The role of galectin-3 in cardiac remodeling and fibrogenesis
Yu, Lili

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2012

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Pharmacological Inhibition of Galectin-3 Protects Against Hypertensive Nephropathy

Lili Yu; Anne-Roos S. Frenay; A. Rogier van der Velde; I. Baudoin–Vreeswijk; Wiek H. van Gilst; Harry van Goor; Herman H.W. Sillijé; Rudolf A. de Boer

Manuscript
Abstract

Background: Galectin-3 activation has been implicated in renal damage and fibrogenesis. Limited data are available to suggest that galectin-3 targeted intervention acts as a potential therapeutic candidate for the prevention of chronic kidney disease (CKD).

Methods: We used homozygous TGR(mREN)27 rats (REN2), which develop severe high blood pressure around 4 weeks of age and heart failure at 13-15 weeks of age. Six-week-old male REN2 rats were treated with a galectin-3 blocking compound, N-acetyllactosamine (Gal3i), for 6 weeks. Untreated REN2 and SD rats served as controls. We measured cardiac function with echocardiogram and invasive hemodynamics prior to sacrifice. Blood pressure and proteinuria were measured at 0, 3 and 6 weeks. Plasma creatinine was determined at 6 weeks. Renal damage was assessed using histological scores: focal glomerular sclerosis (FGS), glomerular desmin expression, glomerular and interstitial macrophage numbers and alpha smooth muscle actin expression. Inflammatory cytokines and extracellular matrix proteinases were quantified by RT-qPCR.

Results: Systolic blood pressure was consistently higher in untreated REN2 rats compared with SD rats and was not affected by Gal3i treatment. Plasma creatinine and proteinuria were significantly increased in untreated REN2 rats and this was reduced by treatment with Gal3i. Parameters of renal damage were also elevated in untreated REN2 rats except for glomerular macrophage scores. All these parameters were reduced upon Gal3i treatment. Various inflammatory cytokines were elevated in untreated REN2 rats and attenuated by Gal3i. However, markers of extracellular matrix turnover were marginally altered in untreated REN2 rats as compared to SD rats.

Conclusion: Pharmacological inhibition of galectin-3 attenuates hypertensive nephropathy, as indicated by a reduction in proteinuria, preservation of renal function and a decrease in renal damage. Drugs binding to galectin-3 may be potential therapeutic candidates for the prevention of chronic kidney disease (CKD).

Keywords
Chronic kidney disease, fibrosis, Galectin-3, renin-angiotensin system
Introduction

Renal impairment is frequently observed in cardiovascular disease (1, 2). Chronic kidney disease (CKD), defined as progressive loss of renal function over a period of month or years, is characterized at the level by glomerular sclerosis and interstitial fibrosis (5). CKD represents a significantly global health problem (3, 4). Early detection and prevention of CKD could improve both renal and cardiovascular morbidity and mortality. Glomerular sclerosis is an important factor in the progression of chronic kidney injury. Accordingly, drugs that aim to protect against glomerular injury can be of great value.

Galectin-3 belongs to the galectin-3 family of mammalian lectins and is characterized by a carbohydrate recognition domain (CRD) that has affinity for β-galactosides. Galectin-3 mediates cell-cell and cell-matrix interactions by binding to lactosamine-containing glycoconjugates via its CRD (6). There is accumulating evidence that galectin-3 plays an important role in inflammatory and fibrotic processes (7). Upregulation of galectin-3 is involved in various types of organ fibrosis. Macrophage derived galectin-3 induces myofibroblast activation, promotes collagen synthesis, deposition and subsequently leads to fibrosis. Moreover, previous research showed that binding of modified citrus pectin (MCP) to the galectin-3 CRD exerts beneficial effects in experimental acute kidney injury (8).

Transgenic TGR (mRen2)27 (REN2) rats exhibit persistent high blood pressure, progressive proteinuria and nephropathy that strongly resembles the human situation: injury and dysfunction of glomerular endothelial cells, micro-inflammation, excessive production of extracellular matrix (ECM), which eventually results in glomerular sclerosis (9, 10). This collective of inflammation, glomerular sclerosis, tubular interstitial fibrosis, and proteinuria are all early markers for progressive renal dysfunction in CKD (11, 12). Previous experimental and clinical studies demonstrate that proteinuria caused by hypertension could almost be totally reversed by angiotensin converting enzyme (ACE) inhibitor (13, 14), whereas other experimental studies showed that reduction in proteinuria was partially independent of blood pressure level (15-17).

In the present study, we examined the effects of pharmacological inhibition of galectin-3 by N-acetyllactosamine (N-lac, Gal3i) on progressive glomerulosclerosis and proteinuria in hypertensive REN2 rats.

Materials and methods

Animals

We studied 6-week-old, male, homozygous, TGR (mRen2)27 rats (denoted as REN2). These rats show a phenotype of severe hypertension and left ventricular (LV) hypertrophy, leading to heart failure (HF) at 13-15 weeks of age (18). Rats were bred at the Max Delbrück Center for Molecular Medicine (Berlin, Germany). Male age-matched Sprague-Dawley (SD) rats were used as control (Harlan, The Netherlands). Animals were housed under standard conditions. The study was approved by the Animal Ethical Committee of the University of...
Groningen, the Netherlands, and was conducted in accordance with existing guidelines for the care and use of laboratory animals.

**Experimental design**

To study the effects of galectin-3 inhibition on renal protection, we allocated SD rats and REN2 rats to control treatment or treatment with the galectin-3 inhibitor. Rats were treated with an established inhibitor of galectin-3, N-Acetyllactosamine (N-Lac), in a final dose of 5 mg/kg/day. Injections were administered intraperitoneally, three times per week. Three different groups were studied: SD-control (N=5), Ren-2-control (N=5), Ren-2-Gal3-inhibitor (N=10). Rats were placed in metabolic cages during 24 hours at baseline and at 3 and 6 weeks and urine samples were collected for determination of urinary protein. Systolic blood pressure (SBP) was measured using a noninvasive tail-cuff method using a computer-assisted oscillatory detection device (Apollo 179; IITC Life Science, Woodland Hills, California, USA) at week 0, 3 and 6 after treatment, Rats were sacrificed 6 weeks after initiation of the experiment.

**Measurement of cardiac and hemodynamic function**

Cardiac function was assessed by echocardiography at baseline and prior to sacrifice (Vivid 7, GE Healthcare, Chalfont StGiles, UK; equipped with a 10-MHz (rats) phase array linear transducer, as described (19). Hemodynamic function was assessed invasively, as previously described (20), by introducing a 1.4 French microtip pressure-volume transducer (Millar Instr. Inc., Houston, TX, USA) via the right carotid artery into the aorta. A three-minute period was allowed for stabilization before systolic and diastolic blood pressure and heart rate (HR) were recorded (average of 20 heart cycles). Other parameters that were measured include peak systolic blood pressure (SBP) and LV end diastolic pressure (LVEDP).

**Tissue and plasma processing**

After measuring hemodynamics, arterial blood was drawn and collected. Samples were centrifuged at 3000 rpm for 15 min at 4°C, and plasma was frozen for creatinine analysis. Kidneys were removed and their weight was determined. Transversally cut kidneys were snapping frozen or fixed in buffered formalin (3.7%) for 24 hours, dehydrated and embedded in paraffin.

**Immunohistochemistry**

Paraffin sections were dewaxed and subjected to an antigen retrieval procedure by overnight incubation at 80°C in 0.1M Tris/HCL, pH 9.0. Sections were subsequently washed three times with PBS, endogenous peroxidase was blocked with 0.075% H2O2 in phosphate buffered saline (PBS, pH 7.4) for 30 minutes and incubated with the following primary antibodies: the myofibroblast marker alpha smooth muscle actins, α-SMA (clone 1A4, Sigma Aldrich, St.Louis, MO, USA); the macrophage marker ED1(#MCA341R, AbDSerotec,
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Oxford, UK); the tubular damage marker Kim-1 (a gift from Victoria Bailly, Principal Scientist, Cambridge, UK); and the glomerular epithelial marker desmin (Dakopatts, DAKO, Glostrup, Denmark). All incubations with primary antibodies were in 1% BSA/PBS for 1 hour at room temperature. Binding was detected using sequential incubation with appropriate peroxidase labeled secondary and tertiary antibodies diluted in 1% BSA/PBS buffer and 1% normal rat serum for 30 minutes, peroxidase activity was developed by using 3,3’-diaminobenzidine tetrachloride for 10 min containing 0.03% H₂O₂. Ultimately, counterstaining was performed using Mayer’s hematoxilin. All sections were digitalized using a scanning system at 20x magnification (Nanozoomer 2.0-HT, Hamamatsu, Japan). α-SMA and Desmin staining were quantified with Scanscope software (Aperio Technologies version 9, Vista, CA, USA). Kim-1 staining was similarly analyzed in the entire renal cortex. The number of both glomerular (50 glomeruli) and interstitial macrophages (30 interstitial fields) was manually counted by a blinded observer. Additionally, kidney sections were stained with periodic acid-schiff (PAS) and scored for focal glomerular sclerosis (FGS). FGS was scored positive if all of the following features were present: collapse of capillaries, mesangial matrix expansion, and adhesion of the glomerular visceral epithelium to Bowman’s capsule. A score for the degree of affected glomeruli was applied as follows: unaffected glomeruli were scored as 0; 0-25% affected glomeruli was scored as 1, 25-50% affected was scored as 2, 50-75% affected was scored as 3 and if all glomeruli were positive (75-100%) for FGS, a score of 4 was given. The ultimate score (%) is obtained by dividing the total score by the number of glomeruli times one hundred (21).

Quantitative real-time PCR

cDNA synthesis was performed using 0.5 µg total RNA (Quantitect Rev. Transcriptase kit, Qiagen, Venlo, The Netherlands) as described previously (19, 22). Quantitative real-time PCR (RT-qPCR) was performed using SYBR Green mix (Absolute SYBR Green ROX mix, Thermo Scientific, Breda, the Netherlands) on CFX384 Real-Time PCR Detection System (Bio-Rad Laboratories, Veenendaal, the Netherlands). All targets gene expression level of inflammation cytokines and extracellular matrix turnover proteins (a list of primers used for RT-qPCR showed in supplemental table 1) were evaluated under the same reaction conditions: 95°C for 15 minutes, then 36 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Samples were analyzed with quantification software (Bio-Rad CFX Manager 1.6). mRNA levels were expressed in relative units based on a standard curve obtained with serial dilutions of a calibrator cDNA mixture. Gapdh expression was used to normalize all expression data.

Statistical analysis

Data are expressed as mean ± SEM. Means were compared using one -way ANOVA with a Dunnett post hoc test, using untreated REN2 rats as a comparator. All calculations were made using SPSS computer software, (version 18 SPSS, ll, Chicago, USA).
### Results

#### Table 1. Baseline characteristics of rats

<table>
<thead>
<tr>
<th></th>
<th>SD-con (N=5)</th>
<th>REN2-con (N=5)</th>
<th>REN2-Gal3i (N=10)</th>
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<tr>
<td>BW (g)</td>
<td>377±6*</td>
<td>329±14</td>
<td>347±26</td>
</tr>
<tr>
<td>Kidney weight (mg/gr)</td>
<td>6.61±0.2*</td>
<td>8.98±0.3</td>
<td>8.32±0.3</td>
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<td>SBP (week 0, mmHg)</td>
<td>146±8*</td>
<td>229±13</td>
<td>220±5</td>
</tr>
<tr>
<td>SBP (week 3, mmHg)</td>
<td>151±9*</td>
<td>227±7</td>
<td>232±4</td>
</tr>
<tr>
<td>SBP (week 6, mmHg)</td>
<td>140±11*</td>
<td>216±8</td>
<td>235±19</td>
</tr>
<tr>
<td>Proteinuria (week0, mg/24h)</td>
<td>14.27±0.9*</td>
<td>37.17±8.08</td>
<td>21.63±3.49#</td>
</tr>
<tr>
<td>Proteinuria (week3, mg/24h)</td>
<td>27.83±2.77*</td>
<td>59.26±8.15</td>
<td>22.69±2.96*</td>
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<tr>
<td>Proteinuria (week6, mg/24h)</td>
<td>26.35±1.11*</td>
<td>80.58±18.95</td>
<td>16.1±1.8*</td>
</tr>
<tr>
<td>FS (%)</td>
<td>44±2*</td>
<td>34±1</td>
<td>41±3*</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>4±1*</td>
<td>9±1</td>
<td>5±2*</td>
</tr>
</tbody>
</table>

Gal3i: galectin-3 inhibitor; BW: body weight; Kidney weight: corrected for BW, mg/gr; SBP, systolic blood pressure before treatment; FS: Fractional shortening; LVEDP: Left ventricular end diastolic pressure. * P<0.05 vs. REN2-con, # P= 0.06 REN2-Gal3i vs. REN2-con.
Characteristics of REN2 rats

REN2 rats developed severe hypertension (>200mmHg) at 6 weeks of age; SBP was significantly increased compared to SD rats at all-time points (0, 3, 6 weeks, Table 1 and Figure 1b). There was no difference in SBP between the untreated REN2 and Gal3i treated REN2 rats (217±7 vs. 235±6 mmHg, P=NS) (Figure1b). Ren2 rats had lower body weights compared to SD rats. Cardiac and hemodynamic measurements prior to sacrifice showed a significant decrease in fractional shortening (FS) and an increase in LVEDP in REN2 rats compared to SD rats (34±1% vs. 44±2% and 9±1 vs. 4±1 mmHg, P<0.05) (Table1). The untreated REN2 rats develop LV dysfunction. Treatment with Gal3i attenuated LV dysfunction by interfering with myocardial fibrogenesis (unpublished data) (Table1).

Renal function

Renal function was impaired in REN2 rats, as shown by a marked increase in proteinuria, plasma creatinine and an associated decrease in renal clearance after 6 weeks of the treatment (proteinuria: 81±18 vs. 26±1 mg/24h; plasma creatinine: 45±5 vs. 26±2 µmol/L; renal clearance: 2.1±0.1 vs. 4.0±0.3 mL/24h, P<0.05) (Table 1, Figure 1c-e).
Proteinuria was increased in the untreated REN2 rats compared with SD rats, but the increase was attenuated in the Gal3i treated REN2 rats (16±2 vs. 81±19 mg/24h in untreated REN2 rats, P<0.05) (Figure 1c). Also, a marked increased level of plasma creatinine was found in untreated REN2 rats, and this was also attenuated after treatment with the Gal3i (27±2 vs. 45±5 µmol/L in the untreated REN2 rat, P<0.05) (Figure 1d). Creatinine clearance was clearly decreased in the untreated REN2 rats compared with SD rats, which was attenuated after treatment with Gal3i (2.8±0.4 vs. 2.2±0.1 ml/24h in the untreated REN2 rat, P<0.05) (Figure 1e).

**Galectin-3 inhibition protects renal damage**

In untreated REN2 rats, FGS was significantly higher as compared to SD rats (15 ±4% vs. 0 % in SD rats, P<0.05). Gal3i treatment resulted in a significant attenuation of FGS (3 ±1% vs. 15±4% in untreated REN2 rat, P<0.05) (Figure 2a, b, c and g). Additionally, glomerular desmin expression was markedly increased in untreated REN2 rats (21±1 vs. 5±1 in SD rats, P<0.05), which was attenuated after Gal3i treatment (14±2 vs. untreated REN2 rat, P<0.05) (Figure 2d, e, f and h).

**Galectin-3 inhibition suppresses renal inflammatory response**

Persistent systemic hypertension induces glomerular micro-inflammation (9). We observed that the number of interstitial macrophages was significantly increased in the untreated REN2 rats as compared to SD rats, while the number of glomerular macrophages

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**Figure 2** Glomerular morphology in the rat kidney. Representative pictures of focal glomerular sclerosis with PAS staining are shown for SD, untreated REN2, and Gal3i-treated REN2 rats (a-c). Glomerular desmin expression in SD, untreated REN2, and Gal3i-treated REN2 rats (d-f). The quantified percentages of focal glomerular sclerosis (g) and desmin expression (h). * P<0.05 vs. REN2-con
was not changed in the untreated REN2 group (Figures 3a, 3b, 3d, 3e, 3g, and 3h). Gal3i treatment attenuated macrophage influx in both the glomerular and the interstitial compartment (Figures 3c, 3f, 3g, and 3h). CD68 (macrophages), interleukin-6 (IL-6) and other relevant inflammatory cytokines e.g. galectin-3 and monocyte chemoattractant protein 1 (MCP-1) were subsequently determined by RT-qPCR (Figure 3i-l). Galectin-3, IL-6 and MCP-1 were all significantly increased in untreated REN2 rats and reduced by the treatment with...
Gal3i. Cd68 mRNA level was not altered in the untreated REN2 group as compared to the SD group. Gal3i treatment did, however, attenuate Cd68 expression, which is in line with the decreased glomerular and interstitial influx of macrophages in this animal group.

**Matricellular protein changes and in early stages of kidney injury**

α-SMA expression, as investigated by IHC and RT-qPCR was significantly increased in untreated REN2 rats as compared to SD rats. Gal3i caused a reduction in α-SMA IHC-staining and mRNA levels (Figures 4a-c, 4g and supplemental figure 1a). Also, a decrease in KIM-1 protein expression was observed after Gal3i treatment (Figures 4d-f) and Kim-1 mRNA expression was similarly decreased (Figure 4h). Additionally, the mRNA levels of Tgf-β, Mmp2 and Timp2 were not altered in the untreated REN2 group as compared to the SD group (Supplemental figures 1b, 1c and 1f). Interestingly, Mmp9 tends to show a lower expression in both untreated and treated REN2 groups as compared to the SD group (Supplemental figure 1d). Furthermore, Timp1 expression was significantly increased in the untreated REN2 group and decreased when treated with Gal3i (Supplemental figure 1e). Finally, we found that mRNA expression of extracellular matrix collagen proteins Col1a1 and Col3a1 were not different between the untreated REN2 and the SD groups, but significantly decreased when treated with Gal3i (Supplemental figures 1g and 1h).
Discussion

The major finding of the present study is that targeted inhibition of galectin-3 attenuates renal structural and functional deterioration in REN2 rats with hypertensive end organ damage and increased plasma galectin-3 levels. Over a six weeks course, the untreated REN2 rats developed substantial proteinuria which was associated with the development of glomerulosclerosis. Treatment with Gal3i almost completely prevented the development of proteinuria and attenuated fibrogenesis and inflammation, as determined by histological staining and gene expression analysis. Interestingly, hypertension is not altered in REN2 rats treated with the Gal3i, indicating that the positive effects of the treatment are blood pressure independent.

Galectin-3 is expressed in various organs, including heart, lung, liver and kidney (7). The role of galectin-3 in fibrosis and inflammation has been partially elucidated in recent years. In a mouse model of unilateral ureter obstruction (UUO), characterized by severe hydronephrosis, inflammation and fibrosis galectin-3 disruption (Gal-3 KO mice) resulted in decreased renal inflammation. This was associated with a reduced pro-fibrotic response as evidenced by a decreased collagen production and deposition (23). Another study in UUO-induced renal injury also reported reduced myofibroblast activation as determined by decreased α-SMA staining in Gal-3 KO mice as compared to wild type mice. Surprisingly, they observed increased interstitial collagen deposition in these mice and decreased E-cadherin levels, a surrogate marker for tubular damage (24). Unfortunately, no explanation is given for this paradoxical result of less interstitial myofibroblasts and less procollagen I expression, but more fibrosis (collagen deposition). These remarkable effects may occur at the late stages (2-3 weeks induction) of the UUO model, since Henderson et al., who investigated an earlier time point (7 days) after UUO-induced kidney injury observed both less myofibroblasts and less fibrosis in Gal-3 KO mice. In a model of folic acid induced kidney fibrosis it was shown that treatment with modified citrus pectin (MCP), a compound that binds to the galectin-3 carbohydrate recognition domain (CRD), attenuated macrophage influx and renal fibrosis (8). Together with our results, which reveal Gal3i mediated attenuation of inflammatory responses (e.g. CD68, IL-6 and MCP-1), macrophage infiltration and myofibroblast activation in a hypertensive rat model, there is strong support for a protective role for galectin-3 inhibition in different models of renal damage. Future studies require the analysis of long-term treatment effects and the effects of Galectin-3 inhibition in established kidney disease.

TGF-β is an important mediator of myofibroblast activation (25, 26) and it was demonstrated that macrophage derived galectin-3 directly induces myofibroblast activation resulting in up-regulation of collagen synthesis (23, 27). We did not observe altered renal expression of Tgfβ mRNA in our hypertensive rat model and Gal3i treatment did also not affect TGF-β expression. This is in line with previous observations (23). This suggests that in our model TGF-β may not be the main driving force for fibrogenesis and that galectin-3 does not affect the expression of TGF-β. Gal3i did, however, lower the expression of several other genes involved in extracellular matrix (ECM) remodeling, including Timp1 and Coll1a1 and
Chapter 5

*Col3a1*. The expression of the metalloproteases *Mmp2* and *Mmp9* was not altered by Gal3i treatment, indicating that galectin-3 inhibition alters the expression of a limited number of ECM genes independent of TGF-β modulation.

Numerous studies have shown that anti-hypertensive therapy can slow down the decline in renal dysfunction, (28, 29), although other experimental studies have shown that a reduction in proteinuria can be mediated independent of blood pressure. Intervention with tranilast, an inhibitor of TGF-β, shows beneficial effects on proteinuria and tubulointerstitial damage independent of blood pressure in streptozotocin induced diabetic REN2 rats (15). Also, statin treatment reduces glomerular inflammation and podocyte damage in experimental deoxytocosterone-acetate (DOCA)-salt hypertensive rats (16). Moreover, arrest-specific protein 6 (Gas6) is involved in cardiac and renal injury, and Gas6 deficiency reduced renal inflammation, fibrosis and cardiac remodeling independent of blood pressure (17). Collectively, the above mentioned studies, together with our galectin-3 inhibitory study indicate that renal protection can be reached not only by lowering blood pressure levels, but also by specific anti-fibrotic and anti-inflammatory treatments.

**Conclusion**

Our study shows that pharmacological inhibition of galectin-3 attenuates impaired CKD in a hypertensive rat model. Galectin-3 inhibition attenuates myofibroblast activation and inflammation resulting in reduced fibrogenesis. This improves glomerular filtration function and reduces proteinuria. Therefore, we conclude that galectin-3 inhibition exerts its protective effects by directly acting on the renal glomeruli, parenchyma and tubuli and is independent of blood pressure. These new findings warrant further studies using galectin-3 inhibition as a potential novel supplementation to CKD therapy.
References


## Supplemental

### Table S1. List of primers used for RT-qPCR in rats renal

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<tr>
<th>Gene symbol (name)</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>Lgals3 (Galectin-3)</td>
<td>CCCGCTTCAATGAGAACAAC</td>
<td>ACCGCAACCTTGGAAGTGGTC</td>
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<td>α-SMA</td>
<td>CATCATCGCCTGAGACCTGG</td>
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<td>Cd-68</td>
<td>CTCTCATCATGGCCTGGTC</td>
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<td>Il-6 (Interleukin-6)</td>
<td>CCCACACAGAACGAGAATCA</td>
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<tr>
<td>Mcp-1</td>
<td>CCGACTCATGGGATCATCTT</td>
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<td>Col1a1</td>
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<td>TGGACACCGAGAGAACATGC</td>
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<td>Gapdh</td>
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### Figure S1
Gene expression of ECM proteins changed in rat kidney. α-SMA(a), Tgf-β(b), Mmp2(c), Mmp9(d), Timp1(e), Timp2(f), Col1a1(g), Col3a1(h). *P<0.05 vs. REN2-con