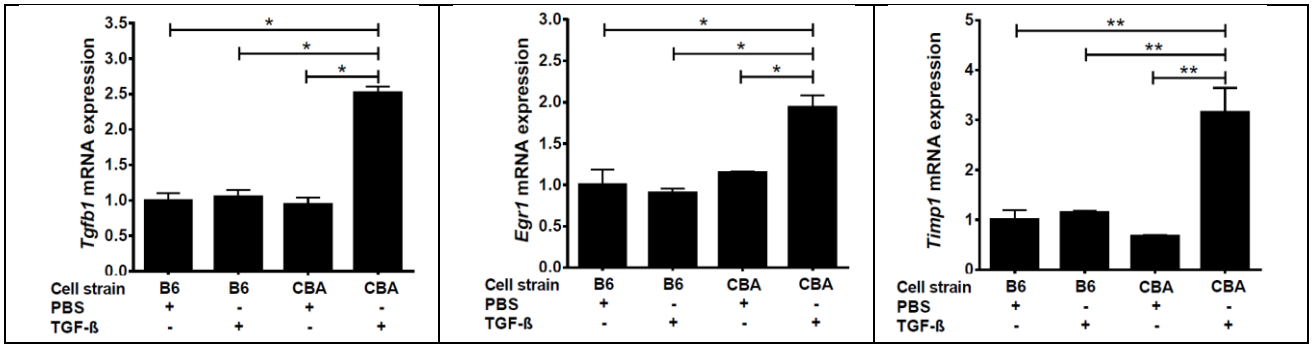
to the array, miR-200a was upregulated in CBAxB6-TGFß kidneys (B6-TGFß: 0.7±0.4, CBAxB6-TGFß: 1.1±0.3).

We have also analyzed possible miRNA expression differences in early stages of fibrosis development after UUO. Six hours after surgery, we observed 1.5-fold and 1.4-fold expression of miR-199 (B6 UUO 6h: 0.9±0.2 vs CBA UUO 6h: 1.5±0.3) and miR-200 (B6 UUO 6h: 1.0±0.2 vs CBA UUO 6h: 1.4±0.1) in CBA UUO vs B6 UUO kidneys, respectively. Interestingly, these differences disappeared at 24 hours after surgery for both miR-199 (B6 UUO 24h: 0.9±0.4 vs CBA UUO 24h: 0.7±0.2) and miR-200 (B6 UUO 24h: 0.8±0.2 vs CBA UUO 24h: 0.6±0.3). However, miR-342 expression was 9-fold higher in CBA UUO vs B6 UUO kidneys at 6 hours after surgery (B6 UUO 6h: 0.7±0.3 vs CBA UUO 6h: 7.4±1.2).

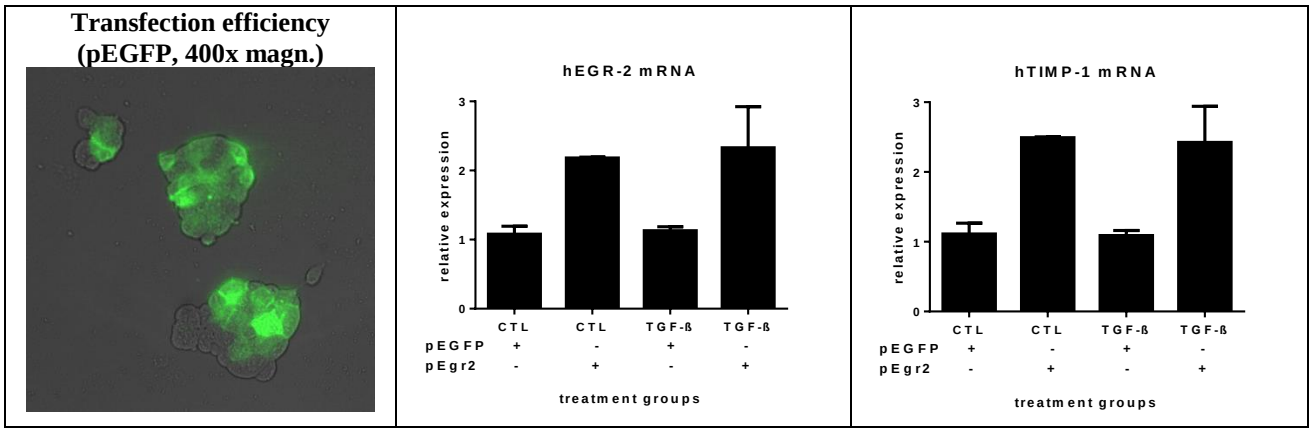
We also wished to investigate the strain differences of TGF-ß induced TIMP-1 response at cellular level, using primary mesangial cells isolated from B6 and CBA mice. First, the mice were perfused with cold sterile PBS containing magnetic bead particles (Dynabeads), kidneys were removed and glomeruli were isolated using magnetic separation technique. After successful characterization (mesangial cells are positive for vimentin and fibronectin, negative for E-cadherin), we used our primary mesangial cells between passages P5 to P8 for all our experiments.



First, we compared the gene expression profile of B6 and CBA mesangial cells upon PBS or TGF-ß treatment (10 ng/ml) for 48 hours. As compared to B6 cells, TGF-ß treated CBA cells showed several fold overexpression of TGF-ß, TIMP-1, EGR-1 (see Figure below), and EGR-2 (B6+PBS: 1.0±0.4; B6+TGFß: 1.5±0.1 vs CBA+PBS: 1.2±0.4; CBA+TGFß: 12.8±2.5). The transcription factor RUNX1, recently identified in our in vivo experiments, increased 3-fold in CBA cells (B6+PBS: 1.0±0.3; B6+TGFß: 2.0±0.1 vs CBA+PBS: 0.9±0.1; CBA+TGFß: 5.8±0.1), accompanied by marked increase in type-I collagen expression (B6+PBS: 1.0±0.3; B6+TGFß: 0.8±0.1 vs CBA+PBS: 1.1±0.2; CBA+TGFß: 8.7±2.3). These results confirmed our previous in vivo results obtained from TGF-ß transgenic mice on B6 or CBAxB6 background.



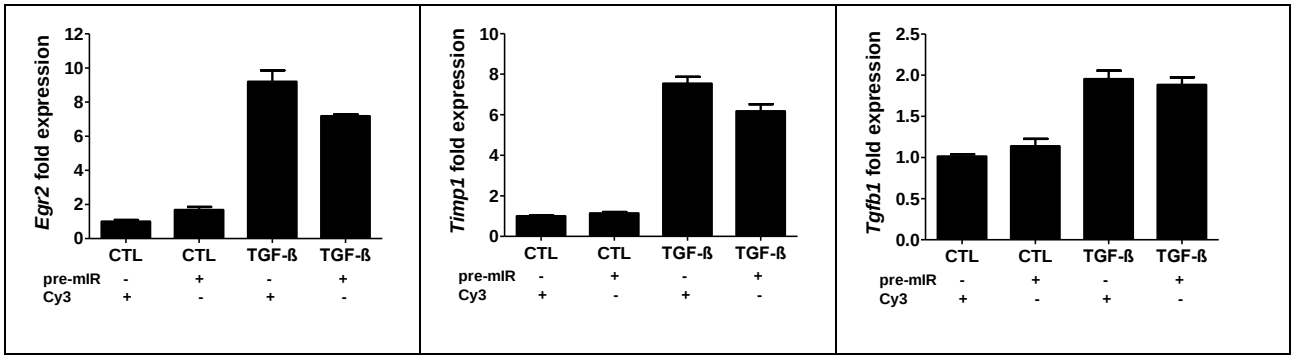
In order to prove our hypothesis based on the strong correlation found in our SNX and UUO experiments that EGR-2 might directly induce TIMP-1 expression, we performed transient transfection experiments on 293T human embryonic kidney cells using a plasmid that contains the full-length murine EGR-2 mRNA coding sequence (synthesized by IDT DNA, USA). 293T cells showed 80-85% transfection efficiency in our experiment, confirmed using an EGFP plasmid construct with similar molecular size to the EGR-2 plasmid (see figure below).



The mEgr2 plasmid transfection resulted in 2-fold increase in hEGR-2 mRNA expression as compared to EGFP-transfected control cells, accompanied by 2.5-fold hTIMP-1 overexpression. Immunoblot experiments revealed that mEgr2 plasmid transfection rose hEGR-2 protein expression 800-fold (pEGFP: 1.0±0.5; pEgr2: 800±200), accompanied by 4-fold hTIMP-1 protein expression as compared to EGFP-transfected controls (pEGFP: 1.2±0.3; pEgr2: 4.1±0.9). We observed a very tight correlation between hTIMP-1 and hEGR-2 mRNA expression of transfected cells (R-square: 0.89, $p<0.0001$). Our transient transfection experiment underlines the hypothesis of a new regulatory pathway for TIMP-1, driven by the transcription factor EGR-2.

In contrast to the miRNA expression profile of TGF-ß transgenic mice, we observed a different miRNA pattern in our primary mesangial cells. TGF-ß treatment resulted in 1.8-fold lower miR-200a expression in B6 cells than in CBA cells (B6+TGFß: 0.5±0.1 vs CBA+TGFß: 0.8±0.2). As the higher miR-200a expression in CBA cells was accompanied by increased EGR-2 expression, or in vitro results support previous reports on EGR-2 induced miR-200 expression. The expression of miR-199 was also upregulated (3-fold) in TGF-ß treated B6 mesangial cells as compared to CBA cells (B6+TGFß: 2.1±0.7 vs CBA+TGFß: 0.7±0.3), in contrast to the observed downregulation in vivo, in B6-TGF-ß transgenic mice.

In order to test the possible effect of miR-200, we transfected CBA mesangial cells with pre-miR-200 in the absence and presence of TGF-ß. The 48h pre-miR-200 treatment reduced both the TGF-ß induced EGR-2 and TIMP-1 mRNA expressions by 20%, but had no effect on TGF-ß mRNA expression. These minor, however significant changes in EGR-2 and TIMP-1 show that miR-200 might partly regulate the expression of these genes.



We also wished to transfect B6 cells in parallel with CBA, but we did not succeed, even with higher doses of Lipofectamine. We have also planned in the workplan to downregulate miR-200 by transfecting the cells with antagonizing miR, however due to the long optimization of previous transfections we ran out of time, unfortunately. Now we are planning to finish these pending experiments. We also wish to investigate mRNA and microRNA expression of the above described molecules in human kidney biopsy semples, to confirm their possible role in human kidney disease.

The manuscript presenting these mouse and primary mesangial cell experiment results *(Genetic susceptibility to renal fibrosis in mice is associated with TIMP-1)* is in preparation.

In summary, our studies revealed that renal TIMP-1 expression is associated with genetic susceptibility to renal fibrosis, linked to EGR-2 and miR-200, but TIMP-1 also correlates to sGC activation in diabetic kidney disease. In addition, we also demonstrated that chronic hyperosmolarity facilitates the renal expression of profibrotic EGR-1 and AP-1 components that may worsen an ongoing kidney disease.

Grant related publications:

1. Kökény Gábor, Amelia Foss, Németh Ágnes, Fazekas Krisztina, Mózes Miklós. EARLY RENAL INFLAMMATORY MOLECULE EXPRESSION DIFFERENCES AFTER UNILATERAL URETER OBSTRUCTION IN MICE. *NEPHROLOGY DIALYSIS TRANSPLANTATION* 33:(Suppl 1) pp. 430-431. (2018)
2. Mózes Miklós, Németh Ágnes, Fazekas Krisztina, Kökény Gábor. GENETIC SUSCEPTIBILITY TO TGF-BETA INDUCED RENAL FIBROSIS IS ASSOCIATED WITH ALTERED MIR-199 EXPRESSION. NEPHROLOGY DIALYSIS TRANSPLANTATION 33:(Suppl 1) p. i434. 1 p. (2018)
3. Czirok S, Fang L, Radovits T, Szabo G, Szenasi G, Rosivall L, Merkely B, Kokeny G. Cinaciguat ameliorates glomerular damage by reducing ERK1/2 activity and TGF-β expression in type-1 diabetic rats. *SCIENTIFIC REPORTS* 7:(1) Paper 11218. 15 p. (2017)
4. Gabor Kokeny, Krisztina Fazekas, Petra Szoleczky, Miklos Mozes. GENETIC BACKGROUND DETERMINES MURINE PRIMARY MESANGIAL CELL RESPONSE TO TGF-BETA. *NEPHROLOGY DIALYSIS TRANSPLANTATION* 32:(Suppl. 3) p. iii444. (2017)

5. Miklos Mozes, Amelia Foss, Krisztina Fazekas, Gabor Kokeny. DELAYED PROGRESSION OF RENAL FIBROSIS IN C57BL6/J MICE IS ASSOCIATED WITH ROBUST MMP ACTIVITY. *NEPHROLOGY DIALYSIS TRANSPLANTATION* 32:(Suppl. 3) p. iii559. (2017)

6. Mozes MM, Szoleczky P, Rosivall L, Kokeny G. Sustained hyperosmolarity increses TGF-beta1 and Egr-1 expression in the rat renal medulla. *BMC NEPHROLOGY* 18:(1) Paper 209. 11 p. (2017)

7. Kokeny G, Nemeth A, Fazekas K, Szenasi G, Rosivall L, Mozes MM. MICRORNA NETWORKS IN THE PROGRESSION OF TGF-BETA INDUCED RENAL FIBROSIS. *NEPHROLOGY DIALYSIS TRANSPLANTATION* 31:(Suppl.1.) p. 1422. 1 p. (2016)

8. Mozes MM, Nemeth A, Szabo L, Fazekas K, Kokeny G. EARLY AND SUSTAINED RUNX1 ACTIVATION AFTER UNILATERAL URETER OBSTRUCTION IN MICE: A POSSIBLE NEW PLAYER IN RENAL FIBROSIS. *NEPHROLOGY DIALYSIS TRANSPLANTATION* 31:(Suppl.1.) p. 1438. 1 p. (2016)

9. Gábor Kökény, Ágnes Németh, Jeffrey B. Kopp, László Rosivall, Miklós M. Mózes. Genetic susceptibility to renal fibrosis in mice is associated with TIMP-1. (2018) ***(in preparation)***