Molecular Pathogenesis of Genetic and Inherited Diseases

Transgenic Overexpression of *Anks6*^(p.R823W) Causes Polycystic Kidney Disease in Rats

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The PKD/Mhm(cy/+) rat is a widely used animal model for the study of human autosomal dominant polycystic kidney disease, one of the most common genetic disorders, affecting one in 1000 individuals. We identified a new gene, *Anks6*, which is mutated (*Anks6*^(p.R823W)) in PKD/Mhm(cy/+) rats. The evidence for a causal link between *Anks6*^(p.R823W) and cystogenesis is still lacking, and the function of *Anks6* is presently unknown. This study presents a novel transgenic rat model that overexpresses the mutated 2.8-kb *Anks6*^(p.R823W) cDNA in the renal tubular epithelium. The transgenic *Anks6*^(p.R823W) acts in a dominant-negative fashion and causes a predictable polycystic phenotype that largely mimics the general characteristics of the PKD/Mhm(cy/+) rats. Cyst development is accompanied by enhanced *c-myc* expression and continuous proliferation, apoptosis, and de-differentiation of the renal tubular epithelium as well as by a lack of translational up-regulation of p21 during aging. Using Northern blot analysis and *in situ* hybridization studies, we identified the first 10 days of age as the period during which transgene expression precedes and initiates cystic growth. Thus, we not only provide the first *in vivo* evidence for a causal link between the novel *Anks6*^(p.R823W) gene mutation and polycystic kidney disease, but we also developed a new transgenic rat model that will serve as an important resource for further exploration of the still unknown function of *Anks6*. (*Am J Pathol* 2010, 177:3000–3009; DOI: 10.2353/ajpath.2010.100569)

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common genetic disorders, affecting one in 1000 individuals.¹ The PKD/Mhm(cy/+) rat was originally developed from a spontaneous mutation in the Han:SPRD strain. Heterozygous PKD/Mhm(cy/+) rats reflect several features of human ADPKD, including autosomal inheritance and slowly progressive massive cyst formation leading to end-stage renal failure. In contrast to human ADPKD, however, cysts arise almost exclusively from proximal tubules. Disease progression is accompanied with progressive renal enlargement to week 10 to 12, followed by a decrease in size before resumption of growth with advanced uremia. Homozygous PKD/Mhm(cy/cy) rats die at approximately 3 weeks from acute renal failure.² Although the cystic phenotype and possible therapeutic interventions are extensively investigated,^{3–7} the gene *Anks6*, which is mutated in cystic PKD/Mhm rats, was identified only recently.⁸ *Anks6*, which is different from any of the known PKD-related genes, consists of 16 exons with 2655 bp coding for an 885-amino acid protein including 10 tandem ankyrin repeats at its N-terminus and a sterile α motif (SAM) domain at its C-terminus.⁸ A missense mutation (C>T) in this gene (*Anks6*^(p.R823W)) accounts for the exchange of a highly conserved arginine residue with a tryptophan residue at amino acid 823 within the SAM domain, and it is tightly linked to the cystic phenotype. The function of *Anks6* is still unknown, but the presence of the ankyrin repeats and SAM domain suggests a function in protein interaction and/or protein binding, such as scaffolding. Recently, *in vitro* studies demonstrated the interaction of *Anks6* with Bicc1, another SAM domain-containing protein involved in murine PKD.⁹

The evidence for the causal relationship of *Anks6*^(p.R823W) with cystogenesis in PKD/Mhm rats is still lacking. Consequently, we developed a new transgenic rat model overexpressing *Anks6*^(p.R823W) cDNA in the renal tubular

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epithelium. These rats exhibit typical PKD that is reflective of the PKD/Mhm(cy/+) phenotype. The level of transgene expression in individual transgenic lines predicts disease severity. Thus, we provide the first *in vivo* evidence for a causal link between *Anks6*^(p.R823W) and the development of cystic lesions. This new transgenic rat model represents a valuable tool for elucidating further molecular mechanisms and developing new therapies for human ADPKD.

Materials and Methods

Construction of the Transgene

The 2.8-kb mutated *Anks6*^(p.R823W) sequence was cloned into a pcDNA4-vector in-frame to a C-terminal c-myc- and 6xHistidine-tag. This tagged sequence was then subcloned into pUCTrans (kind gift from R. Witzgall). This plasmid contains a human cytomegalovirus promoter (hCMV) upstream and an intron as well as a polyadenylation signal of SV40 downstream of an *EcoRI* cloning site.

Generation of Transgenic Rats

The transgenic cassette was removed from the plasmid by using *Clal* and *ApaLI* and injected into the pronuclei of fertilized oocytes of Sprague-Dawley rats as previously described.¹⁰ Rats were genotyped by PCR on tail DNA by using as forward primer 5'-AAC AGT GTC GAG CAG GAG GT-3' and as reverse primer 5'-TGT CCA ATT ATG TCA CAC CAC A-3' corresponding to the 3'-end of the *Anks6* cDNA and the 5'-end of the SV40 region (amplicon 186 bp). Annealing temperature was chosen at 56°C. Southern blotting of *BamHI* or *EcoRI* digested tail DNA by using a ³²P-labeled 1.0-kb cDNA probe coding for 230 bp of the 3'-end of the *Anks6* cDNA and the complete SV40 sequence was performed to evaluate the integration of the complete transgene and to determine the number of integration sites as previously described.¹⁰

Rats received standard rat diet (containing 19% protein) and tap water *ad libitum*. All experiments were conducted in accordance with the German Animal Protection Law and were approved by the local authority (Regierungspräsidium Karlsruhe, Germany).

RNA Analysis by Northern Blotting and Real-Time RT-PCR

Total RNA was extracted from snap-frozen organs by means of guanidium isothiocyanate-caesium chloride centrifugation (Trizol Reagent; Invitrogen, Darmstadt, Germany) according to the manufacturers' instructions and then treated with RNase-free DNase I (Qiagen, Hilden, Germany) and cleaned-up with RNeasy spin columns (Qiagen). For Northern blotting, 30 µg total RNA was size-fractionated on a 1.0% formaldehyde/agarose gel, and blotted onto nylon membranes (Hybond N; Amersham, Braunschweig, Germany). Blots were hybridized

with a random-primer ³²P-labeled 1.0-kb cDNA probe specific to the transgene (as described for Southern blot). For simultaneous detection of transgenic as well as wild-type *Anks6* mRNA, a ³²P-labeled 1.4-kb cDNA probe corresponding to the 3'-end of the *Anks6* cDNA was used. Rat glyceraldehyde-3-phosphate dehydrogenase mRNA levels were used as loading standard. For qualitative RT-PCR the following primers were used: TgAnks6^(p.R823W) as described for genotyping; *Anks6* 5'-CTCTAGCAGTGGCACCATCA-3', 5'-CGCTGCTCTC-AAAGGAAGAG-3'. Genotyping for the endogenous cy allele in PKD/Mhm rats was performed as previously described.⁸ β-actin RT-PCR with primers 5'-CGTAAAGAC-CTCTATGCCAA-3', 5'-AGCCATGCCAAA TGTCTCAT-3' was used as internal control. For quantification, reverse transcription was performed in triplicates in three independent experiments by using M-MLV Reverse Transcriptase (Invitrogen). Identical amounts of reverse transcription product were used in a PCR on Light-Cycler (Roche Diagnostics, Mannheim, Germany) by using the QuantiTect SYBR Green PCR Kit (Qiagen) following the manufacturers' instructions. For each sample three independent experiments were run. For amplification, the following primers were used: p21 5'-GTGAGACACCA-GAGTGCAAGA-3', 5'-ACAGCGATATCGAGACACTCA-3'; c-myc 5'-AGCTCGCCCAATCCTGTAC-3', 5'-TGCT-GGTGAGTAGAGACATGG-3'; Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by using the primer 5'-TGCACCACCAACT-GCTTA-3', 5'-GGATGCAGGGATGATGTT C-3'.

Protein Isolation, SDS-Polyacrylamide Gel Electrophoresis, and Western Blotting

Snap-frozen tissue samples were lysed and homogenized in radioimmunoprecipitation assay buffer (50 mmol/L Tris, 250 mmol/L NaCl, 2% NP-40, 2.5 mmol/L EDTA, 0.1% SDS, 0.5% Deoxycholic acid), subjected to SDS-polyacrylamide gel electrophoresis, and blotted onto polyvinylidene difluoride membranes (Millipore, Schwalbach, Germany). Membranes were blocked in 5% skimmed milk powder in Tris-buffered saline-Tween 20 for 1 hour at RT. The following primary antibodies were used: rabbit anti-p21 (H-164; Santa Cruz Biotechnology, Santa Cruz, CA; 1:3000, 4°C overnight) and mouse anti-β-actin (A5441, Sigma, München, Germany; 1:10000, 45 minutes, room temperature). Specifically bound primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL; numbers 31460 and 31430, respectively) and subsequent chemiluminescence.

Tissue Preparation and Perfusion

Left kidney was removed for RNA and protein preparation by clamping the arteria and vena renalis before anesthetized (xylazine/ketamine) rats were sacrificed by retrograde total body perfusion directly with the fixative 2% paraformaldehyde (in 1× PBS) for 3 minutes at a pressure of 220 mmHg as described previously.¹¹ Perfusion-

fixed organs were subdivided and embedded in paraffin or snap-frozen in liquid nitrogen after saturation in 18% sucrose (in 1× PBS).

Nonradioactive *In Situ* Hybridization

In situ hybridization was performed as previously described⁶ on 3- μ m paraffin sections and 6- μ m cryosections. Briefly, paraffin sections were deparaffinized and then rinsed in PBS, pH 7.4, postfixed in 4% paraformaldehyde in PBS, treated with proteinase K (8 μ g/ μ l), and acetylated in 0.1 M triethanolamine, pH 8.0, containing 0.25% acetic anhydride. Then the slides were dehydrated in graded ethanol up to 95% and air-dried. For *in situ* hybridization sense and antisense, cRNA probes directed against the complete SV40 region of the transgene were generated by *in vitro* transcription by using digoxigenin-11-UTP (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Transcripts were finally subjected to partial alkaline hydrolysis to improve penetration. Sections were hybridized with a solution containing 50% formamid and 3 ng/ μ L hydrolyzed RNA probe overnight at 48°C, followed by several stringent washes. 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 signal. On cryosections co-staining for aquaporin 1 was accomplished as described below.

Histological Staining and Morphological Evaluation

Three-micrometer paraffin sections were subjected to hematoxylin/eosin staining according to standard protocols. Specimens were analyzed by using light microscopy (Leica, Wetzlar, Germany). Cysts were scored as described earlier¹² by using a four-step grading system based on the size and number of cysts.

Immunohistology

Cryosections (6 μ m) were blocked with 2% bovine serum albumin in 1× PBS for 1 hour at room temperature. First antibody (anti-aquaporin 1, 1:200; Santa Cruz Biotechnology) was applied in blocking solution overnight at 4°C and for 1 hour at room temperature, followed by secondary antibody (anti-rabbit-cy3, 1:800; Dianova, Hamburg, Germany) for 45 minutes.

Proliferation and Apoptosis

Proliferating cells were stained with an antibody against rat-Ki-67 immunohistochemically. Paraffin sections were deparaffinized, treated with 0.3% hydrogen peroxide, and subjected to microwave treatment for antigen retrieval (20 minutes, 10 mmol/L citrate buffer, pH 6.0 to 6.2). After blocking with 2% bovine serum albumin in 1×

PBS for 1 hour, incubation was carried out each for 1 hour at room temperature with first antibody (Rat Ki-67, 1:50; Dako, Hamburg, Germany) and then with biotinylated secondary antibody (anti-mouse, 1:200; Sigma). Incubation with avidin-biotin complex reagent and colorimetric detection was accomplished according to the manufacturer's instruction (Vectastain Elite ABC-Peroxidase Kit, DAB Substrate Kit; Vector Laboratories, Burlingame, CA). Apoptotic cells were stained by using a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay according to the manufacturer's instruction (DeadEnd Colorimetric TUNEL System; Promega, Mannheim, Germany). Both apoptotic and proliferating cells were counted in 15 visual fields at a 20× magnification.

Cyst Counting during Aging

All cysts per section were counted and classified according to their size into four groups and according to aquaporin 1 staining (positive/negative) into two groups. Cyst size was related to the average size of a glomerulus in the respective age (~60 μ m, 90 to 100 μ m, and ~125 μ m in animals aged 10 days, 4 weeks, and 8 weeks, respectively), and groups were defined as follows: group 1: smaller than two glomeruli; group 2: two to four glomeruli; group 3: four to six glomeruli; and group 4: larger than six glomeruli. Five animals were evaluated for each age. Cysts were counted at 10× magnification on a Leica DMR microscope.

Evaluation of Kidney Function

Blood was collected from the retro-orbital plexus of anesthetized (isoflurane) rats in heparinized tubes (Sarstedt, Nümbrecht, Germany). Serum creatinine¹³ and serum urea were determined with an HITACHI 911 Autoanalyzer (CREA Plus number 1775669, UREA/BUN number 11489364; Roche Diagnostics).

Statistics

Statistic evaluation was performed with SAS one-way analysis of variance (Version 8.02; SAS Institute, Cary, NC). Level of significance was set to $P < 0.05$ unless stated differently.

Results

Overexpression of Anks6^(p.R823W) Leads to Renal Cyst Formation

To study the function of Anks6^(p.R823W) *in vivo*, we developed four transgenic rat lines TGR-hCMV/Anks6^(p.R823W) carrying the 2.8-kb mutated Anks6^(p.R823W) cDNA under the control of the promoter/enhancer region of hCMV (Figure 1A), referred to as TGR/395, TGR/400, TGR/402, and TGR/405, respectively. Northern blotting revealed transgenic Anks6^(p.R823W) mRNA in various tissues, though expression was strongest in the kidney in all

Figure 1. Transgenic construct, its expression, and renal phenotype. **A:** The transgene contains the cytomegalovirus (CMV) promoter/enhancer sequence upstream and the intron and polyadenylation signal [poly(A)] of the SV40 downstream of the 2.8-kb c-myc-epitope/His₆-tagged mutated *Anks6*^(p.R823W) cDNA. **B:** Typical autoradiograph of Northern blot analysis of *Anks6*^(p.R823W) mRNA (20 μ g/lane) in various tissues (TGR/405, aged 8 weeks) using a probe specific to the transgene. Specificity is verified by the fact that no signal was detected in kidneys of wild-type littermates (Nc). GAPDH mRNA was detected as a loading control. Transgene expression is strongest in kidney. K, kidney; Li, liver; Sp, spleen; H, heart; Lu, lung; B, brain; SM, smooth muscle; Ag, adrenal gland; Nc, negative control (RNA from kidney of wild-type littermates); Pc, positive control (RNA from kidney of TGR/405). **C:** Expression levels of mutant *Anks6*^(p.R823W) and total *Anks6* in 8-week-old males of different transgenic lines and the PKD/Mhm(cy/+) rat. RT-PCR using primers specific to both transgenic and endogenous mutant *Anks6*^(p.R823W) demonstrate substantial higher levels of *Anks6*^(p.R823W) in TGR/400 and TGR/405 and lower levels in TGR/395 and TGR/402 when compared with PKD/Mhm(cy/+) rats. Northern blot using a probe corresponding to both the mutant and wild-type *Anks6* show comparable low levels of total *Anks6* in PKD/Mhm(cy/+), TGR/395, and TGR/402, and strong signals in TGR/400 and TGR/405. TGR/400 and TGR/405 show a strong transgene expression and develop a cystic phenotype. H&E-stained sections are shown. **D:** The table shows the absolute number of cysts classified into different grades in transgenic rats from four transgenic lines aged 8 to 10 weeks. **E:** Macroscopic picture of a kidney of a 4-week-old TGR/400 is shown in comparison to an age-matched wild-type kidney. **F:** Kidney function of 8-week-old rats is shown by plasma urea and creatinine levels (level of significance, $P < 0.05$; wild-type: $n = 5$; TGR/400: $n = 5$; TGR/405: $n = 7$; PKD/Mhm(cy/+): $n = 5$). TGR/400 with the strongest phenotype report with poor kidney function. TGR/405 shows no influence of the phenotype on kidney function due to the mild grade of cysts. Plasma urea and creatinine levels of age-matched PKD/Mhm(cy/+) animals is shown in comparison.

transgenic lines (Figure 1B). Nevertheless, individual transgenic lines differed strikingly in renal transgene expression. The *Anks6*^(p.R823W) mRNA levels of TGR/395 and TGR/402 were even lower than in PKD/Mhm(cy/+) rats and did not result in phenotypic changes (Figure 1, C and D). In contrast, TGR/400 and TGR/405, expressed notable *Anks6*^(p.R823W) mRNA levels and developed PKD (Figure 1, C–F). TGR/400 rats exhibited the highest renal *Anks6*^(p.R823W) mRNA levels among the four lines and had massively enlarged kidneys due to multiple expanded cysts throughout the whole renal cortex. The disease was accompanied by deteriorating renal function, as indicated by increased plasma urea and creatinine levels. The severity of the cystic lesions and renal function deterioration resembled, or even exceeded, that of age-matched PKD/Mhm(cy/+) rats. In contrast, TGR/405 rats, which had lower renal *Anks6*^(p.R823W) mRNA

levels, exhibited numerous small cystic tubules, predominantly in the innermost cortex and extending into the outer stripe of the medulla. Kidney function was not affected at a given age. Further studies were conducted by using TGR/400, which is referred to hereafter as TGR.

Time Course of Renal Transgene Expression and Cyst Development

To our knowledge, renal *Anks6* expression was not previously evaluated over the course of kidney development and aging. Consequently, we determined the renal *Anks6* expression in wild-type rats and the transgenic *Anks6*^(p.R823W) mRNA in TGR at E15 and E18 and detected both as early as at E15. Quantification of total *Anks6* expression at the age of 0 and 10 days as well as 4 and 8 weeks demonstrates that

Figure 2. Time course of *Anks6* and transgenic *Anks6*^(p.R823W) mRNA expression and of cyst grading. **Top:** RT-PCR for the transgenic *Anks6*^(p.R823W) in TGR/400 and for the wild-type *Anks6* in wild-type (WT) rats using specific primers show that both the endogenous *Anks6* and the transgenic *Anks6*^(p.R823W) are already expressed in E15. Nc, wild-type kidney as negative control. Northern blot analysis: A ³²P-labeled probe corresponding to a 1-kb sequence of the *Anks6* cDNA detects both the transgenic *Anks6*^(p.R823W) and the endogenous *Anks6* mRNA in TGR and the endogenous *Anks6* mRNA in WT rats. *Anks6*/GAPDH mRNA ratios were calculated and blotted against age. Endogenous *Anks6* mRNA was equally expressed throughout life with a slight increase in 8-week-old rats. Cyst number classified according to their origin (male TGR/400); cyst size was estimated morphometrically and graded by using the average glomerular size as an internal parameter. Grade 1: < two glomeruli; grade 2: two to four glomeruli; grade 3: four to six glomeruli; grade 4: > six glomeruli. Cysts number and size increase from 10 days to 8 weeks of age. Cysts arise predominantly from proximal tubules (positive for aquaporin1) with later involvement of small cysts from other origin. Five rats per age group were analyzed. **Bottom:** *In situ* hybridization (ISH) of transgenic *Anks6*^(p.R823W) mRNA in kidney sections of TGR/400 (A–G, I–L). Strong transgene expression was found in developing tubules at E18 (A). No cysts but dilated tubules are present at 0 days (B and E). At 10 days of age, first cysts primarily adjacent to the medulla were formed (C and F). In neonatal transgenic rats, *Anks6*^(p.R823W) is heavily expressed in epithelial cells of primitive tubules and in glomerular podocytes [G] (E) preferential in the mid-cortical layer as well as in the medulla (B). *In situ* hybridization for transgenic *Anks6*^(p.R823W) (G) and immunohistochemistry for the proximal tubule marker aquaporin1 (H) at the same section of 0-day-old kidney reveal that even though cysts evolve from the proximal tubules transgenic *Anks6*^(p.R823W) was also detected in cells of tubules from other origin. *Anks6*^(p.R823W) mRNA is often noted in most, or all cells in a particular tubular section not from proximal origin and only in fewer cells lining tubular profiles of proximal origin. In 10-day-old transgenic rats, the transgene expression is restricted to the tubular epithelium and disappeared in glomeruli and medulla (C and F). Transgene expression is most prominent in normal appearing tubules and small cysts rather than in larger cysts (C, D, K, and L). In 4- and 8-week-old TGR/400, transgene expression is found predominantly in smaller cysts and normal appearing tubules (D, K, and L) 4-week-old TGR cysts with single and multilayered proliferating epithelial cells highly expressing the transgene. *Anks6*^(p.R823W) mRNA positive cells are shed into the lumen (I and J). At 8 weeks of age, large cysts with flattened epithelium do not express the transgene (K and L). Transgene was detected predominantly in the nucleus since a specific cRNA probe directed against the 3'-untranslated region of the transgene was used. Its specificity was approved by the fact that no signal was detected in the kidney of wild-type littermates and by using the sense probe. **Arrow** indicates transgene positive cell shed into the lumen. G, glomeruli; **Asterisk**, proximal tubule; **Arrowheads**, not proximal tubule; **P* < 0.05. Original magnifications: ×5 (D); ×10 (B, C, and L); ×20 (G, H, and K); and ×40 (A, E, F, I, and J).

the endogenous *Anks6* is steadily expressed in wild-type rats (Figure 2, top). Data support the view that wild-type *Anks6* may play a role in kidney development as well as in the maintenance of mature tubular structures. In TGR, the total *Anks6* mRNA levels (endogenous plus transgenic mutated *Anks6* mRNA) were increased roughly eightfold rela-

tive to the wild-type rats, and they remained almost constant throughout the observation period.

To examine the relationship between transgene expression and cyst formation, we evaluated the renal expression pattern of transgenic *Anks6*^(p.R823W) mRNA by *in situ* hybridization and histomorphology of cyst formation

in rats at 0 and 10 days as well as 4 and 8 weeks of age, representing important developmental stages. Unlike humans, the development of the rat kidney continues after birth until the kidney is terminally matured at the age of 3 weeks. The last nephron anlagen to be formed develop into functional nephron by 10 days after birth.^{14,15} Rats enter puberty at the age of 4 weeks and reach sexual maturity at 8 weeks. We used an *in situ* probe specific to the transgene as well as a probe that detected both the transgenic and endogenous Anks6 mRNA. Although the Anks6 mRNA levels in wild-type rats were below the detection level of *in situ* hybridization, in TGR both probes stained strongly the same morphological structures even at the age of E18 (Figure 2A).

The kidney of newborn TGR strikingly expressed transgenic Anks6^(p.R823W) mRNA in the tubular epithelium and glomerular podocytes of the renal cortex, toward the medulla but not immediately beneath the capsule, containing a rim of metanephrogenic mesenchym and early metanephrogenic forms, such as comma- and S-shaped bodies (Figure 2B; Figure 2E, bottom). Morphologically, in the newborn rat kidney some nephrons became slightly dilated; cystic growth, however, was first observed by 10 days after birth, predominantly in proximal tubules of the innermost cortical region containing the oldest functional nephron (Figure 2 top; Figure 2C; Figure 2F, bottom). Concomitantly, transgene expression disappeared from the medulla and glomeruli and individual cyst lining cells (Figure 2, C, E, and F). Notably, only a few individual cells lining a particular proximal tubular profile expressed the transgene, whereas each of the cells lining other tubular segments were strongly stained for Anks6^(p.R823W) mRNA, as demonstrated by co-staining with the proximal tubule marker aquaporin1 (Figure 2, E–H).

With further aging, all TGR displayed a progressive cystic phenotype with an increased number and size of cysts, which were dispersed across the whole cortex (Figure 2D, I–L) similar to what was described by Nagao et al¹⁶ for the PKD/Mhm(cy/+) rat. At 4 weeks of age, numerous cysts were lined with hyperplastic Anks6^(p.R823W) mRNA positive cells, which were occasionally arranged in several layers (Figure 2, I and L). Large cysts with flattened epithelium did not express transgenic Anks6^(p.R823W) mRNA (Figure 2L). In advanced disease stages, Anks6^(p.R823W) mRNA was found predominantly in intact tubules and small cysts (Figure 2, D and K). As shown in Figure 2 top, cysts were predominantly proximal in origin with a later involvement of other tubular segments.

To verify whether an increased Anks6^(p.R823W)/Anks6 ratio exacerbates the cystic disease, we crossed hemizygous TGR (now referred to as tgCMVAnks6^(p.R823W)) with PKD/Mhm(cy/+) rats (now referred to as PKD/MhmAnks6^(p.R823W/+)) to generate crTGRtgCMVAnks6^(p.R823W)/Anks6^(p.R823W/+) rats carrying both the transgenic tgCMVAnks6^(p.R823W) gene and at the Anks6 locus one wild-type Anks6⁽⁺⁾ plus one mutated Anks6^(p.R823W) allele. Their littermates, carrying either the tgCMVAnks6^(p.R823W) alone or the Anks6^(p.R823W/+) locus only, and the wild-type rats were used for a direct comparison. The expression ratio of transgenic

Figure 3. Comparison of cystic phenotype in H&E-stained kidney sections and relative kidney weight of 10-day-old TGR/400PKD/Mhm(cy/+) F1 littermates with the genotype tgCMVAnks6^(p.R823W)/Anks6^(p.R823W/+) (TGR;cy/+) (A), tgCMVAnks6^(p.R823W)/Anks6^(+/+) (TGR; B), Anks6^(p.R823W/+) (cy/+) (D), and PKD/Mhm(cy/cy) (Anks6^(p.R823W/p.R823W)) rats (C). The rats were genotyped with RT-PCR by using primers specific to the transgene and primers specific to the endogenous mutation as described in Brown et al.⁸ **P* < 0.01; ***P* < 0.001.

Anks6^(p.R823W) driven by the strong CMV promoter versus endogenous wild-type Anks6 (controlled by two Anks6 alleles) is eight in TGR (tgCMVAnks6^(p.R823W)) and more than doubled in the crTGRtgCMVAnks6^(p.R823W)/Anks6^(p.R823W/+) due to the loss of one wild-type Anks6 allele. In contrast, the Anks6^(p.R823W)/Anks6 mRNA ratio in PKD/MhmAnks6^(p.R823W/+) rats is only 1:1. Indeed, 10-day-old crTGRtgCMVAnks6^(p.R823W)/Anks6^(p.R823W/+) rats exhibited a dramatically enhanced cystic phenotype when compared with their littermates. The aggravated morphology was associated with an extremely increased renal hypertrophy (Figure 3, A–D).⁸

Deregulation of Apoptosis and Proliferation in the Transgenic Kidneys

Increased proliferation and apoptosis of epithelial cells is a hallmark of human and rodent ADPKD, and it is regarded as a prerequisite of cyst formation.^{17,18} Thus, we evaluated these processes in TGR and wild-type rats. Apoptotic figures and mitotic cells were a prominent feature of the developing kidney up to the age of 3 to 4 weeks in both lines and were barely seen in the wild-type

Figure 4. Co-localization of proliferating (immunohistochemical staining for Ki-67) and *Anks6*^(p.R823W) mRNA (*in situ* hybridization) expressing cells in 8-week-old male TGR/400. **A and B:** Distribution pattern of *Anks6*^(p.R823W) mRNA predominantly in epithelial cells lining small cysts and normal appearing tubules, but not large cysts resemble those of Ki-67-positive (proliferating) cells. Ki-67- and *Anks6*^(p.R823W) mRNA-positive cells were found in multilayered epithelium and in cells shed into the tubule lumen (**C and D**), in a patchy distribution among cysts lining cells (**E and F**). Smaller cysts were also often lined by TUNEL-positive cells, which were shed into the lumen (**G and H**). **I and J:** Time course of c-myc and p21 expression in kidneys of male TGR/400 and wild-type littermates. **I:** Quantitative evaluation of c-myc and p21 mRNA expression related to *GAPDH* as internal standard. Real-time RT-PCR (three independent RT reactions and runs per sample) indicates a significant increased expression of c-myc and p21 in TGR when compared with wild-type littermates; 0 day: TGR *n* = 5, WT *n* = 3; 10 days: TGR *n* = 9, WT *n* = 5; 4 weeks: TGR *n* = 4, WT *n* = 7; 8 weeks: TGR *n* = 7, WT *n* = 6. **J:** Western blot analysis of p21 protein related to β -actin as an internal standard shows increased protein levels with aging in wild-type rats but not in TGR; 10 days: TGR *n* = 5, WT *n* = 5; 4 weeks: TGR *n* = 15, WT *n* = 6; 8 weeks: TGR *n* = 5, WT *n* = 5. **P* < 0.05; ***P* < 0.001. Original magnifications: $\times 10$ (**A** and **B**); $\times 40$ (**C–H**).

kidney thereafter. In the 8-week-old TGR, the proliferation detected by Ki-67 immunostaining was roughly twofold higher compared with wild-type littermates (TGR: 13 ± 0.6 ; wild-type: 6.9 ± 2.0 ; *P* < 0.0023). Apoptosis detected by the TUNEL assay was increased 4.8-fold (TGR: 2.9 ± 1.9 ; wild-type: 0.6 ± 0.4 ; *P* < 0.0027). Histomorphologically, the distribution pattern of Ki-67- and TUNEL-positive epithelial cells paralleled that of transgene expressing cells in the adult TGR. We detected Ki-67- and TUNEL-positive cells in numerous epithelial cells lining smaller cysts and in normal appearing tubules. In contrast, large cysts with flattened de-differentiated epithelium did not stain for mutated *Anks6*^(p.R823W) or Ki-67 (Figure 4, A and B). Similar to what was reported for the PKD/Mhm(cy/+) rats, we found cysts of varying size with hypertrophic/hyperplastic-appearing epithelium that were regionally composed of densely packed cells

occasionally arranged in several layers. These cells were shed into the lumen and stained for Ki-67 or TUNEL, and even for *Anks6*^(p.R823W) (Figure 4, C, D, G, and H). In addition, *Anks6*^(p.R823W) mRNA positive cells, which were sporadically distributed among cyst-lined epithelial cells, often appeared larger, cuboidal-shaped, and morphologically resembled Ki-67-positive cells (Figure 4, E and F).

To explore the mechanisms that mediate cystic growth in TGR in more detail, we compared the c-myc and p21 expression in TGR and wild-type rats in the first 8 weeks of life. Both, c-myc and p21 are principally capable of controlling proliferation, apoptosis, or differentiation, depending on the cellular context. Relative to wild-type rats, in TGR the c-myc expression was significantly increased throughout life even at birth; p21 was not differentially expressed during the first 10 days of life. However, in 4- and 8-week-old TGR, p21

mRNA levels were significantly up-regulated and the p21 protein levels were simultaneously down-regulated when compared with age-matched wild-type rats (Figure 4, I and J). As previously reported, p21 protein is down-regulated in the PKD/Mhm(cy/+) rat. We found, however, that the p21 mRNA in 8-week-old PKD/Mhm(cy/+) rats is significantly up-regulated comparable with the TGR (data not shown).

Discussion

In the present study, we provide the first *in vivo* evidence for a causal link between a missense point mutation in the newly identified *Anks6* and cystogenesis in rats. We present a novel TGR model that develops typical PKD due to overexpression of *Anks6*^(p.R823W) cDNA in the renal tubular epithelium. The course of the disease evolves in a predictable manner reflective of the PKD/Mhm rat.

We placed the *Anks6*^(p.R823W) cDNA under the regulatory control of a strong, widely active hCMV promoter. Similar to what we previously reported,¹⁹ this promoter drives high transgene expression, predominantly in the kidney and specifically in tubular epithelial cells. Thus, this CMV promoter seems to be a valuable tool for targeting renal tubular epithelial cells in TGR.

Two of our four TGR lines exhibited marked transgene expression in the kidney, resulting in a typical, progressive PKD arguing against a possible insertion mutagenesis due to transgene integration. The most severe cystic phenotype was observed in the TGR line 400 with highest transgene expression. In this line, the level of transgenic *Anks6*^(p.R823W) mRNA, driven by the CMV promoter, exceeded the mRNA level produced by two alleles of endogenous wild-type *Anks6* by roughly eightfold. To further increase the *Anks6*^(p.R823W)/*Anks6* mRNA ratio, we crossed TGR with PKD/Mhm(cy/+) rats to obtain crTGRtgCMV*Anks6*^(p.R823W)/*Anks6*^(p.R823W/+) carrying both the transgenic tgCMV*Anks6*^(p.R823W) and the heterozygous *Anks6*^(p.R823W/+) alleles at the endogenous *Anks6* locus. Due to the loss of one wild-type *Anks6* allele, the *Anks6*^(p.R823W)/*Anks6* mRNA ratio doubled from 8 to more than 16. Notably, 10-day-old crTGRtgCMV*Anks6*^(p.R823W)/*Anks6*^(p.R823W/+) rats exhibited a dramatically enhanced cystic phenotype when compared with their littermates carrying either the tgCMV*Anks6*^(p.R823W) alone or the heterozygous *Anks6*^(p.R823W/+) alleles at the *Anks6* locus only. This tight correlation between the *Anks6*^(p.R823W)/*Anks6* mRNA ratios and disease severity is particularly striking and supports the view that *Anks6*^(p.R823W) may act in a dominant negative fashion or, at least, has a gene-dose effect.

In a recent *in vitro* study, Stagner et al⁹ provide evidence that *Anks6* self-associates to form homomers that interact via a protein-RNA-complex with Bicc1, another SAM domain containing PKD-related protein. The mutation in the SAM domain of *Anks6*^(p.R823W) prevented its self-association but did not disrupt the association of *Anks6*^(p.R823W) to Bicc1. Thus, we suggest the mutated *Anks6*^(p.R823W) could compete with wild-type *Anks6* homomers for binding to protein-RNA-complexes. Such in-

terference with a possible scaffolding function of *Anks6* might result in altered translation of specific genes.

In the present model, the transgenic *Anks6*^(p.R823W) induces cystic growth, thereby perturbing the orderly function of the matured tubular epithelial cell, but it seems not to interfere with nephrogenesis. Cysts were first observed at the age of 10 days, after the formation of functional nephrons was finalized, despite the fact that transgenic *Anks6*^(p.R823W) mRNA was already found in the embryonic kidney at E15 and was highly expressed in the developing tubules of the neonatal rats. This observation coincides with previous findings from Obermüller et al,⁶ who demonstrated that the tubular epithelium developed in an orderly fashion in PKD/Mhm(cy/+) rats but degenerated thereafter. It is still a matter of speculations whether the *Anks6*^(p.R823W) is functionless or has an altered function compared with wild-type *Anks6*. Unlike homozygous PKD/Mhm(cy/cy) rats that die within the first 3 weeks of life due to cystic deterioration of the kidney, our TGR survive with slowly progressive PKD despite of having much higher *Anks6*^(p.R823W) mRNA levels, arguing against an autonomous function of *Anks6*^(p.R823W). Thus, the presence of two copies of the endogenous wild-type *Anks6* in our rats prevents the dramatic course of PKD but does not prevent the disease at all. We speculate that *Anks6*, as many other PKD-related proteins, may have spatiotemporally multiple functions, some sensitive to interference by the mutated *Anks6*^(p.R823W) others not. In this study we have shown that endogenous *Anks6* is already expressed in early nephrogenesis at E15 and is kept constantly expressed during life. Consequently, *Anks6* might have a function in both developing and mature tubules. In the adult kidney, endogenous *Anks6* expression is restricted to the proximal tubules, as we showed previously.⁸ In TGR, cysts predominantly originated from the proximal tubules, despite the fact that the transgene was also highly expressed in other tubular segments. Therefore, *Anks6*^(p.R823W) may be important, specifically for the maintenance of proximal tubular function, whereas its expression in other tubules does not predictably lead to cysts, only to a lesser extent in later stages. This fact underlines that proximal tubules react differently from other parts of the tubules to different kinds of "injuries," including consequences of specific gene transfer. It is possible that the hitherto mentioned nephron parts are more resistant to, or are capable of compensating for, the changes induced by *Anks6*^(p.R823W) in regards to cyst formation.

We further demonstrated that the cystic phenotype in TGR is accompanied by continuous proliferation and apoptosis, as well as elevated expression of c-myc and p21 mRNA and lowered p21 protein levels in the adults. Even the PKD/Mhm(cy/+) rat exhibits likewise as our TGR increased p21 mRNA levels. C-myc was significantly increased from birth on, indicating a possible direct relation to the *Anks6*^(p.R823W) expression. Contrary, altered p21 expression was detected not until the age of 4 weeks suggesting involvement in later stages of cystic growth. It is of interest that reduced p21 protein levels were associated with increased p21 mRNA levels, suggesting a translational down-regulation of p21 during cystogenesis.

Increased p21 transcription might be a compensatory response to the translational changes. With aging, in wild-type rats c-myc is decreased, whereas the p21 protein but not the p21 mRNA is strongly increased. Contrarily, these age-related changes in the c-myc and p21 protein levels do not occur in the TGR kidney. Compelling evidence exists that a temporal and spatial balance between c-myc induced proliferation and apoptosis is critical to the development of cystic disease. Renal c-myc expression is increased in normal and cystic epithelium in all tested rodent models of PKD such as Han:SPRD(cy/+) rats, transgenic mice overexpressing PKD1, and in humans with ADPKD.^{20,21} Moreover, SBM mice that overexpress c-myc develop PKD,¹⁷ and c-myc antisense oligonucleotide treatment ameliorates murine ARPKD.²² The role of p21 in cystogenesis is only recently indicated and not well established; p21 was found to be transcriptional up-regulated by PKD1 and PKD2 to mediate cell-cycle arrest at G0/G1,^{23,24} and it is repressed by c-myc allowing cell-cycle progression.²⁵ Thus, an attenuation of p21 seems to be related to increased proliferation and apoptosis.

We speculate that Anks6^(p.R823W) plays a role in driving the cell to proliferation or impeding the cell to switch off the developmental program, thus perturbing the critical balance between the opposing processes of cell proliferation and apoptosis. Anks6 may act in concert with other PKD-related proteins to regulate cell fate, and it is possibly integrated in pathways linking cilia function to cell-cycle regulation. In this context are recent studies of interest that place the regulation of cell proliferation, differentiation, and apoptosis under the control of the cilium, the function of which is deregulated in many forms of PKD.^{26,27} Some proteins, such as polycystin 1 and 2, have been shown to act both in the cilium and cell-cycle regulation.^{28–30} An important aspect is that the interaction of Anks6 and Bicc1 mediated via RNA has been demonstrated.⁹ Moreover, a recent study provides evidence that Bicc is involved in establishing the planar alignment of motile cilia and can uncouple Dvl2 signaling from the canonical Wnt pathway, which has been implicated in antagonizing planar cell polarity and activation of cell proliferation via β -catenin signaling. The SAM domain concentrates Bicc in cytoplasmatic structures harboring RNA-processing bodies (P-bodies) and Dvl2.³¹ Bicc is suggested to regulate Dvl possibly by regulating RNA silencing in RNA-processing bodies. Future studies should explore a possible involvement of Anks6-RNA-Bicc1 complexes in these processes.

In summary, with the novel Anks6^(p.R823W) TGR model, we provided the first *in vivo* evidence for a causal link between Anks6^(p.R823W) and the development of PKD, that are predominantly proximal tubule in origin. Cyst development is accompanied by enhanced c-myc expression and continued proliferation, apoptosis, and differentiation of the renal tubular epithelium as well as with a lack in translational up-regulation of p21 during aging. We identified the first 10 days of life as a period in which transgene expression precedes and initiates cystic growth. Thus, this novel TGR model is an ideal tool for further exploration of the still unknown function of the

Anks6 gene product and studying molecular mechanisms early in cystogenesis (E15–10 days of age) in direct relation to the expression of Anks6^(p.R823W) at the individual cellular level, which is a unique advantage over the PKD/Mhm(cy/+) model.

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