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A possible role for metalloproteinases in renal cyst development

NICHOLAS OBERMÜLLER,[1] NATIVIDAD MORENTE,[1] BETTINA KRÄNZLIN,[1]
NORBERT GRETZ,[1] AND RALPH WITZGALL[2]
[1]*Medical Research Center, Klinikum Mannheim, University of Heidelberg, 68167 Mannheim; and*
[2]*Institute for Anatomy and Cell Biology I, University of Heidelberg, 69120 Heidelberg, Germany*

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Obermüller, Nicholas, Natividad Morente, Bettina Kränzlin, Norbert Gretz, and Ralph Witzgall. A possible role for metalloproteinases in renal cyst development. *Am J Physiol Renal Physiol* 280: F540–F550, 2001.—The expansion of cysts in polycystic kidneys bears several similarities to the invasion of the extracellular matrix by benign tumors. We therefore hypothesized that cyst-lining epithelial cells produce extracellular matrix-degrading metalloproteinases and that the inhibition of these enzymes may represent a potential target for therapeutic intervention. Using in situ hybridization, we first analyzed the expression of membrane-type metalloproteinase 1 (MMP-14), an essential matrix metalloproteinase, of its inhibitor TIMP-2, and of the cytokine transforming growth factor (TGF)-β2 in the (*cy*/+) rat model of autosomal-dominant polycystic kidney disease. Upregulated MMP-14 mRNA was predominantly located in cyst-lining epithelia and distal tubules, whereas TIMP-2 mRNA was confined almost exclusively to fibroblasts. TGF-β2, a cytokine known to regulate the expression of matrix metalloproteinases and their inhibitors, was also expressed by cyst wall epithelia. We then treated (*cy*/+) rats with the metalloproteinase inhibitor batimastat for a period of 8 wk. The treatment with the metalloproteinase inhibitor batimastat resulted in a significant reduction of cyst number and kidney weight. Our study suggests that metalloproteinase inhibitors represent a new therapeutic tool against polycystic kidney disease, which should be applicable independently of the background of the disease.

autosomal-dominant polycystic kidney disease; therapy; extracellular matrix; matrix metalloproteinase-14; tissue inhibitor of metalloproteinases-2; transforming growth factor-β2

AUTOSOMAL-DOMINANT POLYCYSTIC kidney disease (ADPKD) represents the most frequently inherited nephropathy. Approximately 50% of all affected individuals reach end-stage renal disease at the age of 60, ultimately requiring expensive renal replacement therapy such as dialysis and transplantation (14, 22, 52). Genetically, this disease is most often caused by mutations in the *PKD1* (72) and *PKD2* (38) genes, which together are mutated in far more than 90% of all ADPKD patients. So far, however, it is only poorly understood how these mutations contribute to cyst formation in the kidney and in other organs. Several studies have investigated the role of abnormal cell proliferation, fluid accumulation within cysts, and alterations of the extracellular matrix in cystogenesis (11, 12, 78), but no conclusive concept has surfaced so far.

It is likely, however, that the basement membrane plays an important role in the formation of cysts, because it represents the interface between the cyst-lining epithelium and its environment. The balance between the increased degradation of collagens, glycoproteins, and proteoglycans on the one hand and the enhanced synthesis and deposition of extracellular matrix components on the other should influence the extent of cyst enlargement and interstitial fibrosis, the latter being typical at advanced stages of the disease (44, 50). Matrix metalloproteinases (MMPs) are a large family of secreted and membrane-bound zinc-dependent endopeptidases, which degrade a wide spectrum of substrates and therefore represent key enzymes in the turnover of the extracellular matrix (8, 37, 40). Most MMPs are released as zymogens before being activated in the extracellular environment; MMP-14, however, 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 (20, 40). Thus net activation of MMP-2 depends on local MMP-14 and TIMP-2 levels, and it therefore follows that the interaction of a cell with the extracellular matrix is critically determined by the concentrations of metalloproteinases and their natural inhibitors. Furthermore, components of the extracellular matrix have long been recognized as transforming growth factor (TGF)-β- and fibroblast growth factor (FGF)-2-binding proteins. Degradation of the extracellular matrix leads to the release of these growth factors (7, 21, 69) and, in turn, may regulate cell division and cyst growth.

Address for reprint requests and other correspondence: R. Witzgall, Institute for Anatomy and Cell Biology I, Univ. of Heidelberg, Im Neuenhimer Feld 307, 69120 Heidelberg, Germany (E-mail: ralph.witzgall@urz.uni-heidelberg.de).

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Some studies have indeed provided circumstantial evidence for the importance of certain matrix metalloproteinases and their natural counterparts, the tissue inhibitors of metalloproteinases (TIMPs), in polycystic kidney disease (23, 42, 55, 56, 61, 64). More direct experimental support for the role of MMPs and TIMPs in the formation of tubular and cystic structures has come from organ culture experiments and from the analysis of the invasive behavior of cells in collagen gels. Murine embryonic kidneys have not only been shown to produce MMPs in vitro, but moreover renal organogenesis is impaired by the inhibition of MMP-9 (35), MMP-14 (31), and possibly of MMP-2 (31, 35). Similar results have arisen from the three-dimensional culture of certain cell lines. When renal epithelial cell lines such as Madin-Darby canine kidney (MDCK) and mIMCD3 cells are grown in collagen gels, they form arborized tubular structures, a phenomenon called branching morphogenesis. In that process they synthesize MMPs such as MMP-1 (59), MMP-2 (33), MMP-9 (33), and MMP-14 (30). The addition of natural (TIMP-1, TIMP-2) and synthetic (batimastat, see below) MMP inhibitors drastically reduces branching morphogenesis (30, 33), thus emphasizing the importance of MMPs.

We therefore performed an in situ hybridization study to localize MMP-14, TIMP-2, and TGF-β2 in polycystic kidneys of the (*cy*/+) rat, a model that closely resembles human ADPKD (16, 17, 26, 34, 44, 62). These experiments demonstrated the distinct expression of all three mRNAs in polycystic kidneys, with MMP-14 mRNA being expressed by cyst-lining epithelia in particular.

Because a local degradation of extracellular matrix components appears necessary for cyst expansion, inhibition of metalloproteinases could be a useful strategy to influence the clinical course of polycystic kidney disease. Many synthetic inhibitors of metalloproteinases presently being studied have been developed as anticancer agents to prevent tumor cell invasion through matrix barriers (10). They essentially are collagen peptide mimetics and in addition contain zinc-binding groups such as hydroxamates to block the active site of the MMPs (6, 79). Batimastat, also known as BB-94, was one of the first synthetic inhibitors of metalloproteinases; it specifically and potently inhibits MMPs without showing major toxicity in animals (79). As a test of principle, we used batimastat as a therapeutic agent against polycystic kidney disease and were able to reduce renal cyst number in the (*cy*/+) rat.

MATERIALS AND METHODS

Animals. Our colony of (*cy*/+) rats is derived from the Han:SPRD rat strain. It has been inbred for over 20 generations in Mannheim and has therefore been registered as PKD/Mhm (for polycystic kidney disease, Mannheim; inbred strains of rats, http://www.informatics.jax.org/external/festing/rat/docs/PKD.shtml). Animals were maintained under the control of N. Gretz at the Animal Care Facility in Mannheim. Only male wild-type and heterozygous rats were used in this study (homozygously affected progeny do not live longer than 3–4 wk). A total of 53 animals originating from 9 different litters were enrolled randomly over a period of 16 days in the treatment protocol. Starting on postnatal *day 14*, rats either received daily intraperitoneal injections of batimastat (British Biotech, Oxford, UK) at a dosage of 25 mg/kg body wt or vehicle (0.9% NaCl containing 0.01% Tween-20) for 6 wk, followed by 3 injections/wk for another 2 wk. The applied daily dosage of batimastat used in this trial was based on previous studies in human subjects. Body weight was monitored weekly, and blood samples were collected 2 wk after the beginning and at the end of the treatment period. The rats received standard rat chow (containing 19% protein) and tap water. Two to three days before the conclusion of the study period, animals were placed into metabolic cages to analyze their urine parameters. All experiments were conducted in accordance with the German Animal Protection Law and were approved by the local government (Regierungspräsidium Karlsruhe, Germany).

Tissue preparation. For histomorphological analysis and histochemical experiments, all animals in the study were subjected to perfusion-fixation. Rats were anaesthetized with pentobarbital sodium (40 mg/kg body wt) and perfused through the distal abdominal aorta with 2.5% freshly depolymerized paraformaldehyde in PBS, pH 7.4, at a pressure level of 180–200 mmHg for 3 min. Subsequently, kidneys were carefully removed and weighed. A complete slice from the midportion was cut from the right kidneys, immersion-fixed overnight in the same fixative, and embedded in paraffin. Four- to five-micrometer-thick paraffin sections were stained with hemotoxylin and eosin and examined morphometrically. For subsequent in situ hybridization experiments, the remaining portions of the right and left kidneys were cut into pieces and incubated in a 18% sucrose solution in PBS for 4 h on a shaking platform at room temperature before being snap-frozen in liqid nitrogen-cooled isopentane. Twelve-week-old, male heterozygous rats, which were used for initial in situ hybridization studies, were perfused in a similar way, and both kidneys were processed as described above.

Albumin ELISA. The concentration of albumin in rat urine was determined by using a competitive two-step ELISA, which was developed in the Medical Research Center in Mannheim. During the first step, a chicken anti-rat albumin antibody (catalog no. 55727, Cappel, Eppelheim, Germany) was incubated with the sample. After complexes between albumin and the anti-rat albumin antibody had formed, this mixture was transferred to a 96-well plate coated with rat albumin (catalog no. A-4538, Sigma, Deisenhofen, Germany). The amount of antibody bound to the albumin in the 96-well plates was determined with a peroxidase-coupled antibody directed against chicken IgG (catalog no. A-9792, Sigma) and subsequent photometric measurement at 450 nm. Each measurement included rat albumin samples with known concentrations, so that the albumin concentration in the urine could be determined from a standard curve.

Morphometry. For morphometric measurements hemotoxylin- and eosin-stained paraffin sections from all animals were analyzed by using the image-analysis system Quantimet 600 (LEICA Q600, Leica Cambridge, Cambridge, UK) and QWin software (v 1.05). To differentiate between normal tubular profiles and cysts, the roundedness and the open diameter of the profiles were taken into account. Thus only profiles with approximately the size of a glomerulus (~9,000 μm^2) and larger were counted as cysts.

Preparation of riboprobes for in situ hybridization. Recombinant plasmids containing a 2.5-kbp cDNA fragment of rat TIMP-2, a 2.4-kbp cDNA fragment of rat MMP-14 (both

kindly provided by Michael T. Crow, National Institutes of Health, Baltimore, MD), and a 1.4-kbp cDNA fragment of mouse TGF-β2 (a kind gift of Clemens Suter-Crazzolara, Institute for Anatomy and Cell Biology III, Heidelberg, Germany) were digested appropriately. Antisense and sense RNA probes were synthesized and labeled by in vitro transcription using digoxigenin-11-UTP according to the protocol supplied by the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). The transcripts were finally subjected to partial alkaline hydrolysis to obtain fragments of a calculated average length of 250 nucleotides.

In situ hybridization. This procedure was essentially carried out as described in detail previously (43). In brief, 6-μm-thick cryostat sections were hybridized with a solution containing 50% formamide and 5–8 ng/μl of hydrolyzed TIMP-2, MMP-14, or TGF-β2 RNA probes. Hybridization was performed overnight at 45°C, followed by several stringent washes [the most stringent wash was in 0.2× standard sodium citrate (SSC) containing 50% formamide at 52°C for 1 h]. The specificity of the obtained in situ hybridization signal was verified by parallel incubation with antisense and sense riboprobes on alternate sections. Throughout all experiments, sense probes did not produce any detectable signals. As further negative controls, some sections were processed without anti-digoxigenin antibody, which also yielded completely negative results.

Preparation of total RNA. The kidneys from 10-wk-old male (*cy*/+) and (+/+) rats were rapidly removed, and total RNA was extracted according to the acid-guanidinium-phenol-chloroform protocol of Chomczynski and Sacchi (13). The resulting RNA pellets were dissolved in diethylpyrocarbonate-treated water, and the yield was measured by spectrometry at 260 nm. Samples were stored at −80°C until further use. The integrity of the extracted RNA was checked by agarose gel electrophoresis.

RNase protection assay. RNAse protection analysis was performed according to standard protocols (3). An ~300-bp rat MMP-14 cDNA fragment, a 361-bp rat TIMP-2 cDNA fragment (both kindly provided by Michael T. Crow), a 290-bp rat TGF-β2 cDNA fragment (kindly provided by Ian McLennan and Kyoko Koishi, University of Otago, Dunedin, NZ), and a 80-bp 18S cDNA fragment (Ambion, Austin, TX) were used for in vitro transcription. Plasmids were digested appropriately, and radiolabeled antisense cRNA probes were synthesized in vitro with viral RNA polymerases (Roche Molecular Biochemicals) in the presence of [α-^{32}P]UTP.

Fifty micrograms each of total RNA were hybridized with the radiolabeled MMP-14, TIMP-2 and TGF-β2 riboprobes, whereas 50 ng of total RNA were hybridized with the radiolabeled 18S riboprobe; tRNA from *Escherichia coli* served as a negative control. Hybridization was conducted overnight at 42°C. After digestion with RNase A and T1, the protected fragments were separated on a 4% polyacrylamide/6 M urea gel. Thereafter, the gel was dried for 2 h and analyzed in Fujifilm BAS-2500 and Bio-Rad GS-525 Phosphorimagers.

Processing of figures. Black-and-white photographs were scanned with a Nikon Coolscan LS-2000 by using Silverfast 4.1 software (LaserSoft, Kiel, Germany) and then processed with Photoshop 5.0 (Adobe Systems, San Jose, CA).

Statistics. Statistical evaluations were performed by using the statistical analysis system (SAS) from SAS Institute (Cary, NC) The following procedures were applied: medians and quartiles were calculated by using the PROC UNIVARIATE software, Wilcoxon tests were carried out with PROC NPAR1WAY, and χ^2-tests were done with PROC FREQ. Statistical significance was considered at a P value ≤ 0.05.

RESULTS

Expression pattern of the MMP-14, TIMP-2 and TGF-β2 mRNAs. By using polycystic kidneys from 12-wk-old male (*cy*/+) rats, the expression patterns of the mRNAs encoding the metalloproteinase MMP-14, the metalloproteinase inhibitor TIMP-2, and the cytokine TGF-β2 were determined by in situ hybridization. MMP-14 mRNA was expressed in tubular profiles of the cortex and outer medulla (Fig. 1, *A* and *B*). Whereas in the renal cortex MMP-14 was prominently expressed in cyst-lining epithelia (Fig. 2*A*), in the inner stripe a number of thick ascending limb profiles were stained for MMP-14 mRNA (Fig. 2*B*). In addition, MMP-14 mRNA expression could also be localized to few fibroblasts in the vicinity of cystic and noncystic tubules (Fig. 2*C*). By contrast, in situ hybridization for TIMP-2 mRNA in polycystic kidneys demonstrated an almost exclusive expression in fibroblasts (Fig. 3). In

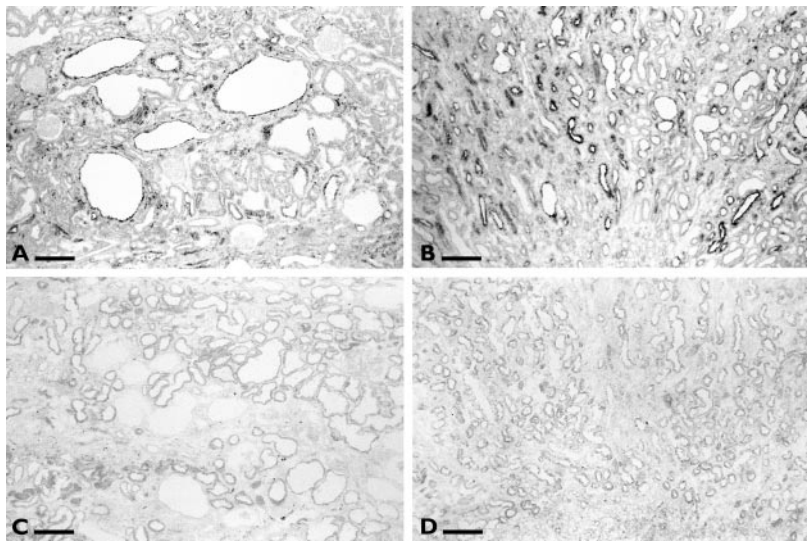

Fig. 1. Overview of membrane-type metalloproteinase 1 (MMP-14) mRNA expression on a kidney section of a 3-mo-old (*cy*/+) rat. In situ hybridization for MMP-14 mRNA was carried out by using digoxigenin-labeled antisense and sense RNA probes. An overview of the cortex (*A*) and the inner stripe (*B*) demonstrates the expression of MMP-14 mRNA in cystic (*A*) and noncystic (*B*) tubular profiles. Because no signal was obtained in the inner medulla, the positive tubules extending into the inner stripe most likely correspond to thick ascending limbs. Hybridization with MMP-14 sense RNA yielded no specific signals in the cortex (*C*) and the inner stripe (*D*). Bars, 200 μm.

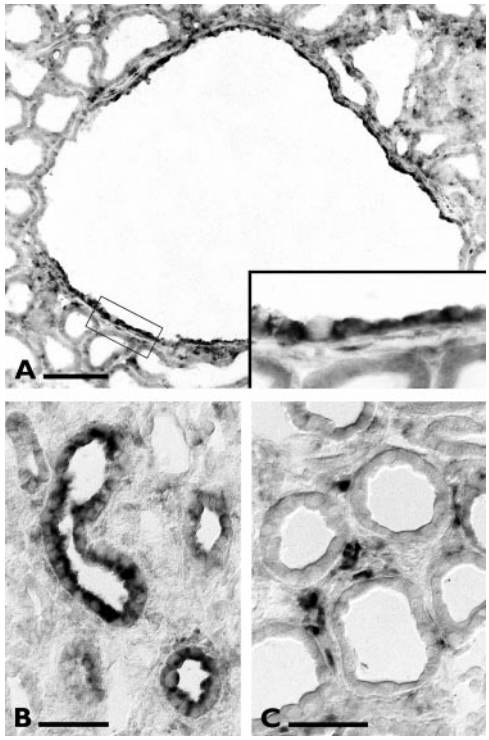

Fig. 2. Detailed analysis of MMP-14 mRNA expression on a kidney section of a 3-mo-old (*cy*/+) rat. Strong expression of MMP-14 mRNA can be clearly seen in cyst-lining epithelial cells in the cortex (*A*) and in thick ascending limb cells in the inner stripe (*B*). *Inset*: expression of MMP-14 in cyst-lining epithelial cells at a higher magnification. In some cases, MMP-14 mRNA was also detected in fibroblasts surrounding tubular profiles in the cortex (*C*). Bars, 100 μm (*A*), 50 μm (*B*, *C*).

rare cases, specific hybridization signals for MMP-14 and TIMP-2 were also observed in the renal capsule, in parietal cells of Bowman's capsule, and in the papillary epithelium (data not shown). In situ hybridization for TGF-β2 in polycystic kidneys resulted in the labeling of a subset of cysts with a mosaic expression pattern (Fig. 4). When kidneys from age-matched wild-type rats were analyzed by in situ hybridization, virtually no specific expression was seen in the case of MMP-14 and TGF-β2, and only scarce expression of TIMP-2 mRNA in fibroblasts (data not shown). To corroborate these findings, RNase protection assays with RNAs isolated from total kidneys were carried out. We were able to detect all three mRNAs in both (*cy*/+) and (+/+) rat kidneys, but there were no pronounced differences between (*cy*/+) and (+/+) kidneys (Fig. 5).

Inhibition of MMP activity leads to reduced cyst number. These results prompted us to study the effect of the metalloproteinase inhibitor batimastat on the progression of cystic disease. Starting at postnatal *day 14* and lasting until postnatal *day 70*, a total of 53 animals were treated with batimastat or vehicle; no mortality was observed during the study. Treatment with batimastat was associated with a statistically significant reduction in absolute kidney weights (Table 1) in both (*cy*/+) rats (median, 4.98 vs. 6.05 g, $P = 0.007$) and wild-type (+/+) animals (median, 2.38 vs. 3.13 g, $P = 0.001$). The reduction in kidney weight was still statistically significant when corrected for body weight. Body weights were only moderately lowered in the case of batimastat-treated (*cy*/+) rats, whereas in (+/+) animals treatment with batimastat led to a statistically significant reduction in body weights (Table 1). Morphometric analysis revealed that treatment with the metalloproteinase inhibitor was associated with a marked and statistically significant reduction in cyst number in (*cy*/+) animals (375 vs. 474, $P = 0.026$; Table 1). When the distribution of different cyst sizes in batimastat- and placebo-treated (*cy*/+) rats was analyzed, it could be seen that batimastat treatment resulted in a decreased number of cysts of all sizes (Table 2), although it had a more pronounced inhibitory effect on the occurrence of smaller cysts (Table 2).

Renal function as assessed from serum urea and creatinine values was unaltered in batimastat compared with placebo groups, and treatment with the

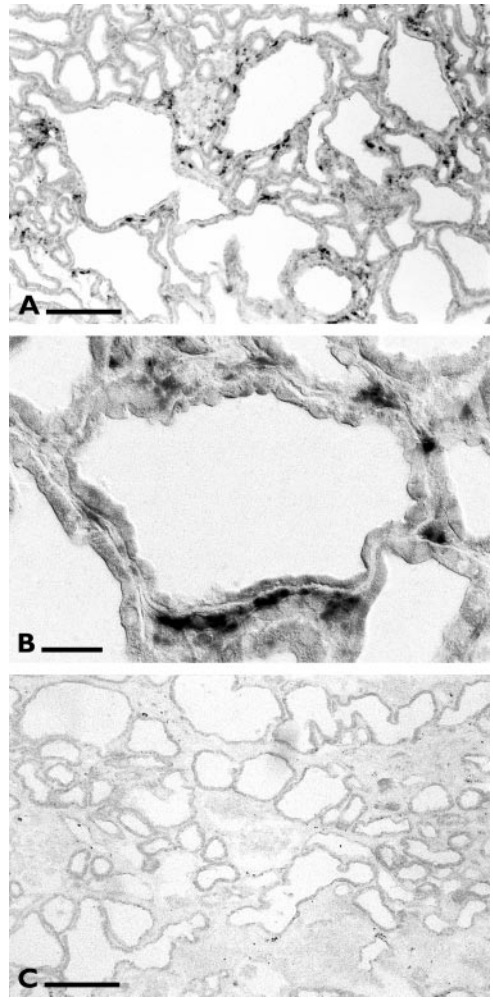

Fig. 3. Nonradioactive in situ hybridization for tissue inhibitor of metalloproteinases (TIMP)-2 mRNA in a 3 mo-old (*cy*/+) rat kidney. An overview through the renal cortex (*A*) shows numerous cells expressing TIMP-2; on closer examination, these cells were identified as fibroblasts located in the close vicinity of cortical cysts (*B*). Hybridization with TIMP-2 sense RNA yielded no specific signals (*C*). Bars, 200 μm (*A*, *C*), 50 μm (*B*).

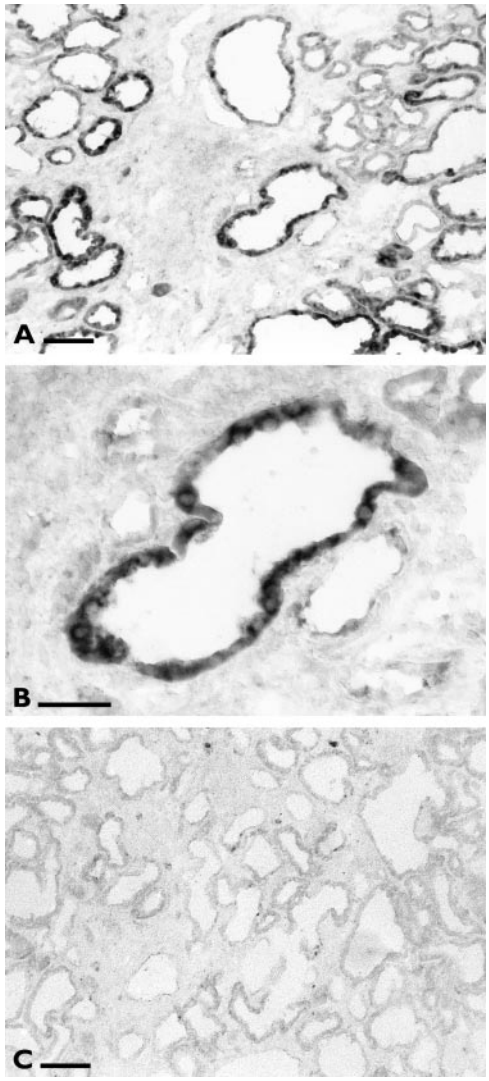

Fig. 4. Nonradioactive in situ hybridization for transforming growth factor (TGF)-β2 mRNA in a 3-mo-old (*cy*/+) rat kidney. TGF-β2 was strongly expressed by portions of the cyst wall epithelium, whereas no signal could be demonstrated in epithelial cells of noncystic tubular profiles nor in the interstitium [overview (*A*), higher magnification (*B*)]. Hybridization with TGF-β2 sense RNA yielded no specific signals (*C*). Bars, 100 μm (*A*, *C*), 50 μm (*B*).

metalloproteinase inhibitor also had no effect on the absolute and relative 24-h urine volume production in (*cy*/+) and wild-type animals (Table 3). However, sodium excretion per 24 h was significantly increased in batimastat-treated vs. placebo-treated (*cy*/+) rats (0.44 vs. 0.29 mmol $Na^+ \cdot 24\ h^{-1} \cdot 100$ g body wt^{-1}, $P = 0.009$; Table 3). This effect was not found in the wild-type animals. No statistically significant differences were encountered in batimastat vs. placebo groups with regard to the excreted amounts of other electrolytes; moreover, total urinary excretion of osmolytes was also unchanged (Table 3).

One side effect of the batimastat treatment was a higher urinary protein excretion, although this difference was statistically significant only in wild-type rats (4.87 vs. 4.09 mg protein$\cdot 24\ h^{-1} \cdot 100$ g body wt^{-1}, $P = 0.003$; Table 3), while reaching borderline significance in (*cy*/+) rats (4.95 vs. 4.40 mg protein$\cdot 24\ h^{-1} \cdot 100$ g body wt^{-1}, $P = 0.065$; Table 3). A similar, but more pronounced effect could be found, when the urinary albumin excretion was analyzed; in both the (*cy*/+) (1.11 vs. 0.79 mg albumin$\cdot 24\ h^{-1} \cdot 100$ g body wt^{-1}, $P = 0.013$; Table 3) and the wild-type (0.55 vs. 0.28 mg albumin$\cdot 24\ h^{-1} \cdot 100$ g body wt^{-1}, $P < 0.001$; Table 3) rats, the albumin excretion in the urine was significantly higher in the batimastat groups compared with the respective placebo groups.

During the removal of the renal capsules after the perfusion procedure, it was noticed that the renal capsular tissue of most batimastat-treated (*cy*/+) and wild-type rats had lost its typical transparency and appeared as an opaque capsule with a rigid consistency. The expression of MMP-14, TIMP-2, and TGF-β2 mRNA was also studied after treatment. There was no obvious difference between the batimastat-treated and placebo-treated animals (data not shown).

DISCUSSION

Expression pattern of the MMP-14, TIMP-2, and TGF-β2 mRNAs. Our results show that the mRNA for a crucial membrane-bound metalloproteinase, MMP-14, is expressed in cyst-lining cells of polycystic kidneys, whereas in normal kidneys expression levels were below the detection limit of the in situ hybridization experiments. Previous analysis of patients (23, 41, 42) and (*cpk*/*cpk*) mice (55, 56) already has provided evidence for the upregulation of secreted MMPs, whereas one publication found a downregulation of MMP expression in tubular cultures from polycystic kidneys (61). Furthermore, there also is direct evidence for an increased degradation of the extracellular matrix in patients with polycystic kidney disease (41, 64). We believe that our study makes an important contribution to a better understanding of cystogenesis, because we are the first to identify the MMP-synthesizing cells in polycystic kidneys in situ. MMP-14, a membrane-bound metalloproteinase, plays a key role in the turnover of the extracellular matrix (20, 40), which is demonstrated by the severe phenotype of the *MMP14*-knockout mice (27, 83) and the rather mild phenotype of the *MMP2* (29)- and *MMP9* (76)- knockout mice. The importance of MMP-14 is further emphasized by the striking invasive phenotype of MDCK cells overexpressing MMP-14 compared with other MMPs (28). Nevertheless, we cannot rule out the possibility that other matrix-degrading enzymes also play an important role for the expansion of cysts.

In addition to the detection of MMP-14 mRNA in cyst-lining epithelial cells, MMP-14 mRNA expression was seen in a portion of thick ascending limb profiles, which, however, was not or only moderately dilated. Interestingly, clusterin, a molecule upregulated in states of cell injury, is also strongly expressed by noncystic distal tubules in heterozygous rat kidneys (44). The expression of MMP-14 in noncystic distal tubules

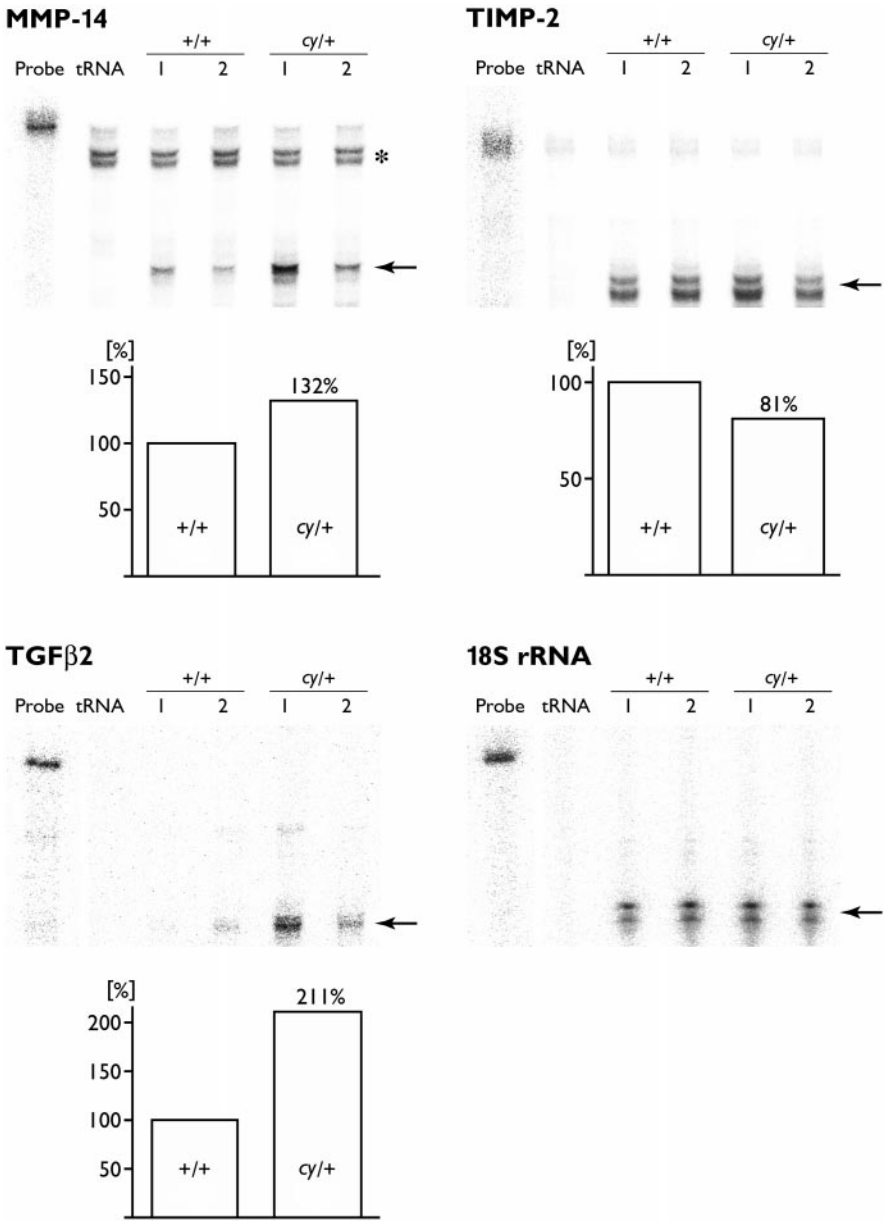

Fig. 5. RNase protection assay for the detection of MMP-14, TIMP-2, and TGF-β2 mRNA. Two different (+/+) and (*cy*/+) rats, each 10 wk old, were used to isolate RNA from whole kidneys. Fifty micrograms of total RNA were hybridized with MMP-14, TIMP-2, and TGF-β2 antisense RNA, respectively, whereas 50 ng of total RNA were hybridized with antisense RNA directed against the 18S subunit of rRNA. The expression levels were determined by quantitating the protected bands (indicated by an arrow in all 4 cases) with a Phosphorimager and normalizing the expression of MMP-14, TIMP-2, and TGF-β2 to that of 18S rRNA. Bar graphs below the gels represent the mean values obtained from (+/+) and (*cy*/+) rats [the level in (+/+) rats was arbitrarily set as 100%]. Although in the case of TGF-β2 a >2-fold change could be detected, no drastic changes were observed with MMP-14 and TIMP-2. *, In the protection assay with MMP-14 antisense RNA, 2 nonspecific bands were observed, which survived the RNase digest probably due to a particular intrinsic property of the probe.

is another indication that the underlying defect in the (*cy*/+) kidneys also affects the distal portion of the nephron, but at this point it is not understood why in this model cysts almost exclusively develop in the proximal tubule. It is possible that additional defects, e.g., in cell-matrix or cell-cell contacts, are present in the proximal tubule. Alternatively, because MMP-14 activates other metalloproteinases, it is conceivable that proximal tubules but not thick ascending limbs express additional metalloproteinases, which, on activation, contribute to cyst expansion.

Our study also shows the increased cell-specific expression of TIMP-2 mRNA in kidneys of the (*cy*/+) rat, again a finding in agreement with previous reports analyzing human (42), mouse (56), and rat (61) polycystic kidneys. Whereas MMP-14 mRNA was expressed predominantly by epithelial cells, TIMP-2 mRNA was almost exclusively located in fibroblasts. The membrane-bound protease MMP-14 directly influences pericellular proteolysis, and it therefore is conceivable that an imbalance of MMP-14 over TIMP-2 contributes to cell migration and cyst enlargement in the early stages of cyst development. In contrast, the observed upregulation of endogenous MMP inhibitors such as TIMP-2 may represent a defense mechanism of the surrounding interstitium to diminish the effects of MMPs, thus neutralizing soluble MMPs and leading to fibrosis at advanced stages of the disease (44). This hypothesis is also supported by the recently generated MMP-14-knockout mouse, hallmarks of which are impaired connective tissue growth and fibrosis of soft tissues (27, 83).

The finding that TGF-β2 is expressed by cyst-lining epithelia points to its possible role in the progression of

Table 1. *Median values for body weights, kidney weights, and morphometric data obtained from batimastat- and placebo-treated (cy/+) and (+/+) rats*

	(*cy*/+)			(+/+)		
	Batimastat (n = 14)	Placebo (n = 18)	P	Batimastat (n = 10)	Placebo (n = 11)	P
Body wt, g	337(309;348)	345(340;347)	0.128	316(308;324)	345(334;349)	0.001*
Kidney wt, g	4.98(4.67;5.75)†	6.05(5.61;6.61)	0.007*	2.38(2.27;2.51)‡	3.13(2.80;3.29)	0.001*
Kidney wt, g/100 g bw	1.54(1.51;1.76)†	1.76(1.61;1.90)	0.026*	0.78(0.72;0.79)‡	0.91(0.87;0.94)	0.002*
Cyst no.	375(331;445)	474(383;532)	0.026*	NA	NA	
Cyst area/kidney area, %	4.27(3.92;4.60)	3.91(3.45;4.64)	0.247	NA	NA	
Mean cyst area, μm^2	18,403(18,020;19,032)	17,863(17,317;18,339)	0.060	NA	NA	

Data are presented as medians; the 25th and 75th percentiles are given in parentheses. bw, Body weight; NA, not applicable. The ratio of cyst area to kidney area was calculated by determining the area covered by cysts and the area of the respective kidney section. Mean cyst area was obtained by dividing the cyst-covered area by the total no. of cysts on a given kidney section. *Statistically significant at $P \leq 0.05$; †n = 11 because of technical reasons; ‡n = 7 because of technical reasons.

polycystic disease. The regulation of matrix metalloproteinases and their inhibitors by growth factors has been described (19, 58). TGF-β inhibits the synthesis of matrix metalloproteinases and increases the expression of TIMPs, therefore promoting matrix deposition and fibrosis (9, 59), which is a prominent feature in polycystic kidneys from old (*cy*/+) rats (44). Among the three TGF-βs, TGF-β2 may be of particular importance in the kidney, because renal alterations have only been described in mice lacking a functional TGF-β2-encoding gene and not in mice, in which the genes coding for TGF-β1 and TGF-β3 were inactivated (60). Under physiological conditions, TGF-β2 is synthesized only during nephrogenesis in the kidney, where its mRNA is found in tubules (63) and the protein is detected in the tubular basement membrane (53). Our observation that TGF-β2 mRNA is synthesized by cyst wall epithelia reiterates the phenomenon that cyst-lining epithelial cells dedifferentiate and reactivate genes that have been transcribed earlier in development.

The result that by RNase protection analysis we were not able to detect any drastic differences in the expression levels of MMP-14, TIMP-2, and TGF-β2 mRNAs between (*cy*/+) and (+/+) kidneys may not be too surprising considering the following facts. It is estimated that only a minor percentage of cortical proximal tubules become cystic in the (*cy*/+) rat (16, 44, 62). Because there already is a baseline expression of all three mRNAs in (+/+) rat kidneys, and because MMP-14 and TGF-β2 are expressed only in a subset of cysts, even a pronounced increase of expression in some cysts would only lead to a moderate increase of expression as judged from analyzing RNA from whole kidneys. The finding that according to the RNase protection assay the expression of TIMP-2 even decreased to a small degree shows that data obtained by extracting RNA from a whole organ have to be interpreted with a lot of caution.

Inhibition of matrix metalloproteinase activity leads to reduced cyst number. The second important result of our study is the evidence that treatment with the synthetic metalloproteinase inhibitor batimastat leads to decreased kidney weight and a reduced number of cysts in (*cy*/+) rat kidneys. Although the 21% decline in cyst number was statistically significant, one might have hoped for a larger reduction. On the other hand, treatment with batimastat did not commence until 2 wk after birth, when renal cyst development is well underway (Obermüller and Witzgall, personal observations), and therefore a complete disappearance of cysts could not be expected. An analysis of the serum creatinine and serum urea levels showed no change under

Table 2. *Distribution of cyst sizes in the kidneys of batimastat- and placebo-treated (cy/+) rats*

Cyst Size, μm^2	Total Cyst No. (Cyst No./Kidney) After Batimastat Treatment (n = 14)	Total Cyst No. (Cyst No./Kidney) After Placebo Treatment (n = 18)	Relative Group Size After Batimastat Treatment, %	Relative Group Size After Placebo Treatment, %	Difference Among Group Sizes
9,000–9,999	730(52)	1,279(71)	13.7	15.3	−1.6
10,000–12,499	1,335(95)	2,159(120)	25.0	25.9	−0.9
12,500–14,999	767(55)	1,217(68)	14.3	14.6	−0.3
15,000–19,999	896(64)	1,338(74)	16.8	16.1	0.7
20,000–29,999	829(59)	1,255(70)	15.5	15.1	0.4
≥30,000	788(56)	1,088(60)	14.7	13.1	1.6
Total	5,345	8,336	100.0	100.0	

Cyst sizes were arbitrarily divided to obtain approximately equal group sizes of at least 700 cysts/group in the batimastat-treated rats and of at least 1,000 cysts/group in the placebo-treated rats. Batimastat treatment led to a reduction in cyst numbers independent of the cyst sizes (*columns 2* and *3* list the total no. of cysts in a given group and the mean no. of cysts/kidney section). The strength of the inhibitory effect of batimastat, however, depended on the size of the cysts, because the reduction of the number of smaller cysts was more pronounced than the decrease in the number of larger cysts (*columns 4, 5,* and *6*). A χ^2-test confirmed that the distribution of cyst sizes in the batimastat-treated and placebo-treated rats was significantly different (P = 0.008).

Table 3. *Plasma and urine parameters of batimastat- and placebo-treated (cy/+) and (+/+) rats*

	(*cy*/+)			(+/+)		
	Batimastat (n = 14)	Placebo (n = 18)	P	Batimastat (n = 10)	Placebo (n = 11)	P
Serum creatinine, mg/dl	0.50(0.47;0.53)	0.50(0.47;0.51)	0.848	0.40(0.40;0.43)	0.42(0.40;0.45)	0.318
Serum urea, mg/dl	74.0(67;78)	76.5(70;80)	0.648	47.0(44;51)	47.0(44;50)	0.546
Urine volume, ml/24 h	24.5(21.2;25.6)	22.9(20.7;27.1)	0.506	12.8(12.2;14.0)	16.7(12.3;18.7)	0.067
Urine volume, ml·24 h^{-1}·100 g bw^{-1}	7.31(6.82;7.83)	6.72(5.98;7.97)	0.106	4.03(3.85;4.61)	4.81(3.82;5.60)	0.218
Sodium excretion, mmol/24 h	1.34(1.21;1.68)	1.00(0.86;1.46)	0.046*	1.23(1.04;1.40)	1.16(1.08;1.39)	1.000
Sodium excretion, mmol·24 h^{-1}·100 g bw^{-1}	0.44(0.38;0.50)	0.29(0.25;0.42)	0.009*	0.40(0.34;0.42)	0.34(0.31;0.41)	0.418
Osmolyte excretion, mosmol/24 h	16.69(14.87;19.72)	15.86(13.67;20.87)	0.531	18.61(15.70;19.78)	17.69(16.90;19.67)	0.751
Protein excretion, mg/24 h	16.54(14.04;26.13)	15.43(10.21;17.98)	0.231	15.04(14.00;16.07)	13.78(12.10;14.70)	0.084
Protein excretion, mg·24 h^{-1}·100 g bw^{-1}	4.95(4.39;7.44)	4.40(3.10;5.25)	0.065	4.87(4.46;4.97)	4.09(3.42;4.24)	0.003*
Albumin excretion, mg/24 h	3.53(2.66;5.71)	2.77(2.30;3.38)	0.050*	1.73(1.45;2.12)	0.90(0.71;1.06)	0.001*
Albumin excretion, mg·24 h^{-1}·100 g bw^{-1}	1.11(0.86;1.63)	0.79(0.68;0.98)	0.013*	0.55(0.47;0.73)	0.28(0.21;0.30)	<0.001*

Data are presented as medians; the 25 and 75th percentiles are given in parentheses. *Statistically significant at $P \leq 0.05$.

batimastat treatment. The creatinine values were still in the normal range, and therefore the lack of a decrease is not surprising. In our experience, the serum urea levels in (*cy*/+) rats are not a very accurate indicator for the progression of polycystic kidney disease; in rats at such an early age and considering the relatively modest reduction of cyst number, we therefore would not have expected a drop in serum urea levels. Our therapeutic study was intended to provide proof of principle, that treatment with a metalloproteinase inhibitor can have a beneficial effect on the progression of polycystic kidney disease. Clearly, dose-finding and longer-lasting studies are needed to corroborate our initial findings.

Somewhat surprisingly, the ratio of cyst area to kidney area and the mean cyst area increased by 9 and 3%, respectively, after batimastat treatment (these changes were not statistically significant). The slight increase in the ratio of cyst area to kidney area can be explained by the fact that treatment with batimastat led not only to a reduction of cyst number but also to a decrease in kidney weight by 18%. In addition, batimastat treatment changed the distribution of cyst sizes, because the percentage of larger cysts grew (although the absolute numbers of both smaller and larger cysts decreased), and this effect also caused an increase in the mean cyst area. From these data it can be hypothesized that batimastat exerts a more pronounced inhibitory effect on the initial phase of cyst development, for which there may be several reasons. First, although batimastat inhibits a number of MMPs (MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, and MMP-14) with comparable efficiency (6, 82), it is possible that in the course of cyst development the cyst-lining epithelial cells start to express MMPs that are not inhibited by batimastat. Second, the results presented in this study demonstrate that epithelial cells from normal proximal tubules and from small cysts did not show detectable amounts of MMP-14 mRNA by in situ hybridization, whereas large cysts contained numerous epithelial cells expressing MMP-14 mRNA. In recent publications, a downregulation, but not a complete loss, of MMP-14 mRNA expression was demonstrated during postnatal development of the normal rat kidney (31, 51, 71), which is also corroborated by our RNase protection data. We therefore speculate that normal tubules and smaller cysts produce amounts of MMP-14 mRNA that are below the detection limit for nonradioactive in situ hybridization. According to this hypothesis, the dosage of batimastat used in our study would have been more efficient in inhibiting the small levels of MMP-14 present in normal tubules and small cysts, but too low to effectively block the higher amounts of MMP-14 being expressed in larger cysts. If this scenario were true, the application of higher doses of batimastat should exert a stronger inhibitory effect on the growth of larger cysts as well.

The application of higher doses of batimastat, however, may be limited by the observed side effects, in particular, albuminuria. It was beyond the scope of the present manuscript to determine the origin of albuminuria, but it could, for example, result from an altered turnover of the glomerular basement membrane. A solution to this problem could be the use of more specific metalloproteinase inhibitors, which may show fewer side effects, or slightly lower doses of the drug. Already a slowed progression of the disease would be of great therapeutic value: because ~50% of ADPKD patients reach end-stage renal disease by the age of 60 (14, 22, 52), a delay of 10 yr or longer would mean that a number of patients would not require renal replacement therapy in their lifetimes. The decreased kidney weight also in the wild-type rats probably results from the fact that the therapy was already started on postnatal *day 14*, when the kidney is still growing. Because it is very well possible that metalloproteinases play a role in renal growth (31, 35), their inhibition may result in smaller kidneys. We also want to point out that previous studies did not report these side effects. In a human phase I trial with batimastat, no adverse

effects of batimastat on renal function were observed, although the authors did not elaborate on how renal function was examined (5). Because we started the administration of batimastat already on postnatal *day 14*, the kidney was not yet fully developed, which may have rendered it more susceptible to the action of batimastat. The same probably is true for the weight loss, which was observed in the (+/+) rats in our study but not in other animal studies, where the treatment with batimastat did not commence until postnatal *weeks 6–8* (e.g., Ref. 77).

At this point, we tend to believe that the beneficial effect of batimastat is due to the inhibition of matrix degradation by metalloproteinases, which is consistent with previous studies demonstrating a strong inhibitory effect of batimastat on the invasion of cells into collagen gels (28, 30). It is also possible, however, that batimastat exerts a negative effect on cell proliferation. The epidermal growth factor family of proteins has been shown to be important for cystogenesis at least under some circumstances (57, 66–68). Unless many other secreted proteins, EGF family members are synthesized as membrane-bound precursors, and the inhibition of metalloprotease activity has been shown to reduce EGF receptor-mediated signaling in general (18, 54), and cell proliferation and migration in particular (18) by preventing the cleavage of these membrane-bound precursors. Further experiments are needed to determine the role of the EGF receptor in cyst formation in this particular rat model of polycystic kidney disease.

In summary, our present study describes a completely novel experimental approach to positively influence the progression of polycystic kidney disease. Previous investigations have focused on the potential benefits of protein restriction (1, 2, 4, 15, 48, 74), other dietary changes (45–47, 70, 75), and also pharmaceutical intervention (24, 25, 32, 36, 39, 49, 65, 67, 80, 81). Although some of these animal studies have demonstrated beneficial effects, they may not be easily transferred to human patients (73), and certain therapeutic strategies only work in some animal models but not in others. Because it is possible that cyst expansion in general depends on the removal of extracellular matrix by matrix-degrading enzymes, a therapeutic strategy based on inhibitors of those enzymes should be independent of the underlying cause of the disease and therefore would be widely applicable.

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