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Cyst Formation in Kidney via B-Raf Signaling in the *PKD2* Transgenic Mice*

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The pathogenic mechanisms of human autosomal dominant polycystic kidney disease (ADPKD) have been well known to include the mutational inactivation of *PKD2*. Although haploinsufficiency and loss of heterozygosity at the *Pkd2* locus can cause cyst formation in mice, polycystin-2 is frequently expressed in the renal cyst of human ADPKD, raising the possibility that deregulated activation of *PKD2* may be associated with the cystogenesis of human ADPKD. To determine whether increased *PKD2* expression is physiologically pathogenic, we generated *PKD2*-overexpressing transgenic mice. These mice developed typical renal cysts and an increase of proliferation and apoptosis, which are reflective of the human ADPKD phenotype. These manifestations were first observed at six months, and progressed with age. In addition, we found that ERK activation was induced by *PKD2* overexpression via B-Raf signaling, providing a possible molecular mechanism of cystogenesis. In *PKD2* transgenic mice, B-Raf/MEK/ERK sequential signaling was up-regulated. Additionally, the transgenic human polycystin-2 partially rescues the lethality of *Pkd2* knock-out mice and therefore demonstrates that the transgene generated a functional product. Functional strengthening or deregulated activation of *PKD2* may be a direct cause of ADPKD. The present study provides evidence for an *in vivo* role of overexpressed *PKD2* in cyst formation. This transgenic mouse model should provide new insights into the pathogenic mechanism of human ADPKD.

ADPKD[4] is a common systemic disease that affects multiple organs and cell types (1, 2). ADPKD affects one in 1,000 individuals, primarily through the occurrence of large, fluid-filled renal cysts that ultimately lead to renal failure (3). Approximately 85% of ADPKD cases are associated with mutations in the *PKD1* gene (ADPKD1), and the rest are hypothesized to be due to mutations in *PKD2* (ADPKD2) (4–7).

Polycystin-2 is encoded by the *PKD2*, and composed of six putative transmembrane domains with intracellular N and C termini (8). Its transmembrane region is homologous to polycystin-1 and to voltage-activated and transient receptor potential channel subunits (9). Polycystin-2 was implicated in signal transduction and Ca^{2+} regulation (10–12). *In vitro* studies suggested that polycystin-2 forms a homodimer and interacts with many other proteins including: polycystin-1 (13, 14), α-actinin (15), CD2AP (16), mDia 1 (17), Id2 (18), inositol 1,4,5-triphosphate receptor (19), phosphofurin acidic cluster sorting protein-1 and -2 (20), polycystin-2 interactor, Golgi- and endoplasmic reticulum-associated protein 14 (21), tropomyosin-1 (22), troponin I (23), and transient receptor potential channel 1 (24).

ADPKD is inherited in a dominant manner. Mutation screening has shown that a myriad of alterations can occur over the entire region of the *PKD2* locus. Indeed, missense, nonsense, frameshift, deletion, and aberrant splicing variations have been described, and these mutations probably result in truncated products and inactivate gene function (8, 25–28). At the cellular level, ADPKD can be explained by a recessive mechanism, leading to the complete loss of function through somatic mutations in the normal *PKD2* allele (29). The "two-hit" model of cyst formation has been supported by a *Pkd2* mutant mouse model (WS25) in which the somatic rearrangement of an unstable allele to a null allele leads to cyst formation (30). However, polycystin-2 is frequently observed in renal cystic epithelium of human ADPKD (31). This finding indicates that functional loss

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[4] The abbreviations used are: ADPKD, autosomal dominant polycystic kidney disease; MEF, mouse embryonic fibroblast; PCNA, proliferating cell nuclear antigen; PKD, polycystic kidney disease; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

or complete deletion of *PKD2* is not enough to explain all cases of human ADPKD, and suggests that more studies should be conducted to clarify the effect of *PKD2* overexpression *in vivo*. Recently, overexpression of human *PKD2* caused tubular dysfunction and centrosome overduplication (32, 33).

To investigate the physiological effects of enhanced *PKD2* function *in vivo*, we generated transgenic mice overexpressing human *PKD2*. Interestingly, all established mice reproducibly displayed cystic phenotypes closely resembling human ADPKD. The present study provides evidence for an *in vivo* role of overexpressed *PKD2* in cyst formation. This transgenic mouse model should provide new insights into the pathogenic mechanism of human ADPKD.

EXPERIMENTAL PROCEDURES

Generation of PKD2 Overexpressing Mice—For the construction of *PKD2*-overexpressing transgenic mice, a human *PKD2* cDNA composed of the 5′- and 3′-untranslated regions and full coding sequence of polycystin-2 (gift from Dr. Somlo in Yale University), was subcloned into the pCAGGS plasmid using XbaI and XhoI sites. The human *PKD2* transgene was isolated by digesting the construct with PvuI and StuI and standard techniques were used to generate transgenic mice (34). Briefly, the transgene was injected into pronuclei of fertilized eggs of FVB/NJ female mice. The injected eggs were then transferred into the oviducts of pseudo-pregnant ICR mice. Founder mouse screening and routine genotyping were conducted by PCR using genomic DNAs extracted from tails as templates and primer pairs specific for human *PKD2* cDNA (5′-TGCTCAGTTGGCATACCTTG-3′ and 5′-CTCCTCAGAGTCATCCAGGC-3′). For Southern blot analyses, genomic DNAs were digested with XbaI and XhoI, resolved on an agarose gel, transferred to a nylon membrane, and hybridized to a ^{32}P-labeled transgene-specific probe. The probe was created by PCR using a primer pair specific for human *PKD2* cDNA (forward, 5′-AGACCTGTTTGGCTTTGCTATT-3′ and reverse, 5′-ATCTCAAAGGGAAGGGCCATACTG-3′). The transgene copy number was measured by a real-time PCR as previously described (35). The primer pairs, which are common and exist on single exon 6 of both human and mouse *Pkd2*, were used for this analysis: 5′-ATTTCTTCCTGGCAGCCTGT-3′ and 5′-TCACAACATCCAGCAATTCCA-3′.

Reverse Transcriptase-PCR—Total RNAs were isolated from kidneys of all transgenic and wild mice using RNeasy mini (Qiagen). The cDNA was generated from total RNA (2 μg) using Moloney murine leukemia virus reverse transcriptase according to the manufacturer's protocol (Promega). The primer pairs used for analysis were: 5′-AGACCTGTTTGGCTTTGCTATT and 5′-ATCTCAAAGGGAAGGGCCATACTG-3′ for human *PKD2* (470 bp); 5′-GCAGCTTCGCAAACAGGTCAT-3′ and 5′-GAAAGTATGCTGAGTTGCTGCCCT-3′ for mouse *Pkd2* (419 bp); and 5′-CGGTGCTGAGTATGTCGTGGAG-3′ and 5′-TGTCATCATACTTGGCAGGTTTC-3′ for mouse *Gapdh* (internal control, 503 bp). PCR products were separated by 1% agarose gel electrophoresis.

Western Blot Analysis—Renal tissues isolated from sacrificed mice and mouse embryonic fibroblasts (MEFs) were homogenized in lysis buffer (Intron Biotechnology). Protein concentrations were determined by BCA assay (Sigma). Samples were subjected to SDS-PAGE, and proteins were immunoblotted with primary antibodies against the following proteins: polycystin-2 (a gift from Dr. Wu, Vanderbilt University), phosphorylated B-Raf, Raf-1, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK), extracellular signal-regulated kinase (ERK), Akt, p38, c-Jun N-terminal kinase (JNK1/2), total MEK, ERK, Akt, p38, JNK1/2 (Cell Signaling), B-Raf, Raf-1 (Santa Cruz), and β-actin (Sigma).

Histological and Immunohistochemical Analyses—Tissue samples were fixed in 4% buffered paraformaldehyde (Sigma), and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) for histological examination. The number of cysts in each kidney was documented by scoring a single representative longitudinal section. Histological images were captured using a Nikon Eclipse E400 microscope, and cyst size was measured using Scion Image 1.62. To calculate the sizes of cysts and normal tubules, images were analyzed using the Axiovision 3.1 software (Carl Zeiss). Sections from transgenic and normal kidneys were stained with antibodies specific for proliferating cell nuclear antigen (PCNA) (Sigma), polycystin-2, Tamm-Horsfall protein (Santa Cruz), and pERK (Cell Signaling), and the signals were detected using horseradish peroxidase-conjugated secondary antibodies (Dako) and Nova red (Vector Lab). Counterstaining was carried out with hematoxylin, and coverslips were mounted using aqueous mounting medium (Zymed Laboratories Inc.). Lectin studies were performed as previously described (36). TUNEL assay was performed with the In Situ Cell Death Detection Kit according to the manufacturer's instructions (Chemicon).

MEF Culture and Transfection—MEFs were derived from each line of *PKD2* transgenic embryos (E13.5). Whole embryos were minced and dispersed in 0.05% trypsin, and were then incubated at 37 °C for 15 min. MEFs were plated in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma), and were incubated at 37 °C in 5% CO_2 to 70–80% confluence. MEFs were transfected with B-Raf small interfering RNA (Santa Cruz) using Lipofectamine RNAi MAX according to the manufacturer's protocol (Invitrogen). B-Raf small interfering RNA was used at a concentration of 10 nM and transfection continued for 48 h. MEFs were treated for 30 min with the MEK inhibitor PD98059 (Calbiochem). For transient transfection, MEFs were subcultured 1 day before and transfected using Effectene (Qiagen).

Statistical Analyses—Data were presented as mean ± S.D. and were analyzed by one-way analysis of variance (ANOVA). An error probability of less than 5% ($p < 0.05$) was considered to be significant.

RESULTS

Cytogenesis in PKD2 Transgenic Mice—To analyze the physiological consequences of *PKD2* overexpression *in vivo*, we generated transgenic mice that overexpress *PKD2* under the control of the chicken β-actin promoter and cytomegalovirus immediate early enhancer (Fig. 1*A*). Four transgenic mouse lines were established, and the *PKD2* transgene copy number was determined by Southern blot analysis (data not shown) and real-time PCR using primer pairs common to both human and

mouse *Pkd2* orthologues (Fig. 1*B*). The results showed that transgenic mouse lines carried multiple copies of the transgene. To determine transgene expression reverse transcriptase-PCR was conducted using a primer pair specific for the human orthologue of *PKD2*. The results showed that significant *PKD2* transcript levels were detected in kidneys of all transgenic mouse lines (Fig. 1*C*). Consistently, expression levels of polycystin-2 were also up-regulated in transgenic kidneys (Fig. 1*D*).

To characterize the physiological effects of *PKD2* overexpression, we performed histological analyses. Adult kidneys from all transgenic mouse lines exhibited cyst formation and were affected bilaterally (Table 1). When severely affected, kidneys showed numerous cystic lesions, visible at the surface, and fluid-filled cysts (Fig. 2*A*). Cysts developed from tubules in the cortical and medullar regions (Fig. 2*B*), and most were found in the cortical region. In addition, the glomeruli were dilated and the morphology of Bowman capsules was altered (Fig. 2*C*). These cystic phenotypes were commonly observed in all four transgenic lines. The development of renal cysts in *PKD2* transgenic mice was classified into distinct stages based on cellular characteristics during progressive cyst formation (34). At the early stage, cysts were lined with a single layer of cuboidal cells that were proliferative and hyperplastic (Fig. 2*D*). At the intermediate stage, most cyst-lining cells were still cuboidal (Fig. 2, *E* and *F*), but several cells had transformed to flat cells (Fig. 2*F*, *black arrowhead*). At the late stage, cyst-lining cells were completely changed into flat cells (Fig. 2, *G* and *H*). Therefore, all *PKD2* transgenic mice displayed progressive cystic phenotypes that were variable in location and stage.

To determine whether cyst-lining cells express the *PKD2* transgene, we immunohistochemically localized polycystin-2 expression in transgenic kidneys. Consistent with previous reports, prominent polycystin-2 expression was detected in distal tubules of wild-type kidney cells (Fig. 3, *A* and *B*). As expected, polycystin-2 was strongly expressed in large cyst-lining cells (Fig. 3, *C* and *D*). Therefore, our data indicated that the overexpression of *PKD2* is directly involved in renal cyst formation in mice.

In human ADPKD patients, cysts arise from multiple tubule segment types (37). However, the cysts of *PKD2*-mutated animal models frequently originate from specific tubule segments. The origins of cysts were distal tubule segments in *Pkd2* knock-out mice and proximal tubules in truncated *PKD2* transgenic rats (30, 38). To investigate the origin of renal cysts, we conducted immunohistochemical analyses using nephron segment-specific lectins including: *Lotus tetragonolobus* (*LTA*, proximal tubule), *Dolichos biflorus* (*DBA*, collecting tubule), and Tamm-Horsfall protein (*THP*, distal tubule) (Fig. 4). All of cysts originating from proximal tubules and collecting ducts displayed continuous lectin staining unlike cysts in *Pkd2*-deficient mouse kidneys (30). Large cysts and dilated tubules developed from proximal tubules (Fig. 4, *A* and *B*). Small numbers of cysts originated from collecting tubules (Fig. 4, *C* and *D*). Cysts from distal tubules were also detected in the transgenic kidney (Fig. 4, *E* and *F*). Therefore, cysts in *PKD2* transgenic mice developed

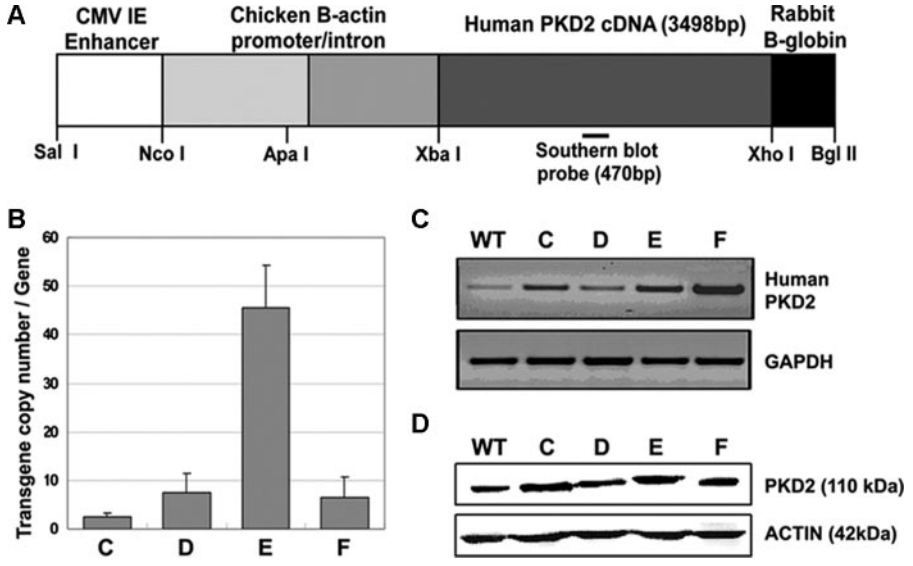

FIGURE 1. ***PKD2* transgenic mice.** *A*, schematic map of the human cytomegalovirus enhancer and the human *PKD2* cDNA construct. Restriction enzyme sites and probes used for Southern hybridization are indicated. *B*, copy number in *PKD2* transgenic mice. Genomic DNA was subjected to real-time PCR and signals were normalized using *Gapdh* as an internal control. Endogenous signals were ruled out from the average of triplicate experiments, and transgene copy number per genome was expressed from triplicate experiments. *Error bar*, standard deviation. *C*, semiquantitative reverse transcriptase-PCR analysis was performed using a human *PKD2*-specific primer pair. The expression of the *PKD2* transgene was determined in kidneys from four-independent transgenic mouse lines. *D*, Western blot analysis of polycystin-2 in kidneys of *PKD2* transgenic mice. Polycystin-2 expression was evaluated in four-independent transgenic mouse lines. The polycystin-2-specific antibody could detect both human and mouse orthologues. *WT*, wild-type mouse; *C*, *D*, *E*, and *F*, transgenic mice lines.

TABLE 1
The numbers and mean sizes of cysts in *PKD2* transgenic mice

PKD2 transgenic mouse line	Age	Cyst size in a diameter (mean ± S.D., μm)		
		Total mean size	Cyst number (mean size)	
			<200 μm	<200 μm
	Months			
C line (n = 14)	6–18	130.04 ± 58.22	40 (128.37 ± 57.83)	4 (250.72 ± 27.98)
D line (n = 6)	6–18	161.87 ± 97.50	10 (104.00 ± 31.30)	5 (277.60 ± 77.16)
E line (n = 5)	2–18	170.33 ± 84.70	12 (134.76 ± 37.61)	4 (277.04 ± 102.18)
F line (n = 3)	7–18	257.59 ± 182.59	4 (151.35 ± 29.62)	3 (399.24 ± 214.54)

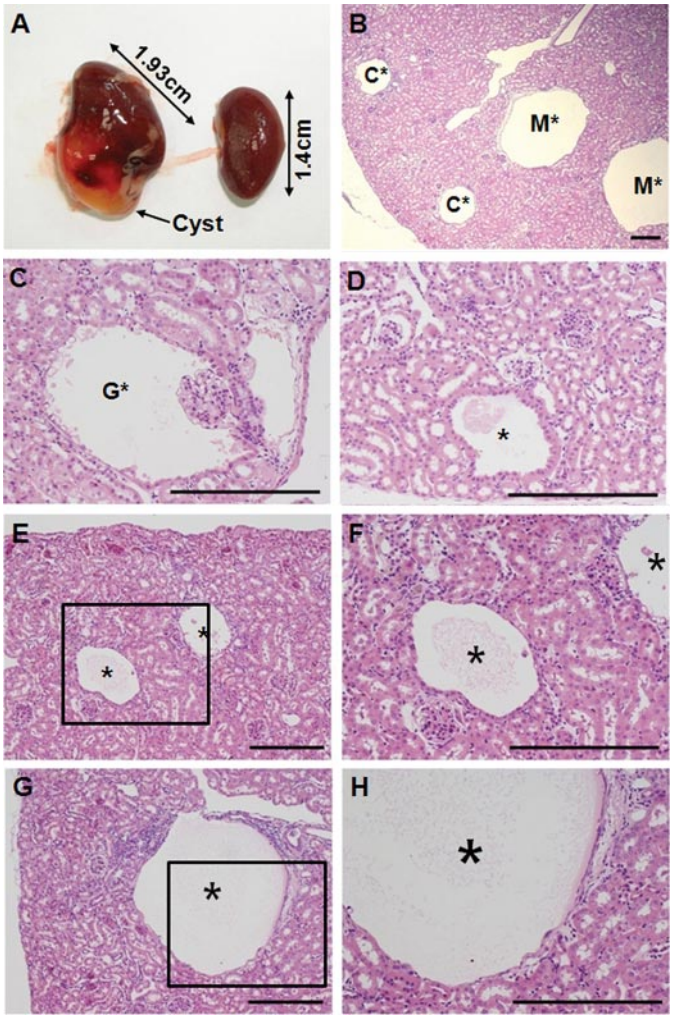

FIGURE 2. **Cystic phenotype in kidneys of *PKD2* transgenic mice.** H&E staining of *PKD2* transgenic mouse kidney. *A*, the enlarged kidney filled with fluid from an 18-month-old *PKD2* transgenic mouse. *B* and *C*, cysts were generated in variable regions of the kidney. *B*, a kidney section of an affected 18-month-old *PKD2* transgenic mouse shows variable cyst size. Cysts arose in the cortex (*C**) and medulla (*M**). *C*, the morphological change of glomeruli (*G**) in an 18-month-old *PKD2* transgenic mouse. *D–F*, morphological changes of cyst-lining epithelial cells in the *PKD2* transgenic mouse kidney during the development of renal cysts. *D*, at the early stage of cyst formation, tubules lined by cuboidal epithelium. *E*, cyst at the intermediate stage of formation, where epithelial cells are transformed into flattened cells (*black arrowhead*). *F*, magnification of the intermediate stage cyst. *G* and *H*, at the later stage of cyst formation. *G*, the cuboidal epithelium is completely transformed into flat, single-layered lining cells. *H*, magnification of the late stage cyst. *C**, cortical cyst; *M**, cyst in medullar region; *G**, glomerular alteration; *scale bar*, 50 μm.

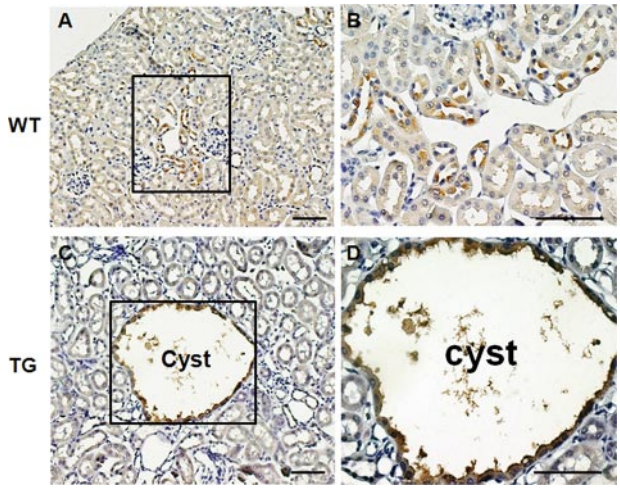

FIGURE 3. **Sustained expression of polycystin-2 in the renal cysts of *PKD2* transgenic mice.** Immunohistochemical staining of polycystin-2 in kidneys of wild-type (*WT*) (*A* and *B*) and *PKD2* transgenic (*TG*) mice (*C* and *D*). *Boxed* regions (*A* and *C*) were magnified (*B* and *D*). Polycystin-2 expression was detected in basolateral regions of distal tubules in wild-type (*WT*) kidney (*A* and *B*). Polycystin-2 was strongly expressed in the large cyst of the transgenic mouse (*C* and *D*). *Scale bar*, 50 μm.

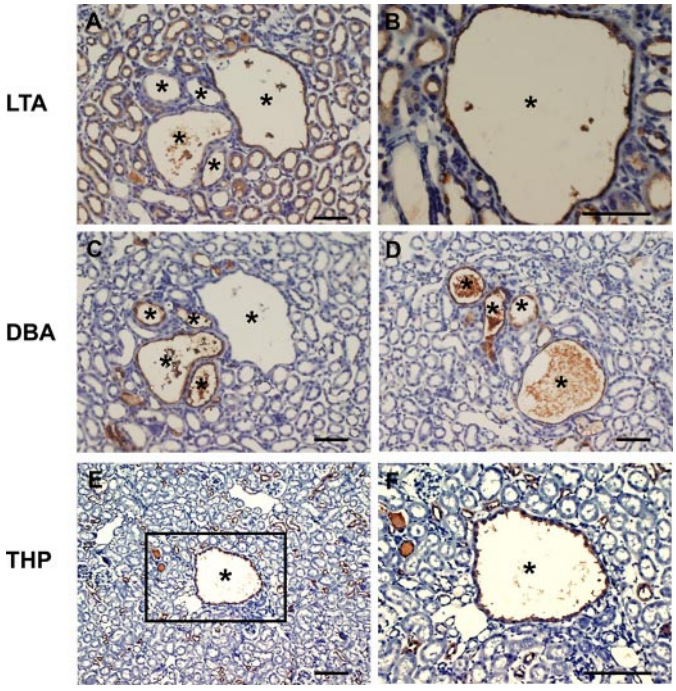

FIGURE 4. **Variable origins of cysts in *PKD2* transgenic mice.** The origins of the cystic lesions in *PKD2* transgenic mice were immunohistochemically defined. *A* and *B*, expression of *L. tetragonolobus* (*LTA*, the proximal tubule marker) was detected in one of the cysts and surrounding dilated tubules (*A*). *C* and *D*, the cyst that originated from collecting tubules was identified by detecting the expression of *D. biflorus* (collecting tubule marker). Note that the cysts on serial sections were alternatively stained by *L. tetragonolobus* and *D. biflorus* (*A* and *C*). *E* and *F*, the large cyst positively stained with Tamm-Horsfall protein (*THP*)-specific antibody (distal tubule marker). *Boxed* regions (*E*) were magnified (*F*). *, cyst; *scale bar*, 50 μm.

from a range of segments in kidneys, resembling the clinical features of human ADPKD.

Renal cysts first appeared at six months of age in all transgenic lines. Therefore, we examined cystic phenotypes in mice from 6 to 18 months old. Indeed, the cystic phenotype appeared at a relatively late age and correlated with the aging process. Cyst size appeared to increase as transgenic mice aged (6-month-old, 190.27 $\pm$ 79.15 μm diameter; 18-month-old, 318.47 $\pm$ 129.82 μm) (Fig. 5). The number of cysts greater than 200 μm also increased in aged transgenic mice (6-month old, $n = 4$; 18-month old, $n = 11$). These results indicated that the cystic phenotypes of *PKD2* transgenic mice are progressive with aging.

Deregulation of Apoptosis and Proliferation in Kidneys of PKD2 Transgenic Mice—Because apoptosis and tubular proliferation are prerequisites for cyst formation (39, 40), we evaluated immunohistochemically these processes in the kidneys of *PKD2* transgenic mice (Fig. 6). Most PCNA-positive cells were detected around a cyst and especially at late stage cysts (Fig.

PKD2 Tg	6m (C,D,E,F, n=5)	18m (C, D, E, F, n=8)
<200 ㎛	109.47 ± 38.09 (cyst n=29)	132.74 ± 42.97 (cyst n=28)
>200 ㎛	190.27 ± 79.15 (cyst n=4)	318.47 ± 129.82 (cyst n=11)

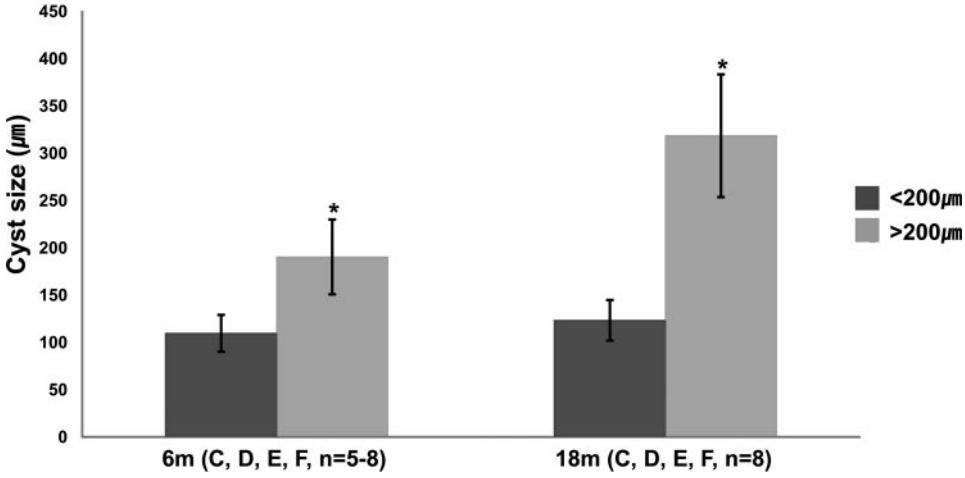

FIGURE 5. **Progressive deterioration of cystic phenotypes with aging.** The mean sizes of cysts are shown for all *PKD2* transgenic mouse lines according to age. Note that more cysts exceeding 200 μm were found in aged transgenic mice (18 months) than in young transgenic mice (6 months). Cystic tubules larger than 50 μm in diameter were counted, and the morphology of epithelial cells lining the cysts was evaluated (*, $p < 0.01$).

6*A*). PCNA-positive cell number per tubule was significantly increased in all *PKD2* transgenic mice (Fig. 6*B*). Compared with PCNA-positive cells, apoptotic cells were mainly detected at the intermediate stage (Fig. 6*C*). In addition, the number of apoptotic cells per tubule increased (Fig. 6*D*). Coincidentally, the patterns of bromodeoxyuridine incorporation were similar to those of PCNA staining (data not shown). Therefore, both proliferation and apoptosis were involved in cyst formation in *PKD2* transgenic mice.

ERK, a Downstream Effector of PKD2, Is Activated via B-Raf Signaling in Renal Cyst Formation—Progression of cystogenesis can be delayed *in vivo* by suppressing ERK activity (41), which was reported to be regulated by calcium signaling (42). Polycystin-2 binds to polycystin-1 forming a heterodimer, which is essential for calcium signaling (13, 14). Therefore, proliferative properties of renal cells may be altered by disrupting calcium regulation through the overexpression of a polycystin-1 C-terminal fragment (42). Indeed, levels of phosphorylated ERK are up-regulated in other ADPKD models (32). It is plausible that *PKD2* overexpression may contribute to renal cystogenesis by modulating ERK activity through calcium regulation (42–44).

To test this hypothesis, we confirmed he level of the B-Raf/MEK/ERK sequential signaling pathway using Western blot analysis. Interestingly, phosphorylation of B-Raf/MEK/ERK was activated in *PKD2* transgenic mice kidneys but not phosphorylated Raf-1 (Fig. 7*A*). Fig. 7*B* shows the quantification data of Fig. 7*A*. The levels of phosphorylation of Akt/total Akt was higher than in NHK cells compared with ADPKD cells (42). The Akt, upstream of B-Raf, was also down-regulated in *PKD2* transgenic mice kidney similar to previous data (Fig. 7*C*). To confirm the ERK phosphorylation increase by B-Raf signaling, we checked the expression change of phosphorylated ERK that was critical for the development of cyst by B-Raf inhibition using B-Raf small interfering RNA in *PKD2* transgenic MEFs. The phosphorylation of ERK was reduced by inhibition of B-Raf and these data were similar to the result of MEK inhibitor treatment (15 μM, PD98058) (Fig. 7*D*). Finally, we checked activation of other MAPK pathways on the effect on cyst formation. The ERK phosphorylation level was also elevated in *PKD2* transgenic mice but the phosphorylation level of p38 MAPK and JNK1/2 were unaffected by *PKD2* overexpression (Fig. 7*E*). We investigated that the phosphorylation status of ERK via B-Raf signaling may be critical for development of cyst in *PKD2* transgenic mice.

We next examined the status of ERK, as a downstream effector of *PKD2* in *PKD2* transgenic mice kidney. As shown in Fig. 8*A*, the phosphorylation of ERK was induced in kidneys of *PKD2* transgenic mice relative to wild-type of similar age. Because the inactivation of *Pkd2* causes the cystic phenotype in mice (30), the level of phosphorylated ERK was measured in *Pkd2*-deficient MEFs (Fig. 8*A*). Similar to *PKD2* transgenic MEFs, the level of phosphorylated ERK was increased in *Pkd2*-deficient MEFs (Fig. 8*A*). These results suggest that the activation of ERK may be the common step leading to the induction of ADPKD because of either the deficiency or overexpression of *PKD2*. To evaluate the phosphorylation of ERK more precisely, we carried out immunohistochemical analysis with cystic tissues of the transgenic kidneys. ERK was phosphorylated in cyst-lining cells and cyst-surrounding tubules in cystic kidneys of *PKD2* transgenic mice (Fig. 8, *B–D*).

In addition, to confirm the ERK phosphorylation increase by *PKD2* overexpression, we transiently overexpressed exogenous human *PKD2* in wild-type MEFs cells from *PKD2* transgenic mice. As shown in Fig. 8*E*, the phosphorylation of ERK was induced by exogenous expression of *PKD2*, which was consistent with observations from *PKD2* transgenic MEFs. This result indicates that ERK activation is induced by *PKD2* overexpression and may be associated with renal cyst formation in *PKD2* transgenic mice.

The PKD2 Transgenic Product Is Functionally Active in Mice—As nullizygosity of *Pkd2* results in embryonic lethality (30), we hypothesized that the transgenic overexpression of *PKD2* should rescue the lethal phenotype of *Pkd2*-deficient mouse embryos, at least partially, if the transgenic polycystin-2 is functional *in vivo*. We introduced the *PKD2* transgene into the $Pkd2^{+/-}$ genetic background strain, and then crossed them with $Pkd2^{+/-}$ mice to determine whether transgenic overex-

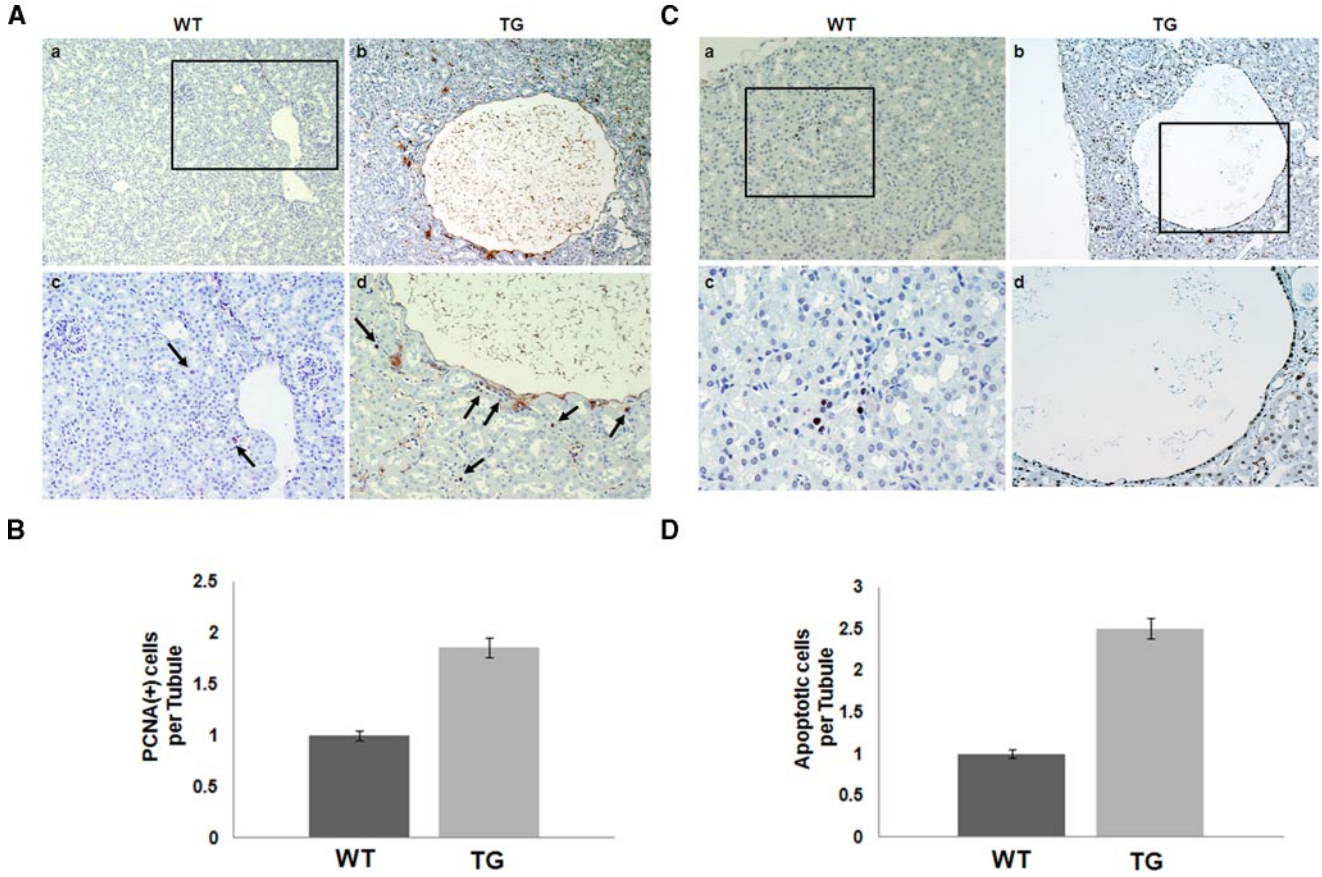

FIGURE 6. **Increase of tubular cell proliferation and apoptosis in the cystic kidneys of *PKD2* transgenic mice.** PCNA-positive cells in the tubules of the cortex and medulla were significantly increased in *PKD2* transgenic mice. Specifically, considerable numbers of PCNA-positive cells were also cyst-lining cells, and were observed in surrounding regions. *A*, *a* and *c*, kidney of a wild-type mouse. *b* and *d*, kidney of a *PKD2* transgenic mouse. *Boxed* regions are magnified *panels c* and *d*. Note that flattened cells in the cyst were stained with PCNA. *B*, quantification of PCNA positive cells in wild-type (*WT*) and *PKD2*-transgenic (*TG*) kidneys, showing that the number of PCNA-positive cells per tubule was significantly increased in the *PKD2* transgenic kidney compared with that of wild-type mice. The apoptotic index was evaluated by TUNEL assay. The number of apoptotic cells in the cysts and tubules around cysts were significantly increased in the *PKD2* transgenic mouse compared with that of wild-type mice. *C*, *a* and *c*, kidney of a wild-type mouse. *b* and *d*, kidney of a *PKD2* transgenic mouse. *Boxed* regions were magnified (*c* and *d*). Note that, similar to cell proliferation, apoptotic cells were enriched in cyst-lining cuboidal cells. *D*, quantification of TUNEL positive cells.

pression of *PKD2* would rescue the embryonic lethality of $Pkd2^{-/-}$ mouse embryos. As shown in Table 2, the lethal phenotype of $Pkd2^{-/-}$ mouse embryos was partially rescued by transgenic overexpression of *PKD2*. Table 2 show that Mendelian ratios were observed for the *PKD2* positive offering of theses crosses demonstrating that the transgenic human polycystin-2 could partially rescue the lethality of *Pkd2* knock-out mice and therefore that the transgene generated a functional product. The rescued mice remained viable from 28 to 34 days ($n = 5$). *Pkd2* knock-out mice with the *PKD2* transgene remained alive more than 3–4 weeks although mice kidney had cysts. This result emphasized that polycystin-2 expressed from the exogenous *PKD2* transgene is functional *in vivo*.

DISCUSSION

Mutations in *PKD2* genetically predispose people to ADPKD, and extensive studies have focused on its loss of function mutations (45). *Pkd2* deficiency may result in embryonic lethality and is critical for the onset of ADPKD in mice (30, 35). *Pkd2* is haploinsufficient and cyst-lining epithelial cells are negative for *Pkd2* in renal cysts of $Pkd2^{+/-}$ mice (31), suggesting that the functional loss of *PKD2* and its down-regulation by somatic mutations (the two-hit model) or heterozygosity itself constitute the main causes of ADPKD.

Recently, kidney-specific overexpression of *PKD1* induced severe cystic phenotypes in mice (46) even though its deficiency is a leading cause of renal cyst formation in mice (47). However, there is no definitive explanation for the contribution of deregulated *PKD2* expression to renal cystogenesis without the mutational loss of *PKD2* functions in kidneys. Indeed, *PKD2* is continuously expressed in human ADPKD renal cysts (48). Expression of *PKD2* was sustained in spontaneously developed renal cysts of *PKD2* transgenic mice (Fig. 3), closely resembling human ADPKD phenotypes.

Renal cysts developed from all tubule segments including proximal, collecting, and distal in human ADPKD (37). However, renal cysts predominantly originate from both distal and collecting tubules of *Pkd2* mutant kidneys where *Pkd2* expression disappears (30). Glomerular cysts are frequently observed in other animal models. Renal cysts of *PKD2* transgenic mice developed from proximal, collecting, and distal tubules (Fig. 4). Our model also observed glomerular cysts in *PKD2* transgenic mice. Therefore, the mouse model we present is clinically comparable with human ADPKD and may be useful for understanding the pathophysiology of ADPKD.

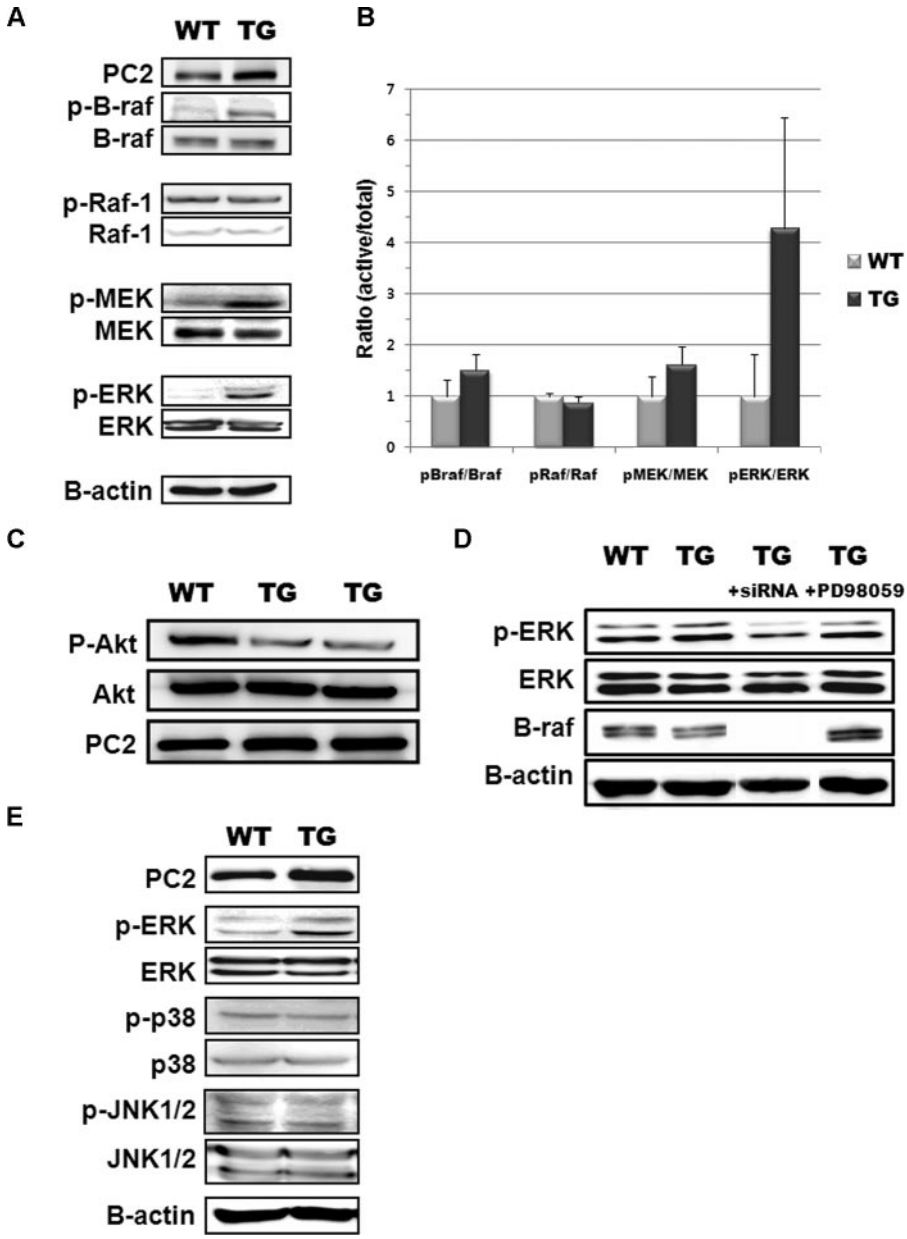

FIGURE 7. **Activation of B-Raf/MEK/ERK signaling in the cystic kidneys of *PKD2* transgenic mice.** *A*, B-Raf/MEK/ERK sequential signaling was induced in *PKD2* transgenic (*TG*) mice kidneys. The levels of phosphorylated and total B-Raf/MEK/ERK were examined by Western blot analysis. β-Actin was used as a loading control. *PC2*, polycystin-2. *B*, the graph shows quantification data of *A*. *C*, the statue of Akt phosphorylation was altered in *PKD2* transgenic mice kidneys. *D*, the levels of phosphorylated ERK was reduced by transfection of B-Raf small interfering RNA and these levels were similar to the results of the MEK inhibitor treatment (PD98059). *E*, ERK phosphorylation level was also elevated in *PKD2* transgenic mice but the phosphorylation level of p38 MAPK and JNK1/2 were unchanged in *PKD2* transgenic mice. *WT*, wild type.

Because polycystin-2 forms complexes with many signaling molecules involved in Ca^{2+} cascade (49), the abnormally high level of polycystin-2 may disrupt the strict molar ratios of the component required for efficient assembly. On the other hand, it may lead to the formation of functionless or dysfunctional complexes. Moreover, *PKD2* overexpression may alter downstream signaling and induce abnormalities in regulating renal cell cycle and apoptosis that are the basis for cyst formation (39, 40).

Cystic phenotypes of *PKD2* transgenic mice were associated with the significant increase of ERK activation via signaling *in vitro* and *in vivo* (Figs. 7 and 8). Interestingly, phosphorylation of B-Raf/MEK/ERK was activated in *PKD2* transgenic mice kidneys similar to a previous report (44). In addition, phosphorylated Akt was down-regulated in the *PKD2* transgenic kidney. But the phosphorylation levels of p38 MAPK and JNK1/2 were unaffected by *PKD2* overexpression when we checked other MAPK pathways. These MAPK pathways excluded contribution to the cyst formation. We suggest that cell proliferation related to B-Raf/MEK/ERK signaling is important in cystogenesis. Furthermore, we confirmed the ERK phosphorylation increases by *PKD2* overexpression, because ERK-mediated signaling is closely associated with cell cycle progression and apoptosis in various cell types (50), polycystin 2-induced ERK activation may be critical for the development of cystic disease in kidneys. In fact, the proliferation of the cystic epithelium can be suppressed by inhibiting ERK activation (41, 43).

Polycystin-2, a calcium-permeable, nonselective cation channel, has been known to function in both calcium entry and release (51). Because polycystin-1 regulates the function of polycystin-2 (52), it is plausible that polycystin 1-free polycystin-2 may accumulate and alter intracellular calcium signaling upon *PKD2* overexpression. Indeed, the overexpression of full-length polycystin-2 leads to an increase of intracellular Ca^{2+} (38). Furthermore, these alternations can determine the sensitivity or fates of renal cells to growth-stimulating signals by modulating ERK-mediated pathways (42). Therefore, it is likely that the deregulated expression or aberrant activation of *PKD2* may induce cystogenesis through ERK activation by changing calcium homeostasis and/or calcium signaling.

As shown in Table 2, the lethal phenotype of $Pkd2^{-/-}$ mouse embryos was partially rescued by the transgenic overexpression of *PKD2*. Mendelian ratios were observed for the *PKD2* positive offering of theses crosses demonstrating that the transgenic human polycystin-2 could partially rescue the lethality of *Pkd2* knock-out mice. The rescued mice remained viable from 28 to 34 days although mice kidney have cysts (data not shown). We proposed that the *PKD2* transgene can delay lethality induced

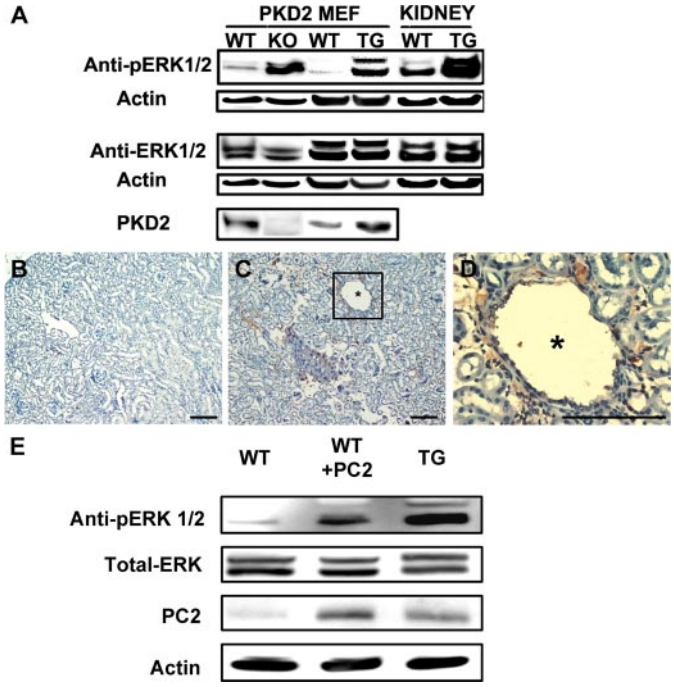

FIGURE 8. **Abnormal activation of ERK signaling in the cystic kidneys of *PKD2* transgenic mice.** *A*, the statue of ERK phosphorylation was altered in *PKD2*-transgenic MEFs and kidneys. The levels of phosphorylated and total ERK were examined by Western blot analysis. β-Actin was used as a loading control. MEFs were derived from each *PKD2* transgenic line (F embryo and *Pkd2* deficient embryo). *B–D*, immunohistochemical analysis of phosphorylated ERK1/2 of wild-type (*WT*) kidneys in the control mouse (*B*) and cystic kidneys (*C* and *D*) in the *PKD2* transgenic mouse. *Boxed* region in *C* was magnified in *D*. Note that phosphorylated ERK1/2, localized in nuclei, was detected in cyst-lining epithelium and tubules around the cysts. *Scale bar*, 50 μm. *E*, ERK1/2 was activated by exogenous *PKD2* expression in wild-type MEFs.

TABLE 2

Results of the rescue experiments: number of viable PKD2-positive offering

PKD2 Tg	*Pkd2*		
	+/+	+/−	−/−
Wild type	4 (23.5)	13 (76.5)	0 (0)
Transgenic	9 (30.0)	11 (36.7)	10 (33.3)

by pkd deficiency. The results suggest that polycystin-2 expressed from the exogenous *PKD2* transgene is functional *in vivo*. *TPK1* and *TPK3* transgenic lines rescued the $Pkd1^{del34/del34}$ phenotype (53). If this were the case, the observed phenotype may be due to a dominant negative effect of the transgenic product, creating a high level of functionless polycystin-1-containing complexes (53). We proposed that the transgene of polycyctin-2 also plays a role on the dominant negative effect in the *PKD2* transgenic mice.

Our results suggest that polycystin-2-induced ERK activation via B-Raf signaling may be important for renal cystogenesis. Therefore, the mouse model that we present may provide an important resource for the elucidation of the mechanism of cystogenesis, and may serve as a valuable model system for developing new therapeutic strategies for ADPKD.

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Cyst Formation in Kidney via B-Raf Signaling in the *PKD2* Transgenic Mice

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