Transgenic overexpression of prothymosin α induces development of polycystic kidney disease[1]

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Background. Polycystic kidney disease (PKD) is a genetic disorder characterized by development of renal cysts and progressive renal dysfunction. Renal tissues from both PKD patients and rodent models of PKD show elevated c-myc expression. Prothymosin α (ProT) is positively regulated by c-myc through binding to the E box of its promoter. Through creating transgenic mice and clinical studies, we sought to investigate whether ProT overexpression contributes to PKD development.

Methods. ProT heterozygous and homozygous transgenic mice were generated and characterized. Morphologic, histologic, immunohistochemical, and biochemical analyses of the transgenic mice were performed.

Results. Two transgenic lines that represented integration at two different loci of the chromosomes were generated. ProT overexpression in the kidneys of homozygous transgenic mice induced a PKD phenotype, which included polycystic kidneys, elevated blood urea nitrogen (BUN), and lethality at about 10 days of age. Similar overexpression pattern of ProT was noted in cystic kidneys of the transgenic mice as well as in human autosomal-recessive PKD (ARPKD) and autosomal-dominant PKD (ADPKD) kidneys. ProT protein levels in the kidneys and urine as well as renal mRNA level of epithelial growth factor receptor (EGFR) of homozygous ProT transgenic mice were significantly higher than heterozygous or nontransgenic littermates. Furthermore, the heterozygous transgenic mice at 17 months of age also developed mild cystic kidneys.

Conclusion. Transgenic mice overexpressing ProT represent a novel model for PKD and may provide insights into PKD development. ProT, like c-myc and EGFR, may contribute to the development of renal cysts and may be a potential noninvasive diagnostic molecule of PKD.

Polycystic kidney disease (PKD), which is potentially fatal, is one of the most common genetic disorders. The massive enlargement of the kidneys due to the build-up of fluid-filled cysts eventually leads to functional failure in a high proportion of cases. Autosomal-recessive PKD (ARPKD) and autosomal-dominant PKD (ADPKD) are the two major inheritable forms of PKD in humans [1]. ARPKD is a rare defect, and infants born with ARPKD die from renal failure shortly after birth. In ADPKD, cyst formation also occurs during development; however, the disease is usually only detected in the third decade of life. By late middle age, patients with ADPKD may also suffer from renal failure. The neoplastic nature of renal cysts has been recognized for several years [2]. A number of animal models for ADPKD and ARPKD, including DBA-2J-*cpk/cpk*, C57BL/6J-*cpk/cpk*, CD-1*cpk/cpk*, BALB/c-*bpk/bpk*, and BALB/c-*cpk/cpk* mice, as well as Han:SPRD *cy/+* rats, have been established [3–8]. Among many genes examined, elevated c-myc expression was observed in all forms of PKD in humans and animal models [8–13].

Prothymosin α (ProT), which was first isolated from rat thymus [14, 15], is a highly acidic protein with widespread tissue distribution. As ProT was thought to be the putative precursor of thymosin α1, earlier studies have focused on its immunomodulatory effect, especially on T cells [16]. However, accumulating evidence indicates that ProT is an essential protein related to cell proliferation [17–19]. Our previous study also showed that overexpression of ProT accelerates cell proliferation by shortening the duration of the G_1 phase of the cell cycle [20]. ProT may exert diverse biologic functions through its interaction with histone H1 protein to affect chromatin remodeling [21, 22], and with procaspase 9 to affect cell death [23]. In addition, ProT gene is one of the target genes of myc that is encoded by the proto-oncogene c-myc [24]. Myc directly regulates the expression of ProT gene by binding to the E box of its promoter [25, 26]. Although many biologic functions of ProT have been revealed by these studies, the physiologic roles of ProT in vivo remain largely unclear.

Table 1. Primer pairs used for polymerase chain reaction (PCR) or reverse transcription (RT)-PCR

Specificity	Nucleotide sequence (5′ to 3′)	Location	Fragment (bp)
ProT	RAP-f[a] (TGAGCGAGCCGGAGCCAA)	β-actin promoter	857
	ProT-r[b]1 (GTAGTTTTGGAGGGGTGGTGGAGAG)	ProT gene	
ProT	MCS-f (TCTAGAGGATCCCCGGGCGAGCTC)	Multiple cloning site in pJ6Ω-ProT minigene construct	875
	ProT-r2 (ACGCTCTGAAGGCTGGTTTGGTC)	ProT gene	
c-Myc	c-Myc-f (AGCCCTATTTCATCTGCGACGAG)	c-myc gene	1100
	c-Myc-r (GGATGTAGGCGGTGGCTTTTTTG)	c-myc gene	
EGF	EGF-f (CTCGCCTTCCTGCTGGTGTTTTT)	EGF gene	640
	EGF-r (ACCCAGAAGAGCCGTTTGTCCAG)	EGF gene	
EGFR	EGFR-f (AGTTTTCTTTGGCGGTCGTTGGC)	EGFR gene	730
	EGFR-r (TGGAAGAAACTGGAAGGTGAGAGGG)	EGFR gene	
18S rRNA	18S rRNA-f (TATGGTTCCTTTGGTCGCTCG)	18S rRNA gene	415
	18S rRNA-r (ATTTTTCGTCACTACCTCCCCG)	18S rRNA gene	

Abbreviations are: ProT, prothymosin α, EGF, epithelial growth factor; EGFR, epithelial growth factor receptor.
[a]Denotes forward primer; [b]Denotes reverse primer.

c-myc regulates the expression of ProT gene [25–27] and elevated c-myc expression is observed in all forms of PKD in humans and animal models [8–13]. Therefore, we speculated that overexpression of the ProT gene may contribute to the development of PKD. In this study, we describe the generation of transgenic mice with ProT overexpression driven by the β-actin promoter. The homozygous transgenic mice reproducibly developed morphologic and functional alterations characteristic of PKD, and died 10 days after birth. The relevance of this model to the human disease is further supported by ProT overexpression noted in both human ARPKD and ADPKD kidneys. Our results suggest that ProT overexpression may play a role in the development of PKD. ProT transgenic mice represent a novel model for PKD and may provide insights into potential pathogenetic mechanisms resulting in PKD.

METHODS

Construction and identification of transgenic mice with overexpression of a ProT minigene

The ProT minigene construct (Fig. 1A) used to generate transgenic mice was pJ6Ω-ProT, which contained a complete 1.2 kb murine ProT cDNA together with the rat β-actin promoter and SV40 polyadenylation tail [20]. The ubiquitous β-actin promoter has been reported to direct widespread gene expression in transgenic mice [28, 29]. The minigene, which was excised from pJ6Ω-ProT by *Pvu*II and *Sca*I digestions, was microinjected into the pronucleus of FVB zygotes, and the injected eggs were transferred into the oviduct of pseudopregnant recipients. Genomic DNA of founder mice was extracted from tail biopsies and the integration of the transgenic ProT minigene was detected by polymerase chain reaction (PCR) using RAP-f and ProT-r1 primers (Table 1). The PCR reaction was performed at an annealing temperature of 60°C for 35 cycles. Analysis for the presence

of the transgene was performed by dot blot analysis of genomic DNA using ^{32}P-labeled murine ProT cDNA as the hybridization probe. The copy number of the transgene was determined by comparing the intensity resulting from 0.5 μg, 1 μg, and 2.5 μg genomic DNA among wild-type, nontransgenic, and transgenic founders, respectively, on dot blot analysis by densitometry. All animal experiments were performed following the guidelines approved by the Laboratory Animal Care and Use Committee of the National Cheng Kung University Medical College.

Quantification of transgene expression by reverse transcription (RT)-PCR

Total RNA from a variety of mouse tissues was isolated with RNeasy Mini Kit (Qiagen, Hilden, Germany). To avoid genomic DNA contamination, RNA was treated with DNase I for 15 minutes, and reverse transcribed at 47°C using oligo-dT (0.5 μg/μL) and reverse transcriptase in a total volume of 20 μL. The RT reaction product (3 μL) was mixed with 10 × reaction buffer (5 μL) containing 5 mmol/L deoxynucleoside triphosphate (dNTP) mix, *Taq* DNA polymerase, and 3 μmol/L of each oligonucleotide primer. The primer pairs used for amplification of ProT, c-myc, epithelial growth factor (EGF), EGF receptor (EGFR), and 18S rRNA mRNA serving as the internal control for RT-PCR are shown in Table 1. All amplified products were confirmed by DNA sequencing. RT-PCR products were quantified by densitometric scanning of the electrophoresis bands and related to the band intensity of the 18S rRNA gene.

Flow cytometry

Staining of cell surface antigens was performed by incubating splenocytes or thymocytes of ProT heterozygous (7 weeks old), homozygous (10 days old) transgenic, or age-matched wild-type mice for 60 minutes at 4°C in the presence of NaN$_3$, with fluorochrome-conjugated rat antimouse monoclonal antibodies, including

145-2C11 (anti-CD3e)-phycoerythrin-Cy5 (Pharmingen, San Diego, CA, USA), GK1.5 (anti-CD4)-R-phycoerythrin (Pharmingen), and 53–6.7(anti-CD8)-fluorescein isothiocyanate (FITC) (Pharmingen). The percentage of cell subpopulation was determined using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and CellQuest software (Becton Dickinson). At least 10,000 gated events were acquired per antibody analyzed.

Quantification of ProT protein by enzyme-linked immunosorbent assay (ELISA)

Lysate was prepared from homogenization of whole kidney in 300 μL radioimmunoprecipitation assay (RIPA) buffer [1% Nonidet P-40, 0.5% deoxycholic acid, and 0.2% sodium dodecyl sulfate (SDS) in phosphate-buffered saline (PBS)] with complete protease inhibitor cocktail (Roche, Penzberg, Germany). The insoluble fraction was removed by centrifugation at 12,000 rpm for 15 minutes. The protein content of each kidney lysate was measured using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Urine samples were collected from mice for ProT measurements after depriving them of liquid for 12 hours.

We have previously generated mouse monoclonal and rabbit polyclonal antibodies against recombinant human ProT produced in *Escherichia coli*. They were employed in an antibody-sandwich ELISA for determining renal and urinary ProT contents in mice [20]. In brief, 96-well-microtiter plates (MaxiSorp, Nunc, Roskilde, Denmark) were coated with rabbit anti-ProT polyclonal antibody (2 μg/mL in 0.2 mol/L carbonate-bicarbonate buffer, pH 9.6) at 37°C for 5 hours and blocked with 1% bovine serum albumin (BSA) diluent/blocking solution (KPL, Gaithersburg, MD, USA) at 37°C for 2 hours. After removal of the blocking agent, mouse urine (15 μL) diluted 1:2 in 1% BSA was added in duplicate and plates were incubated overnight at 4°C. After washings with PBS-T [0.05% (vol/vol) Tween-20 in PBS], anti-ProT monoclonal antibody (ascites, 5 μg/mL, 100 μL) was added at 37°C for 2 hours. After washings with PBS-T, horseradish peroxidase–conjugated goat anti-mouse IgG + IgM antibody (KPL) was added and the plates were incubated at 37°C for 2 hours. The plates were washed and 3, 3′, 5, 5′-tetramethyl benzidine was added. After a further incubation at room temperature for 40 minutes, the reaction was stopped by adding 2 mol/L H_2SO_4 and the absorbance at 450 nm was measured by an ELISA reader. Twofold dilutions of recombinant human ProT protein ranging from 0.001 to 0.01 μg/mL were used as the standard in the assay.

Histologic, immunohistochemical, and biochemical analyses of kidneys

Mice were sacrificed via cervical dislocation, and perfusion was performed with calcium- and magnesium-free PBS. Kidneys were removed and fixed in 10% formalin at 4°C overnight. The tissues were dehydrated and embedded in paraffin, and sectioned at 5 μm for immunostaining or hematoxylin-eosin staining. Segment-specific localization of renal cysts was assessed immunohistologically using biotinylated *Dolichos biflorus* agglutinin (1:500) (Vector Laboratories, Burlingame, CA, USA) specific to collecting tubule [30], rabbit antibody against human Tamm-Horsfall protein (1:500) (Biomedical Technologies, Stoughton, MA, USA), a marker for distal tubule [31], and rabbit antibody against human lysozyme (1:300) (Dako, Glostrup, Denmark), a marker for proximal tubule [32]. Three human kidney specimens from PKD cases obtained from the Department of Pathology, National Cheng Kung University Hospital were immunohistochemically investigated for the presence of ProT protein. Case 1 had ADPKD with chronic renal failure due to severe tubulointerstitial nephritis, while cases 2 and 3 had ARPKD. Case 2 at 30 weeks of gestational age was diagnosed premature ARPKD with mega-kidney and terminated due to polycystic kidney. Case 3 at 31 weeks of gestational age was diagnosed prenatal ARPKD with mild congenital hepatic fibrosis. To detect ProT expression, kidney sections from ProT transgenic mice and from clinical specimens were blocked with an avidin/biotin blocking kit (Vector Laboratories) after deparaffinization and rehydration, and incubated overnight at 4°C with rabbit anti-ProT antibody (1:100) (Calbiochem, San Diego, CA, USA). Immunostaining was performed using a commercial streptavidin-biotin alkaline phosphatase complex Dako LSAB 2 Kit (Dako) according to the manufacturer's instructions, using aminoethyl carbazole (AEC) staining and hematoxylin counterstaining. Masson trichrome stain was used to determine the extent of interstitial fibrosis in the kidney and liver. Serum samples were collected for determination of blood urea nitrogen (BUN) by a colorimetric assay kit (Ecoline 25) (Merck, Darmstadt, Germany).

Statistics

Data are expressed as mean ± SD. Statistical analysis was conducted by using the two-tailed Student *t test*. Statistical significance was defined as $P < 0.05$.

RESULTS

Generation and identification of transgenic mice overexpressing ProT

To generate ProT transgenic mice, microinjection of the linearized ProT minigene (Fig. 1A) into the pronuclei of fertilized FVB embryos was performed using standard techniques. Genomic DNA from tail biopsies was isolated and examined for the presence of ProT transgene by PCR using RAP-f and ProT-r1 primers, which were
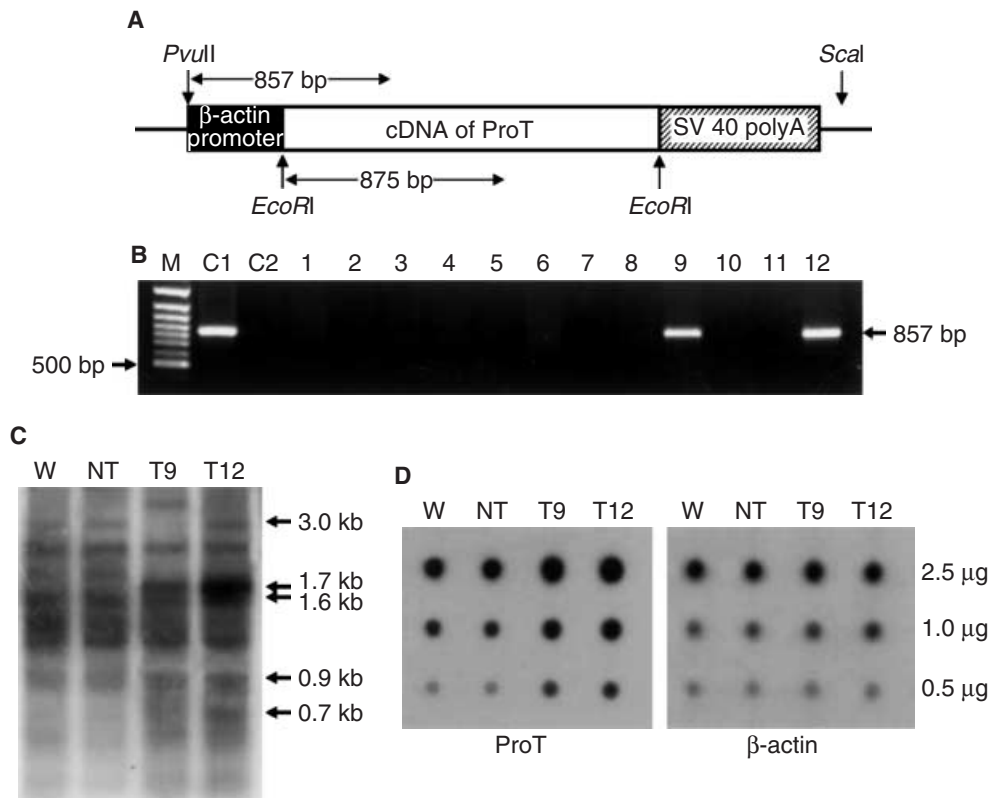
Fig. 1. Generation and characterization of prothymosin α (ProT) transgenic founder lines. (*A*) Schematic diagram of the ProT transgene construct used to create transgenic mice. A transgene construct consisting of the rat β-actin promoter, full-length mouse ProT cDNA, and the SV40 large T antigen polyadenylation signal sequence was generated. Restriction sites for excision of the construct from the cloning vector (*Pvu*II/*Sca*I), for generation of radiolabeled probe (*Eco*RI), and for digestion of genomic DNA for Southern blot analysis (*Pvu*II) are indicated. Locations of primers for amplifying 857 bp (RAP-f and ProT-r1) and 875 bp (MCS-f and ProT-r2) products in polymerase chain reaction (PCR) and reverse transcription (RT)-PCR analyses are shown, respectively. (*B*) PCR screening of the transgene using tail DNA. The presence of an 857 bp PCR product indicates the ProT transgenic founder. Lane C1 represents the mixture of linear transgene and genomic DNA from normal mice, which served as a positive control. Lane C2 represents genomic DNA from normal mice, which served as a negative control. Numbers 1 to 12 represent 12 different offspring. (*C*) Localization of the transgene (T) in two identified founders was determined by Southern blot analysis. Different *Pvu*II restriction patterns were observed in T9 and T12 compared with wild-type (W) and nontransgenic (NT) mice. (*D*) The copy number of transgene integrated into the genome of T9 and T12 founder lines ranged between three and five copies per haploid genome, as determined by densitometric comparison on DNA dot blot analysis. β-actin served as an internal control.

designed to exclude amplification of endogenous ProT gene. The expected 857 bp PCR product should be detected in the F_0 offspring. Occasionally, embryonic lethality was encountered. Among 12 offspring examined, two male founders (T9 and T12) that revealed positive genomic integration of the transgene were established (Fig. 1B). The authenticity of the PCR product was further confirmed with DNA sequencing (data not shown). Localization of the minigene in T9 and T12 founders was determined by Southern blot analysis. As shown in Figure 1C, different restriction patterns between T9 and T12 were noticed, suggesting that these two founders were independent lines and ProT transgene was localized at different loci. The copy number of transgene integrated into the genome of these mice ranged between three and five copies per haploid genome, as determined by densitometric comparison on DNA dot blot analysis (Fig. 1D). Transgenic founder mice were used to establish transgenic mouse lineages. Unless otherwise noted, the fifth (F5) and sixth (F6) generation mice at 10 weeks of age were used throughout the experiments.

Analysis of ProT gene expression in transgenic mice

To determine whether ProT minigene was expressed in transgenic mice, we analyzed ProT mRNA expressions in the spleen, thymus, lung, liver, and kidney from the offspring of both founder lines by semiquantitative RT-PCR using MCF-f and ProT-r2 primers (Fig. 1A). Expression of ProT transgene was detected in all the tissues examined from T12 transgenic offspring, but not in nontransgenic littermates (Fig. 2A). Similar results were also obtained from T9 transgenic offspring (data not shown). However, no obvious phenotypical alterations were observed in these founder lines and their offspring which were born from the founder mating with wild-type animals. They
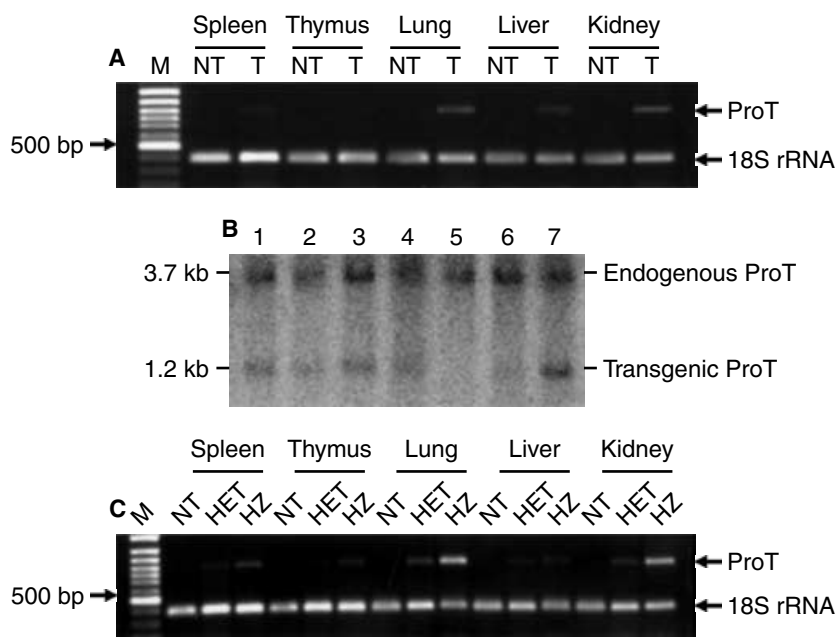
Fig. 2. Transgene expression in prothymosin α (ProT) transgenic mice. (*A*) ProT transgenic RNA expression was detected in various organs in T12 transgenic (T) offspring, but not in nontransgenic (N) littermates by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) using primers specific for the mouse ProT transgene. Similar results were also obtained from T9 transgenic offspring (data not shown). 18S rRNA expression served as the quantitative control. M is 100 bp DNA ladder as molecular size standard. (*B*) Southern blot of *Eco*RI-digested genomic DNA from homozygous (lane 7), heterozygous (lanes 1 to 4 and 6), and nontransgenic (lane 5) mice hybridized with a ProT probe. The 1.2 kb and 3.7 kb bands correspond to transgenic and endogenous ProT, respectively. (*C*) ProT transgenic RNA expression was detected in various organs in homozygous (HZ) and heterozygous (HET) ProT mice, but not in nontransgenic (NT) littermates by RT-PCR using primers specific for the mouse ProT transgene. 18S rRNA expression served as the quantitative control. M is 100 bp DNA ladder as molecular size standard.

were then bred to homozygous state to double the level of transgene expression. Homozygous transgenic mice were generated from heterozygous breeding pairs and the genotype was determined by Southern blot analysis. As shown in Figure 2B, the copy number of transgene in homozygous transgenic mice was doubled compared with that for heterozygous transgenic mice. Expression of the ProT transgene was observed in all the organs examined, including spleen, thymus, lung, liver, and kidney. As expected, the level of ProT transgenic mRNA expression in the homozygous mice was higher than that in their heterozygous littermates (Fig. 2C). Among these organs, higher expression of ProT transgene was detected in the kidney and lung compared with the liver, spleen, and thymus.

Morphologic and immunologic analyses of ProT transgenic mice

As shown in Figure 3A, birth weight of homozygous ProT mice was reduced to approximately 70% of the weight of their heterozygous or nontransgenic littermates. The homozygous mice were fed very poorly during the first few hours of life. Furthermore, they gained less weight and were approximately half the size of their heterozygous or nontransgenic littermates at 10 days of age. Whereas all the homozygous mice inevitably succumbed to death approximately 10 days after birth, the heterozygous mice appeared normal and survived their normal life span. Figure 3B shows that the homozygous mouse was smaller and retarded in growth rate compared with heterozygous or nontransgenic littermates at 10 days of

age. Morphologic examinations revealed that the thymus, lung, liver, and spleen of the homozygous mice were all smaller than the corresponding organs of age-matched heterozygous or nontransgenic littermates. However, on a percentage of body weight basis, the relative weight was significantly increased in the kidney and liver, but remained constant in the lung, spleen, and thymus of homozygous ProT mice (Fig. 3C). The following values were found for the ratio of kidney weight to body weight: homozygous ProT mice, 0.058 ± 0.014 g ($2.350 \pm 0.290\%$) of 2.35 ± 0.32 g; heterozygous ProT mice, 0.060 ± 0.004 g ($1.160 \pm 0.068\%$) of 5.20 ± 0.21 g; and nontransgenic mice, 0.060 ± 0.004 g ($1.136 \pm 0.071\%$) of 5.26 ± 0.22 g. Therefore, the homozygous mice had similar kidney size but increased ratio of kidney weight to body weight, displaying renal enlargement with smooth surfaces (Fig. 3D). The 10-day-old homozygous ProT mice had no obvious abnormalities in the spleen and thymus, nor did their splenic CD4^{+} and CD8^{+} T cells change in relative percentage (data not shown). Analysis on thymus and spleen cells of 7-week-old heterozygous ProT mice did not reveal any major differences in populations of CD4^{+} and CD8^{+} T cells in either cell numbers or relative percentage (data not shown). However, it remains to be determined whether T-cell functions are affected in ProT transgenic mice. Notably, although the morphology of lungs from newborn homozygous ProT mice appeared normal, emphysematous changes and slight fibrosis of the lung were observed when they were 10 days of age [Li KJ, Shiau AL, and Wu CL, manuscript in preparation]. However, little apparent change in the gross morphology of the lung was found in heterozygous ProT mice.
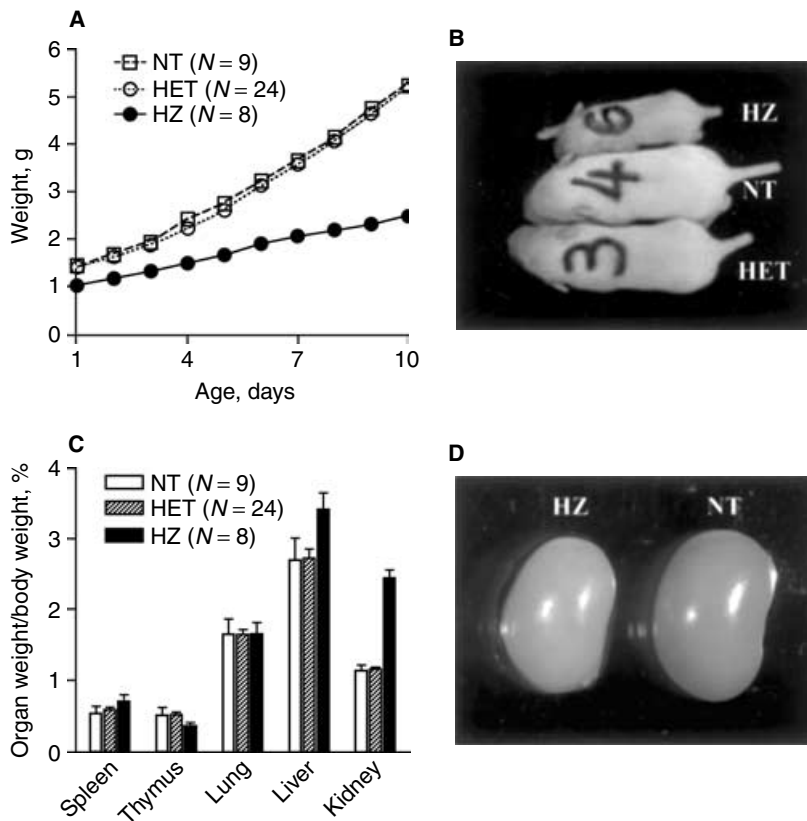
Fig. 3. Gross phenotypes of prothymosin α(ProT) transgenic mice. (*A*) Growth curves for body weight and (*B*) photographs of homozygous (HZ), heterozygous (HET), and nontransgenic (NT) littermates. (*C*) Ratios of organ weight to body weight for the spleen, thymus, lung, liver, and kidney in homozygous, heterozygous, and nontransgenic mice. (*D*) Photographs showing the kidneys of homozygous (0.0286 g) and nontransgenic (0.0298 g) littermates at 10 days of age.

Histologic analysis of ProT transgenic mice

Abnormalities in both kidney and lung were noticed in the homozygous mice upon histological analysis. A total of 16 kidneys from 16 homozygous mice, 12 at 10 days of age, and 4 at 1 day of age, as well as equivalent samples from heterozygous mice and their nontransgenic littermates underwent histologic analysis. Independent of age, all kidney specimens from homozygous ProT mice displayed a polycystic phenotype (Fig. 4A to D) in contrast to the normal appearing kidneys from heterozygous (data not shown) and nontransgenic littermates (Fig. 4E to H). Bilateral diffusely distributed renal cysts and hyperplasia of renal epithelium were observed in the homozygous mice. The cysts of newborn mice exclusively occurred in the inner cortex and medulla (Fig. 4A and B), and then predominantly developed in medulla generally at 10 days of age (Fig. 4C and D). Furthermore, the cysts were larger in the homozygous mice at 10 days of age (Fig. 4C and D) compared with those in the newborn mice (Fig. 4A and B). At birth, cysts were localized throughout collecting (Fig. 5A), distal (Fig. 5C), and proximal (Fig. 5E) tubular segments, whereas at 10 days in the terminal stages of the disease, cysts presented prominently in collecting (Fig. 5B) and distal (Fig. 5D) tubules, but not in proximal tubules (Fig. 5F). In addition, slight renal fibrosis of homozygous ProT mice was detected as assessed by Masson trichrome staining (data not shown). However, liver fibrosis, a feature associated with some human forms of PKD, was not noted in these mice (data not shown).

We also examined four 17-month-old heterozygous ProT transgenic mice in each independent line. Morphologic examination revealed that their lungs, livers, and spleens were not different from those of nontransgenic littermates, whereas all of their kidneys displayed a small degree of phenotypic alterations. Visible cysts, albeit to a lesser extent compared to young homozygous mice, were observed in their kidneys (Fig. 6A), which were confirmed by histologic analysis (Fig. 6B). As detailed examinations of the lung in the aged heterozygous mice have not yet been carried out, histologic changes in the lungs cannot be ruled out and need to be investigated further.

Increased levels of ProT production in the kidney and urine of homozygous ProT transgenic mice

To determine whether PKD found in ProT transgenic mice was associated with an increase of ProT, we determined the ProT levels in the kidneys of ProT transgenic mice. As shown in Figure 7A, the ProT level, corrected for protein content, in the kidneys of homozygous ProT mice was more than two times higher than that of heterozygous or nontransgenic littermates (Fig. 7A). Furthermore, the concentration of ProT in urine (52.29 ± 2.8 pg/μL) was approximately tenfold higher in the homozygous mice than that of heterozygous or nontransgenic littermates
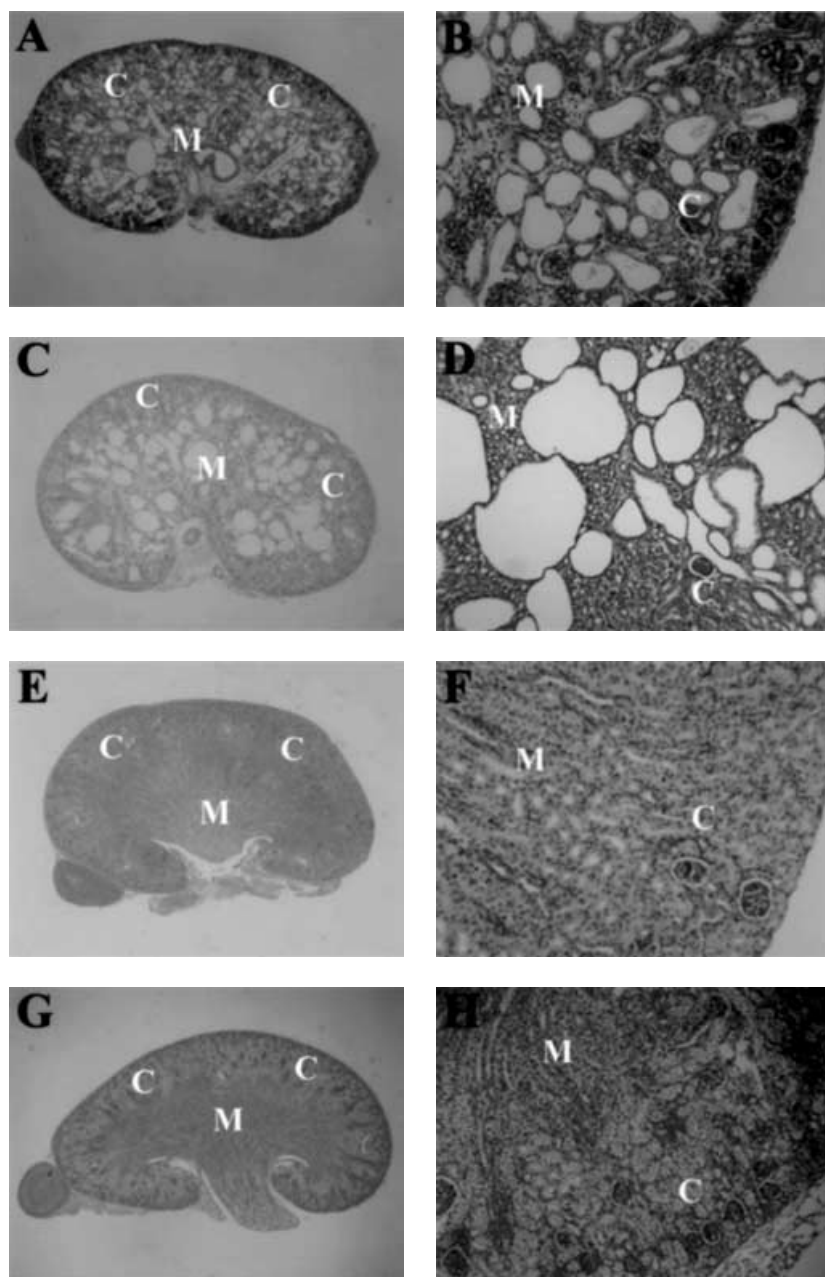
Fig. 4. Formation of renal cysts in homozygous prothymosin α (ProT) transgenic mice. Hematoxylin-eosin stains on kidney sections from newborn and 10-day-old homozygous and nontransgenic littermates. Cysts presented in the kidneys of homozygous ProT mice at newborn (*A* and *B*) and at 10 days of age (*C* and *D*), whereas the kidneys of nontransgenic littermates, either at newborn (*E* and *F*) or at 10 days of age (*G* and *H*), appeared normal [original magnification ×10 (A, C, E, and G) and ×200 (B, D, F, and H)]. C is cortex; M is medulla.

(Fig. 7B). These results indicate that polycystic kidney found in ProT transgenic mice was associated with increased expression of ProT.

Correlation between the murine PKD model induced by ProT overexpression and human PKD

To understand the pattern of ProT overexpression in cystic kidneys of homozygous and aged heterozygous ProT mice, ProT expression in renal tissue was examined by immunohistochemistry. ProT expression was localized abundantly in the cystic epithelium of homozygous ProT mice (Fig. 8A), in contrast to low expression in renal tubular epithelium of nontransgenic littermates (Fig. 8C). To

further explore whether PKD induced by ProT overexpression in transgenic mice resembled human PKD, renal specimens of three PKD cases were examined for ProT expression. Interestingly, the expression patterns of ProT in the clinical specimens (Fig. 8I) were similar to those of ProT homozygous (Fig. 8A) and aged heterozygous transgenic mice (Fig. 8E), whereas ProT was hardly detectable in the kidney of normal individuals (Fig. 8K). The tissue sections shown in Figure 8I and J were from case 2 (ARPKD). Similar expression patterns and levels of ProT were also noted in cases 1 and 3 suffering from ADPKD and ARPKD, respectively (data not shown). These clinical findings suggest that increased expression
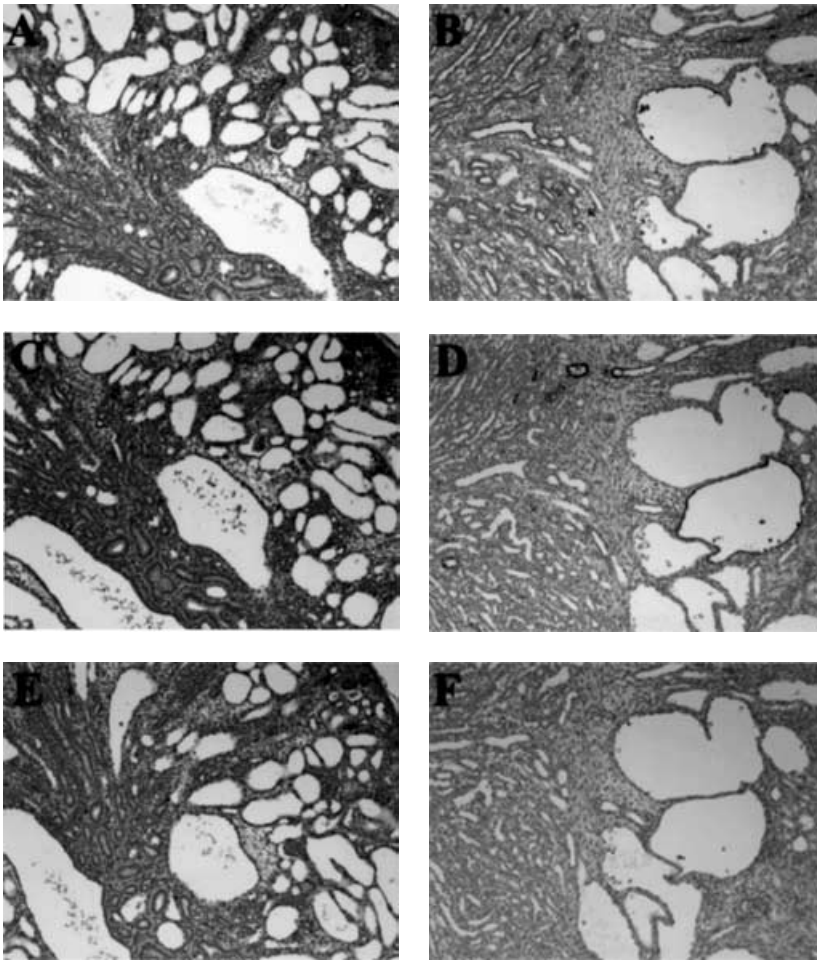
Fig. 5. Location of renal cysts in homozygous prothymosin α (ProT) transgenic mice. Segment-specific localization of cysts from the kidneys of the newborn (*A, C,* and *E*) and 10-day-old (*B, D,* and *F*) homozygous ProT mice was assessed immunohistologically using *Dolichos biflorus* agglutinin specific to collecting tubule (A and B), antibody that recognizes Tamm-Horsfall protein, a marker for distal tubule (C and D), and antibody that recognizes lysozyme, a marker for proximal tubule (E and F) (original magnification ×200).

of ProT may be a common feature of different forms of PKD. Collectively, ProT was present abundantly in the cytoplasm of cystic epithelium in both animal models and clinical specimens of PKD, but scarcely in renal tubule epithelium from nontransgenic mice and normal individuals. These results suggest that overexpression of ProT may lead to the development of PKD.

To evaluate whether the lethality in the homozygous mice was due to the deterioration of renal function, serum levels of BUN, another major characteristic of PKD in humans and in congenital mouse models, were measured. As shown in Figure 9, the levels of BUN in heterozygous ProT mice (24.79 ± 1.25 mg/dL) and nontransgenic mice (23.93 ± 1.01 mg/dL) were similar. In contrast, BUN values of the homozygous mice were nearly three times higher than those of heterozygous or nontransgenic littermates. Notably, the BUN levels in the homozygous mice killed in a moribund state were approximately five times higher than those of heterozygous or nontransgenic littermates, reaching a value of 120 mg/dL. These results suggest that renal failure, at least in part, might be a possible cause of death in homozygous ProT transgenic mice.

Up-regulation of EGFR expression in homozygous ProT transgenic mice

To further investigate how ProT overexpression induced PKD, expressions of several genes thought to be associated with PKD, namely c-myc, EGFR, and EGF, were examined by RT-PCR in the kidneys of ProT transgenic mice obtained from both T9 and T12 lines. The results shown are representative of four independent experiments (Fig. 10). As expected, the expression level of ProT transgenic RNA in the homozygous mice was higher than that in the heterozygous mice. The expression levels of c-myc and EGF in homozygous ProT mice remained unchanged compared with those in heterozygous or nontransgenic littermates. However, in accordance with PKD in humans [33] and in congenital mouse models of PKD [34, 35], significant up-regulation of EGFR mRNA expression was detected in homozygous ProT transgenic mice. Comparative densitometric analysis of various RT-PCR products relative to 18S rRNA product revealed significant augmentation of renal mRNA expression of EGFR in homozygous ProT mice (Fig. 10B).
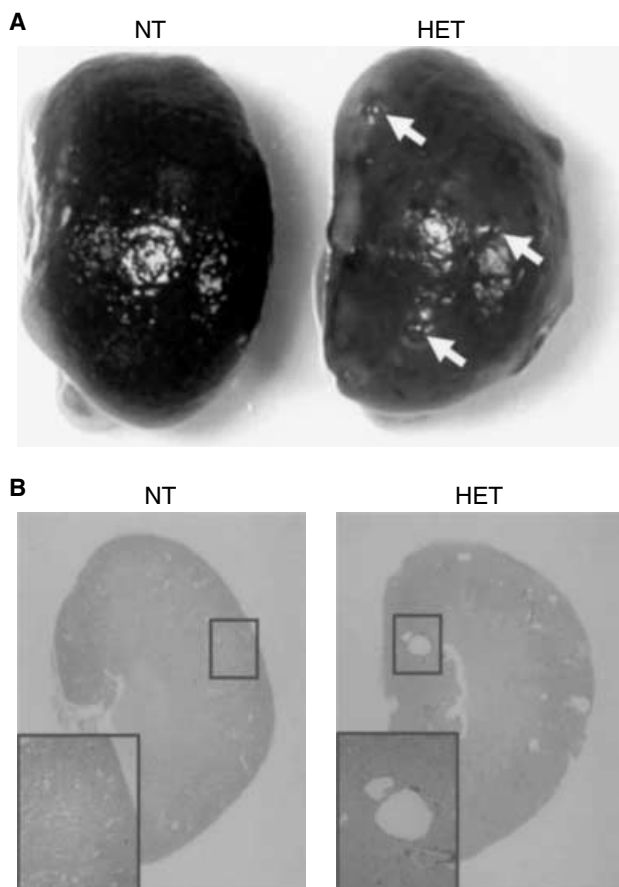
Fig. 6. Gross appearance of the kidneys from aged heterozygous pro-thymosin α (ProT) transgenic and nontransgenic mice. (*A*) Photographs showing the difference of the kidneys between heterozygous (HET) and nontransgenic (NT) mice at 17 months of age. Cysts were consistently observed in the kidneys of aged heterozygous but not in age-matched nontransgenic mice. The arrows indicate the location of cysts. (*B*) A few cysts of various sizes were always seen in the hematoxylin-eosin–stained kidney section from aged ProT transgenic mice, but not in nontransgenic mice (original magnification ×10). The insets represent the magnified area (original magnification ×100).

DISCUSSION

In this study, we describe the generation and characterization of a novel animal model for PKD by transgenic overexpression of ProT. Similar to other congenital PKD mice [3], homozygous ProT transgenic mice were fed very poorly during the first few hours of life, and their body sizes were smaller than heterozygous or nontransgenic littermates. The homozygous ProT mice also revealed growth retardation with age, and finally died about 10 days after birth, while congenital PKD mice die at the age of 3 to 4 weeks [3–7]. Based on histological analysis, bilateral diffusely distributed renal cysts and hyperplasia of renal epithelium were observed in homozygous ProT transgenic mice. Particularly, cyst development in the ProT transgenic mice is very similar to other congenital PKD mouse models, in which renal cyst forma-

tion occurs in the proximal tubules and collecting ducts at birth and predominantly in collecting ducts at the age of 10 days [3–7]. Of note, renal cysts also presented in the distal tubules of homozygous ProT mice. Furthermore, although the kidney size remained largely unchanged, the kidney weight/body weight ratio of homozygous ProT mice was greater compared with that of heterozygous or nontransgenic littermates, suggesting that overexpression of ProT resulted in progressive kidney enlargement of postnatal mice and increased fatality in infants. The moribund homozygous ProT mice had fivefold higher BUN levels than did age-matched heterozygous or nontransgenic littermates. In some congenital PKD mouse models [3, 5–7], in addition to renal pathology, hepatic cysts and bile duct cysts are the most common extrarenal pathology. However, these pathological changes were not noted in homozygous ProT transgenic mice. Although the homozygous mice presented high levels of BUN, they displayed only a small degree of renal fibrosis. Notably, large emphysematous air spaces were observed in the lungs, which may have reduced air exchange efficiency in alveolar duct, leading to respiratory failure. Therefore, in addition to impaired renal function, pulmonary emphysema may have contributed to the early death of homozygous ProT mice. It is of interest to note that higher levels of ProT mRNA were expressed in the kidney and lung than in the liver, spleen, and thymus in homozygous ProT mice, which correlated with phenotypic alterations found in the kidney and lung. The ProT transgene was driven by the ubiquitous β-actin promoter to give widespread expression in transgenic mice. However, it has been shown that β-actin gene may not be constitutively expressed under some circumstances. In the folic acid–induced regenerative hyperplasia model in rabbits, kidney epithelial cells revealed rapid, transient increases in β-actin mRNA [36]. This suggests that higher expression of transgenic ProT driven by the β-actin promoter may have occurred in proliferating cells, such as cystic epithelial cells, of ProT transgenic mice. ProT expression may further promote cell proliferation, resulting in a positive feedback regulation on ProT expression. Furthermore, in mouse tissues, lung and kidney are the two nonlymphoid tissues to express the highest level of endogenous ProT [37], which accords with the highest expression of ProT mRNA in ProT transgenic mice. Our results demonstrate that renal and pulmonary functions of homozygous ProT transgenic mice deteriorated rapidly after birth and all the mice inevitably died approximately 10 days after birth.

In the present study, similar PKD phenotypes were consistently observed in two independent transgenic lines, suggesting that the phenotype of homozygous ProT transgenic mice was attributable to ProT overexpression, rather than inactivation of genes associated with transgene integration. The two founder lines that we established contained three to five copies of the ProT
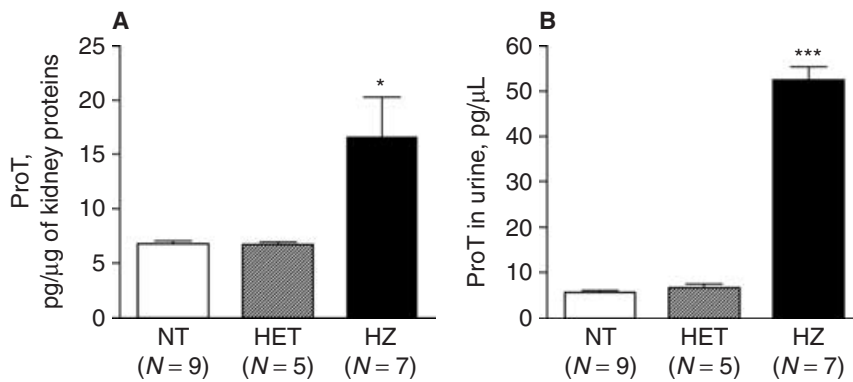
Fig. 7. Increased expression of renal and urinary prothymosin α (ProT) protein in homozygous ProT transgenic mice. ProT protein level was analyzed by enzyme-linked immunosorbent assay (ELISA) in renal extraction (*A*) and urine (*B*) from homozygous (HZ), heterozygous (HET), and nontransgenic (NT) mice. *$P < 0.05$ for homozygous vs. nontransgenic; ***$P < 0.001$ for homozygous vs. nontransgenic or heterozygous.
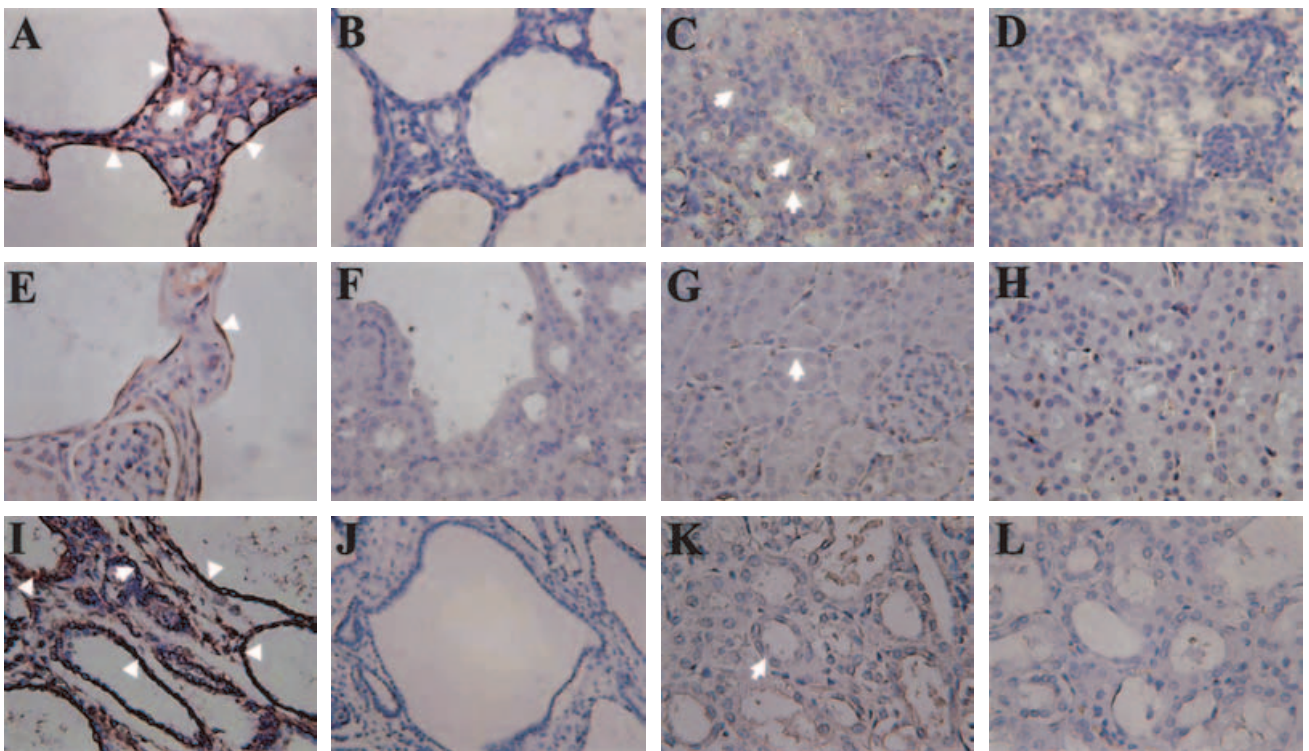

Fig. 8. Immunohistochemical examination of prothymosin α (ProT) in the kidneys of homozygous ProT transgenic mice and human polycystic kidney disease (PKD). ProT expressions in representative tissue sections (original magnification ×400) of the kidneys from 10-day-old homozygous ProT (*A* and *B*) and nontransgenic mice (*C* and *D*), from 17-month-old heterozygous ProT (*E* and *F*) and nontransgenic mice (*G* and *H*), and from human autosomal-recessive PKD (ARPKD) case (*I* and *J*) and normal individuals (*K* and *L*) were determined by immunostain with rabbit antibody against ProT (A, C, E, G, I, and K) or with control rabbit serum (B, D, F, H, J, and L). The arrowhead indicates dark staining for ProT in cystic epithelial cells from ProT transgenic mice and from human ARPKD case, while the arrow indicates pale staining for endogenous ProT in renal tubule cells from nontransgenic mice and from normal individuals.

transgene. However, we were unable to obtain transgenic lines containing higher copy numbers of the transgene. It is conceivable that very high levels of ProT expression may have been detrimental to mice, resulting in embryonic lethal phenotype or early mortality. As a result, heterozygous mice overexpressing ProT but not to a level high enough to cause phenotypic alterations would grow normally and were expected to be selected as founders in our initial screening of heterozygous transgenic mice. They were then bred to homozygous state to double the level of transgene expression. All of the homozygous

ProT mice developed PKD and died around 10 days after birth. Interestingly, although young heterozygous ProT mice appeared normal and had no obvious phenotypical alterations, 17-month-old heterozygous mice did develop mild PKD with ProT expression detectable in their cystic epithelium. This result suggests that intracellular accumulation of ProT increased as ProT transgenic mice grew older, which might lead to PKD. The same ProT expression pattern was detected in the kidneys of ProT transgenic mice that displayed slight renal fibrosis, as well as in those of human ARPKD and ADPKD. Taken
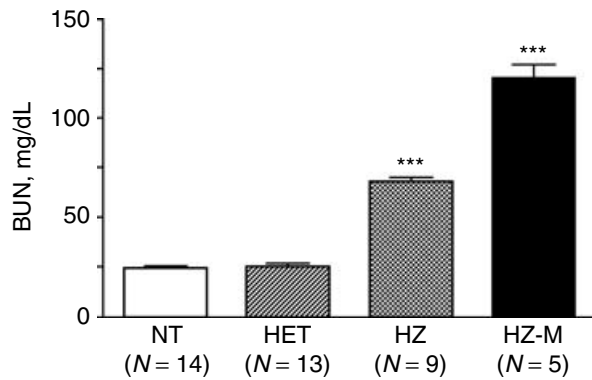
Fig. 9. Increase of blood urea nitrogen (BUN) in homozygous prothymosin α (ProT) transgenic mice. The levels of BUN in 10-day old homozygous (HZ), moribund homozygous (HZ-M), heterozygous (HET), and nontransgenic (NT) mice as determined by a colorimetric assay. ***$P < 0.001$ for homozygous vs. nontransgenic or heterozygous, and moribund homozygous vs. nontransgenic or heterozygous.
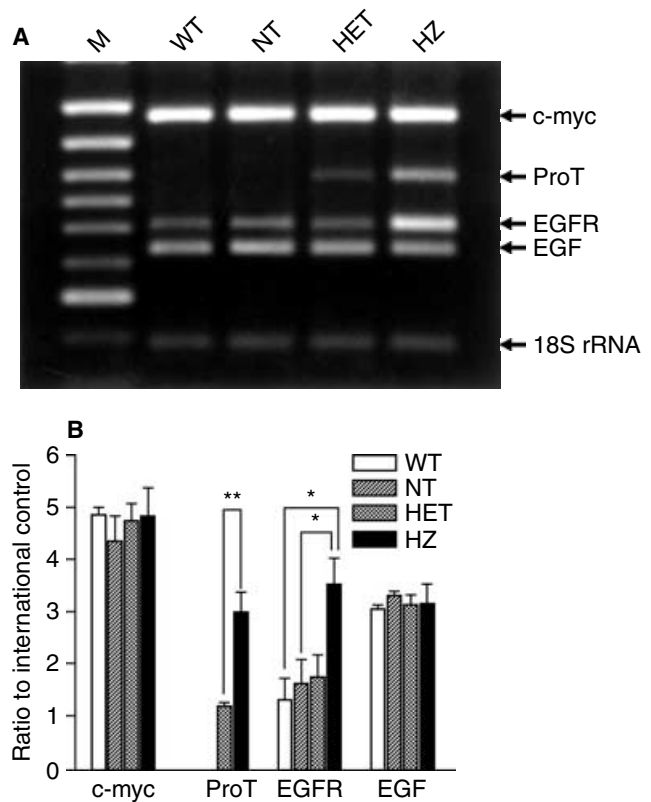

Fig. 10. Effects of prothymosin α (ProT) overexpression on mRNA expressions of c-myc, epithelial growth factor receptor (EGFR), and epithelial growth factor (EGF) in the kidney. Total renal RNA was extracted from T9 homozygous (HZ), heterozygous (HET), and nontransgenic (NT) mice, as well as from wild-type (WT) mice. *(A)* Expressions of c-myc, EGFR, and EGF mRNA, as well as ProT transgenic mRNA were examined by reverse transcription-polymerase chain reaction (RT-PCR). 18S rRNA served as an internal control. M is 100 bp DNA ladder as molecular size standard. *(B)* Bar chart showing comparative densitometric analysis of various RT-PCR products relative to 18S rRNA in renal tissues of the mice. The expression analysis was repeated three times. **$P < 0.01$; *$P < 0.05$.

together, these results suggest that ProT overexpression appeared to be associated with PKD rather than with uremia/azotemia. However, as end-stage kidneys from other forms of chronic kidney disease were not examined in this study, whether ProT overexpression is also related to uremia/azotemia is unclear, which warrants further investigation.

The proto-oncogene c-myc encodes a transcription factor that plays a pivotal role in cell proliferation, differentiation, and apoptosis. Overexpression of c-myc induces PKD in transgenic mice [11, 12]. In human ADPKD, renal c-myc expression is elevated up to 15-fold, which correlates with increases of both proliferation and apoptosis in cystic epithelium [13]. Increasing evidence supports an important role for EGF/transforming growth factor-α (TGF-α)/EGFR axis in promoting tubular epithelial cell proliferation and cyst formation in PKD. EGFR and Erb-B2 (an EGFR-related tyrosine kinase receptor) are overexpressed and mislocalized to the apical membrane of the cystic epithelium in human ADPKD and ARPKD and in murine models of these diseases [33–35, 38]. The abnormally expressed apical receptors are capable of high-affinity EGF binding and autophosphorylation and initiate a signaling cascade that results in cell proliferation [35]. To this end, our results demonstrate that EGFR expression was increased, while c-myc and EGF expressions were unchanged in the kidney of homozygous ProT transgenic mice. This suggests that renal expression of EGFR may be affected by ProT overexpression. Since c-myc directly regulates the expression of ProT gene by binding to the E box of its promoter [25, 26], whether c-myc regulates EGFR expression through ProT in vivo remains to be elucidated. Furthermore, the lack of significant alteration in c-myc expression provides further evidence that the PKD phenotype noted in ProT trans-

genic mice did not result from c-myc activation, probably arising from an "integration position effect." It is well documented that ProT promotes cell proliferation [17–20]. In ProT transgenic mice described here, renal cyst development always occurred in newborn mice and cyst number and size increased with age, suggesting that overexpression of ProT may affect kidney development through promoting tubular epithelium proliferation during embryo development, resulting in the development of PKD. However, the underlying molecular mechanism still awaits further investigation. Recently, EKI-785, an EGFR tyrosine kinase inhibitor, has been demonstrated to attenuate PKD development in a mouse ARPKD model [39] and a rat ADPKD model [40]. Since our data indicate that ProT overexpression leads to EGFR up-regulation in the kidneys of homozygous ProT transgenic mice and that renal ProT is overexpressed in the clinical specimens of ARPKD and ADPKD cases,

suppression of ProT overexpression might be a considerable approach for ameliorating the symptoms of PKD. So far, there is no proper molecular maker available for the diagnosis of PKD. We have demonstrated abundant distribution of ProT not only in cystic kidneys but also in the urine of homozygous ProT transgenic mice that resemble human ARPKD. In particular, the content of ProT protein in the urine of homozygous ProT transgenic mice was ten times greater compared with heterozygous or nontransgenic littermates. Therefore, ProT might represent a candidate molecular marker for noninvasive diagnosis of PKD if further studies using sufficient samples prove its feasibility.

Taken together, ProT transgenic mice represent a novel PKD animal model resembling human ARPKD. The relevance of this model to the human disease is further supported by ProT overexpression observed in both human ARPKD and ADPKD kidneys. However, the role of ProT in the cystogenesis still remains to be clarified.

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