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Research Article

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Epidermal Growth Factor Receptor Activity Mediates Renal Cyst Formation in Polycystic Kidney Disease

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Abstract

A consistent phenotype observed in both human patients and several different mouse models of autosomal recessive polycystic kidney disease (ARPKD) is an increased activity of the epidermal growth factor receptor (EGFR) in the affected kidneys. To determine whether this increased activity of the EGFR is a functional event that is directly part of the disease pathway of renal cyst formation, we used a genetic approach to introduce a mutant EGFR with decreased tyrosine kinase activity into a murine model of ARPKD. We found that the modified form of the EGFR could block the increase in EGFR-specific tyrosine kinase activity that normally accompanies the development of renal cysts, and this correlated with an improvement in kidney function and a substantial decrease in cyst formation in the collecting ducts. These results suggest that changes in the expression of the EGFR contribute to the formation of cysts in the collecting ducts, and that drugs that target the tyrosine kinase activity of the EGFR may potentially be therapeutic in ARPKD. (*J. Clin. Invest.* 1998. 101:935–939.) Key words: epidermal growth factor receptor • tyrosine kinase activity • polycystic kidney disease • mouse mutations

Introduction

Autosomal recessive polycystic kidney disease (ARPKD)[1] is a devastating genetic condition that affects as many as 1:10,000 individuals in the human population. Mutations in a single gene appear to be responsible for ARPKD (1, 2), while mutations in several other genes in the mouse are known to cause forms of ARPKD (3). Currently there are no therapies for ARPKD other than renal dialysis or kidney transplantation. Establishing clinical treatments for ARPKD will be greatly facilitated by an understanding of how individual gene products participate in the disease pathway.

A common feature of the development of ARPKD in humans and mice is a distension of the renal collecting tubules caused by a localized proliferation and aberrant secretion of epithelial cells (4–6). The expanding structures develop into cysts that are filled with fluid containing biologically active ligands for the epidermal growth factor receptor (EGFR) such as EGF and TGF-α (7–9). The EGFR, normally localized at the basolateral surfaces of the collecting tubule epithelium, becomes mislocalized to the apical surface on the cells lining cystic structures. This mislocalization of the EGFR is known to occur in both humans and mice and is a common end point associated with several different forms of polycystic kidney disease that are initiated by mutations in different genes (9–13). Along with this mislocalization to the apical surface there is an increase in mRNA, protein, and tyrosine kinase activity of the EGFR in the diseased kidneys (10, 11, 13).

It is possible that the increased EGFR tyrosine kinase activity in the collecting tubule is part of an autocrine/paracrine cycle that drives the cellular proliferation which is required for cyst formation and enlargement (6). To test this possibility and to establish the role of increased EGFR tyrosine kinase activity in the formation of renal cysts in vivo, we used a genetic approach to decrease the tyrosine kinase activity of the EGFR in mice that are programmed to develop ARPKD. By developing mice which carry both the *orpk* cystic mutation (14) (in which increased EGFR activity is associated with renal cysts [11, 12]) and the *wa2* mutation (15) (in which a point mutation decreases EGFR tyrosine kinase activity) we demonstrate that decreasing tyrosine kinase activity of the EGFR diminished collecting tubular cyst formation in murine ARPKD.

Methods

Mice. Matings between animals heterozygous for the *orpk* mutation and the *wa2* mutation generated compound heterozygous animals that were then used to generate double mutant animals. Animals carrying the *orpk* mutation were identified genotypically (14), whereas

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1. *Abbreviations used in this paper:* ARPKD, autosomal recessive polycystic kidney disease; CI, cystic index; DBA, *Dolichos biflorus*; EGFR, epidermal growth factor receptor.

animals homozygous for the *wa2* mutation were identified phenotypically. The wavy hair phenotype of the *wa2* mutation was unchanged in the double mutant animals.

Histological analysis. For histological analysis, kidneys were harvested and one kidney was fixed in 4% paraformaldehyde for 30 min at 4°C. 3 μM sections were then cut and stained with hematoxylin following standard techniques. Immunohistochemical staining of the EGFR was conducted using the rabbit polyclonal anti-EGFR antibody RK-2 (kindly provided by Dr. Ben Margolis, New York University Medical Center, New York) (16). Cyst localization was studied using segment-specific lectin binding as described previously with *Dolichos biflorus* (DBA) as a marker for collecting tubules and *Lotus tetragonolobus* as a marker for proximal tubules (17). The contralateral kidney was processed for Western analysis of EGFR tyrosine kinase activity.

Tyrosine phosphorylation of the EGFR. Total kidney extracts were prepared by homogenizing whole kidneys in RIPA buffer including protease and phosphatase inhibitors. Equal amounts of protein were immunoprecipitated with an anti-EGFR antibody (RK-2) (2 μg antibody/mg protein). The immunoprecipitate was immunoblotted using standard techniques and the Western blot was probed with a horseradish peroxidase–conjugated antiphosphotyrosine antibody. Monoclonal antiphosphotyrosine RC20:HPPO (Transduction Laboratories, Lexington, KY) was used in Western analysis of activated EGFR.

Morphometric analysis. The severity of renal cyst formation was determined by the cystic index (CI) using 6–10 regularly spaced, 3 μM sections. Cyst severity was graded on a scale from 0 (no cysts) to 5 (cysts > 0.2 mm) (18, 19).

Results

To decrease tyrosine kinase activity of the EGFR in vivo, we used the *waved-2* (*wa2*) mouse mutation, in which a point mutation of a T to a G results in the substitution of glycine for a valine at position 743. This single amino acid change in the EGFR is in the third subdomain of the protein kinase sequence and causes a decrease in tyrosine kinase activity of the receptor (15, 20). The *wa2* single mutant animals have normal kidneys but develop wavy fur and curly vibrissae. We crossed the *wa2* allele into mice carrying the recessive *orpk* (Oak Ridge Polycystic Kidneys) mouse mutation to generate double homozygous mutant animals (here abbreviated *orpk;wa2*). The *orpk* mutation arose by insertional mutagenesis and was referred to previously as *TgN737Rpw* (14, 21). The *orpk* mutant animals develop histopathological abnormalities similar to those in human patients with ARPKD, including the mislocalization of the EGFR (10, 22, 23). Animals that are homozygous for just the *orpk* mutation exhibit an increased tyrosine phosphorylation of the EGFR in postnatal 14- and 21-d-old kidneys (Fig. 1). It is during this time period when cystic lesions begin to develop in the collecting tubules of mutant animals.

Kidneys from *orpk;wa2* double mutants have a significant decrease in cyst formation compared with age-matched mutants carrying only the *orpk* mutation (Fig. 2, *a* and *b*). To quantify the severity of the renal lesions, we performed a morphometric analysis and determined the quantitative CI (18, 19) for the samples. Cysts were scored ranging from 0 (no cysts) to 5 (≥ 0.2 mm in size). Single mutants with just the *orpk* mutation show an average CI of 3.19±0.83, whereas the double mutants showed a significant ($P < 0.001$) CI reduction to 1.19±1.25 (Fig. 3 *a*). The EGFR was still detected on the apical surface of collecting tubules in the double mutants (Fig. 2, *c* and *d*).

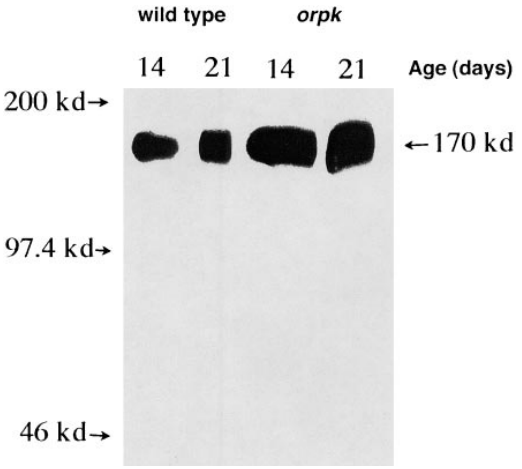

Figure 1. Tyrosine phosphorylation of the EGFR in wild-type and *orpk* cystic kidneys. Kidney extracts from age-matched wild-type (*first and second lanes*) and *orpk* kidneys (*third and fourth lanes*) were immunoprecipitated with an anti-EGFR antibody and then immunoblotted with an antiphosphotyrosine antibody. *First and third lanes*, 14-d-old kidneys; *second and fourth lanes*, 21-d-old kidneys.

To correlate reduced tyrosine kinase activity with decreased cyst severity in *orpk;wa2* mice, we measured phosphotyrosine levels associated with the EGFR in single and double mutant animals using the contralateral kidney from mice in which the CI of the other kidney had been measured (Fig. 4). In kidneys from *orpk;wa2* double mutants, a reduction in EGFR phosphotyrosine levels was observed when compared with *orpk* single mutant mice. Furthermore, this reduction correlated with the CI. All double mutant animals (Fig. 4, lanes *C* and *D*) with reduced EGFR phosphotyrosine levels close to that observed in *wa2* single mutant kidneys (Fig. 4, lane *E*) had a low CI. However, in one double mutant with a high CI of 3.1, the EGFR activity was similar to that in a single mutant *orpk* kidney with a CI of 3.0 (Fig. 4, lanes *A* and *B*), indicating a direct positive correlation between EGFR tyrosine kinase activity and cyst severity in *orpk* mutant animals. The variability of tyrosine kinase activity in double mutants is unclear but is likely due to the known effects of genetic background on *orpk* mice (14) and on mutants that contain a null allele of the EGFR (24, 25).

To determine if the decrease in renal cyst severity in double mutant animals resulted in improved renal tubular function, we compared the ability of single and double mutant animals to concentrate urine by monitoring urine osmolality. The inability to concentrate urine is common in human ARPKD and the *orpk* mutant mouse (23). Double mutants revealed a marked improvement in the ability to concentrate urine compared with *orpk* single mutant animals (Fig. 3 *b*).

Discussion

The EGFR is normally localized specifically to the basolateral surface of renal epithelial cells lining collecting tubules. However, in humans with ARPKD and in several animal models for ARPKD (9–13) the EGFR is also directed to the apical surface of cells lining the collecting tubule cysts. This mislocalization to the apical surface is accompanied by an increase in

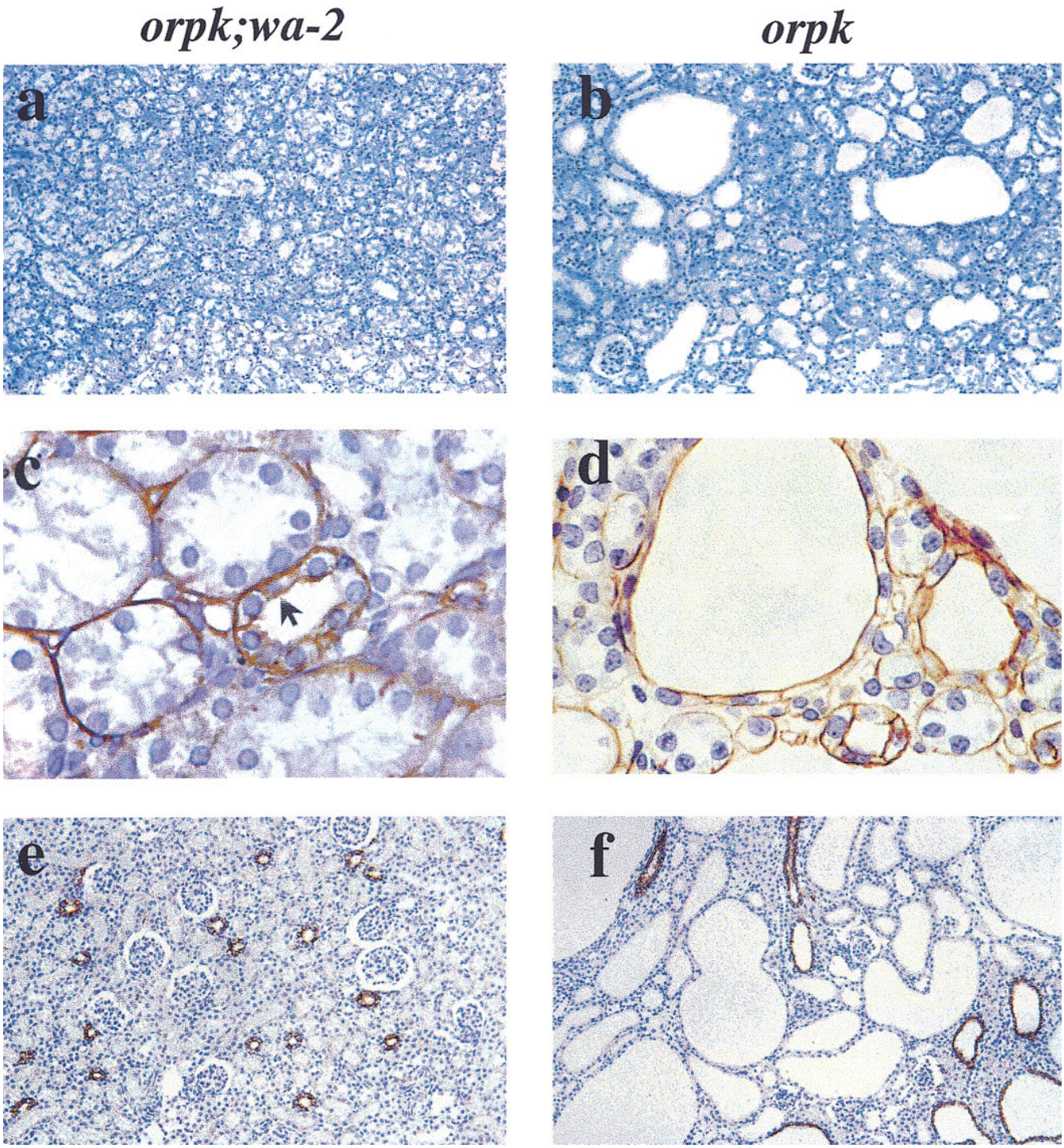

Figure 2. Histological analysis of the cystic phenotype in *orpk;wa2* double mutant and *orpk* animals. (*a*) Hematoxylin-stained section of kidney isolated from a *orpk;wa2* double mutant animal (×25). (*b*) Hematoxylin-stained section of kidney isolated from an *orpk* mutant animal (×25). (*c*) EGFR immunohistochemical staining of *orpk;wa2* kidney. Apical localization of the EGFR in a noncystic collecting tubule indicated with arrow (×100). (*d*) EGFR immunohistochemical staining of *orpk* kidney. (×100). (*e*) Collecting tubule–specific DBA staining of *orpk;wa2* kidney section. (*f*) Collecting tubule–specific DBA staining of *orpk* kidney section.

the level of protein and tyrosine kinase activity of the EGFR in affected kidneys (10, 11). Here we demonstrate that this increase in the level of tyrosine kinase can be partially or completely blocked through the introduction of the *wa2* allele of the EGFR. Most important, the degree to which EGFR tyrosine kinase activity is blocked correlates directly with the CI in mutant kidneys (Fig. 4). The fact that the EGFR in the *orpk; wa2* double mutants is still mislocalized to the apical surface of rare normal tubules and most remaining collecting tubule cysts indicates that increased tyrosine kinase activity, and not the mis-

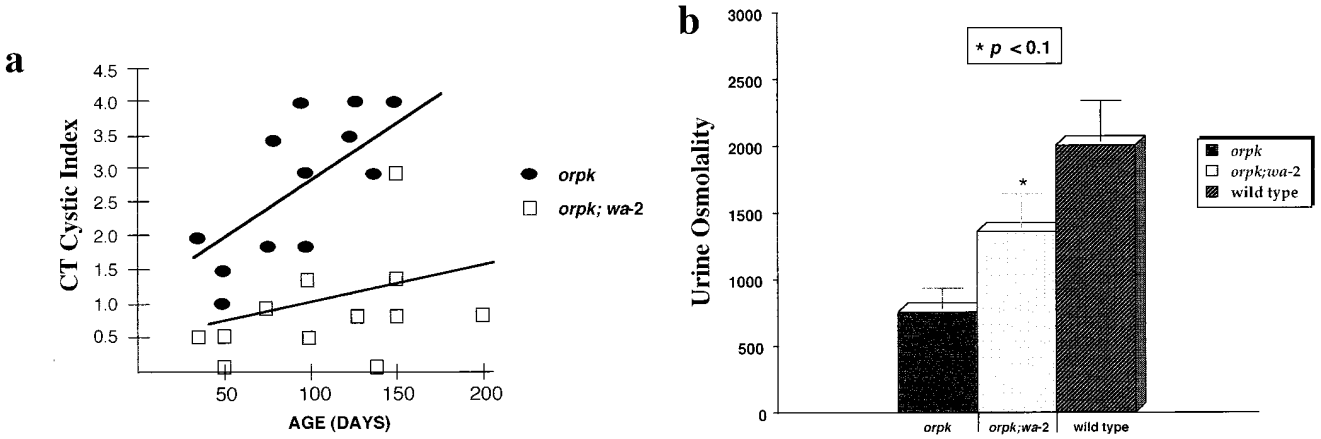

Figure 3. Cystic severity and renal tubular function in *orpk* and *orpk;wa2* mutant animals. (*a*) Scatter graph of collecting tubule CI of *orpk* (n = 12) and *orpk;wa2* double mutant (n = 12) animals. (*b*) Urine osmolality in *orpk, orpk;wa2* and wild-type animals. No significant difference ($P <$ 0.1) in concentrating ability was seen between wild-type and *wa2* mutant animals.

localization of the receptor alone, is associated with the formation of collecting tubule cysts. These data implicate the increased tyrosine kinase activity of the EGFR as a trigger for the cellular proliferation that leads to the formation and enlargement of cysts in the collecting tubules.

Cysts still occur in the kidneys of the *orpk;wa2* double mutants, but most of these cysts are located in the proximal tubules where the EGFR is not mislocalized to the apical surface. Therefore, reduction in the EGFR tyrosine kinase activity appears to have a dramatic effect on collecting tubular, but not proximal tubular, cystic enlargement. This reflects heterogeneity in cyst formation in these two nephron segments as reported previously (26). The fact that the *wa2* mutation has its most potent effect within the collecting ducts, where the EGFR is overexpressed and mislocalized, further supports the possibility for a direct role of increased EGFR tyrosine kinase activity in cyst formation and progression.

Using an in vitro organ culture system with kidney explants isolated from *orpk* mice, we studied previously the role of the EGFR in renal cyst formation. Specifically, we tested the effect of adding TGF-α (20 ng/ml), anti-EGFR blocking antibody, or receptor tyrosine kinase inhibitors (tyrphostin B42, PD153035) on cyst development (27). After stimulation with TGF-α, EGFR tyrosine kinase activity increased and cystic lesions in the collecting tubule enlarged. All effects caused by the addition of TGF-α were inhibited completely by the EGFR antibody or tyrosine kinase inhibitors. In explants treated with a range of tyrphostin B42 and PD 153035 concentrations (19), the effect on cyst development was directly proportional to the degree of inhibition of the EGFR tyrosine kinase activity. In all cases, the CI correlated with the level of tyrosine kinase activity in the kidney.

While our previously reported in vitro results are consistent with the in vivo results reported here, our data are not in agreement with those reported by Gattone et al. (28). These investigators reported that administration of EGF to the *cpk* murine model for ARPKD improved renal function and prolonged survival (28). Their data are difficult to assess in light of the lack of significant morphologic improvement in treated cystic animals and published reports that EGF increases EGFR

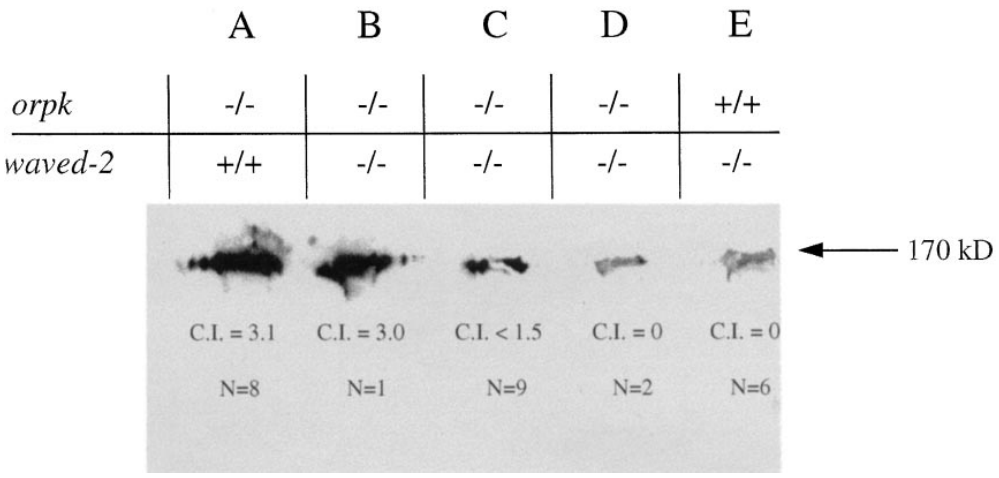

Figure 4. Whole kidney EGFR tyrosine kinase activity. Composite Western depicts the range of EGFR tyrosine kinase activity in *orpk;wa2* double mutants. Equal amounts of protein were immunoprecipitated with an anti-EGFR antibody, and the immunoprecipitate was Western blotted and probed with a horseradish peroxidase–conjugated antiphosphotyrosine antibody. The genotypes of the animals are shown above each lane. The CI of the contralateral kidney is shown below the blot and the number of animals that demonstrate these levels is also shown. *orpk;wa2* mutants (*three middle lanes*) demonstrate a range of tyrosine kinase activity levels which correlate directly with the CI of the contralateral kidney. One *orpk;wa2* double mutant (lane *B*) had a CI of 3.0, however, 11 of 12 *orpk;wa2* mutants (lanes *C* and *D*) had low tyrosine kinase activities and CIs ≤ 1.5.

in animals and humans with chronic renal failure regardless of the cause of the renal failure (29). In addition, these same authors report that overexpression of TGF-α causes polycystic kidney disease (30).

The fact that the tyrosine kinase activity of the EGFR in the *orpk;wa2* double mutants correlates with the CI (Fig. 4) strongly implicates the level of EGFR-specific tyrosine kinase activity as an important mediator of renal collecting tubule cyst formation in ARPKD. A reduction in EGFR tyrosine kinase activity results not only in a decrease in cyst severity, but also a significant improvement in tubular function as measured by urine concentrating ability. These results collectively suggest that the overexpressed EGFR in the collecting tubule may be a promising target for therapeutic agents in the treatment of ARPKD and other renal cystic diseases. Currently, several different compounds are being developed that target the EGFR and lead to an inhibition of its tyrosine kinase activity (19, 31). An important issue for additional research will be to determine whether any of these compounds can decrease EGFR-specific tyrosine kinase activity within the collecting tubules and potentially serve as active agents for the treatment of ARPKD.

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