Restoring multidrug resistance-associated protein 3 attenuates cell proliferation in the polycystic kidney

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Chang E, Park EY, Woo YM, Kang DH, Hwang YH, Ahn C, Park JH. Restoring multidrug resistance-associated protein 3 (MRP3) attenuates cell proliferation in the polycystic kidney. Am J Physiol Renal Physiol 308: F1004–F1011, 2015. First published August 20, 2014; doi:10.1152/ajprenal.00159.2014.—Autosomal dominant polycystic kidney disease (ADPKD) is characterized by abnormal proliferation of renal tubular epithelial cells, resulting in the loss of renal function. Despite identification of the genes responsible for ADPKD, few effective drugs are currently available for the disease. Thus finding additional effective drug targets is necessary. The functions of multidrug- resistance-associated protein 3 (MRP3) have been reported only in the field of drug resistance, and the renal functions of MRP3 are mostly unknown. In this study, we found that MRP3 was significantly downregulated in kidneys of human patients with ADPKD and polycystic kidney disease (PKD) mouse models. Our results suggest that downregulated MRP3 stimulated renal epithelial cell proliferation through the B-Raf/MEK/ERK signaling pathway. In contrast, we found that restoring MRP3 reduced cell proliferation and cystogenesis in vitro. These results suggest that the renal function of MRP3 is related to renal cell proliferation and cyst formation and that restoring MRP3 may be an effective therapeutic approach for PKD.

polycystic kidney; multidrug resistance-associated protein 3 (MRP3); cell proliferation; cystogenesis

AUTOSOMAL DOMINANT POLYCYSTIC kidney disease (ADPKD) is one of the most common genetic disorders, affecting at least 1 in 1,000 individuals. ADPKD is characterized by progressive cyst formation and four to eight times enlarged kidneys compared with those of healthy individuals. Although several therapeutic approaches such as the drugs tolvaptan and octreotide have shown hopeful clinical results, the field of PKD research requires more diverse effective treatment. Only few clinical treatments are available for ADPKD, so most patients with ADPKD develop end-stage renal disease (3, 28). A reduction in intracellular cAMP levels slows renal cyst progression. Ocreotide has been reported to have therapeutic effects with acceptable side effects in patients with ADPKD. Tolvaptan and octreotide reduce levels of cAMP indirectly or directly, respectively. These drugs suppress fluid secretion and cell proliferation by reducing intracellular cAMP; thus both slow renal and hepatic cyst progression. Clinical trials of tolvaptan and octreotide are ongoing. Tolvaptan is a vasopressin receptor 2 antagonist, which is a major regulator of adenyl cyclase activity and cAMP production, and octreotide is a somatostatin analog that inhibits intracellular levels of cAMP (8–13).

Rap1 is an upstream regulator of B-Raf. The functions of Rap1 have been reported as either a mitotic antagonist of Ras or an ERK activator in a cell type-specific manner. Despite the existence of cAMP and PKA, Rap1 functions in the opposite way in a diverse cell environment. In mouse embryonic fibroblasts, cAMP/PKA induces Rap1 to inhibit ERKs but cAMP/PKA stimulates cell proliferation via Rap1 in B-Raf-expressing cells such as PC12 cells (21). Polycystic kidney cells also express B-Raf, and cAMP/PKA turns Rap1 on B-Raf activator in Han:SPRD rat kidneys (13).

Multidrug resistance-associated protein 3 (MRP3) is encoded by the ATP-binding cassette subfamily C member 3 (ABCC3) gene and is a highly conserved protein. MRP3 is expressed in the basolateral membrane of ileal and colonic enterocytes (17), hepatocytes (8, 9), and cholangiocytes (9), as well as in the kidney (19). However, the function of MRP3 in the kidney is unknown. MRP3 is an organic anion transporter and a well-known bile salt efflux pump in hepatocytes. MRP3 also transports xenobiotics in addition to organic conjugates such as bile salts. Most MRP3 studies have focused on drug resistance, particularly as it is related to cancers (1, 8–10, 17). MRP3 is thought to be a target to improve anti-cancer drug efficacy.

In the present study, we assessed the functions of MRP3 in cell proliferation and cystogenesis in polycystic kidney cells in vitro. We demonstrated that 1) MRP3 is significantly downregulated in polycystic kidney tissues; 2) inhibiting MRP3 promotes cell proliferation via B-Raf/MEK/ERK signaling; and 3) MRP3 restoration suppresses the cAMP/PKA-dependent signaling pathway and attenuates cystogenesis. These data suggest that restoring MRP3 may be a potential therapy for ADPKD.
EXPERIMENTAL PROCEDURES

Cell culture and transfection. Mardin-Darby canine kidney (MDCK) cells were grown in DMEM/F-12 medium (Welgene, Daejeou, South Korea) with 5% fetal bovine serum (Welgene) and 1% penicillin-streptomycin in a 5% CO2, 37°C humidified incubator. The Human Renal Cortical Epithelial cell line (HRCE) and WT9-12 cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were grown in REBM (Lonza, Portsmouth, NH) or DMEM (Welgene), respectively. MDCK cells (2 x 10^5) were seeded in media without antibiotics on 10-cm plates for transfection with either RNAi or an hAbcc3/pCMV6-XL4 clone. After 24 h, the cells were transfected with 15 or 30 nM Abcc3 small interfering (si) RNA using Lipofectamine RNAi MAX (Invitrogen, Carlsbad, CA) for 60 h. MDCK cells were transfected with 10 μg of the human (h)ABCC3/pCMV6-XL4 clone (Origene, Rockville, MD) using Fugene (Promega, Madison, WI) for 60 h. Eight micrograms of the ABCC3/pCMV6-XL4 clone was transfected to overexpressing WT9-12 cells.

Kidney tissue samples. This study was performed in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Seoul National University Hospital (H-0701-033-195). Informed consent was acquired from all patients with ADPKD. Cystic tissues removed from the renal cortex of ADPKD patients undergoing nephrectomy were used. As a control, noncancerous renal cortical tissue was obtained from patients undergoing surgery for clear cell renal cell carcinoma (RCC); malignant cell infiltration was excluded by histology. For quantification of ABCC3 mRNA expression, the kidneys of patients with ADPKD were homogenized by lysis buffer.

XTT (WST-1) cell proliferation assay. MDCK (WT9-12) cells were seeded at 3 x 10^3 (2 x 10^4) cells/well in 24-well plates. A mixture of XTT (WST-1)-labeling reagent and electron-coupling reagent (Roche, Mannheim, Germany) was added to each well, and the cells were incubated in a humidified CO2 incubator under dark conditions for 24 h (only 1-h incubation time was needed for the WST-1 assay). Optical density was measured at 570 nm (450 nm) using a microplate reader.

Quantitative RT-PCR. Quantitative (q) RT-PCR was performed using a RG3000 instrument (Corbett Robotics, San Francisco, CA) and the SYBR Green-based procedure, according to the manufacturer’s protocol. The ABI-7500 instrument was used to assess ABCC3 expression of human kidney mRNA. All oligonucleotide primers were designed using DNASTAR. DNA sequences with 100% identity to canine Abcc3 were chosen to measure hABCC3 mRNA expression (data not shown). All qRT-PCR graphs were obtained using comparative Ct (ΔΔCt).

Western blotting and antibodies. A total of 35 μg of protein extract was separated by 8% SDS-PAGE, and the proteins were electrotransferred to nitrocellulose membranes. Western blotting was performed using MDCK cells and antibodies. All antibodies against the B-Raf/MEK/ERK pathway [p-PKA(S96) for the catalytic subunit, PKA, p-B-Raf(S445), p-MEK, MEK, p-ERK, ERK] were purchased from Cell Signaling Technology (Danvers, MA) except Raf B (Santa Cruz Biotechnology, Santa Cruz, CA). The primary anti-MRP3 antibody...
was purchased from Santa Cruz Biotechnology. β-Actin (Bethyl Laboratories, Montgomery, TX) was used as the loading control. The membranes were washed with $1 \times $ PBS/0.1% Tween 20, and bound proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Parsippany, NJ).

**In vitro cystogenesis.** MDCK cells were suspended in equal numbers in collagen type I (BD Biosciences, San Diego, CA) on six-well plates. The cells formed cystic structures for 7 days in collagen and were observed by confocal microscopy (Olympus, Tokyo, Japan). The cystic volume evaluation method was reported previously (15). Collagenase (Sigma, St. Louis, MO) was added for 2–3 h to extract RNA and protein from the cells.

**Immunocytochemistry-fluorescence.** Either RNAi or ABCC3/pCMV6-XL4-transfected MDCK cells were fixed in 4% formaldehyde for 30 min at room temperature, permeabilized with 0.1% Triton X 100 in $1 \times $ PBS, and stained overnight at 4°C with primary anti-proliferating cell nuclear antigen (PCNA) antibody (Santa Cruz Biotechnology). Then, the cells were stained for 15 min with 4',6-diamidino-2-phenylindole (DAPI), incubated for 1 h at room temperature in FITC-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology), and mounted with fluorescence Vectashield mounting medium (Dako, Carpentaria, CA). Images were captured under confocal microscopy (Olympus) and statistically analyzed. Quantitative graphs were prepared by calculating the percentages of bright PCNA-positive cells of the total number of DAPI-stained cells.

**Immunohistochemistry-fluorescence.** Paraffin sections of human (and mouse) kidneys were cut onto slides, deparaffinized in Histo-clear, and rehydrated in a graded ethanol series. Antigens were retrieved with 0.1 M citric acid buffer (pH 6.0). The sections were incubated overnight at 4°C with primary anti-MRP3 antibody (Abcam, Cambridge, MA) and DBA-rhodamine-conjugated antibody (Vector Laboratories, Burlingame, CA). Further steps were performed as in the immunocytochemistry-fluorescence experiment.

**Intracellular cAMP measurement.** cAMP levels were measured in WT9-12 cells. Sample diluent was added after a 20-h incubation for ABCC3/pCMV6-XL4 transfection. Intracellular cAMP concentrations were determined with a Direct Cyclic AMP Enzyme Immunoassay (EIA) kit (Arbor Assays, Ann Arbor, MI). The reactions were stopped and detected immediately with a microplate reader at 450 nm. Sigmoidal graphs of the cAMP EIA were analyzed by the 4PLC method using GraphPad Prism 6.0 (Graphpad Software, La Jolla, CA).

**Statistics.** Student’s t-test was performed with GraphPad Prism 6.0 to analyze tissue expression of ABCC3. $P < 0.05$ was considered significant.

**RESULTS**

**MRP3 is downregulated in polycystic kidneys.** To examine the role of MRP3 in the polycystic kidney, we first checked basal expression and localization of MRP3 in $Pkd1^{\text{flox/flox}}$.
Hoxb7-Cre and Pkd2\textsuperscript{flox/flox}:Hoxb7-Cre mice. In these models, the Pkd1 (Pkd2) gene was inactivated respectively in the renal collecting duct by crossing Pkd1\textsuperscript{flox} mice with Hoxb7-Cre mice that express Cre recombinase in the ureteric bud of the kidney during development (6). Both models develop severe PKD. Postnatal day 7 (P7) is sufficient to analyze the cystic phenotype. Mice at P7 showed cyst enlargement, and cysts developed more rapidly in Pkd1\textsuperscript{flox/flox}:Hoxb7-Cre than those in Pkd2\textsuperscript{flox/flox}:Hoxb7-Cre. The kidneys of Pkd1\textsuperscript{flox/flox}:Hoxb7-Cre and Pkd2\textsuperscript{flox/flox}:Hoxb7-Cre mice were homogenized, and RNA was extracted to evaluate basal expression of MRP3 (Abcc3). The result revealed that Abcc3 mRNA expression was downregulated in the kidneys of both PKD models (Fig. 1, A and C, \( **P < 0.001 \)). Pkd1\textsuperscript{flox/flox}:Hoxb7-Cre mice, which develop a more severe PKD phenotype than that of Pkd2\textsuperscript{flox/flox}:Hoxb7-Cre mice, showed greater decreases in Abcc3 expression. MRP3 is localized in the proximal and distal tubules (19); however, no study has reported localization in the collecting duct. Thus we performed double staining with DBA (red, rhodamine)-positive cells and MRP3 to determine whether MRP3 was localized in the collecting duct. As a result, it was revealed that MRP3 (green, FITC) was expressed in DBA (red, rhodamine)-positive cells in both wild-type and polycystic kidneys (Fig. 1, B and D; bright yellow). In other words, three different types of renal tubules (including distal, proximal, and the collecting duct) expressed MRP3. MRP3 was generally downregulated in Pkd1\textsuperscript{flox/flox}:Hoxb7-Cre and Pkd2\textsuperscript{flox/flox}:Hoxb7-Cre kidneys compared with those in normal (Pkd1\textsuperscript{flox/flox} and Pkd2\textsuperscript{flox/flox}, respectively) mice, and the same patterns were observed in mRNA expression.

Next, we evaluated ABCC3 (MRP3) expression in human ADPKD tissues. All ADPKD kidneys had much lower expression levels of ABCC3 mRNA than that in normal kidneys (Fig. 2A; qRT-PCR, \( *P < 0.001 \)). We performed immunohistochemistry-fluorescence to observe tubular expression and localization of MRP3. Because the kidney is composed of numerous tubules, MRP3 appeared to be ubiquitously expressed in normal tissues (Fig. 2B, a and b). However, MRP3 was downregulated in the cystic epithelial cells (Fig. 2B, c-f). MRP3 expression continued to be observed in the small cysts and dilated tubules. In contrast, the lining cells of large cysts rarely expressed MRP3. These results were also observed in mouse kidneys (Fig. 1). Collectively, these results indicate that MRP3 is significantly downregulated in polycystic kidneys.

Inhibition of MRP3 activates B-Raf/MEK/ERK signaling in vitro. To determine the function of inhibiting MRP3 on renal tubular epithelial cells, we introduced an RNAi system to MDCK renal distal tubular epithelial cells. Then, we performed the XTT assay and PCNA immunostaining to observe the effects of MRP3 downregulation on cell proliferation (Fig. 3, A and B). The graph of Abcc3 knockdown MDCK cells was steeper than that of control cells (Fig. 3A). Cell proliferation rates changed, beginning on the first day of the XTT assay and increased gradually following Abcc3 knockdown. PCNA-positive cells increased approximately twofold (10.23 \(+ \pm\) 2.61\% \( **P < 0.001 \)) in Abcc3 RNAi-treated MDCK cells (Fig. 3B). These results suggest that inhibiting Abcc3 stimulates proliferation of MDCK renal epithelial cells. Western blotting was performed to further evaluate whether downregulating Abcc3 affected cell proliferation through B-Raf/MEK/ERK signaling (Fig. 3C). The results revealed that all kinases involved in the B-Raf/MEK/ERK signaling cascade [p-B-Raf(Ser445)] were gradually phosphorylated after inhibiting Abcc3 in an RNAi dose-dependent manner. Our results show that the potential role of MRP3 is related to cell proliferation through B-Raf/MEK/ERK signaling.
**ABCC3 overexpression reduces cell proliferation and cystogenesis in vitro.** Next, we tested the effects of excess MRP3 on renal epithelial cell proliferation. We performed overexpression experiments to determine whether excess MRP3 repressed the proliferation. Thus we introduced human ABCC3, which was cloned in the pCMV6-XL4 mammalian expression vector (ABCC3/pCMV6-XL4). Abcc3 is a highly conserved gene, and the human ABCC3 sequence shares 86% identity with the canine Abcc3 sequence. We picked primers against the sequences that were identical in humans and canines, to assess the human ABCC3 mRNA level in canine MDCK cells (data not shown). The effects of excess MRP3 on cell proliferation were studied using the XTT assay and PCNA immunostaining.

The results revealed that ectopic MRP3 repressed cell proliferation with an approximate 15% reduction on the third day of the XTT assay (Fig. 4A). PCNA staining also showed that ABCC3 overexpression suppressed cell proliferation (Fig. 4B). The PCNA-positive population of ABCC3-transfected MDCK cells decreased to less than half compared with that of the control (from 17.54 ± 1.76 to 7.83 ± 0.35%, **P < 0.001). A Western blot analysis showed that B-Raf/MEK/ERK kinases were involved in the intracellular signaling triggered by ABCC3 overexpression. The decreased proliferation reduction could have been due to repression of the B-Raf/MEK/ERK signaling cascade (Fig. 4C).

Consistent with these results, we next examined whether overexpressing ABCC3 would affect enlargement of cysts under three-dimensional (3D) conditions. The 3D culture model provided an environment for development of cysts. To culture cystic structures, we first suspended MDCK cells in a collagen matrix and then induced cyst formation using forskolin (5 μM) during a 7-day incubation. The results revealed that ABCC3-transfected MDCK cells developed smaller cysts compared with those in control cells. ABCC3 mRNA expression was significantly increased following ectopic expression of ABCC3/pCMV6-XL4 in MDCK cells (Fig. 5C, **P < 0.001). ABCC3 overexpression resulted in a 32.3% reduction in cyst growth (Fig. 5, A and B; n = 60 each; **P < 0.001). After the 7-day 3D culture period, collagenase was added to the 3D-cultured MDCK cells, and the cells were lysed to obtain protein. Then, we performed Western blotting, which revealed that B-Raf/MEK/ERK signaling was reduced in 3D-cultured cells (Fig. 5D). Taken together, restoring MRP3 reduced the enlarged cysts by repressing cell proliferation through dephosphorylation of B-Raf/MEK/ERK kinases.

MRP3 is downregulated in human ADPKD cell line and restored MRP3 represses cell proliferation through a cAMP/PKA-dependent pathway. We next wondered whether the same mechanism could be applied to human patients with ADPKD. Thus we checked basal expression of MRP3 in human ADPKD cells compared with that in normal renal epithelial cells. WT9-12 cells, which originated from renal distal and proximal tubules, had only 3.3% basal expression of ABCC3 compared with that in HRCE cells (Fig. 6A). Next, we introduced ectopic ABCC3/pCMV6-XL4 into WT9-12 cells and then performed the WST-1 assay to examine the effects of restoring MRP3 on

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**Fig. 4.** Influences of ABCC3 overexpression on cell proliferation in MDCK cells. MDCK cells were transfected with ABCC3/pCMV6-XL4 (10 μg of DNA on 10-cm plates) for 60 h and then harvested for extraction of RNA and protein. All of the results were obtained from >5 independent transfection experiments. A: results of the XTT assay were obtained with a microplate reader at 570 nm. Values are means ± SD of 5 independent experiments. **P < 0.001. B: ICC-IF with anti-PCNA antibody in ABCC3-overexpressed MDCK cells. The percentages of PCNA-positive cells were calculated by the fraction of DAPI. C: Western blotting was used to analyze for the levels of phosphorylation of the B-Raf/MEK/ERK signaling pathway by ectopic ABCC3. Rap1A/B and phosphorylated PKA were also detected. β-Actin was used as the loading control.
cell proliferation (Fig. 6B). Cell proliferation decreased in ABCC3/pCMV6-XL4-transfected WT9-12 cells (\*\*P < 0.05, \*\*\*P < 0.001). The previous figures showed that the levels of B-Raf/MEK/ERK kinase phosphorylation were regulated by MRP3, so we checked whether intracellular cAMP levels were altered by restoring MRP3. As a result, intracellular cAMP content decreased significantly in WT9-12 cells after restoring ABCC3 (*P < 0.05). Decreases in phosphorylation of the B-Raf/MEK/ERK signaling kinases might lead to reduced proliferation in WT9-12 cells. At the same time, it was revealed that restoring ABCC3 (MRP3) decreased phosphorylation of the PKA catalytic subunit (Ser 96) and expression of Rap1, which is an ERK activator in B-Raf-expressing cells that is upregulated in the Han:SPRD (13) (Fig. 4D). Collectively, human ADPKD cells had significantly lower MRP3 expression, and restoring MRP3 attenuated cell proliferation through the cAMP/PKA-dependent signaling pathway.

### DISCUSSION

Inhibiting MRP3 has been used as a strategy for cancer therapy because MRP3 has been mainly characterized by drug resistance (27, 29). However, we suggest novel MRP3 functions in the kidney related to proliferation. Our results demonstrate that downregulating MRP3 expression is related to disease progression in human patients with ADPKD. The kidneys of patients with ADPKD showed significant decreases in MRP3 expression. This study provided evidence that inhibiting MRP3 might drive the cell proliferation signal through the B-Raf/MEK/ERK pathway. MRP3 was significantly downregulated in polycystic kidneys of the PKD mouse model and human patients with ADPKD. Additionally, restoring ABCC3 suppressed cell proliferation in MDCK and WT9-12 cells, which may affect downstream PKA signaling. Although the renal functions of MRP3 are unknown, we found that regulating MRP3 expression affected cell proliferation.

Interestingly, MRP3 was still expressed in small cyst-lining cells or almost-normal tubules in polycystic kidneys, whereas MRP3 was completely downregulated in larger cysts, which might enhance disease severity. MRP3 is expressed in renal tubular epithelial cells under normal physiological conditions. However, MRP3 is downregulated in the pre-disease state, and inhibiting MRP3 contributes to cyst formation by stimulating B-Raf/MEK/ERK signaling.

Numerous drugs are currently under development for PKD, but disappointing clinical results have been reported due to negative feedback. For example, the mammalian target of...
Rapamycin (mTOR) inhibitor rapamycin was thought to be a powerful drug for treating ADPKD, but it has not been effective (5, 14, 23). In recent reports, tolvaptan and octreotide have shown therapeutic effects in ADPKD, and both drugs target intracellular cAMP accumulation (4, 7, 12, 16, 18, 24). In our study, ABCC3 was remarkably downregulated in polycystic kidney cells, and restoring ABCC3 decreased cell proliferation by reducing intracellular cAMP content. We suggest another way to target cAMP-dependent signaling by regulating MRP3 expression.

The cAMP-dependent proliferation pathway is the putative signaling pathway for cyst enlargement in ADPKD. Under the disease condition, cAMP stimulates PKA, which activates the cellular RAS protein, and RAS activation turns on the B-Raf/MEK/ERK signaling cascade in a Rap1-dependent manner (2, 20, 25, 26). In the present study, MRP3 restoration reduces cell proliferation through repression of PKA and Rap1A/B in the polycystic kidneys.

Here, we wondered what the mediator is between MRP3 and cAMP. We found that MRP3 regulated the levels of intracellular cAMP. Whether MRP3 regulates cAMP directly or indirectly should be investigated in further studies. Although there are no published reports about the relationship between MRP3 and cAMP, MRP3 might interact with other proteins at the cellular level. MRP4 interacts physically with the cystic fibrosis transmembrane conductance regulator (CFTR) through PDZ domain-containing protein (PDZK1). MRP4 (ABCC4) is a member of the ATP-binding cassette subfamily C, which includes MRP3, and it acts as a cAMP regulator in smooth muscle cells and gut epithelia. MRP4 effluxes cAMP, thus altering local concentrations of cAMP. Then, CFTR, which physically interacts with MRP4, recognizes the change in cAMP content (11). MRP3 also has a PDZ domain-containing protein, suggesting that MRP3 may have the potential to bind other proteins.

MRP3 is also expressed in cholangiocytes, which develop hepatic cysts in polycystic liver disease (PLD). If cholangiocytes express a low level of MRP3 in PLD, a similar mechanism might occur in hepatic cysts when MRP3 is restored.

In conclusion, restoring MRP3 could be a therapeutic target for ADPKD because it suppresses cell proliferation via cAMP/PKA-dependent signaling in polycystic kidney cells, which is
the putative pathway causing PKD progression. To apply this to clinical trials, we suggest that the accurate mechanism involving MRP3 and cAMP must be further elucidated. Additionally, developing an MRP3 agonist (activator) may represent one clinical approach of targeting MRP3.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: E.C. performed experiments; E.C. drafted manuscript; E.Y.P., Y.m.w., D.-H.K., Y.-H.H., and C.A. interpreted results of experiments; J.H.P. edited and revised manuscript; J.H.P. approved final version of manuscript.

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