Antifibrotic Effect of the HMG-CoA-Reductase Inhibitor Cerivastatin in a Mouse Model of Progressive Renal Fibrosis

Marie-Louise Koepke¹, Manfred Weber¹, Eckhard Schulze-Lohoff¹, Bogdan Beirowski², Stephan Segerer³, Oliver Gross¹,⁴,

¹Department of Internal Medicine I, Cologne General Hospital, Merheim Medical Center
²Department of Anatomy I, University of Cologne,
³Medizinische Poliklinik-Innenstadt, University of Munich, Munich
⁴Department of Nephrology & Rheumatology, University of Göttingen, Göttingen, Germany

corresponding author:
PD Dr. Oliver Gross, MD, FASN
Department of Nephrology & Rheumatology
University of Göttingen
Robert-Koch-Str. 40
D- 37075 Göttingen, Germany
phone  +49 551 39 6331
fax  +49 551 39 8906
e-mail  gross.oliver@med.uni-goettingen.de

Short title: renoprotection with cerivastatin
Keywords: Statins, Alport syndrome, renal fibrosis, extracellular matrix, type IV collagen
Abstract

**BACKGROUND:** Alport syndrome is caused by mutations in genes encoding for the $\alpha3$, $\alpha4$ or $\alpha5$ chain of type IV collagen leading to excessive production of fibrotic tissue and end stage renal failure. HMG-CoA-reductase-inhibitors exhibit pleiotropic effects by which they modulate the production of connective tissue. The aim of this study was to examine the anti-fibrotic effect of the HMG-CoA-reductase-inhibitor cerivastatin in COL4A3 knockout mice, an animal model of Alport syndrome with progressive renal fibrosis.

**METHODS:** 40 homozygous COL4A3 knockout mice received cerivastatin, starting 28 or 49 days after birth. Mice were sacrificed at day 52 or 66 after birth. Immunohistochemistry against laminin and fibronectin was performed. Inflammatory cell infiltration was determined by F4/80- and CD3-staining. Myofibroblasts were identified by $\alpha$-smooth muscle actin staining. Expression of the profibrotic cytokines TGF-ß1 and CTGF were determined by immunoblot.

**RESULTS:** Lifespan of treated COL4A3 knockouts was increased by 28% compared to untreated animals (71+/-6 versus 91+/-9 days, p<0.01). Early cerivastatin treatment reduced cholesterol levels (113+/-13 versus 141+/-19 mmol/l in untreated animals, p<0.05) and serum urea (164 versus 235 mmol/l, day 66, p<0.05). Treatment also decreased proteinuria (5.5 versus 12 g/l at day 66, p<0.05). Deposition of laminin and fibronectin, expression of TGF-ß and CTGF was reduced. Infiltration of T cells and macrophages as well as myofibroblasts appeared to be reduced in kidneys from cerivastatin-treated mice.

**CONCLUSION:** Cerivastatin prolongs lifespan of COL4A3 knockout mice, reduces proteinuria and delays uremia. These effects are associated with decreased renal fibrosis and a reduction of inflammatory cell infiltration.
INTRODUCTION

Kidney fibrosis associated with renal failure is known to be the most common final stage of progressive renal disease. Alport syndrome (AS) is a hereditary disorder associated with progressive renal failure and kidney fibrosis. This disease is characterized by mutations in genes encoding for either the α3, α4 or α5 chain of type IV collagen, leading to the absence of all three chains forming the triple helical collagen monomers [1, 2]. Thus, the glomerular basement membrane in AS consists of only the α1 and α2 chains of type IV collagen, making this altered basement membrane more susceptible to endoproteolysis [3]. In the course of the disease, focal thickening and splitting of the basement membrane occurs, associated with hematuria and proteinuria and later progressive renal fibrosis leads to loss of renal function.

Several animal models of AS have been established, one of which is a COL4A3 knockout mouse model developed by Cosgrove et al. [4]. This model allows examination of a large number of animals exhibiting a phenotype similar to human AS. To date, there is no specific therapy for Alport syndrome and the only treatment at present is dialysis and renal transplantation. To evaluate the effect of a now established therapy in renal disease, COL4A3 knockouts have been treated with an ACE-inhibitor and AT1–antagonist, these achieved a prolongation of lifespan in the mouse model, paralleled by reduction of proteinuria and renal fibrosis [5, 6].

Inhibitors of HMG-CoA-Reductase (statins) are potent inhibitors of cholesterol synthesis. However, effects beyond lipid reduction have become increasingly recognized. Statins improve endothelial dysfunction and reduce thrombogenicity, while in the kidney, statins inhibit proliferation of mesangial cells and mesangial matrix deposition [7, 8]. Statins intervene in the regulation of apoptosis, modify monocyte infiltration and production of the profibrotic cytokine TGF-ß1 [9, 10]. Zoja et al. found that renal injury in passive Heymann nephritis could be ameliorated by an ACE-inhibitor and AT1-antagonist. Further adding a statin to that treatment significantly improved proteinuria, inflammatory and fibrotic processes [11]. Abbate et Remuzzi argue that these numerous beneficial effects of statins in different experimental settings make them an interesting target for evaluation of their nephroprotective potential in the COL4A3 knockout model of renal fibrosis [12].
METHODS

Mice
Heterozygous COL4A3 knockout mice (Jackson Immunoresearch Laboratories, Westgrove, PA, USA) were crossbred on a SvJ/129 background. Treatment protocols for the mice were previously approved by local German authorities and supervised by veterinarians. The genotype of the mice was determined by PCR as described previously [5]. To exclude artifacts, PCR was repeated in all homozygous mice. In addition, tail biopsy and PCR were repeated in all homozygous animals living longer than 84 days. No animals died from infection or obvious side effects. Mice were either sacrificed after 32, 42, 52 or 66 days, or left alive to monitor lifespan.

Administration of cerivastatin
Cerivastatin (Bayer, Leverkusen, Germany) was mixed to standard western diet (20 mg per kilogram body weight following recommendations by the manufacturer). 76 COL4A3 -/- animals were divided into different treatment groups.

1. untreated animals (n = 36)
2. early cerivastatin treatment, starting at day 28 (n = 24)
3. late cerivastatin treatment, starting at day 49 (n= 16)

A subgroup of mice in each group was used to monitor lifespan (no treatment group (1) n = 20; early treatment group (2) n = 8; late treatment group (3), n = 8).

Eight wild type mice were sacrificed at 52 days of age, four wild type mice at 66 days of age.

Statistics
Data were analysed by Log Rank statistic (survival analysis) and one-way analysis of variance (ANOVA). All results are given as mean and standard deviation.

Urinary protein analysis, serum cholesterol and urea
To obtain urine samples, mice were placed in metabolic cages. Proteinuria was measured using a gradient polyacrylamide gel, stained with Coomassie Blue and analysed by densitometry, a semiquantitative method described before [5]. Serum for measurements of
urea and cholesterol was taken, using heparin-covered capillaries on a Hitachi 917 Automatic analyser (Boehringer Mannheim, Germany).

**Light and electron microscopy**

Mice were sacrificed, transcardially perfused with a solution of paraformaldehyde and glutaraldehyde and kidneys were immersion-fixed as described previously [5]. Semithin and thin sections were taken on a Reichert Ultracut UCT ultramicrotome. A Zeiss Axiophot and a Zeiss EM 902 microscope (Göttingen, Germany) were used for histological documentation.

**Immunohistochemistry**

For paraffin embedding, fresh kidneys were fixed in 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS) and vacuum-embedded in a Shandon Citadel 1000 (Pittsburg, PA, USA). Sections of 4-5 µm were cut on a Micron HM355S (Walldorf, Germany). Sections were rehydrated in graded alcohol, treated with 1% Trypsin for 7 min and blocked with 5% BSA at room temperature. The primary antibodies were allowed to incubate overnight at 4°C (rabbit-anti-mouse EHS-laminin and rabbit-anti-mouse fibronectin, 1:1000, gift from M. Paulsson, Cologne, Germany). Goat anti-rabbit Cy3 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) served as secondary antibody. POX-conjugated anti-α-smooth muscle actin (DAKO, Hamburg, Germany) was used to stain activated fibroblasts. Staining of CD3 positive T cells and F4/80 positive monocytes / macrophages (rat anti-CD3, 1:100, clone CD3-12, Serotec, Raleigh, NC and rat anti-F4/80, 1:25, clone A3-1, Serotec, Raleigh, NC) was performed on paraffin-embedded material as previously described [13]. Sections were analysed on a Zeiss Axiophot microscope (Göttingen, Germany). Immunohistochemistry was repeated three times.

**Immunoblotting**

3-4 kidneys from different animals were pooled to minimise individual differences. Protein was extracted in a TBS solution, using protease inhibitors phenylmethylsulfonyl-fluoride (PMSF) and n-ethylmaleimide (NEM). Aliquots with 30 µg protein as shown by BCA protein assay were dissolved in SDS-sample buffer, separated by electrophoresis on a 4-
12% NuPage Novex Bis-Tris Gel (Invitrogen, Carlsbad, USA), transferred to a polyvinylidenfluorid-membrane, and blocked with 5% BSA and milk powder. Mouse anti-TGF 1 (1:100, R&D Systems, Minneapolis, USA) and rabbit anti-CTGF (1:5000, Abcam Limited, Cambridge, United Kingdom) were incubated for 60 min. The membrane was then incubated with the secondary antibody conjugated with HRP (Dako, Hamburg, Germany). The blot was developed using chemoluminescence. Protein expression was analysed by densitometry using a gel-pro analyser software (Media Cybernetics, USA). Immunoblots were repeated three times.
RESULTS

Treatment with cerivastatin extends lifetime of COL4A3 knockout mice
Lifespan of untreated COL4A3 -/- animals was 71.3 days (mean; standard deviation +/- 5.8). Homozygous knockout mice to which cerivastatin was given pre-emptively from day 28 on (early treatment) lived 91.3 days (+/- 9.4) which was an increase of 28.0% over untreated COL4A3 -/- mice (p<0.01). Mice treated with cerivastatin from day 49 on (late treatment) lived 90.5 days (+/- 6.2), an increase of 26.9% (p<0.01). Differences of the increased lifespan between the two treatment groups were not significant (Fig. 1).

Cerivastatin reduces cholesterol levels and proteinuria and delays uremia
Cholesterol levels were elevated in COL4A3 knockouts when compared to wild type mice 52 days of age (131.1 +/-15.7 mmol/l vs . 96.8 +/- 12.1 mmol/l, p<0.05). Treatment with cerivastatin reduced cholesterol levels in the knockouts almost to untreated wild type level (99.6 +/- 17.2 mmol/l, p<0.05). However it should be noted that wild type mice treated with cerivastatin had a slight reduction in cholesterol levels (90.9 +/- 10.7 mmol/l). At the age of 66 days, cholesterol in untreated knockouts was 140.8 +/-19.3 mmol/l, cholesterol in the knockouts treated with cerivastatin was reduced to 112.8 +/-12.7 mmol/l, p<0.05 (Fig. 2A). Cholesterol in untreated and treated wild type animals had also increased (105.5 +/-14.4 mmol/l and 103.5 +/-16.0 mmol/l, respectively).

Proteinuria developed at the age of 42 days in untreated knockout mice and increased from 1 g/l to 4.5 g/l at the age of 52 days, mirroring the beginning of morphologic changes in the kidneys of COL4A3 -/- mice. By 66 days of age, just before death, proteinuria was 12 g/l in untreated animals. Early as well as late treatment with cerivastatin had an antiproteinuric effect (Fig. 2B). In the animals treated pre-emptively, urinary protein was 2.5 g/l at 52 days (vs. 4.5 g/l in untreated animals, p<0.05) and 5.5 g/l at 66 days of age (vs. 12 g/l in untreated animals, p<0.05). In animals receiving late treatment, an antiproteinuric effect could be seen by day 66 (7g/l vs. 12 g/l in untreated animals, p<0.05). The difference between early and late treatment was not significant.

Blood urea began to rise after 52 days in COL4A3 -/- mice. However at this age, urea had increased only mildly when compared to wild type animals. No significant differences could be seen between treated and untreated animals (52.5 +/- 1.6 mg/dl in untreated COL4A3 knockouts, 50.8 +/- 8.4 mg/dl in the early treatment group; 39.6 +/- 4.9 mg/dl in wild type...
mice). By 66 days of age, blood urea had increased to 235.4 +/- 21.8 mg/dl in the untreated animals. Mice treated with cerivastatin had significantly lower serum urea concentrations (163.6 +/- 45.7 mg/dl in the early treatment group, 151 +/- 37 mg/dl in the late treatment group, p<0.05). No significant differences could be seen between early and late treatment (Fig. 2C).

Deposition of extracellular matrix molecules is reduced in mice treated with cerivastatin

In 52 days-old knockout animals, light microscopy showed glomerular hypercellularity and thickening of the Bowman’s capsule as morphological correlates with the beginning of proteinuria and disturbed renal function. By 66 days, severe glomerulosclerosis was seen in most of the glomeruli. Fibrosis and tubular atrophy dominated in the interstitium. These morphologic changes were less pronounced in mice treated with cerivastatin, as glomerular architecture appeared intact and less interstitial fibrosis could be seen (Fig. 3). At day 66, glomeruli of 12 different kidney sections of three different animals in each group were evaluated for glomerulosclerosis by a blinded observer. Glomerulosclerosis was defined as loss of more than 50% of glomerular lumen due to extracellular matrix accumulation. One out of 80 (1.3 %) glomeruli of healthy controls showed sclerosis, whereas 76 out of 92 (83.0%) did in untreated COL4A3 -/- knockouts. In contrast, only 26 out of 82 (31.7%) of glomeruli in the early treatment group and 35 out of 87 (40.2 %) of glomeruli in the late treatment group showed sclerosis. Tubulointerstitial fibrosis was evaluated in a similar matter by grading 12 different kidney sections of three different animals per experimental group (total of 36 sections) into zero, 1+ and 2+ accumulation of extracellular matrix by a blinded observer. Healthy controls showed no accumulation of extracellular matrix in any section; untreated COL4A3 -/- showed an average matrix score of 1.78. Early cerivastatin treatment reduced the matrix score to 1.02 , in the late treatment group, the matrix score was 1.11.

Electron microscopy showed characteristic thickening and splitting of the glomerular basement membrane in COL4A3 knockouts irrespective of treatment. There was complete effacement of podocyte foot processes at day 66 in untreated animals (Fig. 3). However, the typical changes in the glomerular foot processes appeared to be attenuated by statin treatment.
These findings were confirmed in immunofluorescence analysis (Fig. 4). Fibronectin staining was used as an indicator of fibrosis. Staining was markedly less pronounced in sections from animals treated with cerivastatin, not very dissimilar to wild type animals which showed only scant fibronectin. However kidney sections from untreated COL4A3 -/- mice showed a distinct fibronectin staining within the glomerulus and in the periglomerular matrix. Laminin was stained with a polyclonal antibody reacting with the three subunits of laminin 1, the $\alpha_1$, $\beta_1$ and $\gamma_1$ chain. Again, untreated COL4A3 -/- mice showed marked laminin staining within the glomeruli, in the periglomerular region and interstitially, whereas wild type animals displayed only a fine staining of the glomerular and tubular basement membranes. Compared to untreated COL4A3 -/- mice, the periglomerular and interstitial signal was markedly reduced upon statin treatment (Fig. 4).

**Reduction of the profibrotic cytokines TGF-β1 and CTGF by cerivastatin**

To elucidate the pathways by which cerivastatin acts on the deposition of matrix molecules and renal fibrosis, the profibrotic cytokine TGF-β1 and its downstream mediator CTGF were analysed by Western blot. At the age of 66 days, TGF-β1 and CTGF were upregulated in untreated knockouts compared to wild-type littermates. TGF-β1 signal was reduced to 35.7% (integrated optical density (IOD) 3.43) at day 66 in the knockouts receiving early cerivastatin treatment compared to untreated knockouts (IOD 9.61). CTGF was reduced to 56.1 % (IOD 3.12) compared to untreated knockouts (IOD 5.56). Reduction of the profibrotic cytokines was not as pronounced when treatment is initiated at day 49, late treatment reduced TGF-β1 and CTGF expression to 78.6% (IOD 7.56) and 80.6% (IOD 4.48), respectively (Fig. 5).

**Cerivastatin reduces the accumulation of macrophages, inflammatory cells and activated fibroblasts**

To evaluate the role of inflammatory cell infiltration, macrophages were stained with an antibody to the F4/80 antigen, which is specifically expressed by macrophages. Fig. 6 shows accumulation of macrophages in the interstitium of 66 days-old homozygous COL4A3 -/- mice. In kidneys of mice treated with cerivastatin the accumulation of macrophages was reduced, though there was still an increase in macrophage accumulation compared to wild type animals.
Interstitial T-cell infiltration was evident in untreated COL4A3 knockouts by the age of 52 days, as shown by immunohistochemical staining against CD 3. T-cells were seldomly observed in wild type as well as in pre-emptively treated animals. At the age of 66 days, interstitial T-cells were not as numerous as in kidneys of 52 days-old animals (Fig. 6). However, no striking difference in T-cell infiltration between treated and untreated knockouts was noted, consistent with the observation that infiltration of inflammatory cells is one of the first steps in the pathogenesis of fibrosis (20). Interstitial myofibroblasts play a crucial role in the pathogenesis of fibrosis, as they contribute to matrix deposition into the tubulointerstitial space. Therefore, staining for \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA) as a marker of activated fibroblasts was performed. \( \alpha \)-SMA positive cells were abundant in the periglomerular region and in the interstitium of untreated 66 days-old COL4A3 knockouts. The number of activated cells appeared markedly reduced in animals treated with cerivastatin, as figure 6 shows. There was only scarce staining of \( \alpha \)-SMA positive cells in wild type animals.
DISCUSSION
Statins are inhibitors of HMG-CoA-reductase and are potent drugs in the treatment of hypercholesterolemia. However, apart from their cholesterol-lowering action, numerous pleiotropic effects have also been identified. It has been shown that statins exert anti-proliferative, anti-inflammatory and anti-fibrotic effects on a wide variety of tissues. There is growing evidence from in vitro and in vivo experiments suggesting beneficial effects of statins in progressive renal disease. Lovastatin inhibits proliferation of mesangial cells in cell culture and attenuates the expression of proinflammatory mediators [10]. Pravastatin and simvastatin diminish the accumulation of extracellular matrix molecules by mesangial cells [8]. In rat glomeruli, lovastatin inhibits TGF-β1 expression [10]. Statins ameliorate renal injury in many models of renal disease such as 5/6 nephrectomy Sprague Dawley rats [14], and obese Zucker rats [15].

In this study, we examined the effects of cerivastatin on the development of renal disease in the COL4A3 knockout mouse model of human Alport syndrome. Untreated COL4A3 knockout mice develop hematuria at the age of 28 days. Proteinuria occurs at 42 days of age. Blood urea begins to rise from day 49 and mice die of uremia after about 70 days. In our study, 40 COL4A3 -/- animals were treated with cerivastatin starting either at day 28 (early, pre-emptive treatment) or 49 (late treatment). The fully synthetic cerivastatin was chosen because of its lipophilicity. In vitro experiments suggest that hydrophilic statins such as pravastatin may be devoid of antiproliferative effects, presumably due to their poor cell membrane permeability [16]. However, it is not yet clear whether lipophilicity is an important factor for the exhibition of pleitropic effects. The aim of our study was to investigate whether treatment with cerivastatin has a nephroprotective effect in the COL4A3 knockout model of fibrotic renal disease.

The lifespan of COL4A3 knockout mice treated with cerivastatin was increased by 28% compared to untreated knockouts. Consistent with this, we found that serum urea at 66 days of life was significantly decreased in knockouts treated with cerivastatin. Light microscopy showed that fibrotic changes in the kidney were also much improved by cerivastatin treatment. Immunohistochemistry revealed a significantly marked increase in laminin and fibronectin staining during the course of disease, this was greatly reduced
upon cerivastatin treatment. Cerivastatin also reduced proteinuria in COL4A3 −/− mice. Interestingly, cerivastatin did not affect the structure of the GBM, which showed characteristic thickening and splitting. However, effacement of podocyte foot processes appeared somewhat improved by cerivastatin treatment as seen by electron microscopy. As cerivastatin treatment had no major effect on restructuring the pathological GBM yet reduced fibrosis and proteinuria, we speculated that statins could interfere in the secondary events leading to fibrosis in Alport syndrome. Two important mechanisms have been identified in the COL4A3 model. TGF-β1, a profibrotic cytokine, contributes to the focal thickening of the GBM and renal fibrosis. Antagonising the effects of TGF-β1 ameliorates focal thickening of the GBM, but has no effect on podocyte foot process effacement in COL4A3 knockouts [17]. Cosgrove et al. hypothesized that α1β1-integrin, the most abundant integrin on the surface of mesangial cells, may be responsible for foot process effacement by mediating the deposition of non-native laminin isoforms in the GBM. These isoforms, namely laminin 2 and 4, might mask binding of the podocytes to their preferred ligand, laminin 11, leading to loss of focal adhesion and finally to foot process effacement [17]. This group also demonstrated that inhibition of the TGF-β1 pathway by a TGF-β1 antibody attenuated interstitial fibrosis but did not extend lifetime, whereas α1-integrin/COL4A3 double knockouts lived about 70% longer and showed less GBM damage [17]. The inflammatory reaction preceding fibrosis might be important in this context.

In our study, the expression of TGFβ1 and its downstream mediator CTGF was markedly reduced. The expression of profibrotic cytokines was significantly reduced in the early treatment group compared to the late treatment group. Infiltration of CD3-positive T cells, macrophages and activated fibroblasts was attenuated by cerivastatin treatment. Though one would expect that treatment initiation before onset of nephron damage should be more efficient, no difference was seen between the early and late treatment group. Only TGF-β1 was significantly reduced in the early treatment group compared to the late treatment group. However, early TGF-β1 reduction had no influence on lifespan, uremia and proteinuria. Data of the Cholesterol and Recurrent Events (CARE) Trial suggest that treatment with pravastatin may delay loss of renal function in patients with chronic renal
insufficiency, but only in an advanced stage of the disease [18]. The reasons for this phenomenon remain unknown.

How statins exert their pleiotropic effects in the kidney is not yet entirely clear. Animal studies have shown that hyperlipidemia itself contributes to renal injury and progressive scarring, perhaps by mimicking some of the atherosclerotic mechanistic pathways. Dietary hypercholesterolemia leads to deposition of lipids in the glomerulus and tubulointerstitium and induces interstitial inflammation and fibrosis in rats. It has been shown that additional presence of glomerular disease aggravates nephron damage in this case [19]. In vitro experiments examining the potential mechanism of lipid-induced renal damage revealed a crucial role for LDL. LDL activates mesangial cells and in turn becomes oxidized to cytotoxic oxLDL. LDL leads to increased production of matrix molecules and chemoattractant proteins [20]. Lipid reduction prevents glomerular injury in 5/6 nephrectomized rats [14]. However, a large body of evidence suggests that statins exert beneficial effects independent of their lipid lowering properties. Inhibition of HMG-CoA-reductase not only impairs cholesterol synthesis, but also the production of intermediates derived from the same metabolic pathway. Besides cholesterol, the mevalonate pathway leads to the formation of non-sterol isoprenoids that are involved in the lipid modification of a number of intracellular proteins, e.g. small G-proteins and proteins from the Rho, Ras, Rab and Rap family. This process, named isoprenylation, is necessary for their binding to the cell membrane. Thus, statins regulate the cellular signalling pathways involved in many processes within the cell [20]. Some of the statins’ pleiotropic effects are reversed by adding downstream metabolites in the cholesterol cascade such as mevalonate, geranylgeranyl- or farnesyl-pyrophosphate [7].

In conclusion, our data show that cerivastatin reduces proteinuria and delays uremia and hence prolongs lifespan of COL4A3 knockout mice. These effects are associated with decreased renal fibrosis and a reduction of inflammatory cell infiltration. There was no significant difference in the course of disease between beginning treatment either early or late. The molecular mechanisms underlying the beneficial effects of cerivastatin in the COL4A3 knockout model still need further investigation. Both, reduction of cholesterol levels as well as direct anti-fibrotic and anti-inflammatory effects may contribute to the beneficial effect of cerivastatin observed in this model of fibrotic renal disease. However,
our data support a new treatment option for children with Alport syndrome and, possibly, other fibrotic renal diseases.

ACKNOWLEDGMENTS
This work was supported by a grant of the German Kidney Foundation (O.G.). We thank Dr. Odenthal and Dr. Fries from the department of Pathology, Cologne University, for the α-SMA staining, the staff from the animal house of the Center of Molecular Medicine Cologne for maintenance of the animals, Oya Goektas for her technical assistance, and R. Lefering for statistical analysis. S. Segerer is supported by the Else Kröner Fresenius Foundation.
REFERENCES


Fig. 1: Analysis of lifespan of cerivastatin-treated animals compared to untreated knockouts. Lifespan of untreated COL4A3 knockouts was 71.3 days (+/- 5.8). Treatment with cerivastatin from day 28 on increased lifespan by 28%. Homozygous COL4A3 knockouts treated with cerivastatin from day 28 of life on lived 91.3 days (+/- 9.4, P<0.01). Mice treated with cerivastatin from day 49 on (late therapy) lived 90.5 days (+/- 6.2, P<0.01).

Fig. 2A: Serum cholesterol levels, 2B Proteinuria, 2C Serum urea
2A Treating COL4A3 +/- animals with cerivastatin (▽) had no significant effect on serum cholesterol levels. (* p<0,05, compared to wild type controls).
2B Proteinuria is significantly reduced by early and late cerivastatin treatment (*p<0,05, compared to untreated knockouts). The difference between early and late treatment is not statistically significant.
2C Reduction of serum urea by early and late cerivastatin treatment is significant (*p<0,05, compared to untreated COL4A3 -/-).
Symbols are: (▼) untreated COL4A3 -/-, (▼) COL4A3 -/-, late cerivastatin treatment, (♦) COL4A3 -/- early cerivastatin treatment (○) COL4A3+/+.

Fig. 3: Light and electron microscopy.
(a-c) COL4A3 +/-; (d-f) COL4A3 -/-, early cerivastatin treatment; (g-i) COL4A3 -/- late cerivastatin treatment; (k-m) untreated COL4A3 -/- All animals are 66 days old. Electron microscopy shows the typical trilaminar structure of the glomerular basement membrane (GBM) in wild type animals (a). Thickening and splitting of the GBM occurs in untreated COL4A3 knockouts (k). In light microscopy, interstitial fibrosis and glomerular sclerosis are seen (l,m). Thickening and splitting of the GBM is not attenuated by early (d) or late (g) cerivastatin treatment, though glomerular architecture is preserved in more glomeruli and there is less tubulointerstitial fibrosis.
Electron microscopy, original magnification: upper row, 15000-20000fold, light microscopy: middle row, 600-800fold; lower row 400fold.

Fig. 4: Deposition of the extracellular matrix molecules laminin and fibronectin at 66 days of age. Upper row: immunohistochemical staining for laminin 1; (a) wild type; (b) COL4A3 -/-, early cerivastatin treatment; (c) untreated COL4A3 -/- The lower row shows immunohistochemistry for fibronectin: (d) wild type; (e) COL4A3 -/- early cerivastatin treatment; (f) untreated COL4A3 -/-.
Original magnification: 1:100

Fig. 5: Western blot analysis of TGF-ß1 and CTGF. Lanes 1-4 show TGF-ß1 and CTGF expression in kidneys of 66 days old animals: (1) COL4A3 -/- late cerivastatin treatment, (2) untreated COL4A3 -/-, (3) COL4A3 -/- early cerivastatin treatment, (4) untreated wild type. Lanes 5-7 represent kidneys at day 52 of life: (5) untreated COL4A3 -/-, (6) COL4A3 -/- early cerivastatin treatment, (7) untreated wild type.
IOD stands for integrated optical density.

Fig. 6: Infiltration of CD 3 positive T-cells (a-d), F4/80 positive macrophages (e-f) and α-smooth muscle actin (α-SMA) positive activated fibroblasts (h-j).
CD3-positive T cells in the interstitium at the age of 52 days in COL4A3 knockouts: (a) no treatment, (b) early cerivastatin treatment (c) 66 days old untreated COL4A3 -/-, (d) 66 days old COL4A3 -/- receiving early cerivastatin treatment. Figures (e) and (f) show
mature macrophages, stained with an F4/80 antibody, in the interstitium of untreated COL4A3 knockouts at day 66 (e) and in 66 days old knockouts receiving early cerivastatin treatment (f). (g) COL4A3 +/+, (h) untreated COL4A3 -/-, (i) COL4A3 -/-, early cerivastatin treatment, (j) COL4A3 -/-, late cerivastatin treatment. Original magnification: 1:100, 1:400 (glomeruli)