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# MicroRNA-17 post-transcriptionally regulates polycystic kidney disease-2 gene and promotes cell proliferation

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**Abstract** To identify the possible microRNAs (miRNAs) which target the polycystic kidney disease-2 gene (PKD2), and clarify effects of the miRNAs on PKD2. We preliminarily used bioinformatics to analyze 3′UTR (3′untranslated regions) of PKD1 and PKD2 in order to predict the potential microRNAs targeted on them. Subsequently, the stable cell lines with overexpression of microRNA-17 (miR-17) were screened, and luciferase assay combined with the mutation 3′UTR of PKD2 were performed to verify PKD2 is the target of miR-17. Moreover, RT-PCR and Western Blotting were used to determine the post-transcriptionally regulation of PKD2 by miR-17. Finally, MTT cell assays allied with PKD2 rescued strategy were employed to evaluate cell proliferation effects. Our study firstly found that the 3′UTR of PKD2 was more conservation than that of PKD1, and microRNA-17 directly targets the 3′UTR of PKD2 and post-transcriptionally repress the expression of PKD2. Moreover, our findings also demonstrated that overexpression of miR-17 may promote cell proliferation via post-transcriptionally repression of PKD2 in HEK 293T. This suggested that microRNA might be a novel mechanism for cystogenesis as well as a potential therapeutic target for the cell proliferation of autosomal dominant polycystic kidney disease (ADPKD).

**Keywords** microRNA · miR-17 · PKD2 · Polycystin-2 · Cell proliferation

## Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common inherited renal cystic disorders (incidence 1:600–1:1000), characterized primarily by the progressive enlargement of numerous fluid-filled cysts in kidney that destroy the kidney architecture ultimately resulting in renal failure [1, 2]. Genetically, mutations in the polycystic kidney disease-1 gene (PKD1) account for 85% of ADPKD, whereas mutations in polycystic kidney disease-2 gene (PKD2) are responsible for the remainder [3]. PKD2 encodes an integral protein, termed polycystin-2 (PC-2), which is composed of two cytoplasmic termini and six membrane-spanning domains [4]. Due to the pathogenesis of ADPKD is largely unknown, there is still lack of available treatments for prevention ADPKD from kidney failure [5].

Aberrant expression of PC-2 cause abnormal proliferation in renal tubular and biliary epithelial cells, eventually to cystogenesis [6, 7]. Several molecular mechanisms have been proved to be involved in the cell proliferation of ADPKD, however, the global mechanism remains elusive [8]. Recently, it is interesting to note that PKD2-D511V mutation is sufficient to cause cell proliferation in ADPKD at the mRNA level rather than loss of channel activity or protein–protein interactions [9], supporting the hypothesis

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that there may exist post-transcriptional mechanisms in the cell proliferation of ADPKD. Of all post-transcriptional mechanisms such as traditional mRNA processing (5′ capping, 3′ polyadenylation, splicing, and so on) and novel silence mechanisms of microRNA (miRNA) and small interfering RNA (siRNA), microRNAs are the currently popular hotspot [10, 11]. MicroRNAs belong to a newly found class of endogenous short single-stranded RNA molecules containing approximately 21–23 nucleotides in length, which are competent to suppress mRNA translation or mediate mRNA degradation by typically targeting the 3′untranslated regions (3′UTR) of the mRNA [12]. MicroRNAs play essential roles in development, differentiation, cell proliferation, apoptosis, and metabolism [13]. Aberrant expression of microRNAs may lead to cell proliferation, although, relatively little is known about the roles of microRNAs in cell proliferation [14].

Of microRNAs, microRNA-17 (miR-17) is capable to target E2F1, NCOA3 as well as RBL2, and promotes cell proliferation through post-transcriptionally modulating these genes [15]. Preliminary bioinformatics analysis showed that there exist two putative 7mer seed sites of miR-17 in 3′UTR of PKD2; moreover, the two-targeted regions are highly conservative in evolution across mammals. Since epithelial cell proliferation is an invariable component of cystogenesis in ADPKD [16]. Hence, we wondered whether miR-17 is involved in the cell proliferation of ADPKD.

Here, we demonstrated that miR-17 directly targets the 3′UTR of PKD2 and post-transcriptionally represses the expression of PKD2, which leads to cell proliferation. It was for the first time to validate PKD2 which one of the major ADPKD causing genes is targeted by the specific microRNA, miR-17. Furthermore, our findings suggested that microRNAs might be novel mechanisms as well as potential therapeutic targets for the cell proliferation of ADPKD.

## Materials and methods

### Plasmid construction

To overexpress miR-17 gene in HEK 293T cells, the 297 bp genomic fragment that encodes miR-17 flanked up-stream by 92 nt and down-stream by 121 nt was cloned by PCR (miR-17 Forward Primer: 5′-TGTTAGAGTTTGAGGTGT TAATTC-3′; miR-17 Reverse Primer: 5′-CACTTAGGGC AGTAGATGCT-3′). The miR-17 gene was inserted into downstream of the cytomegalovirus (CMV) promoter in a pcDNA3.1 (+) (Invitrogen, Carlsbad, CA, USA) plasmid containing the neomycin resistance gene which render resistance of neomycin analogue G418. Plasmid pcDNA3.1-Luc was constructed from pcDNA3.1 (+) by inserting firefly luciferase gene cloned from pGL3-Basic vector (Promega, Madison, WI, USA). Reporter plasmids of target genes (pcDNA3.1-Luc-WT, pcDNA3.1-Luc-Mut) were constructed by inserting 3′UTR of each target into down-stream of luciferase gene in pcDNA3.1-Luc. A schematic diagram of the luciferase reporter constructs shows in Fig. 2a. The artificial synthesized 3′UTR of PKD2 cassette in pcDNA3.1-Luc was inserted into pcDNA3.1 (+) as Fig. 4a demonstrated. The sequence of 3′UTR oligonucleotides are as follow: 3′UTR of PKD2 wild type (WT): GATTGCTAATCTTCTGCACTTTAATTTAGATTGCTA ATCTTCTGCACTTTAATTTA and 3′UTR of PKD2 mutation type (Mut): GATTGCTAATCTTCTGACTCTTA ATTTAGATTGCTAATCTTCTGACTCTTAATTTA (the underlined letters indicate the mutation sites).

### Cell culture and transfections

HEK 293T cells were cultivated in DMEM (Gibico, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Gibico, Carlsbad, CA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells cultivated at a humidified atmosphere of 5% $CO_2$ at 37°C. All transfections were used Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. HEK 293T cells stably expressing pcDNA3.1-miR-17 and pcDNA3.1 were harvested by selection with 1000 μg/ml of G418 in DMEM supplemented with 10% FBS for weeks. Single-clones were isolated, expanded, and maintained in G418-containing medium for further experiments. Cell transfected pcDNA3.1-miR-17 and pcDNA3.1 empty vector were maintained in DMEM (10% FBS) with 500 μg/ml of G418 (Gibico, Carlsbad, CA, USA) [17].

### RT-PCR

Total RNA isolated from cells using Trizol (Invitrogen, Carlsbad, CA, USA). PKD2 was detected by RT-PCR using the sense primer 5-ACATCAAATGTGGAGGTGCT-3 and anti-sense primer 5′-TGATGTTCATGTTCGGTCAG-3′ to yield a 743-bp fragment corresponding to the coding region. RT-PCR was performed by AccessQuick™ RT-PCR Kit according to the manufacturer's instructions (Promega, Madison, WI, USA).

### Western blotting

After incubation, the cells were washed three times with 100 μl of ice-cold PBS buffer, cell lysised by RIPA Lysis Buffer (Beyotime, Jiangsu, China) immediately and placed on ice for 30 min. Total protein concentration of each

extract was determined via the bicin choninic acid (BCA) protein assay using the microplate procedure (Beyotime, Jiangsu, China). After adjusted the concentration of protein in samples and boiled at 100°C for 5 min, 30 μl of the supernatant was loaded into 8% SDS–PAGE gel and subsequently electrotransferred it to PVDF membrane (Millipore, Bedford, MA, USA). The membranes were blocked with 5% BSA in Tris-buffered saline (TBS)/Tween 20 for 1 h. Primary antibodies specifically against polycystin-2 (1:5000, Santa Cruz, CA, USA), or $\beta$ actin (1:5000, Santa Cruz, CA, USA) were incubated with the membranes overnight at 4°C in 5% BSA with TBS/Tween 20. Washed membranes and incubated for 1 h at room temperature with horseradish peroxidase-linked anti-mouse IgG (1:1000, Santa Cruz, CA, USA) which prepared in blocking solution. After washing, the Western Blot Luminol Reagent (Zhongshan Bio, Beijing, China) was applied for antibody detection with X-ray film [18].

### Luciferase assays

For luciferase assays, cells were plated $1 \times 10^4$ per well in a 24-well plate 24 h before transfection. The plasmids of pcDNA3.1-Luc-WT, pcDNA3.1-Luc-Mut, and pcDNA3.1-Luc cells were transfected respectively into HEK 293T pcDNA3.1-miR-17 and pcDNA3.1 empty vector stable expression cell lines using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). Seventy-two hours later, luciferase assays were performed by Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. All experiments were performed in triplicate and normalized to *Renilla* luciferase activity.

### The MTT assays

Cells were washed twice with PBS and treated with approximately 50 μl of the MTT (3-(4, 5-dimethyl-2-tiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) (Merck, Darmstadt, Germany) solution (0.5 mg/ml PBS). After mixing, they were incubated at 37°C for 4 h. The tubes containing the MTT-cell mixtures were centrifuged ($400 \times g$ for 10 min) to deposit the cells. The supernatant MTT solution was pipetted out and 150 μl dimethyl sulfoxide (DMSO) was then added to the colored cell deposits. After reacted for 5 min, 100 ml of the purple-blue colored supernatant that contained the dissolved formazan in each sample was transferred to a well in a 96-well plate and examined at 490 nm using an ELISA reader (Sunrise, Tecan, Austria). DMSO only group were used as a blank control.

### Statistical analysis

The results are presented as the mean ± standard error of the mean (SEM). Each assay performed in triplicate when appropriate. Statistical analyses were performed with the Prism 4 (GraphPad, San Diego, CA, USA). Data was analyzed by Student's t test and repeated measures analyses of variance or by one-way analysis of variance (ANOVA) with Tukey's post hoc comparisons. Values of $P < 0.05$ were considered statistically significant.

## Results

### Bioinformatics predicted microRNAs target PKD1 and PKD2

In order to explore whether there exists any potential microRNA associated with the ADPKD causing gene, we analyzed the 3′UTR of PKD1 and PKD2 via UC Santa Cruz Santa genome database (UCSC, genome.ucsc.edu). The data showed that the 3′UTR of PKD1 and PKD2 were 1016 and 2082 bp in length, respectively, and the 3′UTR of PKD2 was more conservation than that of PKD1 (Fig. 1). Therefore, we selected the PKD2 gene for further study. To identify the candidate microRNAs associated with PKD2, we utilized the microRNA database (www.microrna.org) to predict the microRNAs target PKD2 [19]. As a result, there were 85 seed sites that are precisely complementary to microRNA 2–8 positions in the 3′UTR of PKD2. Of the 85 seed sites, the oncogene miR-17 occupies two putative seed sites. After retrieved 3′UTR of *Homo sapiens*, *Mus musculus* and *Rattus norvegicus* from the Ensembl Database (www.ensembl.org), the alignments with miR-17 demonstrated in Fig. 2. Especially, in miR-17 target region, the seed site 1 is more highly conserved compared with the seed site 2.

### MiR-17 directly targeted PKD2 3′UTR

To examine whether PKD2 is directly targeted by miR-17 in vitro, two copy-seed-sites fragments of the 3′UTR of PKD2 containing wild type and mutated miR-17 complementary sites were inserted into the 3′UTR of a luciferase reporter gene (Fig. 3a). Three luciferase report plasmids were transfected into the HEK 293T pcDNA3.1-miR-17 and pcDNA3.1 stable cell lines, respectively. Among these, the pcDNA3.1-Luc acted as a positive control, and the pcDNA3.1-Luc-Mut served as a negative control, the luciferase assays were preformed in triplicate and the data were observed three times after transfected 48 h. For comparative purpose, reporter activities were normalized to

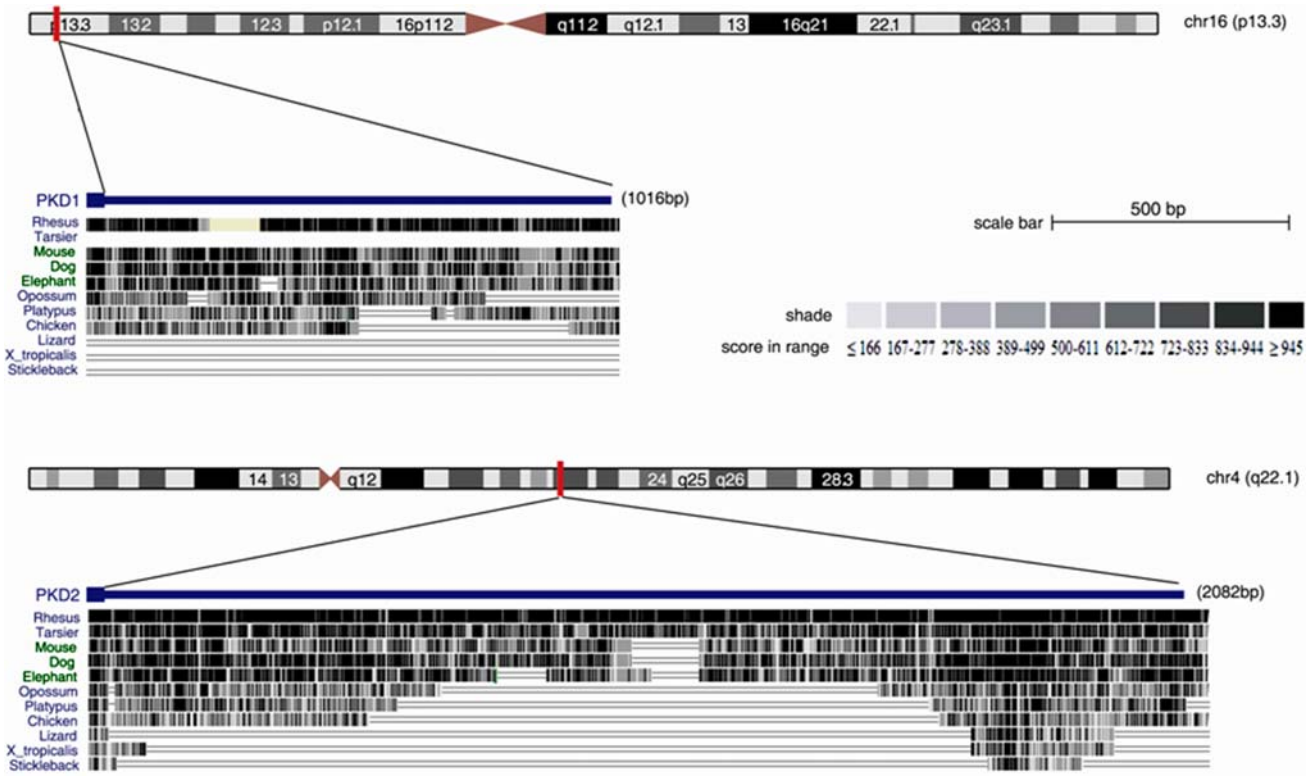


**Fig. 1** Bioinformatics analysis of the 3′UTR of the PKD1 and PKD2 via UC Santa Cruz Santa genome database. The figure showed that the 3′UTR of the PKD1 and PKD2 were 1016 and 2082 bp in length, respectively. Moreover, the 3′UTR of PKD2 was more conservation than that of PKD1. The chromosome tracks show the 3′UTR location of the PKD1 and PKD2, respectively. In the dense display tracks, conservation shown in grayscale using darker values to indicate higher levels of overall conservation as scored by phastCons. The scale bar indicates 500 bp

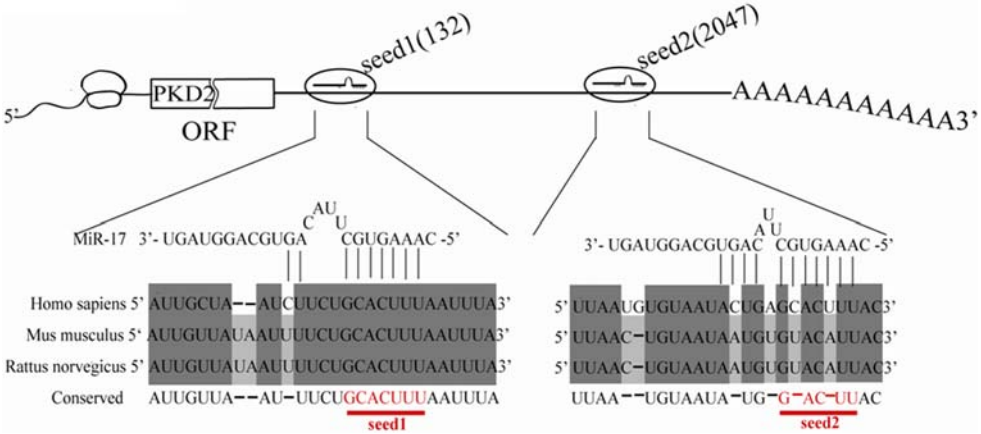


**Fig. 2** Schematic diagrams of the 3′UTR of PKD2 and the predicted binding sites for the miR-17. Upper panel shows two putative seed sites predicted on PKD2 and lower panel shows multiple-sequence alignment of miR-17-5p with the two binding sites on 3′UTR of PKD2 from *Homo sapiens, Mus musculus and Rattus norvegicus.* Part of the alignment shows here. Nucleotides with black boxes indicate those base sequences that appear in all three organisms. The frame Homo sapiens segment is the PKD2 genomic sequence from the human. The underlined bases reflect the location of predicted miR-17 seed sites and spaces (−) are added where needed to facilitate the alignment. The number in bracket indicates the location of miR-17 potential banding sites. The banding sites are located within highly conserved region

the HEK 293T cotransfected with pcDNA3.1-Luc-WT and pcDNA3.1. As showed in Fig. 3b, in HEK 293T cotransfected with miR17 precursor and PKD2 WT construct, luciferase reporter activities reduced by 31% compared with that in HEK 293T cotransfected with PKD2 WT and pcDNA3.1 construct. However, in HEK 293T cotransfected

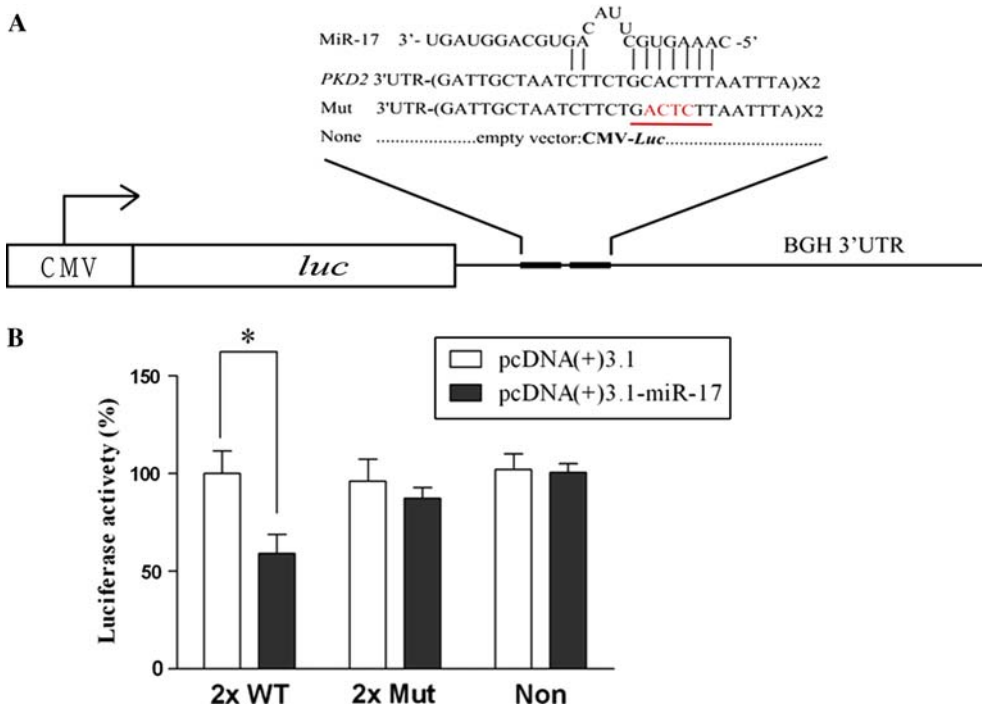


**Fig. 3** Evidence that the 3′UTR of PKD2 is targeted by miR-17. **a** Schematic diagram of luciferase assay strategy. Reporter constructs with two-copy-seed-sites fragments of the PKD2 3′UTR containing wild-type or mutated miR-17 complementary sites were generated. The underlined bases indicate the region of putative miR-17 seed sites and reflect mutation sites. **b** PKD2 WT and PKD2 Mut 3′UTR were tested in the luciferase assay. Luciferase activities measured and normalized to the level of control Renilla luciferase. Bars represent the mean ± SD from three independent experiments. *Statistical significant ($P < 0.05$). *WT* wild type 3′UTR seed; *Mut* mutation type 3′UTR seed; *Non* not insert 3′UTR seed sequence; *BGH* the 3′UTR of bovine growth hormone

with miR17 precursor and PKD2 mutant construct, expression of luciferase reporter was not blocked. We also did not observe any significant changes in expression of luciferase reporter in HEK 293T cotransfected with miR17 and pcDNA3.1-Luc without miR-17 target sites. These data suggested that miR17 may regulate expression of PKD2 protein through directly binding to the 3′UTR region of PKD2.

## MiR-17 post-transcriptionally regulated PKD2 expression

To further explore the effects of miR-17 directly target the 3′UTR of PKD2 on the expression of PKD2, we constructed miR-17 overexpression vector as described previously in material and methods. The structure of pcDNA3.1 (+)-miR-17 depicted in Fig. 4a. The CMV (cytomegalovirus) promoter drives the transcription of miR-17, and the BGH (bovine growth hormone) polyA tail was added in the end.

We extracted RNA and protein form miR-17 and pcDNA3.1 (+) stable HEK 293T cells. Although, as anticipated, miR-17 did not decrease the mRNA levels of PKD2 compared between miR-17 and pcDNA3.1 (+) stable expression cell lines (Fig. 4b), the protein level of PKD2 was slightly decreased (Fig. 4c), suggesting that miR-17 may post-transcriptionally regulate the PKD2.

## Expression of miR-17 promoted cell proliferation

To evaluate the effects of miR17 on cell proliferation, we established stable cell line (HEK 293T pcDNA3.1-miR17) with overexpressed miR17, and stable cell line (HEK 293T pcDNA3.1) as a negative control (Fig. 5a). The MTT assay results demonstrated in Fig. 5b, the Y-axis indicated the absorbance value. The absorbance value of HEK 293T pcDNA3.1-miR17 is $0.17 \pm 0.04$, and that of control is $0.14 \pm 0.01$. Student t test between both shows $P = 0.045 < 0.05$, and the difference is statistically significant when set the threshold value 0.05. This finding shows that overexpression of miR-17 may promotes cell proliferation in HEK29T.

## PKD2 rescued the proliferation induced by miR-17 in part

To further address the role of PC2 in miR-17-stimulated cell proliferation, we introduced PKD2 overexpression vector (pcDNA3.1-PKD2) into miR-17 overexpression stable cell line HEK 293T pcDNA3.1-miR17 (Fig. 5a).

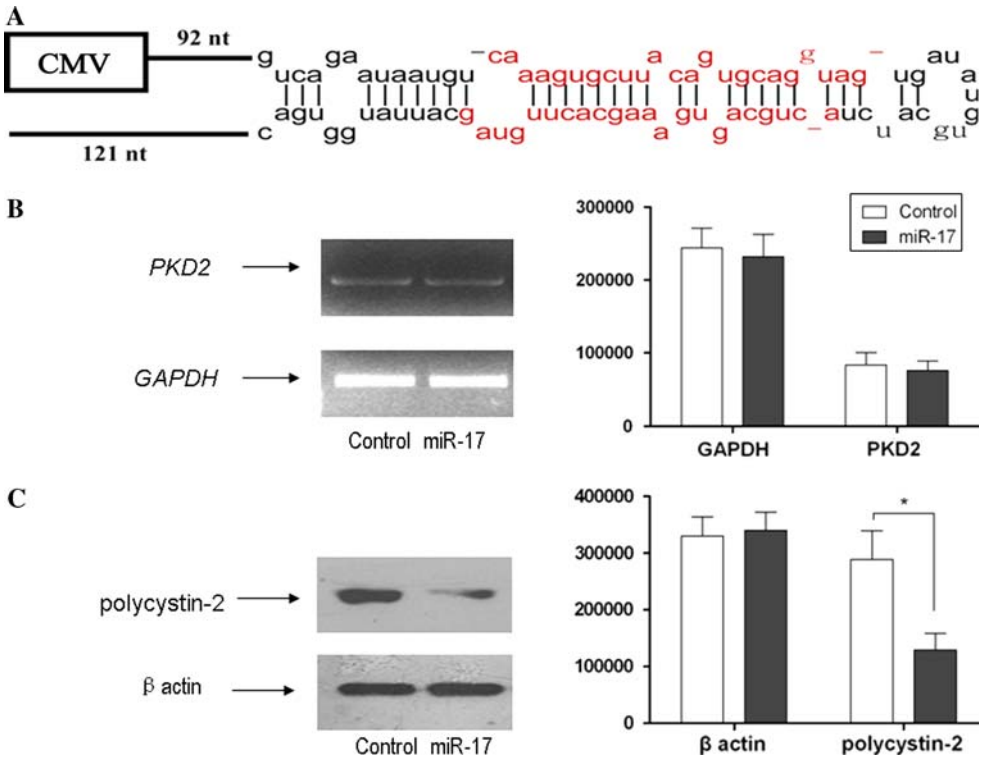


**Fig. 4** miR-17 post-transcriptionally represses PKD2. **a** The construct of miR-17 expression vector: the 297 bp genomic fragment that encodes pre-miR-17 flanked upstream by 92 nt and downstream by 121 nt. The cytomegalovirus (CMV) promoter drives miR-17 in a pcDNA3.1 (+) plasmid containing neo open reading frame (neomycin resistance gene). **b** Detection of PKD2 expression by RT-PCR. Endogenous PKD2 mRNA level was not reduced by miR-17 overexpression. Data were normalized to the GAPDH level. **c** Detection of polycystin-2 expression by western blotting, the protein level was reduced by miR-17 overexpression. Numbers above lanes represent relative levels normalized against $\beta$ actin

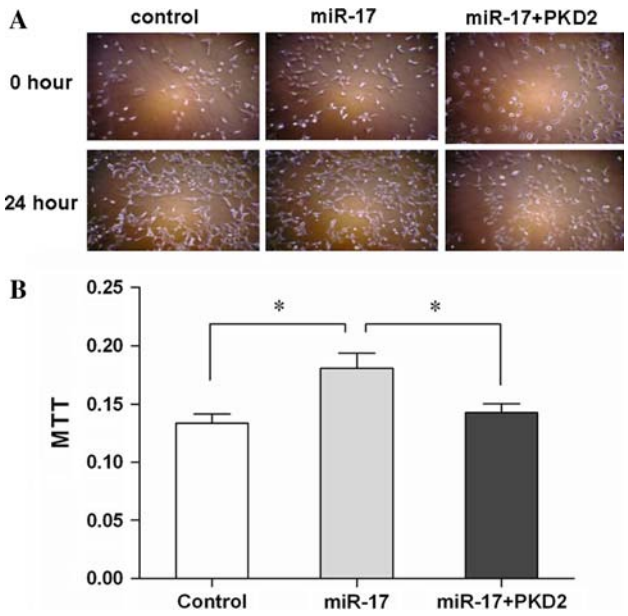


**Fig. 5** Expression of miR-17 promotes proliferation. The cell morphology was assessed by optical microscopy (×200, **a**). The cell proliferation was determined using a MTT test (**b**). The Y-axis represents the absorbance value. These experiments were repeated three independent times and similar results were obtained each time. Panels (**a**) and (**b**) come from two separate experiments. *Statistical significant ($P < 0.05$)

Applying MTT assay described as before, the MTT value of cotransfected PKD2 and miR-17 cells was 0.12 ± 0.02 and the control was 0.17 ± 0.04 (Fig. 5b). There is statistical significant by student t test between them. Taken together, these results suggested that miR-17 may promote cell proliferation, at least partially, through post-transcriptionally regulating PKD2 in HEK 293T.

## Discussion

MicroRNAs are small RNA molecules that widely post-transcriptionally regulate genes expression [20], and up to 30% of human protein coding genes may be regulated by microRNAs [21]. MicroRNAs are essential for divergent biological processes, including development, cell proliferation, differentiation, and apoptosis [22]. ADPKD, as a cell proliferation dysfunction disease, is genetically heterogeneous with two major genes: PKD1 (around 85% cases) and PKD2 (around 15% cases) [3]. So far, many researches focused on the interaction between PKD1 and PKD2, as well as the roles of them in ADPKD. Despite growing knowledge on microRNA biology, there is not any microRNA has been reported to be involved in ADPKD yet. Is there any microRNA-3′UTR target relationship associated with ADPKD, if so, what functions are these? These puzzles are worthy to explore.

To address these issues, we firstly performed a series of bioinformatics analysis to assess the relationships of microRNAs between 3′UTR of PKD1 and PKD2, respectively. According to the results from bioinformatics analysis of the evolutionary conservation of 3′UTR of

PKD1 and PKD2, we initially demonstrated that the 3′UTR length of PKD1 is shorter than that of PKD2 (1016 bp versus 2082 bp, respectively), moreover, there are highly conserved 3′UTR of PKD2 across mammals, whereas this is not the case for PKD1 [23]. Typically, the conservation of seed region in microRNA targets is one of the most prominent characteristics for microRNA [24]. Many microRNA-containing species across large evolutionary distances have the similar microRNAs sequences [25]. This suggested that mechanisms of microRNA are highly conserved. Based on the above-mentioned data, we supposed that the PKD2 would be more likely to be post-transcriptionally regulated by microRNAs. Thus, we for the first time postulated that PKD2 may have much more potential microRNA targeted sites than PKD1. Regardless of the mutations in PKD1 accounts for 85% while the mutations in PKD2 accounts for only 15% in ADPKD, we concentrated on microRNA associated with PKD2 instead of PKD1. Since the functions of microRNAs are mainly performed by complementarity, and now there are clearly defined patterns of complementarity to the 3′UTRs of their targeted transcripts [26]. The 'seed' region, which locates the position from 2 to 7 of microRNAs, is an essential specificity determinant of banding, and required for perfect complementarity [27]. So, we used these principles to predict the theoretical microRNA targets of the 3′UTR of PKD2, furthermore, analyzed the evolutionary conservation of these targeted regions among *Homo sapiens, Mus musculus* and *Rattus norvegicus* [28]. Of these candidate microRNAs, such as miR-20, miR-200c and miR-17, we especially selected the miR-17 which tightly targets on 3′UTR of PKD2 for the further investigation. Our data showed that 3′UTR of PKD2 possesses two putative targeted sites for binding miR-17; moreover, the targeted 3′UTR regions in PKD2 are highly conservative across mammals. These findings implied that miR-17 may potentially target PKD2 via the two putative seed sites.

However, the two theoretical candidate sites were required for further experimental proof [26]. In terms of microRNA genesis process and transcription mechanisms, there are two major ways to overexpress microRNAs: pri-microRNAs are droved by type II promoters or pre-microRNAs are droved by type III promoters [29]. In the present study, we applied type II promoter CMV to drive genomic pri-microRNA-17 to obtain highly expression of mature miR-17, and the pri-microRNA-17 was cloned from human genomic DNA by PCR. Subsequently, Luciferase activities assay confirmed that miR-17 directly targeted the predicted sites. In agreement with the well-established knowledge that perfect complementarity to seed sites is very pivotal for the functions of microRNA [30], our results showed that the 3′UTR of PKD2 containing point mutations in the seed region failed to repress the expression of luciferase, indicating that the target the seed sites indeed binds miR-17. This was consistent with our current understanding on microRNA-guided post-transcriptional regulation mechanisms based on the characteristics of the target recognition [31]. In seed region, a single mismatch would block binding, and even more others extensive match cannot compensate effects of the shorter seed pairing [32]. As for the majority of mammals, miRNA directly target and partly complementary with the 3′UTR of genes would post-transcriptional suppress the expression of target gene [31]. Similar to miR-17 which targets the known genes such as E2F1 [33], our data originally validated that miR-17 post-transcriptionally repressed the translation of PKD2. This means that the regulation of miR-17 may affect the expression of PKD2. Taken into account that aberrant of expression of PKD2 would result in ADPKD, we may infer that miR-17 may be involved in the pathogenesis of ADPKD. This may extend our knowledge of the expression regulations of ADPKD causing genes.

As well known, cell proliferation is one of the most predominant features of ADPKD [16, 34], we further examined the effect of miR-17 post-transcriptionally regulated PKD2 on cell proliferation. More recently, high levels of miR-17-92 have been shown to promote the cell proliferation and inhibits cell differentiation [35]. Furthermore, the overexpression of miR-17 may independently promote cell proliferation and regulate the cell cycle [36]. Therefore, we only focused on the cell proliferation. Our findings demonstrated that miR-17 may enable to promote cell proliferation and were coincidence with the previous investigation that ectopic expression of a single miR-17 is sufficient to drive a proliferative signal in HEK293T cells [33, 36]. Meanwhile, MTT allied with PKD2 rescue strategies confirmed that miR-17 may indeed modulate cell proliferation through post-transcriptionally regulating PKD2 in ADPKD.

In this study, we validated that miR-17 may be a first experimental proved miRNA which plays roles in cell proliferation of ADPKD and elucidated a new potential pathway could contribute to the cell proliferation of ADPKD. The new insights offer novel mechanisms for cystogenesis as well as potential therapeutic targets for the cell proliferation of ADPKD. Of course, miR-17 possesses many other target genes and other more functions. On the other hand, PKD2 has numerous unknown cell functions besides cell proliferation. Furthermore, It is worth to search new microRNAs target PKD1 or PKD2, and to explore novel cell functions for PKD2 and miR-17-PKD2 target relationship as well as the phenotype when knockdown the mir-17. What we plan to do is to generate the overexpression and knockdown miR-17 transgenic mouse for investigation in the functions of the miR-17 and the miR-17-PKD2 relationships in vivo.

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