Autosomal dominant polycystic kidney disease (ADPKD) is characterized by the formation of multiple fluid-filled cysts that expand over time and destroy the renal architecture. Loss or mutation of polycystin-1 or polycystin-2, the respective proteins encoded by the ADPKD genes PKD1 and PKD2, is associated with most cases of ADPKD. Thus, the polycystin proteins likely play a role in cell proliferation and morphogenesis. Recent studies indicate that polycystin-1 is involved in these processes, but little is known about the role played by polycystin-2. To address this question, we created a number of related cell lines variable in their expression of polycystin-2. We show that the basal and epidermal growth factor-stimulated rate of cell proliferation is higher in cells that do not express polycystin-2 versus those that do, indicating that polycystin-2 acts as a negative regulator of cell growth. In addition, cells not expressing polycystin-2 exhibit significantly more branching morphogenesis and multicellular tubule formation under basal and hepatocyte growth factor-stimulated conditions than their polycystin-2-expressing counterparts, suggesting that polycystin-2 may also play an important role in the regulation of tubulogenesis. Cells expressing a channel mutant of polycystin-2 proliferated faster than those expressing the wild-type protein, but exhibited blunted tubule formation. Thus, the channel activity of polycystin-2 may be an important component of its regulatory machinery. Finally, we show that polycystin-2 regulation of cell proliferation appears to be dependent on its ability to prevent phosphorylated extracellular-related kinase from entering the nucleus. Our results indicate that polycystin-2 is necessary for the proper growth and differentiation of kidney epithelial cells and suggest a possible mechanism for the cyst formation seen in ADPKD2.

Cyst formation is believed to require an overproliferation of renal epithelial cells from the walls of kidney tubules (3). Evidence to support this hypothesis has come from microdissection of cystic kidneys, which revealed that cyst size is due to an increase in the number of epithelial cells lining the cyst and not to the stretching of the cyst wall (4, 5). Additionally, cultured epithelial cells from ADPKD cysts display enhanced rates of proliferation, and genes associated with increased proliferation, such as c-Myc, have been found to be overexpressed in cystic epithelium (6, 7).

Recent studies indicate that the protein products of the PKD1 and PKD2 genes, mutations in which account for nearly all cases of ADPKD, may help guard against cystogenesis. Mice with targeted mutations in Pkd1 or Pkd2 develop cystic kidneys during embryogenesis, and ADPKD cysts in humans are associated with mutations in PKD1 or PKD2 (8–11). In addition, polycystin-1, which is encoded by Pkd1, has been implicated in a variety of pathways tied to proliferation, including G-protein signaling and the Wnt, AP-1, and JAK-signal transducers and activators of transcription (STAT) cascades (12–17). Moreover, depletion of polycystin-1 has been shown to increase cell growth, whereas its overexpression has been shown to slow cell growth, indicating that polycystin-1 may exert negative regulation over cell proliferation (13, 18, 19). Polycystin-2, the protein product of PKD2, has also been implicated in regulation of the cell cycle via its calcium channel activity and stimulation of AP-1 (20–24); however, there has been little direct evidence tying polycystin-2 to this process.

To determine what role polycystin-2 plays in cell proliferation, we created a variety of mammalian kidney cell lines with variable polycystin-2 expression. We show that cell proliferation is higher in cells that do not express polycystin-2 versus those that do, indicating that, like polycystin-1, polycystin-2 appears to negatively regulate cell growth. We also show that increased polycystin-2 expression correlates with decreased branching morphogenesis, suggesting polycystin-2 plays an important role in tubule maturation. Utilizing a channel mutant of polycystin-2, we demonstrate that polycystin-2 regulation of the above processes may be at least partially dependent on the calcium channel activity of the protein. Finally, we show that polycystin-2 can influence the subcellular localization of phospho-extracellular-related kinase (ERK), indicating that the protein may exert some of its regulatory effects via the Raf-ERK pathway.
ate stable cell lines from the Pkd2−/− cells (27). Stable cells were selected for and maintained in 300 μg/ml hygromycin (American Bioanalytical). Before splitting for experiments, cells were grown for 5 days at 37 °C without γ-interferon to abrogate expression of the temperature-sensitive SV40 large T immortalizing oncogene (nonpermissive conditions). All assays were performed under the same nonpermissive conditions.

Western Blotting—Cells were lysed in 1% Triton X-100 at 4 °C, the lysate was centrifuged at 14,000 rpm for 5 min, and the soluble protein fraction was recovered from the supernatant. The protein concentration of each sample was determined via a Bio-Rad colorimetric protein concentration assay, and equal amounts of each sample were loaded on an SDS-PAGE gel. The gel was run and electrophoretically transferred to a nitrocellulose membrane (Bio-Rad). This membrane was then probed with a peroxidase-conjugated anti-rabbit secondary antibody (Sigma) in conjunction with ECL (Amersham Biosciences).

Proliferation Assay—For the proliferation assay, 5000 cells from each cell line were seeded into 12 separate wells of a 96-well plate. The cells were serum-starved overnight and then grown with and without hepatocyte growth factor (HGF, 40 ng/ml, R&D) or epidermal growth factor (EGF, 20 ng/ml, Sigma). After 48 h the WST-1 reagent (Roche Applied Science) was added at a 1:10 dilution for 4 hours at 37 °C in 5% CO₂. WST-1 is a slightly red tetrazolium salt that can be cleaved to the dark red dye formazan by mitochondrial dehydrogenases (28). Because differences in cell number correlate directly with the quantity of mitochondrial dehydrogenases available to cleave WST-1, the color intensity of the media bathing cells that have been treated with WST-1 provides a reliable indication of relative cell number. The 96-well plates were then analyzed optically at 415 nm with a reference wavelength of 655 nm using a Bio-Rad Benchmark microplate reader (Bio-Rad).

Branching Morphogenesis and Tubulogenesis Assays—For the short term branching assay, cells were trypsinized, resuspended in type-I collagen (Upstate Biotechnology), and cultured in the presence or absence of HGF (40 ng/ml) or EGF (20 ng/ml) as previously described (29). After a 24-h period of incubation at 37 °C, 40 single cells were scored in a blinded fashion for the number of tubular processes per cell. Each well represents an n = 1, and each experiment was repeated at least 3 times. For the long term tubulogenesis assay cells were suspended in a 70:30 mixture of collagen and growth factor reduced Matrigel (BD Biosciences) as previously described (29) and maintained up to 7 days. The cells were photographed at 20× using a Nikon microscope with Hoffman modulation.

ERK Immunofluorescence—Cells were plated at ~50% confluency on glass coverslips and serum-starved overnight. Selected wells were stimulated at 37 °C in 5% CO₂ with HGF (40 ng/ml) or EGF (20 ng/ml) for 10 or 120 min or with no growth factor. Cells were washed 3 times with cold PBS and fixed in 4% paraformaldehyde for 15 min followed by 100% methanol at ~20 °C for 5 min. Cells were permeabilized in 0.3% Triton X-100, 0.15% bovine serum albumin (perm buffer) in PBS for 15 min at room temperature, then washed three times in PBS and blocked in 16% X-100, 0.15% bovine serum albumin (perm buffer) in PBS for 15 min at room temperature, then washed three times in PBS. Coverslips were mounted in Vectashield (Vector Laboratories) and analyzed on a Zeiss LSM 410 confocal microscope. Cells were focused in the central plane of the nucleus, and the total pixel intensity within nuclear and cytoplasmic regions was determined using a Zeiss software macro. Contrast and brightness settings were chosen to ensure that all pixels were in the linear range, and the same settings were used for all lines.

Statistics—The significance of difference between growth factor-treated and untreated groups was calculated pair-wise using Student’s t test. Where necessary, the significance of difference between various cell lines was calculated using multiple analysis of variance. The increasing number of asterisks in the figures corresponds to higher statistical significance.

RESULTS

Creation of Cell Lines with Variable Polycystin-2 Expression—To test the hypothesis that polycystin-2 plays a role in the regulation of cell proliferation, we created cell lines that manifest various levels of polycystin-2 expression. Two lines were derived from the proximal tubule of a Pkd2WS25/−/− mouse. WS25 is an unstable allele of Pkd2 that undergoes spontaneous rearrangement to produce either a null allele or restore the wild-type Pkd2 allele (10). Thus, both Pkd2−/−/− and Pkd2−/−/− lines were derived from this mouse (26). We refer to these lines as the parental lines and employed two distinct clonal lines of each type, which will be referred to as Null-1 and -2 and Het-1 and -2, respectively. The Pkd2−/− line was stably transfected with an empty pCEP4 vector or with a vector encoding the full-length polycystin-2 protein to create two additional lines, pCEP4 and PKD2-FL, respectively. We refer to these lines as the transfected lines and developed two independent clonal lines of each type, which are designated PCEP4-1 and -2 and PKD2-1 and -2, respectively. The transfected lines were grown in hygromycin-containing medium to ensure retention of the transfected construct, and thus, the pCEP4 line serves as a control for growth in this antibiotic. For the sake of simplicity, only one clone from each line is depicted in the figures of this paper. Results from the additional clones are presented throughout the text under “Results.”

The level of polycystin-2 expression in each line was ascertained via Western blot analysis of total cell lysate. As can be seen in Fig. 1, the Null-1 and pCEP4-1 lines express no polycystin-2, the Het-1 line expresses moderate levels of polycystin-2, and the PKD2-1 line expresses relatively high levels of polycystin-2. Alternate clones of each
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Polycystin-2 Negatively Regulates Cell Proliferation—The cell lines described above were created in the background of a temperature-sensitive mutant of the SV40 large T immortalizing oncogene, which confers enhanced proliferative properties to cells in culture. Because expression of this oncogene would likely confound the results of our assays, all lines were grown for 5 days at 37 °C without γ-interferon to minimize the effect of this factor (30). All assays were also performed under these same “nonpermissive” conditions.

The proliferation rates of each cell line were assessed via a WST-1 assay. An equal number of cells from each line was plated in multiple wells of a 96-well plate and serum-starved overnight to ensure synchronization at the G0 phase of the cell cycle (31). The cells were then induced to proliferate with HGF or EGF, whereas control cells remained in serum-free medium. After 48 h WST-1 was added to the wells, and the color intensity was measured via a plate reader. We found that the basal (serum-starved) level of cell proliferation was approximately twice as high in the Null and PCEP4 cells as was measured in the Het and PKD2 cells (Fig. 2, A and B). In addition, the Null and PCEP4 cells stimulated with HGF or EGF grew at least twice as fast as the HGF and EGF stimulated Het and PKD2 cells, respectively. When the -fold responsiveness of each line to growth factor was calculated, the lines not expressing polycystin-2 exhibited a significantly stronger proliferative response over base line to EGF than their polycystin-2-expressing counterparts (Fig. 2, C and D). No significant difference in -fold HGF stimulation of proliferation was detected between lines. Alternate clones of each line exhibited similar results to their clonal counterparts (data not shown).

The D511V cells displayed a basal proliferation rate that was higher than that of the cells expressing full-length polycystin-2 (PKD2) yet lower than that of the PCEP4 cells (Fig. 4B). A similar pattern was seen after HGF and EGF stimulation. There was no difference in -fold response to HGF over base line between lines; however, both PCEP4 and D511V cells exhibited a significantly stronger proliferative response to EGF than did the PKD2 cells (Fig. 4C). Alternate clones of the PKD2 and PCEP4 lines exhibited similar results to their clonal counterparts with respect to the D511V cells (data not shown). These data suggest that channel activity is required for the full expression of polycystin-2.

The Channel Activity of Polycystin-2 Is Important for Its Regulation of Proliferation and Branching/Tubulogenesis—Polycystin-2 is a transmembrane protein that has been shown to function as a calcium channel in vivo (22–24). To determine whether this channel activity is important for the protein regulation of proliferation and branching, we stably transfected Null cells with D511V, a construct that expresses a naturally occurring mutant form of polycystin-2 (32). The D511V protein contains a single amino acid change in the third transmembrane domain of the protein that abrogates the channel activity of polycystin-2 (23). Western blot analysis of total cell lysate from the D511V line indicates that the expression levels of D511V and polycystin-2 are comparable in their respective lines (Fig. 4A).

The D511V cells displayed a basal proliferation rate that was higher than that of the cells expressing full-length polycystin-2 (PKD2) yet lower than that of the PCEP4 cells (Fig. 4B). A similar pattern was seen after HGF and EGF stimulation. There was no difference in -fold response to HGF over base line between lines; however, both PCEP4 and D511V cells exhibited a significantly stronger proliferative response to EGF than did the PKD2 cells (Fig. 4C). Alternate clones of the PKD2 and PCEP4 lines exhibited similar results to their clonal counterparts with respect to the D511V cells (data not shown). These data suggest that channel activity is required for the full expression of polycystin-2.

Figure 2. Polycystin-2 negatively regulates cell proliferation. 5000 cells from each line were plated in multiple wells of a 96-well plate and grown with and without HGF or EGF for 48 h. Cell number was measured via a WST-1 colorimetric assay. A and B, comparison of relative cell number after 48 h. Cells expressing polycystin-2 show significantly less proliferation when grown with or without growth factor than cells that do not express polycystin-2. C and D, comparison of proliferative response to growth factor as determined by dividing the WST-1 value for cells grown with the growth factor by the WST-1 value for the same cells grown without the factor. There is no significant difference in HGF stimulation of proliferation between cell lines. Under EGF stimulation, however, cells that express polycystin-2 show a significantly lower proliferative response than cells that do not express polycystin-2. * $p < .05; n = 3. **, $p < .002; n = 3. S, starved; S.S., serum-starved.
mediated suppression of proliferation but that other functions of polycystin-2 are likely to contribute to this inhibitory response as well.

When suspended in a three-dimensional type I collagen matrix and grown for 24 h, unstimulated D511V cells exhibited less branching than PKD2 cells (Fig. 4D) and completely failed to respond to HGF (Fig. 4E). Compared with PKD2 cells, the cells expressing D511V had a modest decrease in the responsiveness to EGF as well. Consistent with the single cell-branching responses, Het and D511V cells consistently formed tubular structures only under EGF stimulation (Fig. 4F). In contrast, Null cells were able to form tubules in the unstimulated state as well as in the presence of either HGF or EGF. Occasionally, small stunted structures were observed in both Het-1/Het-2 and D511V cells in the presence of HGF. Thus, the channel activity of polycystin-2 does not appear to play a role in the suppression of branching morphogenesis and tubulogenesis that is imparted by polycystin-2 expression.

Polycystin-2 Regulates the Subcellular Localization of ERK1/2—HGF and EGF stimulation of cell proliferation and branching has been shown to act via the ERK members of the p42/44 mitogen-activated protein kinase pathway (33). Because the subcellular localization of phospho-ERK may determine its activity (34, 35), we investigated whether polycystin-2 could regulate this localization. The transfected lines were plated on coverslips and induced with either HGF or EGF for 10 or 120 min. The cells were then immunostained with propidium iodide and an antibody against phospho-ERK. Pixel intensity measurements taken on a confocal microscope enabled us to compare relative nuclear phospho-ERK levels between lines.

We found that, when unstimulated, all cell lines displayed faint phospho-ERK staining in the nucleus and cytoplasm, although the PCEP4 lines displayed a higher level of basal nuclear ERK than their counterparts (Fig. 5, A and B). When cells were stimulated with HGF or EGF for
10 min, the PCEP4 and D511V cells displayed significantly more nuclear staining than did the PKD2 cells. In fact, the PKD2 cells appeared to display most of their phospho-ERK in the cytoplasm rather than the nucleus, and nuclear exclusion of phospho-ERK was observed in some cells (Fig. 5A). At the 120-min time point, the PCEP4 lines still displayed higher than base-line levels of nuclear phospho-ERK, whereas their counterparts did not. Alternate clones of the PKD2 and PCEP4 lines exhibited similar results to their clonal counterparts (data not shown). As the trend between cell lines seen with the nuclear ERK data closely approximates the trend seen with the proliferation data, polycystin-2 may negatively regulate cell growth by preventing phospho-ERK from translocating to the nucleus. This pathway appears to at least partially depend on the channel activity of the protein, since high levels of nuclear phospho-ERK were detected initially after growth factor stimulation in D511V cells. The elevated basal levels and long term presence of nuclear phospho-ERK in the PCEP4 cells may explain why this line exhibits faster proliferation and tubule formation than its counterparts.

**DISCUSSION**

The association between cystogenesis and the loss or mutation of polycystin-2 has led many to speculate that polycystin-2 functions as a tumor suppressor-like protein. We provide direct evidence linking increased polycystin-2 expression to decreased cellular proliferation, confirming the role of polycystin-2 as a negative regulator of cell growth. We also show that D511V, a channel mutant of polycystin-2, is partially defective in mediating this negative regulation, as D511V cells grow faster than PKD2 cells but slower than PCEP4 cells. Thus, the calcium channel activity of polycystin-2 is important for its control of cell growth. Although the slower growth of the D511V line as compared with the PCEP4 lines may be due to overexpression of the D511V protein, this seems unlikely. The rate of PKD2 cell proliferation is comparable to that of Het cell proliferation, indicating that the quantity of polycystin-2 protein expressed does not correlate with the magnitude of the anti-proliferative effect. Because the D511V and polycystin-2 proteins are expressed at comparable levels in their respective lines, there should be no differences between these cells with respect to nonspecific effects of protein overexpression. A more probable explanation for the reduced proliferation of the D511V line may relate to the capacity of the D511V protein to participate in the normal repertoire of polycystin-2 protein-protein interactions. The D511V protein retains the same sub-cellular distribution as the wild-type protein, indicating that the single amino acid change that abrogates its channel activity is not likely to have significantly altered its three-dimensional structure (23). Thus, we would expect D511V to interact with many of the same binding partners associated with the wild-type protein. Overall, our results suggest that polycystin-2 regulates cell proliferation via multiple means. The protein channel activity may control some growth cascades via calcium signaling, whereas its structure may allow it to bind to and regulate components of other proliferation pathways.

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FIGURE 5. Polycystin-2 reduces nuclear phospho-ERK levels. Transfected cells were grown on glass coverslips, serum-starved overnight, and treated with HGF or EGF for 10 or 120 min. Cells were then stained with an antibody directed against phospho-ERK1/2. A, an immunofluorescence comparison of cell lines indicates stronger nuclear staining in the PCEP4-1 and D511V lines under 10 min of HGF and EGF stimulation than in the PKD2-1 line, which displays significantly more cytoplasmic staining than its counterparts (selected areas of each field are magnified in the insets). Nuclear exclusion of phospho-ERK in the PKD2-1 line can also be observed in some cells (see the arrows). B, quantitation of nuclear staining via pixel intensity measurements obtained with a confocal microscope reveals significantly more nuclear phospho-ERK (pERK) in the PCEP4 and D511V cells than in the PKD2-1 cells under 10 min of HGF and EGF stimulation. In addition, the PCEP4 line has a higher basal level of nuclear phospho-ERK than its counterparts and maintains elevated nuclear phospho-ERK levels up to 120 min.

is consistent with studies implicating it in the activation of protein kinase C and AP-1, both of which can have antiproliferative activities (36, 37). In addition, the proposed polycystin-2 association with polycystin-1 (38–41) suggests that polycystin-2 may be involved in some of the same growth suppression pathways that have been attributed to polycystin-1 (18, 19). Work by Bhunia et al. (13) in particular indicates that polycystin-2 aids polycystin-1 in its antiproliferative function via up-regulation of the cell cycle arrest protein p21waf1 (13). A recent study in lymphoblastoid cell lines suggests that loss of polycystin-2 in these cells is associated with reduced proliferation, a finding that seems at odds with our results (42). The fact that these data were obtained from non-renal cells suggests that polycystin-2 may play specific growth regulatory roles in epithelial cells that are not recapitulated in non-epithelial cell types.

In addition to the polycystin-2 regulation of cell proliferation, we have shown that cells expressing polycystin-2 display significantly less branching under basal and HGF-stimulated conditions than cells not expressing polycystin-2 when grown in a type I collagen matrix. Polycystin-2 involvement in the pathways mentioned above may explain its ability to regulate this phenotype. For example, Johnson et al. (43) demonstrated that a null mutation in c-Jun disrupts proper organ formation, indicating that AP-1 may function in modulating the differentiation states of renal epithelial cells. Our observation that polycystin-2 negatively regulates branching morphogenesis differs from studies of polycystin-1 expression. Both Boletta et al. (19) and Nickel et al. (29) have demonstrated that overexpression of polycystin-1 or its C-terminal tail, respectively, in renal cells results in enhanced branching under conditions similar to those used in our assay. Previous work by our group, however, has shown that polycystin-2 can modulate the subcellular localization of polycystin-1, thereby potentially regulating its function (26). At a high ratio of polycystin-2 to polycystin-1 expression, polycystin-1 is confined to the endoplasmic reticulum of renal epithelial cells, whereas it is present in both the endoplasmic reticulum and cell membrane when this ratio is low. It is also worth noting that recent studies indicate that the C-terminal tail of polycystin-1 can be cleaved and translocated to the nucleus, where it modulates signaling functions (44). Overexpression of polycystin-2 prevents the nuclear translocation of the polycystin-1 tail and blunts its effects on signaling. Thus, the inhibition of branching morphogenesis we found in the Het cells and PKD2-FL lines may be due to a titration effect between polycystin-2 and polycystin-1 that keeps more of the endogenous polycystin-1 in the endoplasmic reticulum or that keeps the polycystin-1 C-terminal tail fragment from entering the nucleus.

This interpretation makes sense in terms of the differences in polycystin expression ratios seen during development. Several groups have reported a pattern of early and intense polycystin-1 expression that decreases during development while reporting a converse pattern for polycystin-2 (for review, see Refs. 45 and 46). More specifically, renal expression of polycystin-1 in mice has been shown to be highest during nephrogenesis, whereas it is present in both the endoplasmic reticulum and cell membrane when this ratio is low. It is also worth noting that recent studies indicate that the C-terminal tail of polycystin-1 can be cleaved and translocated to the nucleus, where it modulates signaling functions (44). Overexpression of polycystin-2 prevents the nuclear translocation of the polycystin-1 tail and blunts its effects on signaling. Thus, the inhibition of branching morphogenesis we found in the Het cells and PKD2-FL lines may be due to a titration effect between polycystin-2 and polycystin-1 that keeps more of the endogenous polycystin-1 in the endoplasmic reticulum or that keeps the polycystin-1 C-terminal tail fragment from entering the nucleus.

Interestingly, we observed that the D511V cells displayed even less branching than the PKD2-FL cells. The ability of the D511V cells to branch and form tubules in response to EGF argues against a branching
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defect in this line. Again, as with polycystin-2 regulation of cell proliferation, these results suggest that the protein functions in more than one pathway. For example, the channel activity of polycystin-2 may be required for normal signaling to mediate branching, whereas its interaction with partner proteins such as the cleaved polycystin-1 C-terminal tail fragment may lead to the inhibition of branching. The importance of the channel activity for HGF-stimulated morphogenesis is supported by the fact that the D511V-expressing cells fail to respond to all HGF stimulation. The diverse nature of the signaling interactions regulated by polycystin-2 is further demonstrated by the finding that expression of polycystin-2 regulates both basal- and EGF-stimulated proliferation while primarily regulating only HGF-stimulated branching. EGF and HGF exert their effects on renal epithelial cell proliferation and morphogenesis through distinct signaling pathways (33). Thus, polycystin-2 likely functions via multiple pathways in exerting its regulatory effects.

To further elucidate some of the pathways via which polycystin-2 exerts its regulatory function, we examined the protein effect on phospho-ERK within the cell. Relatively high expression and a predominant nuclear localization of phospho-ERK are important for its cellular activity. EGF and HGF exert their effects on renal epithelial cell proliferation while primarily regulating only HGF-stimulated branching. The diverse nature of the signaling interactions regulated by polycystin-2 is further demonstrated by the finding that expression of polycystin-2 regulates both basal- and EGF-stimulated proliferation while primarily regulating only HGF-stimulated branching. EGF and HGF exert their effects on renal epithelial cell proliferation and morphogenesis through distinct signaling pathways (33). Thus, polycystin-2 likely functions via multiple pathways in exerting its regulatory effects. For example, the channel activity of polycystin-2 may contribute to the inhibition of branching. The importance of the channel activity for HGF-stimulated morphogenesis is supported by the fact that the D511V-expressing cells fail to respond to all HGF stimulation. The diverse nature of the signaling interactions regulated by polycystin-2 is further demonstrated by the finding that expression of polycystin-2 regulates both basal- and EGF-stimulated proliferation while primarily regulating only HGF-stimulated branching. EGF and HGF exert their effects on renal epithelial cell proliferation and morphogenesis through distinct signaling pathways (33). Thus, polycystin-2 likely functions via multiple pathways in exerting its regulatory effects.

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