The pathogenetic 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 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 Ca2+ 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
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 mice were backcrossed to ICR to be used for analysis. The transgene copy number was measured by a real-time PCR as previously described (36). TUNEL assay was performed as previously described (35).

**Western Blot Analysis**—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 for 30 min with the MEK inhibitor PD98059 (Calbiochem). For transient transfection, MEFs were subcultured 1 day before and 30 min after transfection with the transgene construct. Transfected cells were harvested 48 h after transfection.

**RESULTS**

**Cyto genesis 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. 1A). 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. The transgene copy number was measured by real-time PCR as previously described (36). TUNEL assay was performed as previously described (35).

**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.

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PKD2 Causes Renal Cyst Formation

mouse Pkd2 orthologues (Fig. 1B). 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. 1C). Consistently, expression levels of polycystin-2 were also up-regulated in transgenic kidneys (Fig. 1D).

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. 2A). Cysts developed from tubules in the cortical and medullary regions (Fig. 2B), and most were found in the cortical region. In addition, the glomeruli were dilated and the morphology of Bowman capsules was altered (Fig. 2C). 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. 2D). 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. 2F, 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

**TABLE 1**

The numbers and mean sizes of cysts in PKD2 transgenic mice

| PKD2 transgenic mouse line | Age | Total mean size | Cyst number (mean size) |
|---------------------------|-----|----------------|------------------------|
| C line (n = 14)           | 6–18| 130.04 ± 58.22 | 40 (128.37 ± 57.83)    |
| D line (n = 6)            | 6–18| 161.87 ± 97.50 | 10 (104.00 ± 31.30)    |
| E line (n = 5)            | 2–18| 170.33 ± 84.70 | 12 (134.76 ± 37.61)    |
| F line (n = 3)            | 7–18| 257.59 ± 182.59| 4 (151.35 ± 29.62)     |
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 ± 79.15 μm diameter; 18-month-old, 318.47 ± 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.

PKD2 Causes Renal Cyst Formation

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. 7).
PKD2 Causes Renal Cyst Formation

PKD2 Tg  
6m (C,D,E,F, n=5)  
18m (C, D, E, F, n=8)  

| Condition | Cyst Size (μm) | Count |
|-----------|----------------|-------|
| <200 μm   | 109.47 ± 38.09 (cyst n=29) | 132.74 ± 42.97 (cyst n=28) |
| >200 μm   | 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).

6A). PCNA-positive cell number per tubule was significantly increased in all PKD2 transgenic mice (Fig. 6B). Compared with PCNA-positive cells, apoptotic cells were mainly detected at the intermediate stage (Fig. 6C). In addition, the number of apoptotic cells per tubule increased (Fig. 6D). 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 were 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 the 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 mouse kidneys but not phosphorylated Raf-1 (Fig. 7A). Fig. 7B shows the quantification data of Fig. 7A. The levels of phosphorylation of Akt/total Akt were 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. 7C). 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, PD98059) (Fig. 7D). 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. 7E). 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. 8A, 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. 8A). Similar to PKD2 transgenic MEFs, the level of phosphorylated ERK was increased in Pkd2-deficient MEFs (Fig. 8A). 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. 8B–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. 8E, 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-
expression 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 offspring of these 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.
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 these 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 mouse kidney have cysts (data not shown). We proposed that the PKD2 transgene can delay lethality induced...
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<sup>del134/del134</sup> 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 polycystin-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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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 in wild-type (WT) kidneys (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

| PKD2 Tg | PKD2 KO | PKD2 WT |
|---------|---------|---------|
| Wild type | 4 (23.5) | 13 (76.5) | 0 (0) |
| Transgenic | 9 (30.0) | 11 (36.7) | 10 (33.3) |
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