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Original Article

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Sirolimus ameliorates the enhanced expression of metalloproteinases in a rat model of autosomal dominant polycystic kidney disease

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Abstract

Background. Remodelling of matrix and tubular basement membranes (TBM) is a characteristic of polycystic kidney disease. We hypothesized that matrix and TBM degradation by metalloproteinases (MMPs) could promote cyst formation. We therefore investigated the renal expression of MMPs in the Han:SPRD rat model of autosomal dominant polycystic kidney disease (ADPKD) and examined the effect of sirolimus treatment on MMPs.

Methods. 5-week-old male heterozygous (Cy/+) and wild-type normal (+/+) rats were treated with sirolimus (2 mg/kg/day) through drinking water for 3 months.

Results. The mRNA and protein levels of MMP-2 and MMP-14 were markedly increased in the kidneys of heterozygous Cy/+ animals compared to wild-type +/+ as shown by RT-PCR and Western blot analyses for MMP-2 and MMP-14, and by zymography for MMP-2. Strong MMP-2 expression was detected by immunoperoxidase staining in cystic epithelial cells that also displayed an altered, thickened TBM. Tissue inhibitor of metalloproteinases-2 (TIMP-2) expression was not changed in Cy/+ kidneys. Sirolimus treatment leads to decreased protein expression of MMP-2 and MMP-14 in Cy/+, whereas MMP-2 and MMP-14 mRNA levels and TIMP-2 protein levels were not affected by sirolimus.

Conclusion. In summary, in kidneys of the Han:SPRD rat model of ADPKD, there is a marked upregulation of MMP-2 and MMP-14. Sirolimus treatment was associated with a marked improvement of MMP-2 and MMP-14 overexpression, and this correlated also with less matrix and TBM alterations and milder cystic disease.

Keywords: matrix metalloproteinases (MMPs); polycystic kidney disease (PKD); sirolimus; tissue inhibitors of metalloproteinases (TIMPs)

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Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is a chronic progressive kidney disease, which is characterized by cystic enlargement of both kidneys. Affected individuals usually present in the third to fourth decade of life hypertension, haematuria, polyuria, flank pain, and progress to end-stage renal disease within 5–10 years after the development of renal insufficiency [1]. The presence of numerous fluid-filled cysts is the predominant pathological feature of PKD. In addition to cyst formation, reorganisation of tubular basement membranes (TBM) and the development of fibrosis within the renal interstitium are characteristics of PKD, suggesting that the extracellular matrix (ECM) is intensely remodelled [2]. Tubular epithelial cells play an important role in the synthesis and degradation of ECM components, which in turn play a regulatory role in epithelial cell differentiation and growth [3,4].

Matrix metalloproteinases (MMPs) are a large family of secreted and membrane-bound zinc-dependent endopeptidases, which can degrade a wide spectrum of matrix substrates, and therefore represent key enzymes in the turnover of the extracellular matrix [5–7]. Most MMPs are released as zymogens before being activated in the extracellular environment. However, MMP-14, a membrane-bound metalloproteinase, is processed before its insertion into specific plasma membrane domains. In addition to its matrix-degrading properties, MMP-14 mediates the activation of pro-MMP-2 together with the tissue inhibitor of metalloproteinases TIMP-2, which acts as a cofactor. Activation of pro-MMP-2 from this ternary complex is finally accomplished by a second, non-TIMP-2-bound MMP-14 molecule [7,8]. Thus, net activation of MMP-2 depends on local MMP-14 and TIMP-2 levels, following the general concept that the interaction of a cell with extracellular matrix is critically determined by the concentrations of metalloproteinases and their natural inhibitors.

The purpose of the present investigations was to examine alterations in the expression of MMPs and TIMPs in a rodent model of PKD, namely the Han:SPRD rat, and

to correlate these molecules with changes in matrix and TBM structure. In a previous work, we and others have demonstrated that sirolimus treatment of Han:SPRD rats markedly reduced cyst growth and improved renal histology [9,10]. Here, we investigated the renal expression of MMP-2, MMP-14 and TIMP-2 in the Han:SPRD rat model of PKD, and examined the effect of sirolimus on the expression of these molecules.

Subjects and methods

Animals

The study was conducted in heterozygous (Cy/+) and normal littermate control (+/+) Han:SPRD rats. Only male rats were used since the disease progresses faster in male compared with female rats. A colony of Han:SPRD rats was established in our animal care facility from breeding pairs that were obtained from the Rat Resource & Research Center (Columbia, MO, USA). The regulatory commission for animal studies, a local government agency, approved the study protocol. The rats had free access to tap water and a standard rat diet. Rapamycin (Rapamune® oral solution) was kindly provided by Wyeth-AHP (Schweiz) AG, Switzerland.

Reagents

The following antibodies from Calbiochem were used for western blot analyses: anti-MMP-2 (468–483) (Ab-3) human (mouse), anti-MMP-14 (Ab-4) human (mouse), anti-TIMP-2 (Ab-2) mouse mAb (67–4H11) and as secondary antibody the peroxidase goat anti-mouse IgG. Anti-MMP-2 (Ab-3) clone A-Gel VC2 from Neomarkers (Fremont, CA, USA) was used for immunohistochemistry.

Experimental protocol

Male Cy/+ ($n = 6$) and +/+ ($n = 4$) rats were weaned and were then treated at 5 weeks of age ($n = 10$) with 2 mg/kg/day sirolimus, as described previously [9]. Additional male Cy/+ ($n = 4$) and +/+ rats ($n = 3$) were not treated and served as controls. The drug was administered in the form of a Rapamune® oral solution in the drinking water for 3 months. The concentration of sirolimus (Rapamune® oral stock solution 1 mg/ml) was adjusted according to rat body weight. After 3 months of treatment, the rats were anesthetized with isoflurane, the kidneys were removed, decapsulated and snap frozen in liquid nitrogen. Samples were stored at -80°C.

RNA extraction

Frozen kidney tissues were homogenized in lysis buffer (RNeasy; Qiagen), using Lysing Matrix D tubes and a BIO101 FastPrep® Instrument (QBiogene, Basel, Switzerland). The total RNA isolation (including DNase treatment) was performed from the homogenates according to the manufacturer's instructions (RNeasy; Qiagen). All aliquots of samples were stored at -70°C until use. RNA concentration was measured by using a spectrophotometer, and the quality was analyzed by Bioanalyzer 2100 electropherograms (Agilent, Waldbronn, Germany).

Genechip expression analysis

Affymetrix GeneChip® rat genome 230 2.0 arrays were used according to the manufacturer's instructions (Affymetrix Inc, Santa Clara, CA). Briefly, 5 μg of total RNA from Cy/+ and +/+ rat kidneys was used as starting material to generate biotin-labeled cRNA samples, which includes cDNA synthesis using oligo-dT/T7 primers, followed by *in vitro* transcription (one-cycle labeling protocol). Labeled cRNA samples (15 μg) were randomly fragmented to 35–200 bp and hybridized on arrays for 16 h, and arrays were washed. The fluorescent intensity emitted by the labeled target was measured by an Affymetrix GeneChip Scanner 3000. Finally, the hybridization images were analyzed using Affymetrix GCOSTM 1.2 software.

Reverse transcription and real-time taqman PCR analyses for MMP-2, MMP-14 and TIMP-2

RT and Taqman PCR analyses for MMP-2, MMP-14 and TIMP-2 were performed as described previously [11]. Total RNA was reverse-transcribed and PCR was carried out using TaqMan Universal PCR Master Mix (Applied Biosystems; containing AmpliTaq Gold DNA Polymerase), Gene Expression Assay Mix and cDNA. Real-time PCR analyses were performed with the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland), according to the instructions of Applied Biosystems. The expression levels of the 18S subunit of ribosomal RNA (18S rRNA) were used as a housekeeping gene. Relative quantification of all targets was calculated by the comparative cycle threshold method outlined by the manufacturer (User Bulletin No. 2; Applied Biosystems). For MMP-2, MMP-14, TIMP-2 and 18S, assays-on-demand gene expression products were used as described in the manufacturer's protocol (Applied Biosystems).

Protein extraction

A fresh ice-cold lysis buffer was prepared for protein extraction from frozen rat kidneys. The lysis buffer consisted of 50 mM Tris base, pH 7.4; 150 mM NaCl; 1% deoxycholate; 1% SDS; and 1% Triton X-100. Immediately before use, the lysis buffer was supplemented with 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and a protease and phosphatase inhibitor cocktail. Each sample was homogenized using a Dounce homogenizer. All aliquots of samples were stored at -70°C until use. Protein concentrations were determined using the BCA™ Protein Assay reagent kit (Pierce, Lausanne, Switzerland).

Zymography for MMP-2

Activity of the gelatinase MMP-2 was analyzed by gelatin substrate zymography. Briefly, 20 μg of each sample was loaded on a 7.5% SDS-PAGE gel containing 0.1% gelatin. After migration, gels were first incubated for 1 h in a mixture containing 2.5% Triton X-100 and 50 mM Tris/HCl

pH 8.0, then overnight in 50 mM Tris/HCl pH 8.0, 5 mM $CaCl_2$, 5 μM $ZnCl_2$. After incubation, gels were stained with 0.4% Coomassie blue and destained with 10% acetic acid and 50% methanol. The white bands obtained on the gels corresponding to MMP-2 activity were analyzed by densitometry (Scion Image Corporation) after scanning. The Precision Plus Protein™ Standard (Biorad, Reinach, Switzerland) was used as molecular weight marker, and a recombinant MMP-2 protein from Oncogene™ was used as positive control.

Western blot analysis for MMP-2, MMP-14 and TIMP-2

Equal amounts of protein samples and a reduced loading buffer were incubated for 5 min at 95°C. Samples were loaded on a 7.5% SDS-PAGE gel containing 40% acrylamid and 10% SDS. After migration, the proteins of the gels were transferred onto a supported nitrocellulose membrane (Biorad). The blots were blocked with 5% milk for 1 h at room temperature and were then incubated overnight at 4°C in a blocking buffer containing monoclonal rat anti-α tubulin, or monoclonal mouse anti-MMP-2, anti-MMP-14 or anti-TIMP-2 (dilutions 1:2000, 1:100, 1:25, 1:50). Subsequently, the blots were washed in a PBS-Tween solution and were incubated with a goat anti-mouse HRP-conjugated secondary antibody (dilution 1:1000). Finally, the membranes were incubated for 5 min in an enhanced chemiluminescent (ECL+) reagent (Amersham Pharmacia Biotech), followed by the exposure to Hyperfilm (SuperRX Fujifilm). The molecular weight of the bands of interest was determined by the Precision Plus Protein™ Standard and by recombinant MMP-2 and TIMP-2 proteins from Oncogene™ as positive controls.

Immunohistochemical detection of MMP-2

Rat kidney slices of about 2 mm thickness were cut immediately after the excision. The slices were fixed by immersion for 2 h at 4°C in 4% freshly de-polymerized paraformaldehyde in phosphate-buffered saline (PBS), dehydrated through an alcohol series and embedded in paraffin. For immunohistochemistry, 3 μm thick sections were cut and rehydrated through an alcohol series. They were microwaved for 10 min in a 0.01M sodium citrate buffer, pH 6.0. After blocking with 5% normal goat serum, the sections were incubated overnight at 4°C with the primary antibody. A mouse monoclonal antibody against MMP-2 was diluted 1:500. After three washes in PBS, the binding sites of the primary antibody were revealed with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) using 3,3'-diaminobenzidine tetrahydrochloride as substrate, according to the instructions of the supplier. The sections were then washed in water and were counterstained with the periodic acid-Schiff (PAS) reagent. The nuclei were stained with haematoxylin.

Statistics

All data are expressed as means ± SD and were considered normally distributed. Data were analyzed by applying a *t*-test using the GraphPad Prism software version 4.0 for windows (San Diego, CA, USA). The analysis of Genechip MAS5 normalized expression values was performed using GeneSpring 6.1.1 (Agilent, CA, USA). A *t*-test was carried out between the group of samples belonging to the respective conditions, and p-values <0.05 were considered significant.

Results

Affymetrix gene chip analysis

Microarray analysis revealed an upregulation of several interesting genes in the family of metalloproteinases in Cy/+ kidneys, compared to wild-type +/+ kidneys. In particular, we found a significant upregulation of MMP-2 (3.9-fold, $P < 0.05$) and MMP-14 (3.3-fold $P < 0.05$) in the microarrays (Figure 1A). Sirolimus treatment did not block this upregulation (data not shown). Real-time Taqman PCR analysis of RNA from Cy/+ and control +/+ rat kidneys was performed to determine steady-state mRNA changes for MMP-2, MMP-14 and TIMP-2. Figure 1B shows a significant increase of MMP-2 mRNA (2.2-fold, $P < 0.05$), of MMP-14 mRNA (2.4-fold, $P < 0.05$) and an unchanged TIMP-2 mRNA level in Cy/+ rats compared with +/+ rats, thereby validating the expression data obtained from microarray experiments.

Enhanced MMP-2 expression and activity in Cy/+ kidneys is reduced in sirolimus-treated rat kidneys

The expression of MMP-2 protein was then analyzed by western blot (Figure 2A), and quantified by densitometry (Figure 2B). Both the pro-form (72 kD) and the active form of MMP-2 (66 kD) were detected in wild-type rats, and these forms were not altered by treatment with sirolimus. There was a significant increase in the expression levels of the pro-form and the active form of MMP-2 in untreated heterozygous (Cy/+) vs untreated wild-type (+/+) rats (5.08- and 3.17-fold increase, respectively; $P < 0.05$). Treatment with sirolimus effectively decreased the expression of MMP-2 pro-form and active form by 3.08-fold and 2.57-fold ($P < 0.05$). In Cy/+ rats, respectively, compared to untreated Cy/+ rats (Figure 2B).

Analysis of MMP-2 activity with zymography was then performed to correlate the level of MMP-2 expression with its activity (Figure 3). A weak activity for the 72 kD pro-form of MMP-2 was detectable in +/+ rats; however, a significantly stronger activity was measured in Cy/+ animals (6.65-fold increase of the pro-form activity, $P < 0.01$). Activity for the 66 kD active form could not be detected in +/+ kidneys but was detectable in Cy/+ rats. Treatment with sirolimus was associated with a significant decrease in the pro-MMP-2 activity (1.55-fold decrease; $P < 0.01$), and the activity of the 66 kD active form was again undetectable.

Immunohistochemical staining in Cy/+ kidneys shows predominant tubular upregulation of MMP-2

In +/+ rats, immunohistochemistry revealed a moderate to strong expression of MMP-2 in interstitial cells, in the endothelia of peritubular capillaries and in the epithelium of

A

Gene	Affymetrix probe set	RefSeq/Genbank accession no.	+/+ wild type rats	Cy/+ heterozygous
MMP-2	1370301_at	U65656	0.451 ± 0.338	1.752 ± 0.757*
MMP-14	1378225_at	AI176440	0.532 ± 0.373	1.774 ± 0.447*
TIMP-2	1367823_at	BF523128	0.888 ± 0.352	0.814 ± 0.447

Affymetrix Rat Expression Array 230 2.0 GeneChip data, normalized signal values (mean ± s.d.; *n* = 3 for each group: cy/+ heterozygous and +/+ wild type rats)
* Significant difference (up) compare to +/+ wild type rats (p-values <0.05)

B

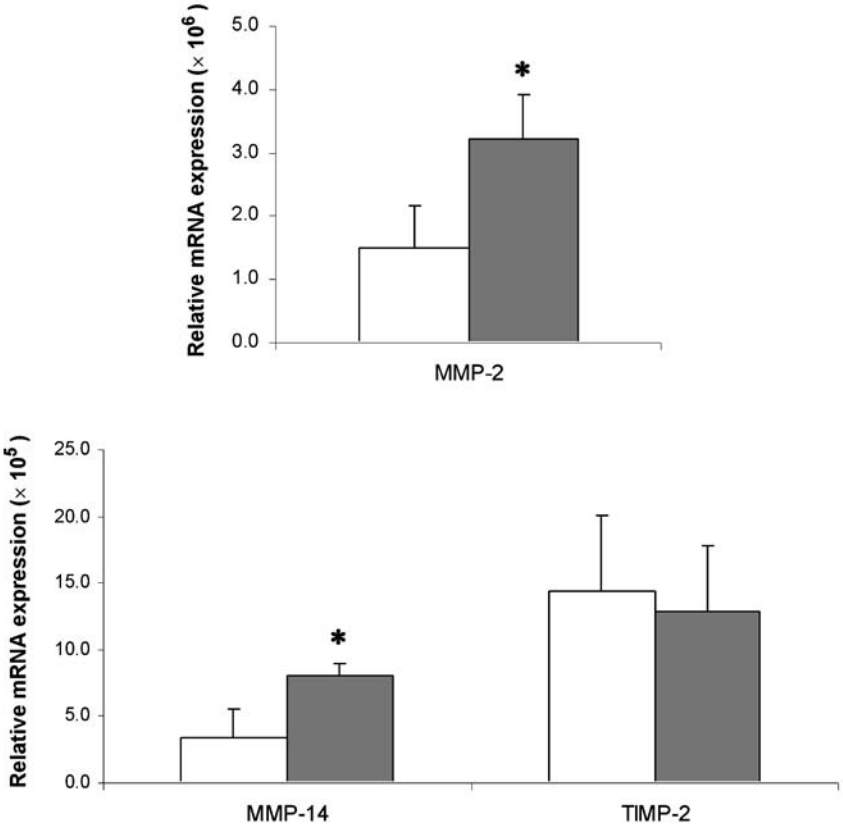

Fig. 1. **(A)** Microarray results of increased levels of MMP-2 and MMP-14 gene expression in Cy/+ heterozygous rats compared with +/+ wild-type rats. TIMP-2 mRNA levels were not modified. **(B)** Real-time TaqMan PCR analysis of MMP-2, MMP-14 and TIMP-2 gene expression in the same set of rats confirmed the original microarray data. The results are expressed as mean ± SD. $^*P < 0.05$ compared to the +/+ group, $n = 4$ to 6 per group.

the capsule of Bowman (Figure 4A). Most of the convoluted part of the proximal tubule showed a weak immunolabeling. MMP-2 was not detected in the late convoluted part, the straight part of the proximal tubule, the whole distal tubule and the collecting duct.

In histologically normal tubular profiles in Cy/+ rats, the expression of MMP-2 was similar to +/+ rats (Figure 4B). In dilated tubules, there was a striking heterogeneity of immunolabeling (Figure 4C). The expression was similar to +/+ rats in epithelial cells that looked morphologically normal. In contrast, MMP-2 staining in Cy/+ was moderate to very strong where the epithelium displayed an abnormally developed tubular basement membrane, as revealed by PAS staining (Figure 4D). The association between a high expression of MMP-2 and a modified basement membrane was found in the proximal tubule, in the capsule of Bowman and in the distal tubule. MMP-2 was expressed in all large cysts, mostly at a high level (Figure 4E).

Cysts in Cy/+ sirolimus-treated rats displayed similar levels of MMP-2 as cysts of placebo-treated rats. Expression of MMP-2 was also focally observed in tubules, which displayed thickening of the basement membrane (Figure 4F).

Effect of sirolimus treatment on MMP-14 and TIMP-2 expression in Cy/+ kidneys

In addition to its matrix-degrading properties, MMP-14 mediates the activation of pro-MMP-2 together with the tissue inhibitor of metalloproteinases TIMP-2, which acts as a

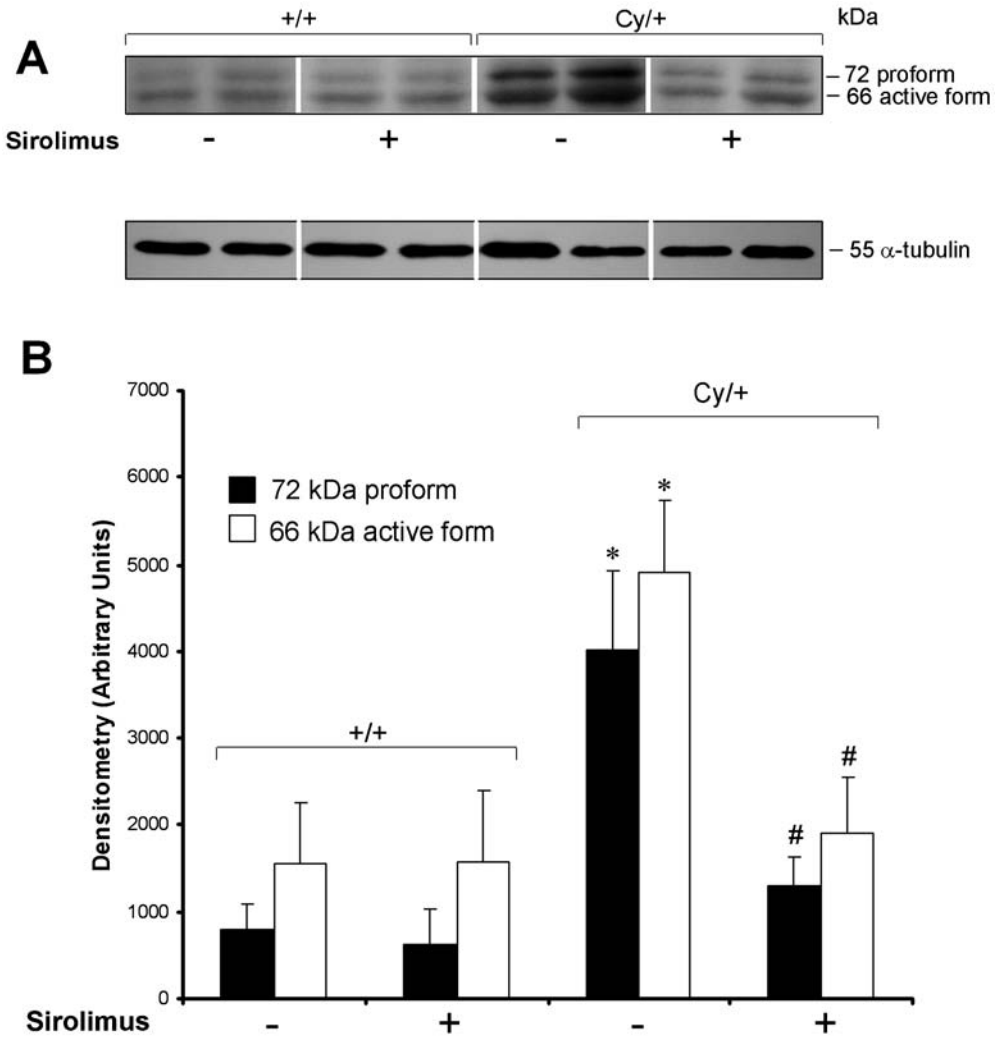

Fig. 2. MMP-2 expression. **(A)** Western blot and **(B)** densitometry confirmed the upregulation of the pro- and active forms of MMP-2 in Cy/+ compared to +/+. Treatment with sirolimus decreased these levels near to those of the wild-type. Two representative samples for each group are depicted. $^{*}P < 0.05$ compared with the untreated or treated wild-type (+/+) rats, $^{\#}P < 0.05$ compared with the untreated heterozygous (Cy/+) rats, $n = 4$ per group.

cofactor. Since MMP-14 and TIMP-2 play decisive roles in MMP-2 activation, we hypothesized that levels of MMP-14 and TIMP-2 would correlate with the increase of MMP-2 in the Cy/+ kidneys of ADPKD rats. The expression of MMP-14 was investigated by western blot analysis. Figure 5 shows that MMP-14 was detected in two different forms, as reflected by the bands at 63 and 58 kD, corresponding to the MMP-14 proform and the active form, respectively. MMP-14 protein expression was stronger in Cy/+ compared to +/+ rat kidneys (Figure 5A). Densitometry (Figure 5B) revealed a 4.55-fold and 6.35-fold increase in both forms of MMP-14 in Cy/+ compared to +/+ rat kidneys ($P < 0.05$). Sirolimus was again associated with reduced MMP-14 protein levels (4.93-fold and 5.73-fold, respectively; $P < 0.05$) down to levels observed in +/+ control rats.

TIMP-2 is involved in MMP-2 activation in conjunction with MMP-14, but can also inhibit activation of MMP-2 [12]. Three different forms of TIMP-2 were found by western blot analysis (Figure 6A). The active form of TIMP-2 at 27 kD and the bimolecular complex of TIMP-2/MMP-14 at 70 kD were not significantly higher in Cy/+ rat kidneys compared with +/+, and were not affected by treatment with sirolimus (Figure 6B). However, the dimeric complex of TIMP-2 at 54 kD increased approximately 1.59-fold ($P < 0.05$) when compared with the untreated +/+ rats, and tended to be reduced (1.45-fold; non significant) in sirolimus-treated rats. Interestingly, of all three forms, the TIMP-2/MMP-14 complex was the least expressed; the TIMP-2 active form had the highest overall expression level of the three forms.

Discussion

Others and we have shown that treatment with the mTOR inhibitors sirolimus and everolimus delays the loss of renal function and retards cyst development in Han-SPRD rats [9,13–15]. Here, we show that treatment of Han:SPRD rats with sirolimus was associated with a marked improvement

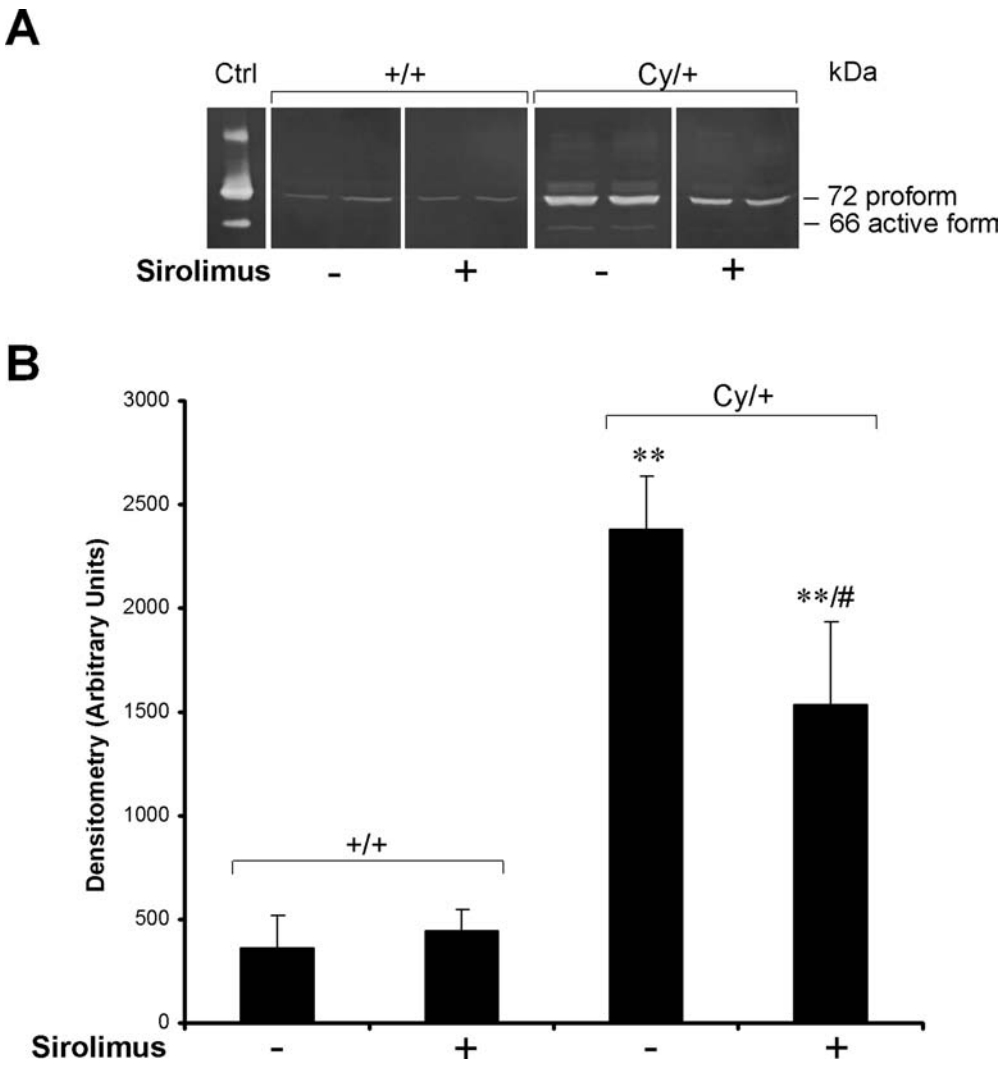

Fig. 3. (A) Zymography showed increased activities of pro- and active forms of MMP-2 (72 and 66 kDa respectively) in untreated heterozygous (Cy/+) rats compared with untreated wild-type (+/+) rats. MMP-2 activities were not modified by sirolimus treatment in +/+, but were decreased in Cy/+ as determined by **(B)** densitometry. Ctrl = purified MMP-2 antigen (Calbiochem, Switzerland) as a reference for MMP-2 identification. Two representative samples for each group are depicted. $^{**}P < 0.01$ compared with the untreated or treated wild-type (+/+) rats, $^{\#}P < 0.05$ compared with the untreated heterozygous (Cy/+) group, $n = 4$ per group.

of MMP-2 and MMP-14 overexpression, and this correlated also with less matrix and TBM alterations and milder cystic disease.

By gene array analysis, we identified MMP-2 and MMP-14 as the two major MMPs, which are markedly enhanced in Han-SPRD rat kidneys with PKD. RT-PCR analysis, and the examination of MMP-2 and MMP-14 protein by western blot and zymography confirmed the upregulation. In agreement with our findings, the abnormalities in the expression of MMPs have been described for human [16,17] and animal models of PKD [18–20]. In humans, increased levels of MMP-2 and -9 have been preferentially localized in tubular epithelia forming renal cysts. In animal models, it has been demonstrated that cystic kidney tubules can synthesize and secrete high levels of MMPs, in particular MMP-2 and MMP-14 [19,21,22]. In contrast to our findings, Schaefer *et al*. found a decrease of MMP-2 expression and activity in Cy/+ rats in early stages of PKD. However, this may be explained by several factors. In our experiments, we used whole kidney homogenate instead of isolated proximal tubules because MMP-2 has been localized to the interstitium and to the epithelial cells of all cyst-forming nephron segments [18]. Also, our rats were at a latter stage of disease progression. The expression of genes involved in fibrosis and inflammation increase with age in Cy/+ rats and further explain our finding of increased MMP-2 expression [20]. MMP-2 expression was prominent in tubular epithelial cells lining cysts and with an altered basement membrane. Those two features remained associated at the different stages of cyst growth, indicating that cystogenesis is associated with tubular induction of MMP-2. The parallel increase of cyst growth and expression of genes involved in inflammation and fibrosis suggests that MMPs may regulate collagen accumulation at sites where cystic transformation occurs, linking the progression of cyst formation with ECM remodelling in PKD.

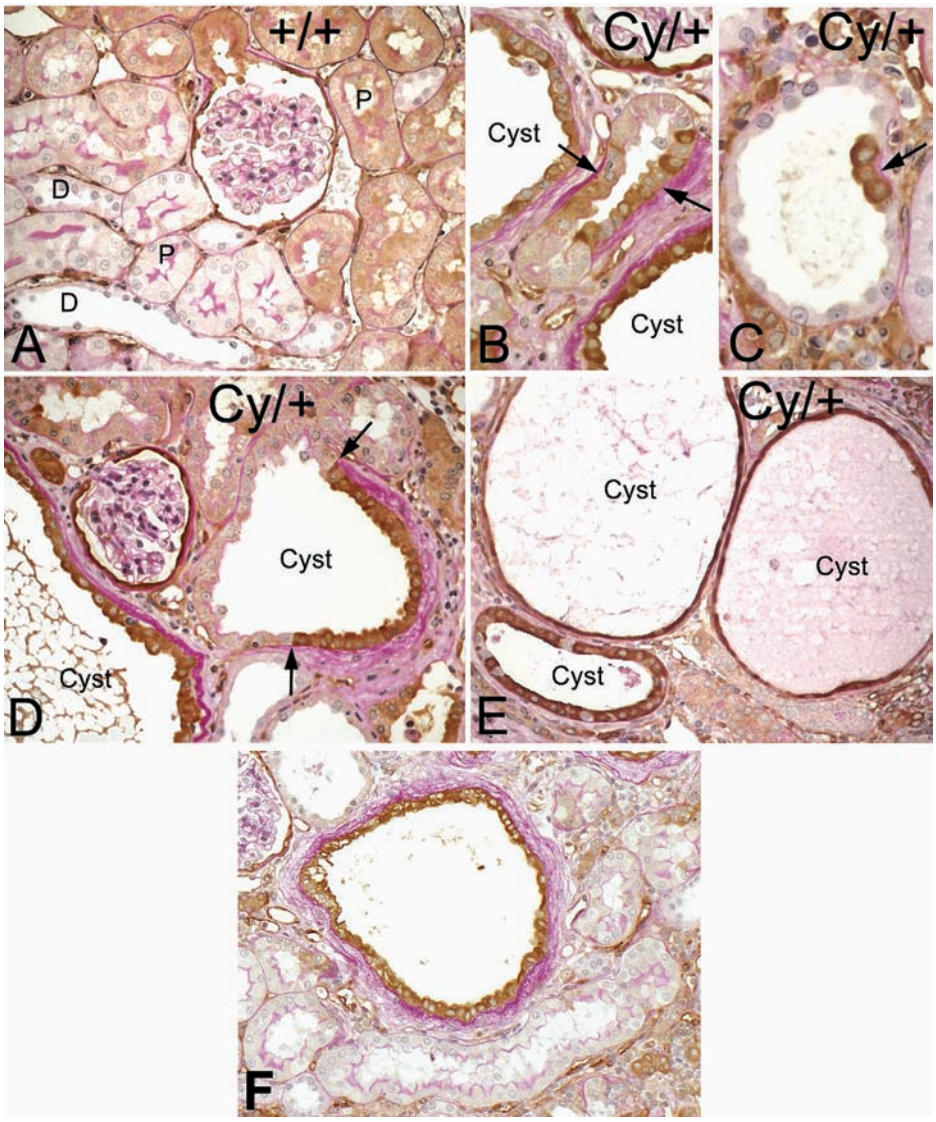

Fig. 4. Immunohistochemistry for MMP-2 in kidney sections. In wild-type (+/+), the segments S1 and S2 of the proximal (P) tubule display a weak immunoreactivity; the segment S3 and the distal (D) tubule are negative **(A)**. Patches of cells that produce excess amounts of basement membrane (arrows in **B** and **C**) represent the earliest histological alteration in **(B)** proximal and **(C)** distal tubules in Cy/+ kidneys. The cells in such patches up-regulate MMP-2. Note the sharp transition in **(D)** between the normal and the altered epithelium with its thickened basement membrane and its high immunoreactivity for MMP-2. **(E)** MMP-2 remains abundant in the large cysts with their thin epithelia. In Cy/+ sirolimus-treated rats, MMP-2 tubular expression is detected in cysts and focally in tubules, which displayed thickening of the basement membrane **(F)**.

Remodelling ECM has been hypothesized as a necessary step for cyst expansion [21]. In a study which also used the Han:SPRD rat, treatment with the global MMP inhibitor batimastat reduced cyst number and kidney weight [18]. Treatment of cystic mice with WAR-1 and WTACE2, MMP inhibitors that share some degree of activity against MMP-9 and MMP-13 slowed disease progression [23], pointing again to an important role of MMPs in cyst formation in PKD. Interestingly, transgenic expression of MMP-2 in proximal tubular epithelial leads to structural alterations in the tubular basement membrane, fibrosis and renal failure [24].

Sirolimus is an immunosuppressant with potent antiproliferative effects on non-lymphoid cells. We and others have previously shown that sirolimus effectively retards renal cyst development in the Han:SPRD rat [9,10]. Shillingford *et al*. have confirmed the potent anticystic effects of sirolimus in additional animal models of PKD [13]. Inhibition of the mTOR signalling pathway with sirolimus most likely inhibits cell proliferation in PKD via control of ribosomal translation. However, this may not be the sole mode of action. The reduced MMP-2 and MMP-14 protein expression, which occurred in response to the sirolimus treatment, was not associated with reduced mRNA levels. Sirolimus controls gene expression at the translational level via inhibition of downstream protein kinases that are required for translation of mRNAs [25]. Effectors of the mTOR pathway are upregulated in the kidneys of Cy/+ rats, and protein expression of MMP-2 and MMP-14 is transduced at least in part by these effectors of the mTOR pathway

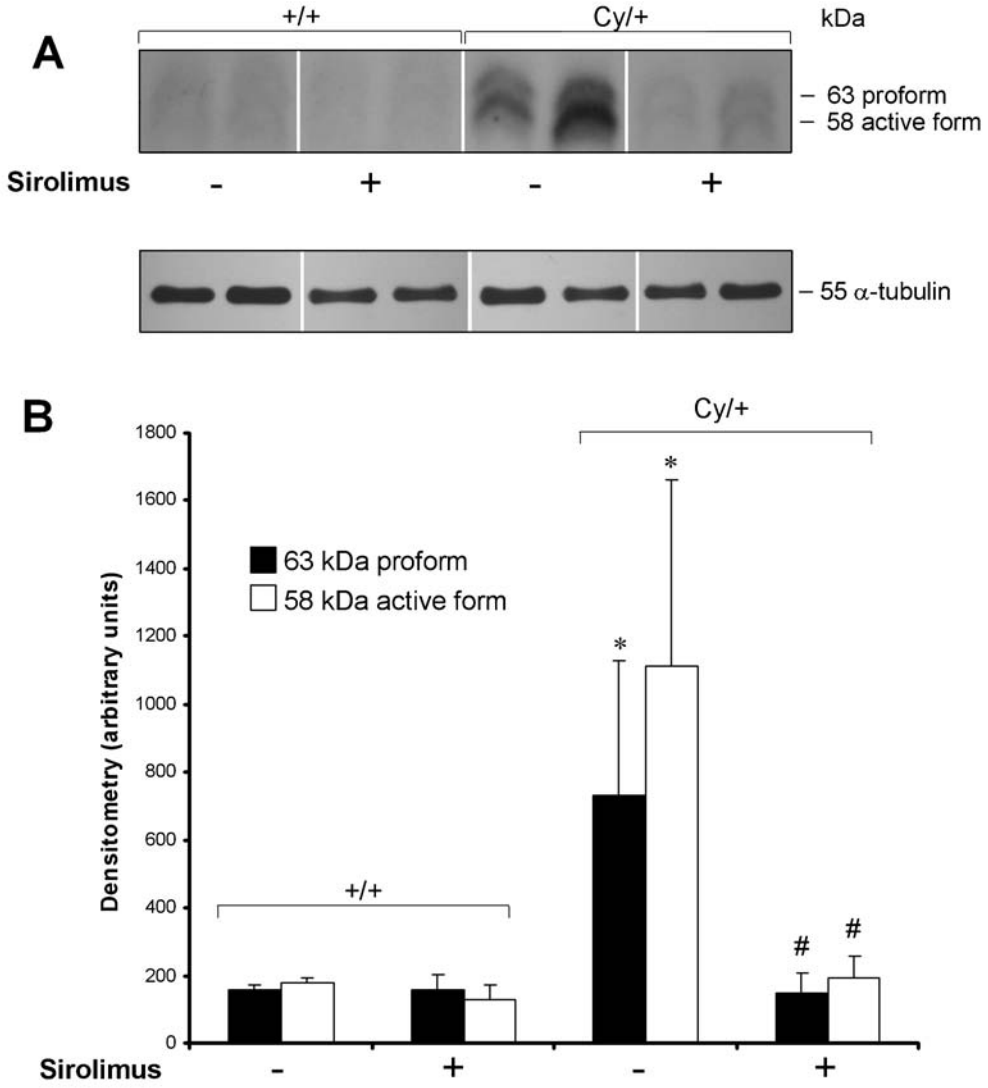

Fig. 5. MMP-14 protein expression. **(A)** Western blot analysis and **(B)** densitometry showed an increase of pro- and active forms of MMP-14 protein (63 and 58 kD respectively) in the kidneys of untreated heterozygous (Cy/+) rats compared with untreated wild-type (+/+) rats. Sirolimus treatment did not affect both MMP-14 forms in +/+ rats. MMP-14 protein levels decreased in kidneys of Cy/+ rats treated with sirolimus. Two representative samples for each group are depicted. $^{*}P < 0.05$ compared with the untreated wild-type (+/+), $^{\#}P < 0.05$ compared with the untreated heterozygous (Cy/+) group, $n = 4$ per group.

in vitro [2,26]. Thus, our results indicate that MMP-2/-14 protein expression in response to sirolimus treatment is mainly due to translational regulation, because mRNA levels of neither protein show a decrease with sirolimus treatment. It is unlikely that the reduction of MMP-2 and MMP-14 in sirolimus-treated rats is mainly due to TIMP inhibition because the active form of TIMP-2 remained unchanged. Increased TIMP-2 expression has been shown in Cy/+ rats by *in situ* hybridization, but when TIMP-2 RNA expression was analysed in total kidney by an RNase protection assay, no pronounced effect was seen and the favourable effect of batimastat on cyst growth was different from TIMP-2 expression [18]. In line with this result is our finding that sirolimus treatment did not change TIMP-2 expression. In 8-week-old Cy/+ rats, an increased expression of TIMP-2 was found in proximal tubules [22]. We cannot exclude that in the early disease stage, locally expressed TIMP-2 is associated with cyst development. Although in our study we cannot prove a direct effect of sirolimus on MMP-2 and MMP-14 expression, it is plausible that part of the beneficial effects of sirolimus might be caused by inhibition of MMPs.

Renal fibrosis is a hallmark of cystic disorder in human ADPKD [27] and in Han:SPRD rats [28]. Fibrosis increases with the progression of disease, indicating a link between cystogenesis and fibrogenesis. It is therefore difficult to deduce the effect of sirolimus on reduced cyst growth from improved MMP activities, and it can either be a consequence of the specific action of sirolimus or the reflection of the overall improvement of the severity of the disease. A number of recent studies show that sirolimus has prominent therapeutic effects on various renal diseases where matrix production and fibrosis are enhanced. In a model for experimental membranous nephropathy, a

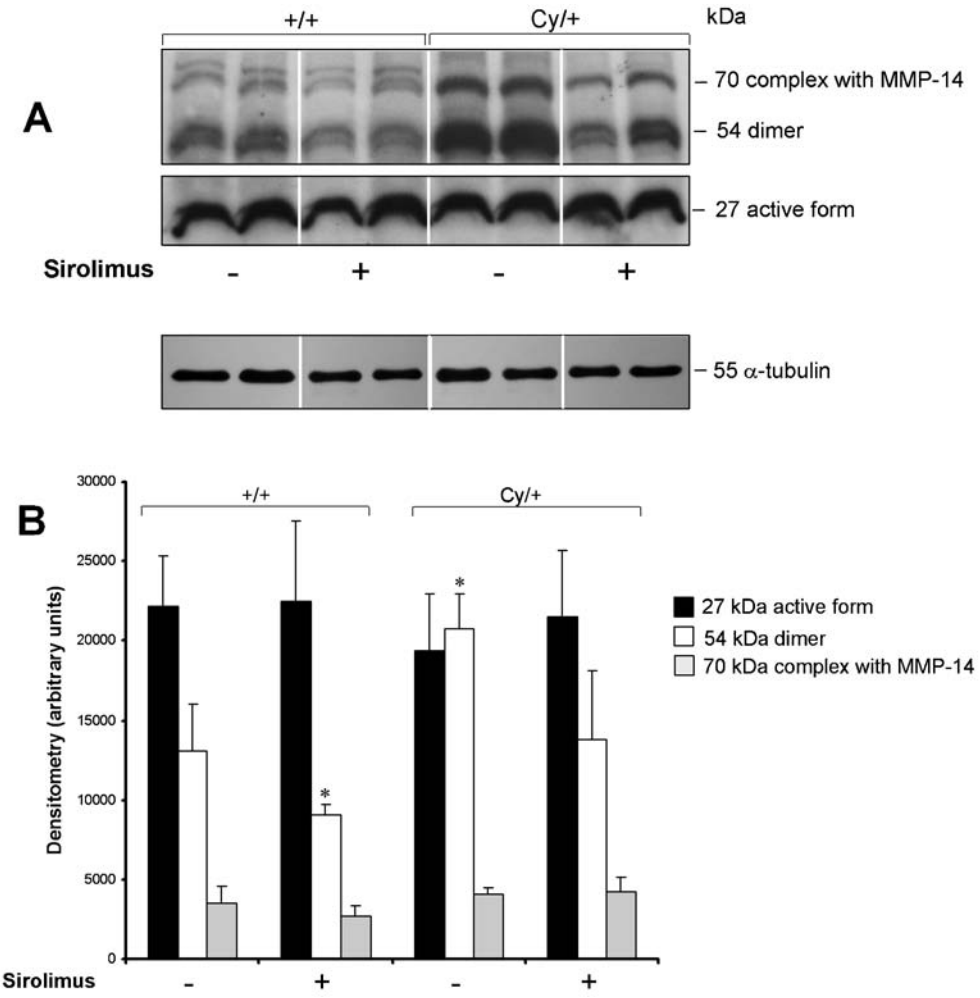

Fig. 6. TIMP-2 protein expression. **(A)** Western blot analyses and **(B)** densitometry revealed three forms of TIMP-2: the active form (TIMP-2 free form), the dimer TIMP-2 and the complex TIMP-2/MMP-14 are depicted by three bands of 27 kD, 54 kD and 70 kD, respectively. Whereas the two complexes appeared to be strongly increased in heterozygous (Cy/+) kidneys, the free form was not differentially expressed compared to the wild-type (+/+). Sirolimus treatment decreased the TIMP-2 complexed forms in +/+ and Cy/+ rats, but did not affect the levels of TIMP-2 active form. Two representative samples for each group are depicted. $^{*}P < 0.05$ compared with the untreated wild-type (+/+) rats, $n = 4$ per group.

relatively low dose of sirolimus resulted in reduced interstitial inflammation and fibrosis [29]. Treatment with sirolimus blunted compensatory renal hypertrophy; a similar effect has been observed in the rat model for hydronephrosis [30]. Sirolimus also blocked tubular epithelial-to-mesenchymal transition, a critical step in fibrogenesis. These results support the hypothesis that the beneficial effect of sirolimus is not only mediated by its antiproliferative effect but by multiple mechanisms.

In conclusion, MMP-2, MMP-14 and TIMP-2 are dysregulated in the Han:SPRD rat model of ADPKD. The activation of MMP-2 and MMP-14 is markedly suppressed *in vivo* by the treatment of Han:SPRD rats with sirolimus, and this is correlated with less matrix and TBM alterations and milder cystic disease.

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References

1. Wilson PD. Polycystic kidney disease. *N Engl J Medr* 2004; 350: 151–164
2. Zhang D, Bar-Eli M, Meloche S *et al*. Dual regulation of MMP-2 expression by the type 1 insulin-like growth factor receptor: the phosphatidylinositol 3-kinase/Akt and Raf/ERK pathways transmit opposing signals. *J Biol Chem* 2004; 279: 19683–19690
3. Martinez-Hernandez A, Amenta PS. The basement membrane in pathology. *Lab Invest* 1983; 48: 656–677
4. Carone FA, Bacallao R, Kanwar YS. Biology of polycystic kidney disease. *Lab Invest* 1994; 70: 437–448
5. Birkedal-Hansen H. Proteolytic remodeling of extracellular matrix. *Curr Opin Cell Biol* 1995; 7: 728–735
6. Massova I, Kotra LP, Fridman R *et al*. Matrix metalloproteinases: structures, evolution, and diversification. *Faseb J* 1998; 12: 1075–1095
7. Nagase H, Woessner JF . Matrix metalloproteinases. *J Biol Chem* 1999; 274: 21491–21494
8. Ellerbroek SM, Stack MS. Membrane associated matrix metalloproteinases in metastasis. *Bioessays* 1999; 21: 940–949

9. Wahl PR, Serra AL, Le Hir M *et al*. Inhibition of mTOR with sirolimus slows disease progression in Han:SPRD rats with autosomal dominant polycystic kidney disease (ADPKD). *Nephrol Dial Transplant* 2006; 21: 598–604
10. Tao Y, Kim J, Schrier RW *et al*. Rapamycin markedly slows disease progression in a rat model of polycystic kidney disease. *J Am Soc Nephrol* 2005; 16: 46–51
11. Berthier CC, Lods N, Joosten SA *et al*. Differential regulation of metzincins in experimental chronic renal allograft rejection: potential markers and novel therapeutic targets. *Kidney Int* 2006; 69: 358–368
12. Brew K, Dinakarpandian D, Nagase H. Tissue inhibitors of metalloproteinases: evolution, structure and function. *Biochim Biophys Acta* 2000; 1477: 267–283
13. Shillingford JM, Murcia NS, Larson CH *et al*. The mTOR pathway is regulated by polycystin-1, and its inhibition reverses renal cystogenesis in polycystic kidney disease. *Proc Natl Acad Sci USA* 2006; 103: 5466–5471
14. Tao Y, Kim J, Faubel S *et al*. Caspase inhibition reduces tubular apoptosis and proliferation and slows disease progression in polycystic kidney disease. *Proc Natl Acad Sci USA* 2005; 102: 6954–6959
15. Wu M, Wahl PR, Le Hir M *et al*. Everolimus retards cyst growth and preserves kidney function in a rodent model for polycystic kidney disease. *Kidney Blood Press Res* 2007; 30: 253–259
16. Harada H, Furuya M, Ishikura H *et al*. Expression of matrix metalloproteinase in the fluids of renal cystic lesions. *J Urol* 2002; 168: 19–22
17. Nakamura T, Ushiyama C, Suzuki S *et al*. Elevation of serum levels of metalloproteinase-1, tissue inhibitor of metalloproteinase-1 and type IV collagen, and plasma levels of metalloproteinase-9 in polycystic kidney disease. *Am J Nephrol* 2000; 20: 32–36
18. Obermuller N, Morente N, Kranzlin B *et al*. A possible role for metalloproteinases in renal cyst development. *Am J Physiol Renal Physiol* 2001; 280: F540–F550
19. Rankin CA, Itoh Y, Tian C *et al*. Matrix metalloproteinase-2 in a murine model of infantile-type polycystic kidney disease. *J Am Soc Nephrol* 1999; 10: 210–217
20. Riera M, Burtey S, Fontes M. Transcriptome analysis of a rat PKD model: importance of genes involved in extracellular matrix metabolism. *Kidney Int* 2006; 69: 1558–1563
21. Rankin CA, Suzuki K, Itoh Y *et al*. Matrix metalloproteinases and TIMPS in cultured C57BL/6J-cpk kidney tubules. *Kidney Int* 1996; 50: 835–844
22. Schaefer L, Han X, Gretz N *et al*. Tubular gelatinase A (MMP-2) and its tissue inhibitors in polycystic kidney disease in the Han:SPRD rat. *Kidney Int* 1996; 49: 75–81
23. Dell KM, Nemo R, Sweeney WE Jr *et al*. A novel inhibitor of tumor necrosis factor-α converting enzyme ameliorates polycystic kidney disease. *Kidney Int* 2001; 60: 1240–1248
24. Cheng S, Pollock AS, Mahimkar R *et al*. Matrix metalloproteinase 2 and basement membrane integrity: a unifying mechanism for progressive renal injury. *Faseb J* 2006; 20: 1898–900
25. Gingras AC, Raught B, Sonenberg N. Regulation of translation initiation by FRAP/mTOR. *Genes Dev* 2001; 15: 807–826
26. Bradley JMB, Kelley MJ, Rose A *et al*. Signaling pathways used in trabecular matrix metalloproteinase response to mechanical stretch. *Invest Ophthalmol Vis Sci* 2003; 44: 5174–5181
27. Zeier M, Fehrenbach P, Geberth S *et al*. Renal histology in polycystic kidney disease with incipient and advanced renal failure. *Kidney Int* 1992; 42: 1259–1265
28. Zhou G, Li C, Cai L. Advanced glycation end-products induce connective tissue growth factor-mediated renal fibrosis predominantly through transforming growth factor beta-independent pathway. *Am J Pathol* 2004; 165: 2033–2043
29. Bonegio RGB, Fuhro R, Wang Z *et al*. Rapamycin ameliorates proteinuria-associated tubulointerstitial inflammation and fibrosis in experimental membranous nephropathy. *J Am Soc Nephrol* 2005; 16: 2063–2072
30. Wu MJ, Wen MC, Chiu YT *et al*. Rapamycin attenuates unilateral ureteral obstruction-induced renal fibrosis. *Kidney Int* 2006; 69: 2029–2036

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