Journal of Biochemistry and Molecular Biology, Vol. 40, No. 4, July 2007, pp. 467-474

JOURNAL OF Biochemistry and Molecular Biology

PKHD1 Gene Silencing May Cause Cell Abnormal Proliferation through Modulation of Intracellular Calcium in Autosomal Recessive Polycystic Kidney Disease

Jiyun Yang[1], Sizhong Zhang[1,*], Qin Zhou[2], Hong Guo[2], Ke Zhang[2], Rong Zheng[2] and Cuiying Xiao[1]

[1]Department of Medical Genetics, West China Hospital, Sichuan University, Guoxue Xiang No. 37, Chengdu 610041, PR China

[1]Division of Human Morbid Genomics, State Key Laboratory of Biotherapy, Chengdu 610041, PR China

[2]Mouse Gene Engineering Center, State Key Laboratory of Biotherapy, Chengdu 610041, PR China

Received 4 December 2006, Accepted 22 January 2007

Autosomal recessive polycystic kidney disease (ARPKD) is one of the important genetic disorders in pediatric practice. Mutation of the polycystic kidney and hepatic disease gene 1 (*PKHD1*) was identified as the cause of ARPKD. The gene encodes a 67-exon transcript for a large protein of 4074 amino acids termed fibrocystin, but its function remains unknown. The neoplastic-like in cystic epithelial proliferation and the epidermal growth factor/epidermal growth factor receptor (EGF/EGFR) axis overactivity are known as the most important characteristics of ARPKD. Since the misregulation of Ca^{2+} signaling may lead to aberrant structure and function of the collecting ducts in kidney of rat with ARPKD, present study aimed to investigate the further mechanisms of abnormal proliferation of cystic cells by inhibition of *PKHD1* expression. For this, a stable *PKHD1*-silenced HEK-293T cell line was established. Then cell proliferation rates, intracellular Ca^{2+} concentration and extracellular signal-regulated kinase 1/2 (ERK1/2) activity were assessed after treatment with EGF, a calcium channel blocker and agonist, verapamil and Bay K8644. It was found that *PKHD1*-silenced HEK-293T cell lines were hyperproliferative to EGF stimulation. Also *PKHD1*-silencing lowered the intracellular Ca^{2+} and caused EGF-induced ERK1/2 overactivation in the cells. An increase of intracellular Ca^{2+} in *PKHD1*-silenced cells repressed the EGF-dependent ERK1/2 activation and the hyperproliferative response to EGF stimulation. Thus, inhibition of *PKHD1* can cause EGF-induced excessive proliferation through decreasing intracellular Ca^{2+} resulting in EGF-induced ERK1/2 activation. Our results suggest that the loss of fibrocystin may lead to abnormal proliferation in kidney epithelial cells and cyst formation in ARPKD by modulation of intracellular Ca^{2+}.

Keywords: Autosomal recessive polycystic kidney disease, Epidermal growth factor, Extracellular signal-regulated kinase, Intracellular calcium, *PKHD1*

Introduction

Autosomal recessive polycystic kidney disease (ARPKD) is an important renal and hepatic disease in neonates and infants, occurring 1 in 20,000-40,000 live births (Zerres *et al.*, 1998). The main pathologic manifestations of the disease are the fusiform dilation of renal collecting ducts and distal tubules as well as dysgenesis of the hepatic portal triad including hyperplastic biliary ducts and congenital hepatic fibrosis (Roy *et al.*, 1997; Zerres *et al.*, 1998). In infancy, the disease results in significantly enlarged polycystic kidneys and pulmonary hypoplasia. Up to 30% of the affected neonates die of secondary respiratory failure shortly after birth (Zerres *et al.*, 1994; Zerres *et al.*, 1996). In subset that survived the perinatal period, the morbidity and mortality are mainly due to severe systemic hypertension, renal failure, and portal hypertension (Zerres *et al.*, 1996; Roy *et al.*, 1997; Fonck *et al.*, 2001). Therefore, the patients with ARPKD are candidates for liver, kidney or combined liver and kidney transplantation (Sairanen *et al.*, 1997).

In 1994, the gene for ARPKD was mapped to human chromosome 6p21-cen(Zerres *et al.*, 1994; Zerres *et al.*, 1994). Then independent groups isolated the gene(Xiong *et al.*, 2002; Ward *et al.*, 2002; Onuchic *et al.*, 2002), and several disease-causing mutations in the *PKHD1* gene were identified (Bergmann *et al.*, 2004; Bergmann *et al.*, 2005). The gene encodes a novel large transmembrane protein with 4074

*To whom correspondence should be addressed.
Tel: 86-28-85164009; Fax: 86-28-85164009
E-mail: szzhang@mcwcums.com; szzhang@vip.163.com

amino acids named fibrocystin or polyductin (Ward *et al.*, 2002; Onuchic *et al.*, 2002). Studies have shown that its mouse gene homologue *Pkhd1* is expressed in ductal structures during development of kidney, liver, lung, pancreas, and vessels (Nagasawa *et al.*, 2002; Xiong *et al.*, 2002). In adult kidney, *PKHD1* is expressed in the epithelia of proximal convoluted tubules, ascending limbs of Henle's loop and collecting ducts. The protein is localized in the basal bodies and primary cilia of renal epithelia (Zhang *et al.*, 2004).

Recently it has been found that fibrocystin interacts with calcium modulating cyclophilin ligand (CAML), a protein that is involved in Ca^{2+} signaling (Nagano *et al.*, 2005), and the calcium influx into cells can change the gene expression and the control of cell growth and differentiation (Li *et al.*, 2005; Kandilci and Grosveld, 2005; Kupzig *et al.*, 2005). Meanwhile loss of cilia also may result in misregulation of Ca^{2+} and elevation of subapical membrane Ca^{2+}. The defect in induced Ca^{2+} signaling leads to aberrant structure and function of the collecting ducts in ARPKD (Liu *et al.*, 2005; Siroky *et al.*, 2006).

The most important characteristics of ARPKD development are neoplastic-like in cell proliferation and the EGF/EGFR axis overactivity (Richards *et al.*, 1998; Veizis and Cotton, 2005). The phosphorylated ERK1/2 is significantly increased in cystic compared with noncystic kidneys and a sustained high level of MAPK signaling may influence cellular proliferation and function of cystic epithelial cells (Veizis and Cotton, 2005). Since abnormal epithelial cell proliferation underlies the cyst formation and renal enlargement in ARPKD, above findings suggest that activation of the MAPK pathway is critical in the development of the cystic changes. Therefore, it may be postulated that fibrocystin, as a membrane receptor, participates in EGF-induced abnormal proliferation of renal epithelial cells in ARPKD through intracellular Ca^{2+} to modulate the MAPK activity. To test this idea, we tried to determine whether the inhibition of *PKHD1* expression by gene silencing could increase EGF-induced cell proliferation and its possible mechanisms.

Materials and Methods

Cells and reagents. Human embryonic kidney (HEK-293T) cells were maintained in DMEM (Gibco) supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Stably transfected HEK-293T cells were maintained in the above DMEM medium with addition of 200 μg/ml G418. The reagents used in the study were purchased as follows. pGenesil-2 plasmid from Wuhan Genesil Biotech Co., Ltd; Rabbit polyclonal antibody to p-ERK1/2 and Rabbit polyclonal to total-ERK1/2 from Santa Cruz Biotech, Inc; Fluo-3/AM, Ionomycin and G418 from Sigma Co., Ltd; Recombinant Human EGF and goat anti-rabbit IgG from Jingmei Biotech Co., Ltd; Lipofectamine 2000, Fetal bovine serum (FCS) from Invitrogen Co., Ltd; Bay K8644 from Alexis; One step Perfect Real Time PCR Kit and Trizol reagents from TaKaRa Biotech Co., Ltd.

Construction of shRNA vectors. Two shRNAs targeting *PKHD1* mRNA (GI: 25777664) were designed according to the standard selection criteria (Reynolds *et al.*, 2004). Double-stranded small hairpin RNA (shRNAs) and control shRNA were synthesized (Table 1), and then were cloned into pGenesil-2 vector (Fig. 1).

Establishment of *PKHD1*-silenced cell lines. According to the manufacturer's protocol for Lipofectamine 2000, vectors with *PKHD1* shRNA, HK-A and pGenesil-2 plasmid were transfected into subconfluent HEK-293T cells for 24 h, 48 h. Expression of *PKHD1* mRNA was measured by quantitative PCR. The vector with the least *PKHD1* mRNA level was selected to establish the stable *PKHD1*-silenced cell lines. In parallel, we used pGenesil-2 plasmid and HK-A shRNA vector to transfect HEK-293T cells to produce respective stable control cell lines. After transfection, G418 (600 μg/ml) selection was commenced and maintained for a week to obtain the G418-resistant clones. The selected cells were then resuspended and seeded in 100-mm^2 culture plates with a cell density of 1×10^3 per plate. The G418-resistant single colonies were picked and transferred onto 24-well plates and the stably transfected HEK-293T cells were propagated in the continual presence of G418 (200 μg/ml).

RNA isolation and real time PCR. Total RNA was isolated from the transient or stable transfected HEK-293T cell lines using Trizol reagents according to the manufacturer's instructions. Real time PCR was performed with the LightCycler system (Roche). The following oligonucleotide primers and Taqman probe were used: *PKHD1*-FP, 5'-GCCACCATGTGAGGATCTATGA-3'; *PKHD1*-RP, 5'-CAGCAGCCAAACGAATGTGT-3'; *PKHD1*-Taqman Probe, FAM-ACCGGCATATTGGAAGTGTACATGTCACG-TAMRA; Humana *β-actin*-FP, 5'-CCTGGCACCCAGCACAAT-3'; Human *β-actin*-RP, 5'-GCTGATCCACATCTGCTGGAA-3'; Human *β-actin*- Taqman Probe; FAM-ATCAAGATCATTGCTCCTCCTGAGCGC-TAMRA.

Cell proliferation assays. Cells were seeded onto 96-well plate in DMEM with 10% FBS and 100 μg/ml penicillin/streptomycin for 24 h and treated with EGF, verapamil, or Bay K8644. Cell proliferation rates were determined by the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay method.

Table 1. shRNAs against Human *PKHD1*

shRNA names	shRNA positions	Sequences
PKHD1shRNA1	Exon4(nt433-453)	AACAATGGCTCTCAATTGGAG
PKHD1shRNA2	Exon5(nt624-644)	AAGCAGTCCAAATCCAGGACC
HK-A(control shRNA)		ACTACCGTTGTTATAGGTG

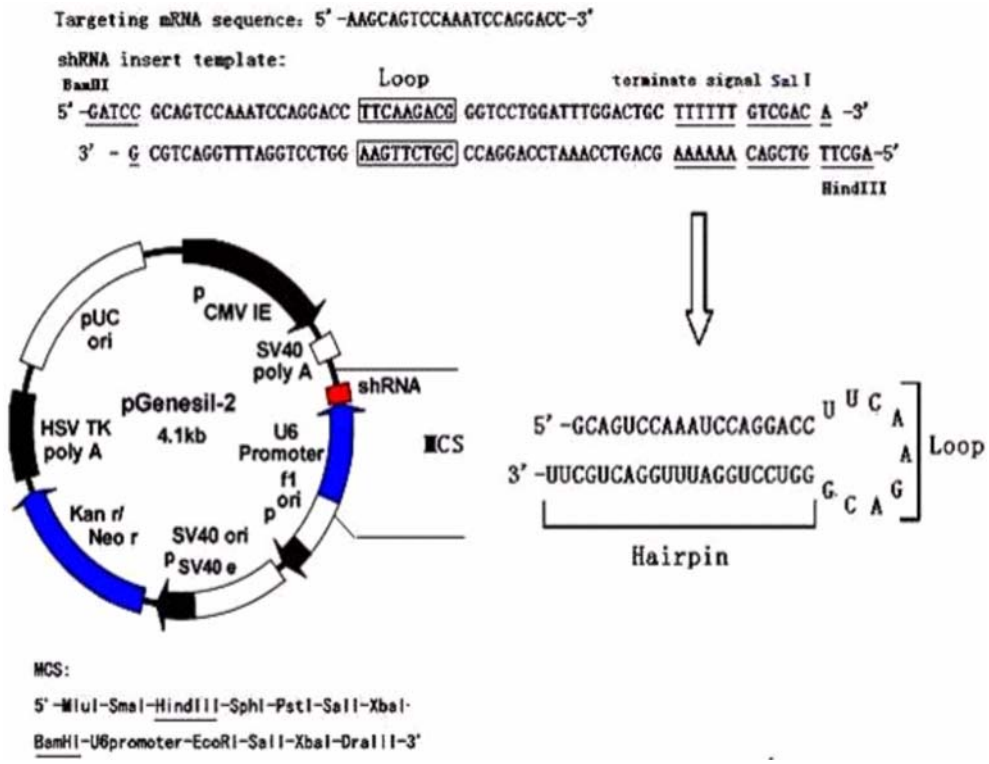

Fig. 1. Construction of shRNA Vectors. Two shRNAs targeting *PKHD1* mRNA and control shRNA cloned into pGenesil-2 vector, which provided the shRNA backbone.

Western blotting. The HEK-293T cells were washed twice with phosphate-buffered saline (PBS) and lysed using RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1% NP-40) with protease inhibitors (0.1 mg/ml aprotinin, 5 μg/ml leupeptin, 50 μg/ml pepstatin, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM NaF) for 30 min on ice. The lysates were centrifuged at 12,000 g for 10 min at 4°C. Supernatant was collected and stored at –20°C for use. 20 μg total protein was run on 12% SDS-PAGE and subsequently transferred onto polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in tris-buffered saline tween-20 (TBS-T) with 3% BSA for an hour at room temperature. After that, they were incubated with the primary antibodies for overnight at 4°C and followed by incubation with the goat-anti rabbit HRP-conjugated secondary antibodies for an hour at room temperature. Immunoreactive bands were identified using SuperSingal west pico chemiluminescent substrate (Pierce) and exposed to X-rays films (Kodak).

Measurement of intracellular Ca^{2+}. After the cells cultured on 6-well plate in DMEM with 10% FBS reached approximately 80% confluent states, they were rinsed with HEPES buffer for three times and loaded with 2 μM Fluo-3/AM at 37°C for 30 min, and rinsed again. Fluorescence intensity was measured with confocal microscope (Leica TCS SP2) at excitation wavelengths of 488 nm, and the emitted wavelengths of 522 nm at five different locations. At the end of each experiment, cells were permeabilized with HEPES buffer containing 10 μM Ionomycin to determine the Fmax, and then HEPES buffer containing 10 μM Ionomycin and 10 mM EGTA was added to determine the Fmin. The fluorescence intensity was converted to Ca^{2+} concentration using the equation $[Ca^{2+}]_i = Kd \times (F - Fmin)/(Fmax - F)$, where the dissociation constant (Kd) of Fluo-3/AM for Ca^{2+} is 400 nM, Fmax and Fmin represent fluorescence intensity for Ca^{2+}-saturating and Ca^{2+}-free conditions respectively.

Statistical analysis. Data are presented as Means ± SE. T-test was used to compare the data, and $p < 0.05$ was taken as the level of significance. All results were analyzed by statistical software SPSS11.0.

Results

Establishment and characterization of stable *PKHD1*-silenced HEK-293T cell lines. As the inhibition of *PKHD1* mRNA expression in the cells transfected with shRNA2 vector was much more significant, it was chosen to establish the stable *PKHD1*-silenced cell lines (Fig. 2A). It was found that the *PKHD1* mRNA level was markedly decreased in the gene-silenced cell lines but not in the controls while the *PKHD1* mRNA level of the controls were similar to that of wild-type cells (Fig. 2B).These results showed that *PKHD1* was down-regulated by the shRNA in the established stable cell lines and the gene silencing is specific.

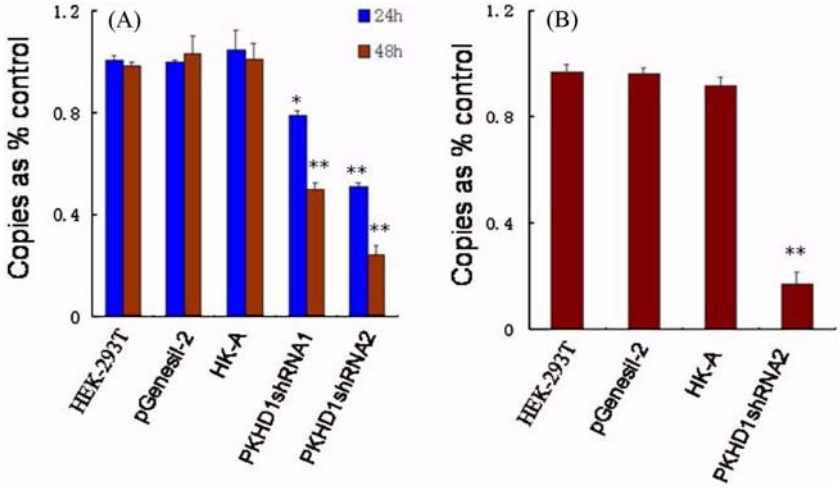

Fig. 2. The expression of *PKHD1 mRNA* after RNA interference. (A) The levels of *PKHD1* mRNA after transfection. From left to right, the wild type, pGenesil-2, HK-A, *PKHD1*shRNA1 and *PKHD1*shRNA2. The mRNA levels of cells transfected with *PKHD1*shRNA1 and *PKHD1*shRNA2 were significantly lower than in the wild-type and HEK-293T transfected with HK-A and pGenesil-2. *PKHD1*-shRNA2 showed the largest amount of inhibition. One asterisk indicates a significant difference at $p < 0.05$ versus HEK-293T. Two asterisk indicates a significant difference at $p < 0.001$ versus HEK-293T (B) The levels of *PKHD1* mRNA in the stable transfected cell lines. *PKHD1* mRNA level was markedly decreased in the *PKHD1*-silenced cell lines ($p < 0.001$), but not in the control cell lines. The *PKHD1* mRNA level of the controls was similar to that of wild-type HEK-293T cells.

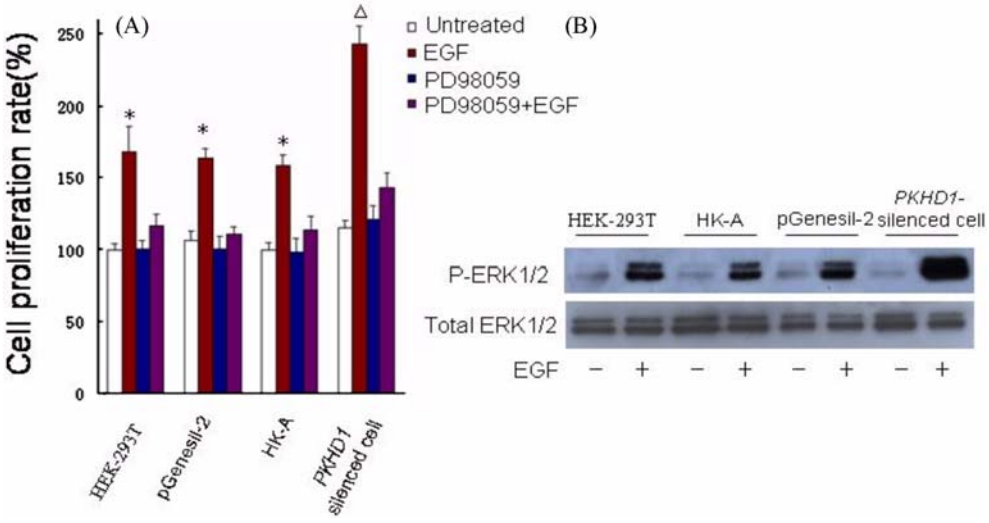

Fig. 3. Proliferation response of *PKHD1*-silenced HEK-293T cells to the EGF stimulation. (A) Four cell groups were treated with EGF (0 ng/ml), EGF (20 ng/ml), PD98059 (20 M), EGF (20 ng/ml) + PD98059 (20 M) respectively for 24 h. Cell proliferation rates were determined by the MTT assay. One asterisk indicates a significant difference at $p < 0.01$ versus control without EGF treatment; triangle indicate $p < 0.01$ versus the HEK-293T cells with EGF treatment. (B) EGF induced ERK1/2 phosphorylation in *PKHD1*-silenced HEK-293T cells. All cell groups treated with EGF show increased ERK1/2 phosphorylation with the highest effect on the *PKHD1*-silenced HEK-293T cells.

Hyperproliferative response of *PKHD1*-silenced cell lines to stimulation with EGF. A significant increase of proliferation rates in *PKHD1*-silenced cells was observed after the stimulation with EGF at concentrations of 20 ng/ml, and the proliferation rates of control groups were similar to those of wild-type cells (Fig. 3A). It has been reported that the EGF treatment can induce proliferation by activating ERK1/2 in many cell types (Falin *et al.*, 2005; Liu *et al.*, 2006; Zhuang and Schnellmann, 2004; Yamamoto *et al.*, 2003). In our study, indeed, the EGF treatment increased the level of ERK1/2 phosphorylation in all cell groups with the highest effect in *PKHD1*-silenced cell lines. And PD98059, an inhibitor for ERK1/2 activation, showed inhibitory effect on EGF-induced cell proliferation in all the four cell groups, (Fig. 3B). Thus the results suggest that the hyperproliferative effect of EGF is mediated by ERK1/2 in *PKHD1*-silenced cells.

Table 2. Intracellular Ca^{2+} Concentration in the silenced HEK-293T Cells and controls

Groups	Intracellular Ca^{2+} Concentration
HEK-293T	82.1 ± 7.1 nM
pGenesil-2	80.7 ± 5.5 nM$^{\Delta}$
HK-A shRNA	81.2 ± 5.3 nM$^{\Delta}$
PKHD1 shRNA	62.4 ± 7.7 nM*

*: $p < 0.001$ versus the HEK-293T cells. Δ: $p > 0.05$ versus the HEK-293T cells.

***PKHD1*-silencing decreases the intracellular Ca^{2+} concentration in HEK 293T cells.** Since fibrocystin interacts with CAML, a protein that is involved in Ca^{2+} signaling, an altered basal Ca^{2+} level may be expected in *PKHD1*-silenced cells. In our experiments, the intracellular Ca^{2+} concentration of them was significantly lower than that of wild-type HEK-293T cells ($p < 0.001$), but no significant difference between control groups and wild-type cells was observed (Table 2).The results suggest that fibrocystin participates in modulating intracellular Ca^{2+} in epithelial cells of autosomal recessive polycystic kidney.

EGF-induced cell excessive proliferation is caused by decreased intracellular Ca^{2+} concentration. To determine whether EGF-induced excessive cell proliferation was caused by change of calcium level, HEK-293T cells were incubated in 1 μM verapamil for 0, 1, 2, 4, 8, 16, 24 h. Then the cells were loaded with Fluo-3/AM and the intracellular calcium was measured. As shown in Fig. 4A, verapamil treatment caused a stable decrease of intracellular calcium after 1 h and this was dose-dependent (Fig. 4B). When cells were treated with verapamil for 8 h, and then incubated in medium with 20 ng EGF for 24 h, the cell proliferation rates were significantly increased (Fig. 4C). Also, the verapamil treatment increased the level of activated ERK1/2 in EGF-treated cells (Fig. 4D).The results imply that decrease of the intracellular Ca^{2+} concentration in wild-type cells by a calcium channel blocker was sufficient for EGF-induced cell overproliferation through stimulating ERK1/2 overactivation.

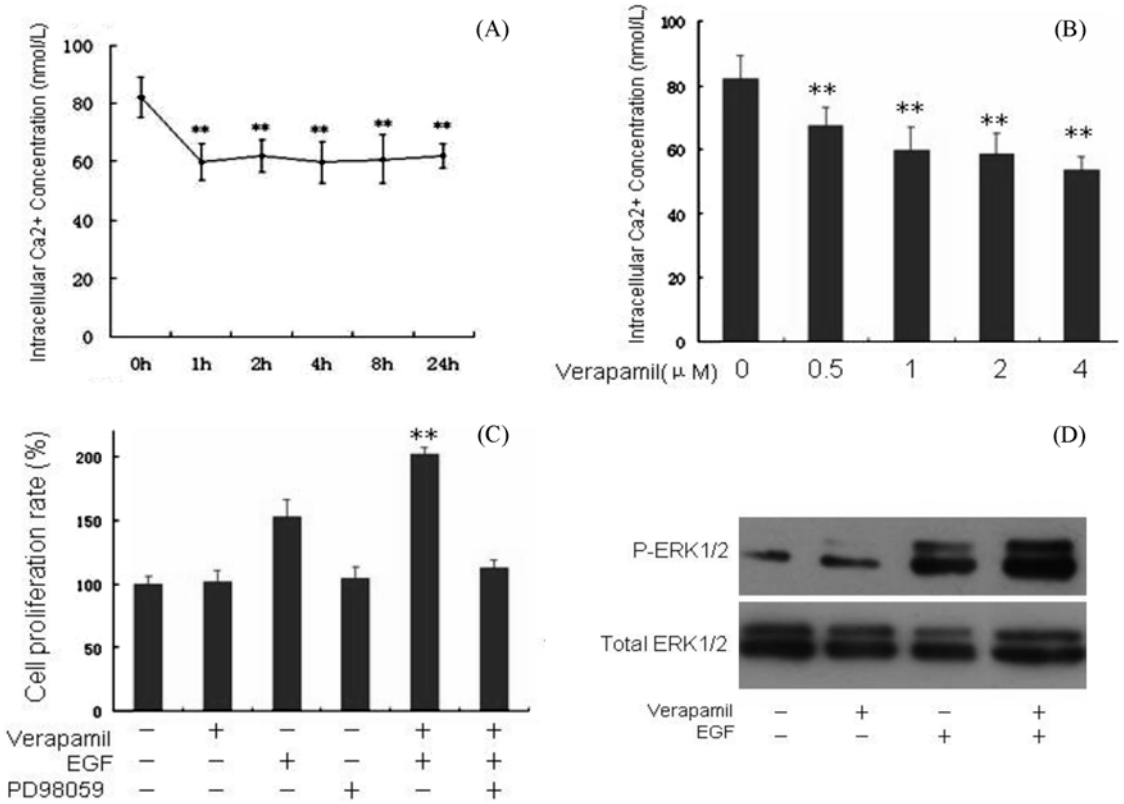

Fig. 4. Decreased intracellular Ca^{2+} concentration caused EGF-induced cell hyperproliferation. (A) Verapamil decreases the intracellular Ca^{2+} concentration of HEK-293T cells after 1 h. Intracellular Ca^{2+} concentration was measured after cells were incubated in 1 μM verapamil for 0, 1, 2, 4, 8, 24 h. (B) Verapamil decreases intracellular Ca^{2+} concentration of HEK-293T cells in dose-dependent. Intracellular Ca^{2+} concentration was measured after incubation in 0, 0.5, 1, 2, 4 μM verapamil for 4 h. (C) Verapamil increases EGF-induced cell proliferation. After cells treated with 1 μM verapamil and PD98059 for 4 h, they were incubated in medium with 20 ng EGF for 24 h and the cell proliferation rates were measured. Corresponding control groups were set up. Two asterisk indicates a significant difference at $p < 0.01$ versus HEK-293T with EGF treatment. (D) Verapamil increases EGF-induced ERK1/2 activation. Cells were treated with 1 μM verapamil and PD98059 for 4 h. Then they were incubated in medium with 20 ng EGF for 1 h. Corresponding control groups were set up.

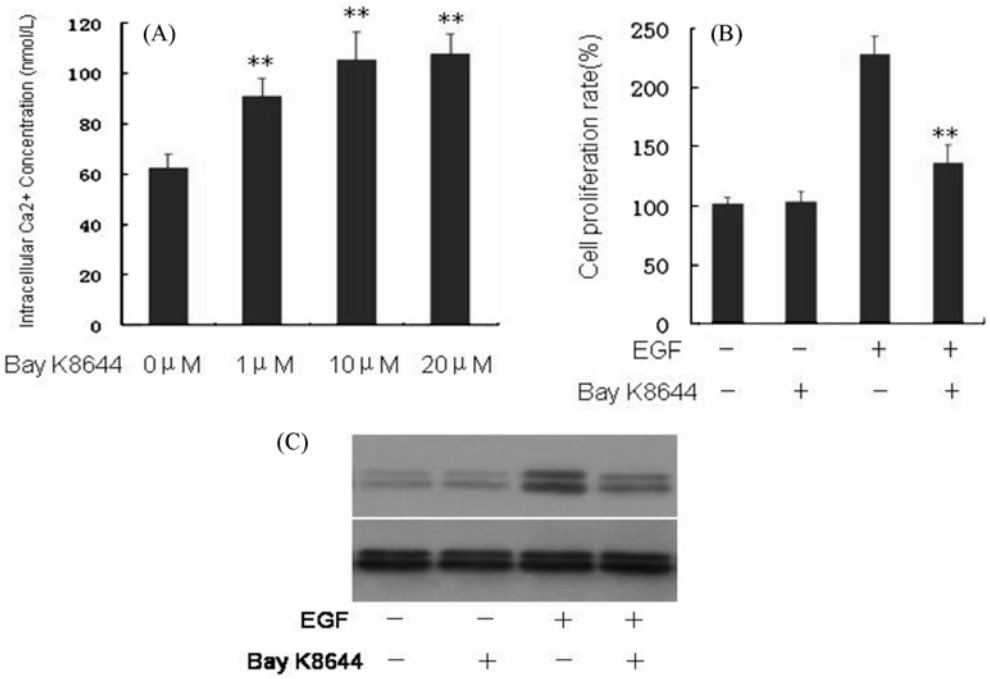

Fig. 5. Elevation in intracellular Ca^{2+} inhibits EGF-induced excessive proliferation of *PKHD1*-silenced HEK-293T Cell. (A) Bay K8644 increases intracellular Ca^{2+} concentration of HEK-293T cells in dose-dependent. The cells were incubated in 0, 1, 10, 20 μM Bay K8644 for 4 h and then intracellular Ca^{2+} concentration was measured. (B) Bay K8644 significantly decreases EGF-induced proliferation in *PKHD1*-silenced cells. Cells were treated with 1 μM Bay K8644 for 4 h and then incubated in medium with 20ng EGF for 24 h. The proliferation rates of the cells and controls were shown. Two asterisk indicates a significant difference at $p < 0.001$ versus the cells with EGF treatment. (C) Bay K8644 repressed EGF-induced ERK1/2 activation. Cells were treated with Bay K8644 for 4 h and then incubated in medium with 20 ng EGF for 1 h. Level of phosphorylated ERK1/2 was measured.

Elevation of intracellular Ca^{2+} inhibits the EGF-dependent excessive proliferation of *PKHD1*-silenced cells. If the mechanism of proliferation by EGF stimulation in *PKHD1*-silenced cells is a consequence of disturbed intracellular Ca^{2+} homeostasis, then the elevation of intracellular Ca^{2+} should inhibit the EGF-dependent excessive proliferation of *PKHD1*-silenced cells. To prove this, we measured the proliferation rates of *PKHD1*-silenced cells treated in EGF alone or in combination with Bay K8644, activator of L-type Ca^{2+} channels. As the results, a dose-dependent increase of intracellular Ca^{2+} was observed in *PKHD1*-silenced cells treated by Bay K8644 (Fig. 5A).The increase of intracellular Ca^{2+} by Bay K8644 showed no effect on the basal proliferation rates but repressed the proliferative response to EGF (Fig. 5B). Thus, the elevation of intracellular Ca^{2+} can repress EGF-dependent excessive proliferation of *PKHD1*-silenced cells.

To explore the relationship between intracellular Ca^{2+} and EGF-induced ERK1/2 activation, level of phosphorlated ERK were measured after Bay K8644 treatment. As the result, ERK1/2 overactivation was repressed by Bay K8644 (Fig. 5C). This suggests that elevation of intracellular Ca^{2+} can inhibit the EGF-dependent excessive proliferation of *PKHD1*-silenced cells though repressing ERK1/2 activation.

Discussion

Fibrocystin is deduced to be a putative membrane receptor-like protein that may involve in ligand-binding, cell-cell, and cell-matrix interactions (Ward *et al.*, 2002; Onuchic *et al.*, 2002). To date, however there have been few studies on the function of the large complex protein. Present study aimed to investigate the effect of fibrocystin on regulation of intracellular Ca^{2+} and EGF-induced MAPK signaling. It was found that disturbed intracellular Ca^{2+} homeostasis due to knock-down of *PKHD1* caused EGF-induced cell overproliferation in the *PKHD1*-silenced cell though increasing ERK1/2 activation.

Fibrocystin is localized to the cilium of renal epithelial cells which plays a role of sensor of mechanical and chemical stimulation from the lumen of renal tubules (Ward *et al.*, 2003). For instance, *in vitro* bending the cilium initiates a transient increase in intracellular Ca^{2+} and loss of cilia abolishes the response of the renal epithelium to fluid flow (Praetorius and Spring, 2001; Praetorius and Spring, 2003). *In vivo*, mutation of the *Tg737*, the gene encoding cilia-associated proteins, leads to attenuation of mechano-regulation of intracellular Ca^{2+} (Liu *et al.*, 2005). Apparently, the misregulation of intracellular Ca^{2+} plays a key role in the pathogenesis of

ARPKD. In the distal nephron, bile ducts, and pancreatic ducts, fibrocystin interacts with CAML which involves in upregulation of intracellular Ca^{2+} concentration (Nagano *et al.*, 2005). Such an increase can modulate cell signaling to activate protein kinase cascades, gene expression, and protein targeting, which eventually change the cellular proliferation, apoptosis, and differentiation in many cell types (Tovey *et al.*, 2001; Mellstrom *et al.*, 2004; Mellstrom and Naranjo, 2001). In ARPKD patients, significant lower basic Ca^{2+} concentration was observed in cystic cells compared with normal kidney cell (Yamaguchi *et al.*, 2006). In present study, we found that inhibition of *PKHD1* by RNA interference led to lowered intracellular Ca^{2+} concentration in the HEK-293T cells.

Proliferation and differentiation of epithelial cell are regulated by various cytokines and growth factors including EGF. EGF induces cell proliferation in a variety of cell types by binding to a prototype transmembrane tyrosine kinase receptor to activate ERK1/2 (Kato *et al.*, 1998). The EGF/EGFR axis overactivity is a feature of cystic tubules in both dominant and recessive polycystic kidney disease (Kato *et al.*, 1998). And inhibition of EGFR slows down the disease progression in animal with ARPKD (Sweeney *et al.*, 2000; Sweeney, Jr. *et al.*, 2003). Treatment with cyst fluid increased the proliferation of cells from cystic collecting tubule. Meanwhile, cyst fluid deprived of EGF decreased the proliferate rate (MacRae *et al.*, 2004). It has been reported that phosphorylated ERK1/2 of cystic kidneys is much higher than that of noncystic kidney (Veizis and Cotton, 2005). These imply that EGF and ERK1/2 play an important role in the development of the cystic formation. In our study, *PKHD1*-silencing caused hyperproliferation and ERK1/2 overactivity in HEK-293T cell treated with EGF.

Our experiment also showed that the EGF-induced proliferation and ERK1/2 activation were increased in HEK-293T cells with Ca^{2+} channel blocker pre-treatment. In contrast, elevation of intracellular Ca^{2+} by Ca^{2+} channel agonist reversed the hyperproliferative and ERK1/2 overactive response to EGF in *PKHD1*-silenced cells. Combined together, these data emphasize the role of Ca^{2+} regulation in pathogenesis of ARPKD and support that the fibrocystin may act as a membrane receptor participating in Ca^{2+} signaling. Thus, it may be postulated that the lowered intracellular Ca^{2+} leads to EGF-induced hyperproliferation in cells with silenced *PKHD1* and the loss of fibrocystin by the gene mutation causes hyperproliferation of kidney epithelial cells and cyst formation through change of intracellular Ca^{2+} concentration in ARPKD.

Acknowledgments This study was supported by National Basic Research Program of China (Grant No. 2004CB518805), National Natural Science Foundation of China (Grant No. 30470960 and 30371491) and the China Medical Board Foundation of New York.

References

Bergmann, C., Senderek, J., Kupper, F., Schneider, F., Dornia, C., Windelen, E., Eggermann, T., Rudnik-Schoneborn, S., Kirfel, J., Furu, L., Onuchic, L. F., Rossetti, S., Harris, P. C., Somlo, S., Guay-Woodford, L., Germino, G. G., Moser, M., Buttner, R. and Zerres, K. (2004) PKHD1 mutations in autosomal recessive polycystic kidney disease (ARPKD). *Hum. Mutat.* **23**, 453-463.

Bergmann, C., Senderek, J., Windelen, E., Kupper, F., Middeldorf, I., Schneider, F., Dornia, C., Rudnik-Schoneborn, S., Konrad, M., Schmitt, C. P., Seeman, T., Neuhaus, T. J., Vester, U., Kirfel, J., Buttner, R. and Zerres, K. (2005) Clinical consequences of PKHD1 mutations in 164 patients with autosomal-recessive polycystic kidney disease (ARPKD). *Kidney Int.* **67**, 829-848.

Falin, R., Veizis, I. E. and Cotton, C. U. (2005) A role for ERK1/2 in EGF- and ATP-dependent regulation of amiloride-sensitive sodium absorption. *Am. J. Physiol Cell Physiol.* **288**, 1003-1011.

Fonck, C., Chauveau, D., Gagnadoux, M. F., Pirson, Y. and Grunfeld, J. P. (2001) Autosomal recessive polycystic kidney disease in adulthood. *Nephrol. Dial. Transplant.* **16**, 1648-1652.

Kandilci, A. and Grosveld, G. C., (2005) SET-induced calcium signaling and MAPK/ERK pathway activation mediate dendritic cell-like differentiation of U937 cells. *Leukemia.* **19**, 1439-1445.

Kato, Y., Tapping, R. I., Huang, S., Watson, M. H., Ulevitch, R. J. and Lee, J. D. (1998) Bmk1/Erk5 is required for cell proliferation induced by epidermal growth factor. *Nature* **395**, 713-716.

Kupzig, S., Walker, S. A. and Cullen, P. J. (2005) The frequencies of calcium oscillations are optimized for efficient calcium-mediated activation of Ras and the ERK/MAPK cascade. *Proc. Natl. Acad. Sci. USA* **102**, 7577-7582.

Li, D. W., Liu, J. P., Mao, Y. W., Xiang, H., Wang, J., Ma, W. Y., Dong, Z., Pike, H. M., Brown, R. E. and Reed, J. C. (2005) Calcium-activated RAF/MEK/ERK signaling pathway mediates p53-dependent apoptosis and is abrogated by alpha B-crystallin through inhibition of RAS activation. *Mol. Biol. Cell.* **16**, 4437-4453.

Liu, L., Xie, Y. and Lou, L. (2006) PI3K is required for insulin-stimulated but not EGF-stimulated ERK1/2 activation. *Eur. J. Cell Biol.* **85**, 367-374.

Liu, W., Murcia, N. S., Duan, Y., Weinbaum, S., Yoder, B. K., Schwiebert, E. and Satlin, L. M. (2005) Mechanoregulation of intracellular Ca^{2+} concentration is attenuated in collecting duct of monocilium-impaired orpk mice. *Am. J. Physiol Renal Physiol.* **289**, F978-F988.

MacRae, D. K., Nemo, R., Sweeney, W. E. and Jr. and Avner, E. D. (2004) EGF-related growth factors in the pathogenesis of murine ARPKD. *Kidney Int.* **65**, 2018-2029.

Mellstrom, B. and Naranjo, J. R. (2001) Mechanisms of Ca(2+)-dependent transcription. *Curr. Opin. Neurobiol.* **11**, 312-319.

Mellstrom, B., Torres, B., Link, W. A. and Naranjo, J. R. (2004) The BDNF gene: exemplifying complexity in Ca^{2+}-dependent gene expression. *Crit. Rev. Neurobiol.* **16**, 43-49.

Nagano, J., Kitamura, K., Hujer, K. M., Ward, C. J., Bram, R. J.,

Hopfer, U., Tomita, K., Huang, C. and Miller, R. T. (2005) Fibrocystin interacts with CAML, a protein involved in Ca^{2+} signaling. *Biochem. Biophys. Res. Commun.* **338**, 880-889.

Nagasawa, Y., Matthiesen, S., Onuchic, L. F., Hou, X., Bergmann, C., Esquivel, E., Senderek, J., Ren, Z., Zeltner, R., Furu, L., Avner, E., Moser, M., Somlo, S., Guay-Woodford, L., Buttner, R., Zerres, K. and Germino, G. G. (2002) Identification and characterization of Pkhd1, the mouse orthologue of the human ARPKD gene. *J. Am. Soc. Nephrol.* **13**, 2246-2258.

Onuchic, L. F., Furu, L., Nagasawa, Y., Hou, X., Eggermann, T., Ren, Z., Bergmann, C., Senderek, J., Esquivel, E., Zeltner, R., Rudnik-Schoneborn, S., Mrug, M., Sweeney, W., Avner, E. D., Zerres, K., Guay-Woodford, L. M., Somlo, S. and Germino, G. G. (2002) PKHD1, the polycystic kidney and hepatic disease 1 gene, encodes a novel large protein containing multiple immunoglobulin-like plexin-transcription-factor domains and parallel beta-helix 1 repeats. *Am. J. Hum. Genet.* **70**, 1305-1317.

Praetorius, H. A. and Spring, K. R. (2001) Bending the MDCK cell primary cilium increases intracellular calcium. *J. Membr. Biol.* **184**, 71-79.

Praetorius, H. A. and Spring, K. R. (2003) Removal of the MDCK cell primary cilium abolishes flow sensing. *J. Membr. Biol.* **191**, 69-76.

Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W. S. and Khvorova, A. (2004) Rational siRNA design for RNA interference. *Nat. Biotechnol.* **22**, 326-330.

Richards, W. G., Sweeney, W. E., Yoder, B. K., Wilkinson, J. E., Woychik, R. P. and Avner, E. D. (1998) Epidermal growth factor receptor activity mediates renal cyst formation in polycystic kidney disease. *J. Clin. Invest.* **101**, 935-939.

Roy, S., Dillon, M. J., Trompeter, R. S. and Barratt, T. M. (1997) Autosomal recessive polycystic kidney disease: long-term outcome of neonatal survivors. *Pediatr. Nephrol.* **11**, 302-306.

Sairanen, H., Jalanko, H., Hockerstedt, K., Salmela, K., Holmberg, C. and Leijala, M. (1997) Organ transplantation in children. *Ann. Chir. Gynaecol.* **86**, 141-148.

Siroky, B. J., Ferguson, W. B., Fuson, A. L., Xie, Y., Fintha, A., Komlosi, P., Yoder, B. K., Schwiebert, E. M., Guay-Woodford, L. M. and Bell, P. D. (2006) Loss of primary cilia results in deregulated and unabated apical calcium entry in ARPKD collecting duct cells. *Am. J. Physiol. Renal. Physiol.* **290**, 1320-1328.

Sweeney, W. E., Chen, Y., Nakanishi, K., Frost, P. and Avner, E. D. (2000) Treatment of polycystic kidney disease with a novel tyrosine kinase inhibitor. *Kidney Int.* **57**, 33-40.

Sweeney, W. E., Jr., Hamahira, K., Sweeney, J., Garcia-Gatrell, M., Frost, P. and Avner, E. D. (2003) Combination treatment of PKD utilizing dual inhibition of EGF-receptor activity and ligand bioavailability. *Kidney Int.* **64**, 1310-1319.

Tovey, S. C., de, S. P., Lipp, P., Thomas, D., Young, K. W., Missiaen, L., De, S. H., Parys, J. B., Berridge, M. J., Thuring, J., Holmes, A. and Bootman, M. D. (2001) Calcium puffs are generic InsP(3)-activated elementary calcium signals and are downregulated by prolonged hormonal stimulation to inhibit cellular calcium responses. *J. Cell Sci.* **114**, 3979-3989.

Veizis, I. E. and Cotton, C. U. (2005) Abnormal EGF-dependent regulation of sodium absorption in ARPKD collecting duct cells. *Am. J. Physiol Renal Physiol.* **288**, F474-F482.

Ward, C. J., Hogan, M. C., Rossetti, S., Walker, D., Sneddon, T., Wang, X., Kubly, V., Cunningham, J. M., Bacallao, R., Ishibashi, M., Milliner, D. S., Torres, V. E. and Harris, P. C. (2002) The gene mutated in autosomal recessive polycystic kidney disease encodes a large, receptor-like protein. *Nat. Genet.* **30**, 259-269.

Ward, C. J., Yuan, D., Masyuk, T. V., Wang, X., Punyashthiti, R., Whelan, S., Bacallao, R., Torra, R., LaRusso, N. F., Torres, V. E. and Harris, P. C. (2003) Cellular and subcellular localization of the ARPKD protein; fibrocystin is expressed on primary cilia. *Hum. Mol. Genet.* **12**, 2703-2710.

Xiong, H., Chen, Y., Yi, Y., Tsuchiya, K., Moeckel, G., Cheung, J., Liang, D., Tham, K., Xu, X., Chen, X. Z., Pei, Y., Zhao, Z. J. and Wu, G. (2002) A novel gene encoding a TIG multiple domain protein is a positional candidate for autosomal recessive polycystic kidney disease. *Genomics.* **80**, 96-104.

Yamaguchi, T., Hempson, S. J., Reif, G. A., Hedge, A. M. and Wallace, D. P. (2006) Calcium restores a normal proliferation phenotype in human polycystic kidney disease epithelial cells. *J. Am. Soc. Nephrol.* **17**, 178-187.

Yamamoto, T., Cui, X. M. and Shuler, C. F. (2003) Role of ERK1/2 signaling during EGF-induced inhibition of palatal fusion. *Dev. Biol.* **260**, 512-521.

Zerres, K., Mucher, G., Bachner, L., Deschennes, G., Eggermann, T., Kaariainen, H., Knapp, M., Lennert, T., Misselwitz, J. and von Muhlendahl, K. E. (1994) Mapping of the gene for autosomal recessive polycystic kidney disease (ARPKD) to chromosome 6p21-cen. *Nat. Genet.* **7**, 429-432.

Zerres, K., Rudnik-Schoneborn, S., Deget, F., Holtkamp, U., Brodehl, J., Geisert, J. and Scharer, K. (1996) Autosomal recessive polycystic kidney disease in 115 children: clinical presentation, course and influence of gender. Arbeitsgemeinschaft fur Padiatrische, Nephrologie. *Acta Paediatr.* **85**, 437-445.

Zerres, K., Rudnik-Schoneborn, S., Steinkamm, C., Becker, J. and Mucher, G. (1998) Autosomal recessive polycystic kidney disease. *J. Mol. Med.* **76**, 303-309.

Zhang, M. Z., Mai, W., Li, C., Cho, S. Y., Hao, C., Moeckel, G., Zhao, R., Kim, I., Wang, J., Xiong, H., Wang, H., Sato, Y., Wu, Y., Nakanuma, Y., Lilova, M., Pei, Y., Harris, R. C., Li, S., Coffey, R. J., Sun, L., Wu, D., Chen, X. Z., Breyer, M. D., Zhao, Z. J., McKanna, J. A. and Wu, G. (2004) PKHD1 protein encoded by the gene for autosomal recessive polycystic kidney disease associates with basal bodies and primary cilia in renal epithelial cells. *Proc. Natl. Acad. Sci. USA* **101**, 2311-2316.

Zhuang, S. and Schnellmann, R. G. (2004) H_2O_2-induced transactivation of EGF receptor requires Src and mediates ERK1/2, but not Akt, activation in renal cells. *Am. J. Physiol. Renal. Physiol.* **286**, 858-865.