

ORIGINAL RESEARCH ARTICLE

MicroRNA501-5p induces p53 proteasome degradation through the activation of the mTOR/MDM2 pathway in ADPKD cells

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Cell proliferation and apoptosis are typical hallmarks of autosomal dominant polycystic kidney disease (ADPKD) and cause the development of kidney cysts that lead to end-stage renal disease (ESRD). Many factors, impaired by polycystin complex loss of function, may promote these biological processes, including cAMP, mTOR, and EGFR signaling pathways. In addition, microRNAs (miRs) may also regulate the ADPKD related signaling network and their dysregulation contributes to disease progression. However, the role of miRs in ADPKD pathogenesis has not been fully understood, but also the function of p53 is quite obscure, especially its regulatory contribution on cell proliferation and apoptosis. Here, we describe for the first time that miR501-5p, upregulated in ADPKD cells and tissues, induces the activation of mTOR kinase by PTEN and TSC1 gene repression. The increased activity of mTOR kinase enhances the expression of E3 ubiquitin ligase MDM2 that in turn promotes p53 ubiquitination, leading to its degradation by proteasome machinery in a network involving p70S6K. Moreover, the overexpression of miR501-5p stimulates cell proliferation in kidney cells by the inhibition of p53 function in a mechanism driven by mTOR signaling. In fact, the downregulation of this miR as well as the pharmacological treatment with proteasome and mTOR inhibitors in ADPKD cells reduces cell growth by the activation of apoptosis. Consequently, the stimulation of cell death in ADPKD cells may occur through the inhibition of mTOR/MDM2 signaling and the restoring of p53 function. The data presented here confirm that the impaired mTOR signaling plays an important role in ADPKD.

KEYWORDS

ADPKD, MDM2 and proteasome, miR501-5p, mTOR signaling, p53

1 | INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is a common inherited renal pathology caused by mutations in either PKD1 or PKD2 genes that encode for polycystin-1 and polycystin-2, respectively (Rangan et al., 2005). This disease is characterized by the development and expansion of fluid-filled renal cysts that lead to destruction of renal parenchyma, ultimately resulting in renal failure

(Lee & Somlo, 2014). ADPKD cyst formation is driven by abnormal proliferation of renal tubular epithelial cells, increased apoptosis, and extracellular matrix alterations (Grantham, Mulamalla, & Swenson-Fields, 2011). In ADPKD cells, these processes are caused by the dysregulation of different signaling pathways including mTOR-related signaling (Boletta, 2009). Consistently, mTOR inhibitors used as specific targeted therapy have already concluded the clinical trials, but this approach gave the best results in ADPKD pre-clinical

models (de Stephanis et al., 2017). Cell cycle and apoptosis in ADPKD cells are also regulated by the tumor suppressor p53, which is downregulated in both human and mice ADPKD kidney cysts (Nishio et al., 2005). The depletion of p53 contributes to de-differentiation of kidney epithelial cells. This phenomenon leads to the formation of flat cells that in turn replace cubic cells, promoting cyst growth and expansion (Nishio et al., 2005). The tumor suppressor p53 seems to be involved in ADPKD cystogenesis, however, the role of this protein is not yet well defined. Currently, it is emerging that cystogenesis of ADPKD kidneys can also be associated with the dysregulation of different microRNAs (miRs). These short untranslated RNAs modulating several target genes can affect cell proliferation of kidney epithelial cells (Tan, Blumenfeld, & Rennert, 2011). In fact, it has been reported that the up-regulation of miR-17~92, an oncogenic miRNA cluster, induces kidney cysts in mouse models of PKD by the inhibition of oxidative phosphorylation. MiR 17~92 may modulate cystogenesis by promoting cell proliferation through the repression of PKD gene expression (Hajarnis et al., 2017; Patel et al., 2013). The silencing of miR-17 attenuates cyst growth in PKD mouse models and suppresses proliferation and cyst growth of primary ADPKD cells (Hajarnis et al., 2017).

We have described that the putative oncogenic microRNA501-5p stimulates cell proliferation in different kidney carcinoma cell lines by the activation of mTOR signaling pathway (Mangolini et al., 2014). Therefore, its differential expression could affect ADPKD molecular pathogenesis as already observed for the oncogenic miR-17~92 cluster (Patel et al., 2013). In fact, we have found that miR501-5p, upregulated in ADPKD cells and tissues, promotes mTOR activity by the reduction of PTEN and TSC1 expression. The activation of mTOR stimulates p53 proteasome degradation enhancing the levels of the E3 ubiquitin-protein ligase MDM2 in a p70S6K-mediated manner.

Rapamycin treatment, miR501-5p silencing and proteasome inhibition lead to the reduction of cell growth and the activation of apoptosis by p53 restoration.

2 | MATERIAL AND METHODS

2.1 | Materials

Culture media and plastic material were obtained from Euroclone (Milan, Italy). Antibodies anti-mTOR, anti-P-mTOR, anti-S6K, anti-P-S6K and anti-Ubiquitin (Cat# 2972 RRID:AB_330978, Cat# 2971 RRID:AB_330970, Cat# 9202 RRID:AB_331676, Cat# 9208S RRID:AB_330990, and Cat# 3933S RRID:AB_2180538) were purchased from Cell Signalling Technologies (Euroclone). Anti-p53, anti-MDM2, anti-PTEN and anti- β -Actin (Cat# sc-98 RRID:AB_628085, Cat# sc-56154 RRID:AB_784462, Cat# sc-133197 RRID:AB_2174350, and Cat# sc-8432 RRID:AB_626630) were bought from Santa Cruz Technologies (D.B.A. Milan, Italy), while anti-TSC1 antibody (Bethyl Cat# A300-316A RRID:AB_2287978) was obtained from BETHYL Laboratories, Inc. (Tema Ricerca, Bologna, Italy). Enhanced chemiluminescent substrates for Western blotting, HRP-conjugated goat anti-rabbit and anti-mouse antibodies (Cat# 7074 RRID:AB_2099233 and

Cat# 7076 RRID:AB_330924) were purchased from Cell Signaling Technology (Euroclone). Rapamycin and the S6K inhibitor PF4708671 were obtained from Sigma-Aldrich (Milan, Italy) and Santa Cruz Technologies, respectively. AntagomiR anti-miR501-5p and scramble sequences were purchased by Ambion (Life Technologies, Monza, Italy), while p53-siRNA sequences were obtained by Santa Cruz Technologies. The recombinant plasmids (PL501) expressing hsa-miR501-5p sequences alone or linked to GFP were generated by OriGene Technologies (Tema Ricerca). Recombinant plasmids p53-GFP and p21-Luc were produced by other laboratories (Xu et al., 2008).

2.2 | Tissue collection and cell line cultures

Fresh cystic kidney tissues were collected from three different ADPKD patients with end-stage renal disease (ESRD) and subjected to radical nephrectomy (Aguari et al., 2009). Fresh normal kidney tissues were obtained by taking the normal part of kidney (histologically characterized) from two adult patients with kidney carcinoma and performing surgical resection. All tissue samples were frozen at -80°C for long-term storage. Human epithelial SW40-transformed control (4/5) and cystic (9.7 and 9.12) kidney cell lines were produced as already reported (Loghman-Adham, Nauli, Soto, Kariuki, & Zhou, 2003). A total of 4/5 kidney control cells were isolated from a non-ADPKD subject. A total of 9.7 ADPKD cystic cells are heterozygous for the PKD1 mutation Q2556X, while 9.12 ADPKD cystic cells carry the same mutation as 9.7 cells and a deletion of the normal allele (Aguari et al., 2009; Loghman-Adham et al., 2003). Control and PKD1 silenced HEK293 stable clones were generated in our laboratory as previously described (Aguari et al., 2008, 2009). All cell lines were cultured at 37°C in DMEM/ F12 (1:1) medium supplemented with 10% FBS by a humidified (95% air-5% CO_2) incubator. The study was in performed in accordance with the guidelines of Helsinki declaration.

2.3 | Analysis of microRNA expression

The expression of miRs was analyzed by microarray and real time RT-PCR procedures. RNA was extracted from normal and ADPKD fresh frozen tissues as well as from normal kidney and ADPKD cell lines by the TRIZOL protocol (Invitrogen; Thermo Fisher Scientific, Milan, Italy). MiRs from control (4/5) and ADPKD (9.7 and 9.12) cells were studied by using an Agilent Human miRNA microarray (G4470B, Agilent Technologies, Milan, Italy) as previously described (Mangolini et al., 2014). Differential miR expression was analyzed through the GeneSpring GX software (Agilent Technologies) using a 1.5-fold expression change as a cut off for miR selection. Different expression levels of selected miRs were also evaluated by real-time RT-PCR. The synthesis of cDNA was obtained by the Taq-Man MicroRNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific) using specific primers for selected miRs and the small nuclear U6B RNA used as endogenous control (reference gene) (Corney, Flesken-Nikitin, Godwin, Wang, & Nikitin, 2007; Mangolini et al., 2014). Quantitative PCR was carried out using the TaqMan method with the Rotor-Gene Q Real time PCR cycler (Qiagen, Milan, Italy).

MiR expression values were expressed as fold change ratio (ADPKD/control samples), calculated by the delta-delta Ct method as previously reported (Aguari et al., 2004; Corney et al., 2007).

2.4 | Cell transfection

Cells, plated overnight in DMEM/F12 medium supplemented with 10% FBS, were transiently transfected using TurboFect Transfection Reagent (Thermo Scientific, Milan, Italy). Cells were transfected with the constructs PL501 (1.5 $\mu\text{g}/\text{ml}$), p53-GFP, p21-Luc as well as with 20 nM p53-siRNA and 30 nM antagomiR sequences as appropriate. The transfection was performed by seeding the cells in 6- or 96-well plates (2×10^5 cells/well and 5×10^3 cells/well, respectively) for 6 hr in DMEM/F12 medium containing 0.4% bovine serum albumin (BSA). After transfection, cells were washed twice with PBS buffer and cultured in DMEM/F12 supplemented with 1% FBS for cell proliferation assay or 0.4% BSA for the other experiments. As a control, cells were transfected with irrelevant plasmids or scramble sequences as appropriate. Expression values of miR501-5p in cells transfected with different plasmids and/or oligonucleotide sequences were inserted in the Supplementary Figure S1.

2.5 | Western blotting and immunoprecipitation

Cells were washed twice in PBS buffer containing a cocktail of protease inhibitors (Sigma-Aldrich), detached by a cell scraper and collected by centrifugation. Fresh frozen kidney tissues were pulverized by a dismembrator. Frozen tissue powders and cell pellets were lysed in TBS 1% Triton X-100 buffer supplemented with a cocktail of protease and phosphatase inhibitors. Thirty micrograms of total protein samples were electrophoresed in 8% SDS-polyacrylamide gel and transferred onto nitrocellulose filters (Euroclone). Filters were blocked at room temperature for 1 hr in PBS-T (PBS with 0.05% Tween 20) containing 5% nonfat dried milk and probed overnight at 4 °C with the specific primary antibody. After washing three times in PBS-T, filters were incubated for 1 hr at room temperature in PBS-T buffer with the appropriate horseradish peroxidase-conjugated secondary antibody and washed again three times in PBS-T. Finally, protein bands were visualized by using Super Signal Femto or Pico chemiluminescence systems (Euroclone). Protein band intensity was detected by film scanning with the GS-700 Imaging Densitometer (BIO-RAD, Milan, Italy). Phosphorylation levels were calculated as the ratio between the phosphorylated form and total protein band intensity. Relative protein abundance was quantified as the ratio between the protein of interest and β -Actin.

Immunoprecipitation of wild-type and ubiquitinated p53 was performed in normal (4/5) and ADPKD (9.7) kidney cells as well as in 4/5 cells transfected with both PL501 and p53-GFP plasmids. Cells were lysed in TBS buffer (50 mM Tris, pH 8.0, 150 mM NaCl) supplemented with SDS (2%), DTT (1 mM) and protease inhibitors. This solution was enriched with the proteasome blocker MG132 (10 μM) and ubiquitin peptidase inhibitor N-ethylmaleimide (10 mM). Cell lysates were centrifuged to exclude cell debris, next total

protein samples were boiled, diluted 10 times in TBS with protease inhibitors and pre-cleared with protein A beads for 1 hr at 4 °C. Protein samples were incubated overnight at 4 °C with an anti-p53 antibody and then with protein A beads for further 2 hr. After washing, immunoprecipitates were analyzed by western blotting with both anti-p53 and anti-Ubiquitin antibodies. The negative control represents cell lysate sample processed in absence of primary antibody.

2.6 | Cell imaging and luciferase assay

Analysis of the proteasome was performed by fluorescence microscopy using specific anti-proteasome antibodies in 4/5 control cells transfected with both PL501 and p53-GFP plasmids. After transfection, cells were cultured for 24 hr in DMEM/F12 0.4% BSA, fixed with 0.4% formalin, and permeabilized in a PBS solution containing 0.2% Triton X-100. Next, cells were washed twice with PBS buffer, blocked in PBS 2% gelatin for 2 hr, and incubated with an anti-proteasome antibody solution for 2 hr at room temperature. After washing, cells were treated for 1 hr with a secondary rhodamine-conjugated antibody (Thermo Fisher Scientific). Finally, cells were washed three times in PBS buffer and fluorescent signals were detected by a fluorescence microscope. Images were acquired at 40 \times magnification using a Zeiss Axiovert 200 fluorescence microscope equipped with a back-illuminated CCD camera (Roper Scientific, Tucson, AZ) and processed by ImageJ software (Mangolini et al., 2014).

Apoptosis was evaluated by Hoechst staining in control (4/5) and ADPKD (9.7 and 9.12) cells cultured in DMEM/F12 0.4% BSA and treated for 48 hr in the presence/absence of rapamycin (500 nM) or MG132 (10 nM). Apoptosis was also analyzed after cell transfection with p53-siRNA sequences in the same culture conditions described above. The formation of apoptotic nuclei was also detected after treatment of 9.7 and 9.12 ADPKD cells with anti-miR (30 nM). After treatment, cells were fixed, permeabilized, and stained with Hoechst 33258 (10 mg/ml) in the dark. Images were acquired at 40 \times magnification by a fluorescence microscope, equipped with CCD camera, as described above.

The activity of p53 was measured by luciferase assay by using the Luciferase Assay System (Promega, Milan, Italy) in 4/5 control cells as well as in 9.7 and 9.12 ADPKD cells. 2×10^5 cells for well were seeded in a six-well plate and cultured overnight in DMEM/F12 medium supplemented with 10% FBS. Next, cells were transiently transfected with 3 μg of p21-Luc and 1 μg of β -galactosidase plasmids for 6 hr using the Turbofect method in DMEM/F12 0.4% BSA. The activity of p21 promoter was also evaluated in 4/5 kidney cells co-transfected with PL501 plasmid and/or p53-siRNA oligonucleotides as previously described. After transfection, cells were washed and cultured for an additional 24 hr in DMEM/F12 medium supplemented with 0.4% BSA. 4/5 cells were also cultured in the presence of 500 nM rapamycin or ethanol (vehicle). After cell lysis, 25 μl of cell lysate was added to 50 μl of luciferase substrate (Promega) and the samples were analyzed using a 20/20n luminometer (Turner Biosystems, Sunnyvale, CA). Data

calculated as relative firefly luciferase units (RLU) normalized by the β -galactosidase units were expressed as the n -fold change ratio (sample/control) (Aguiri et al., 2012).

2.7 | Cell proliferation analysis

Analysis of cell proliferation was performed by the CellTiter method (Promega) plating 5,000 cells/well in 96-well plates. Cells were starved for 24 hr in DMEM/F12 0.4% BSA and transfected with PL501, anti-miR, and relative controls (irrelevant plasmid and scramble sequences, respectively). After transfection, cells were cultured for further 24 and 48 hr in DMEM/F12 medium supplemented with 1% FBS in presence of ethanol (vehicle) or rapamycin (500 nM). Next, cells were incubated at 37 °C for 5 hr with a solution containing tetrazolium salts that were converted by living cells in formazan, a purple colored compound. Cell medium was transferred in a new 96-well plate and color intensity was detected at 490 nm by a plate reader. Color intensity is directly proportional to the number of living cells (Bonon, Mangolini, Pinton, Del Senno, & Aguiari, 2013).

2.8 | Statistical analysis

All data for statistical analysis are inserted in Supplementary Table S1. The statistical analysis was performed using a Student's t -test, except for microarray findings, which were analyzed by an Anova test. Data were

reported as mean \pm Standard Deviation of at least three independent experiments and differences were considered significant at $p < 0.05$.

3 | RESULTS AND DISCUSSION

3.1 | MicroRNA analysis in ADPKD cells and tissues

MiR expression was performed by microarray analysis in 4/5 normal epithelial kidney cells as well as in 9.7 and 9.12 ADPKD cells carrying PKD1 gene mutations (Loghman-Adham et al., 2003). As shown in the table in Figure 1a, six different miRs were differentially expressed in 9.7 and 9.12 cells compared with 4/5 control cells. In particular, hsa-miR368, hsa-miR601 and hsa-miR142-5p were downregulated while hsa-miR202, hsa-miR196b and hsa-miR501-5p were overexpressed. Findings of microarray analysis were validated by real time RT-PCR in different kidney tissues derived from three ADPKD patients. As observed in ADPKD cells, the expression of miR501-5p was increased while the levels of miR601 were lower in ADPKD kidney cysts compared with normal kidney tissues (Figure 1b). Conversely, the expression of the other miRs was not significantly changed in ADPKD tissues compared to controls (Figure 1b). The upregulation of miR501-5p detected in 9.7 and 9.12 ADPKD cystic kidney cells after microarray analysis was confirmed by using real time RT-PCR (Figure 1c). Consistently, increased levels of this miR in stably PKD1-gene silenced HEK293 clones compared with those transfected with scramble sequences were also observed

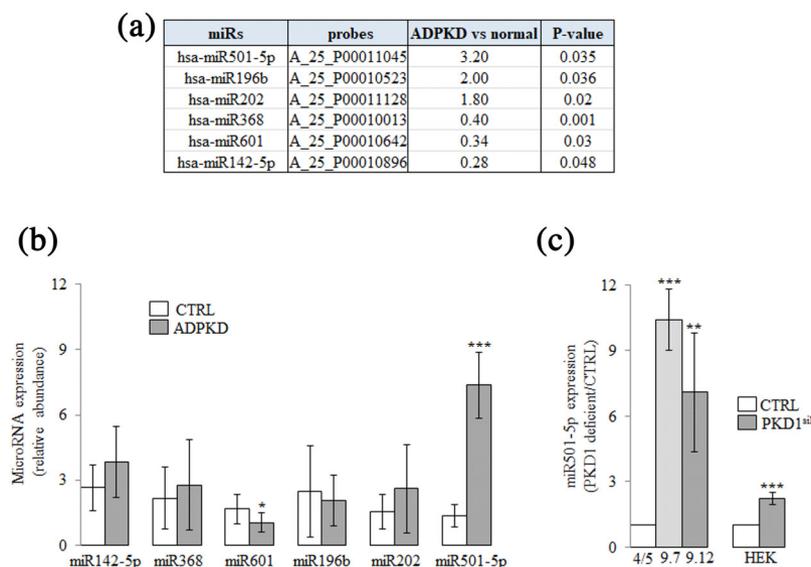


FIGURE 1 Expression of miRs in ADPKD cells and tissues analyzed by microarray and real time RT-PCR. (a) The table shows name, microarray probes, abundance, and statistical significance of six different miRs differentially expressed in ADPKD cells. The analysis of miRNAs was carried out in two ADPKD cell lines (9.7 and 9.12) as well as in control kidney cells (4/5) by the microarray method; miR expression levels were calculated as fold change ratio (ADPKD vs. control) (Mangolini et al., 2014). (b) Real time RT-PCR analysis of six miRNAs changed in ADPKD cells was performed in three ADPKD fresh frozen kidneys (two different cysts for sample, $n = 6$) and in two normal kidney tissues. Relative miR abundance were calculated by the $\Delta\Delta C_t$ method as previously described (Corney et al., 2007; Aguiari et al., 2004). The expression of miR601 is lower, while the levels of miR501-5p are higher in ADPKD tissues than in normal kidneys ($*p < 0.05$ and $***p < 0.001$, respectively). (c) MiR501-5p levels, analyzed by real time RT-PCR, are increased in 9.7 and 9.12 ADPKD cells compared with 4/5 normal kidney cells ($***p < 0.001$ and $**p < 0.01$, respectively). The silencing of the PKD1 gene in HEK293 embryonic kidney cells (PKD1^{sil}) induces the increase in miR501-5p content compared to wild type (CTRL) cells ($***p < 0.001$)

(Figure 1c). Taken together, these data suggest that the depletion/dysfunction of polycystin-1 affects the expression of some miRs, in particular, miR501-5p is strongly upregulated in ADPKD cells and tissues, and therefore could be involved in ADPKD pathogenesis. We have observed that this miR is mostly overexpressed in advanced kidney carcinoma and promotes cell growth (Mangolini et al., 2014). Therefore, the upregulation of miR501-5p in ADPKD cells could affect cell proliferation that is a typical hallmark of this pathology. On the other hand, possible disease-associated effects by the differential expression of others miRs in ADPKD have already been described. For example, the upregulation of miR-17~92, an oncogenic miRNA cluster, in mouse models of PKD stimulated the proliferation of epithelial cystic cells by the post-transcriptional repression of PKD genes and hepatocyte nuclear factor 1 β (Hajarnis et al., 2017; Patel et al., 2013). Moreover, the increased expression of miR199a-5p in ADPKD tissues and cell lines promoted cell proliferation by targeting CDKN1C mRNA (Sun et al., 2015). Finally, increased levels of the oncogenic miR-21 in kidney cysts of ADPKD patients have been reported. The expression of this miR was modulated by cAMP signaling and induced the inhibition of apoptosis in cyst epithelial cells (Lakhia et al., 2016).

Because the increased expression of miR501-5p was strongly significant in both ADPKD cells and tissues, we have investigated the role of this miR in ADPKD molecular pathogenesis.

3.2 | MicroRNA501-5p silencing PTEN and TSC1 genes enhances mTOR signaling that induces MDM2 upregulation and p53 destabilization

The *in silico* analysis that we performed using different software predicting miR recognition sites led to the identification of several ADPKD-related target genes for miR501-5p (Table 1). Because the activation of mTOR signaling is strongly involved in ADPKD pathogenesis, we have investigated PTEN and TSC1 genes that are predicted targets of this miR and can negatively modulate this protein kinase (Song, Salmena, & Pandolfi, 2012). Moreover, the 3'UTR of PTEN and TSC1 transcripts contains multiple putative recognition sites of miR501-5p. Since 9.7 and 9.12 cells showed increased levels of miR501-5p compared to 4/5 cells, it is reasonable to think that the upregulation of this miR may lead to the reduction of PTEN and TSC1 expression and in turn activate mTOR kinase. Actually, reduced PTEN and TSC1 protein levels as well as an increased mTOR phosphorylation in 9.7 and 9.12 ADPKD cells compared with 4/5 control cells were detected (Figure 2a). The activation of mTOR may positively modulate the expression of MDM2, an E3 ubiquitin ligase, that promotes p53 degradation in both glioblastoma and kidney carcinoma cells (Daniele et al., 2015; Mangolini et al., 2014). Consistently, the increased expression of MDM2 as well as reduced levels of p53 in 9.7 and 9.12 ADPKD cells compared with 4/5 control cells were observed (Figure 2a). The transfection of 4/5 and HEK293 non-ADPKD kidney cells with a plasmid expressing miR501-5p sequences (PL501) that enhanced the levels of this miR (Supplementary Figure S1a) also induced the downregulation of both PTEN and TSC1 genes as well as the activation of mTOR kinase (Figure 2b). Moreover, the upregulation

of this miR stimulated the expression of MDM2 as well as the reduction of p53 (Figure 2b). Consistently, the treatment of 9.7 and 9.12 ADPKD cells with specific anti-miR501-5p sequences decreased the expression of this miR (Supplementary Figure S1f) and consequently increased PTEN and TSC1 protein levels leading to the reduction of mTOR phosphorylation (Figure 2c). As expected, the downregulation of miR501-5p enhanced p53 expression and reduced MDM2 protein levels (Figure 2c). Finally, the upregulation of MDM2 as well as the reduction of p53 expression was also found in cystic kidney tissues derived from different ADPKD patients (Figure 2d).

These data indicate that the upregulation of miR501-5p in ADPKD cells causes the reduction of PTEN and TSC1 expression and could affect ADPKD pathogenesis by enhancing mTOR signaling. Interestingly, renal cyst formation caused by the elevated mTOR activity in TSC1 knockout mice was already described (Zhou, Brugarolas, & Parada, 2009). The development of renal cysts by kidney-specific inactivation of TSC1 was associated with polycystin-1 downregulation as well as with the impaired trafficking of polycystin complex to cilia (Pema et al., 2016). Despite PTEN being an upstream effector of mTOR signaling, its involvement in ADPKD pathology is still unclear. In fact, the disruption of the PTEN gene did not generate the ADPKD phenotype in *Nse-cre;Pten^{loxp/loxp}* mice, however, PTEN and TSC1 double knockout mice developed a more severe PKD progression than TSC1 single mutants (Zhou et al., 2009). Therefore, PTEN gene knockout in renal tubular cells is not sufficient to cause ADPKD, but its disruption in combination with TSC1 inactivation enhances disease progression likely by a stronger activation of mTOR signaling.

In order to investigate the role of mTOR signaling on p53 downregulation in ADPKD, we have inhibited this kinase treating normal (4/5) and ADPKD (9.7 and 9.12) cells with rapamycin. As expected, the inhibition of mTOR activity (Figure 3a) decreased p70S6 kinase phosphorylation, induced a strong reduction of MDM2 expression, and enhanced the levels of p53 protein in 9.7 and 9.12 cells, but not in 4/5 control cells (Figure 3a). Similar findings were obtained by the upregulation of miR501-5p transfecting 4/5 and HEK293 control kidney cells with PL501 in presence of rapamycin. In fact, the treatment with rapamycin reduced both mTOR and p70S6 phosphorylation, decreased MDM2 protein and restored p53 expression levels (Figures 3b and S1b). These results confirm that the overexpression of miR501-5p observed in ADPKD cells causes the reduction of p53 expression through the upregulation of MDM2 protein, in a mechanism involving the activation of mTOR kinase. Importantly, the mTOR-dependent MDM2/p53 expression mediated by miR501-5p upregulation was already observed in clear cell renal cell carcinoma (ccRCC) cell lines (Mangolini et al., 2014), confirming that this miR may affect p53 expression through the activation of mTOR signaling. Consistently, it has been reported that rapamycin treatment causes the reduction of MDM2 expression as well as the upregulation of p53 in renal carcinoma (RCC) cells, leading to the induction of p53-mediated apoptosis (Liu, Shen, et al., 2016). Taken together these findings suggest that ccRCC and ADPKD cells could share a common mechanism used to negatively regulate p53 expression.

TABLE 1 MicroRNA501-5p target genes related with ADPKD

Gene acronym	Gene description	Target sites	Function	Signaling	References
BCL2	B-cell CLL/lymphoma 2	1	Apoptosis regulator	JNK/Bcl-2	Yu et al. (2010)
CREB1	cAMP responsive element binding protein 1	3	Transcription factor	cAMP/CREB/AR	Aguiari et al. (2012)
EGFR	Epidermal growth factor receptor	1	Growth factor receptor	EGFR/Raf1/ERK	Yamaguchi et al. (2003)
HGF	Hepatocyte growth factor	1	Growth factor	HGF/cMET/Wnt	Qin, Taglienti, Cai, Zhou, and Kreidberg (2012)
ID2	DNA-binding protein inhibitor	1	Cell cycle regulator	ID2/p21	Li et al. (2005)
JAK2	Janus Kinase	1	Cell growth regulator	JAK2/STAT1/p21	Bhunja et al. (2002)
JUN	Jun proto-oncogene	2	Cell cycle regulator	JNK/AP-1	Parnell et al. (2002)
KRAS	Kirsten rat sarcoma viral oncogene homolog	1	Cell cycle regulator	Ras/Raf1/ERK	Parker et al. (2007)
NRAS	Neuroblastoma RAS viral oncogene homolog	1	Cell cycle regulator	Ras/Raf1/ERK	Parker et al. (2007)
NFAT	Nuclear factor of activated T-cells	1	Transcription factor	NFAT/PKCa	Aguiari et al. (2008)
P2RX7	Purinergic receptor P2X, ligand-gated ion channel 7	1	Cationic permeable channel	P2RX7/ERK	Chang et al. (2011)
PDE4B/D	Phosphodiesterase 4 isophorms B-D	1	Phospho-diesterase, cAMP-specific	cAMP level regulation	Pinto et al. (2016)
PDGF	Platelet-derived growth factor beta polypeptide	1	Growth factor	PDGF/PDGFR	Wilson, Du, Norman and (1993)
PKD2L1	Polycystic kidney disease 2-like 1	1	Calcium channel	Receptor channel complex	Molland et al. (2010)
PKD2L2	Polycystic kidney disease 2-like 2	2	Calcium channel	Plasma membrane channel component	Chen et al. (2008)
PKHD1	Polycystic kidney and hepatic disease like 1	1	Membrane receptor	Plasma membrane receptor	Hogan et al. (2003)
PTEN	Phosphatase and tensin homolog	2	Protein phosphatase	PTEN/PI3K/Akt	Zhou et al. (2009)
RICTOR	RPTOR independent companion of mTOR	2	Cell growth regulator	mTORC2	Ravichandran et al. (2014)
SP1	Transcription factor Sp1	1	Transcription factor	Polycystin-1 expression	Jeon, Yoo, and Park (2007)
STAT1	Signal transducer and activator of transcription 1	1	Transcription factor	JAK/STAT1/p21	Bhunja et al. (2002)
STIM1	Stromal interaction molecule 1	2	Calcium sensor	STIM1/IP3R	Santoso, Cebotaru, and Guggino (2011)
TSC1	Tuberous sclerosis 1	3	mTOR inhibitor component	TSC1/TSC2/mTOR	Zhou et al. (2009)
TRPC3	Transient receptor potential channel, subfam. C, member 3	1	Calcium channel	TRPP2/TRPC3/TRPC7	Miyagi et al. (2009)
TRPC4	Transient receptor potential channel, subfam. C, member 4	2	Calcium channel	TRPP2/TRPC4	Du, Ding, Sours-Brothers, Graham, and Ma (2008)

The molecular targets of miR501-5p were identified by using different software including TargetScan Human 6.0, miRBas, miRNAMap and Miranda. Gene name, site number, function, biological processes, and relative bibliography are indicated.

However, the inhibition of p53 could also affect ADPKD pathogenesis. In this regard, the downregulation of the p53 gene was already observed in HEK293 cells with silenced PKD1 after UV light irradiation (Kim, Bae, Jeong, Ahn, & Kang, 2004), as well as in epithelial cells derived from renal cysts of ADPKD mice (Nishio et al., 2005). Furthermore, the deficiency of polycystin-1 by reducing both p53 and p21 expression leads to the progression of the cell cycle in kidney epithelial cystic cells (Nishio et al., 2005).

Data here shown demonstrate for the first time that there is a cross-link between mTOR activation and MDM2 upregulation that affects the expression of p53 in ADPKD cells.

3.3 | The reduction of p53 expression occurs by proteasome degradation

The activation of mTOR kinase induces the upregulation of MDM2, which may lead to the ubiquitination and degradation of p53 (Pant & Lozano, 2014). Actually, an increase in high molecular weight forms of p53 in 9.7 kidney cystic cells compared with 4/5 control cells was observed (Figure 4a). The overexpression of miR501-5p in 4/5 cells transfected with PL501 plasmid (Supplementary Figure S1c) also enhanced the levels of p53-ubiquitin conjugates compared with 4/5 control cells (Figure 4b). Moreover, 4/5 cells

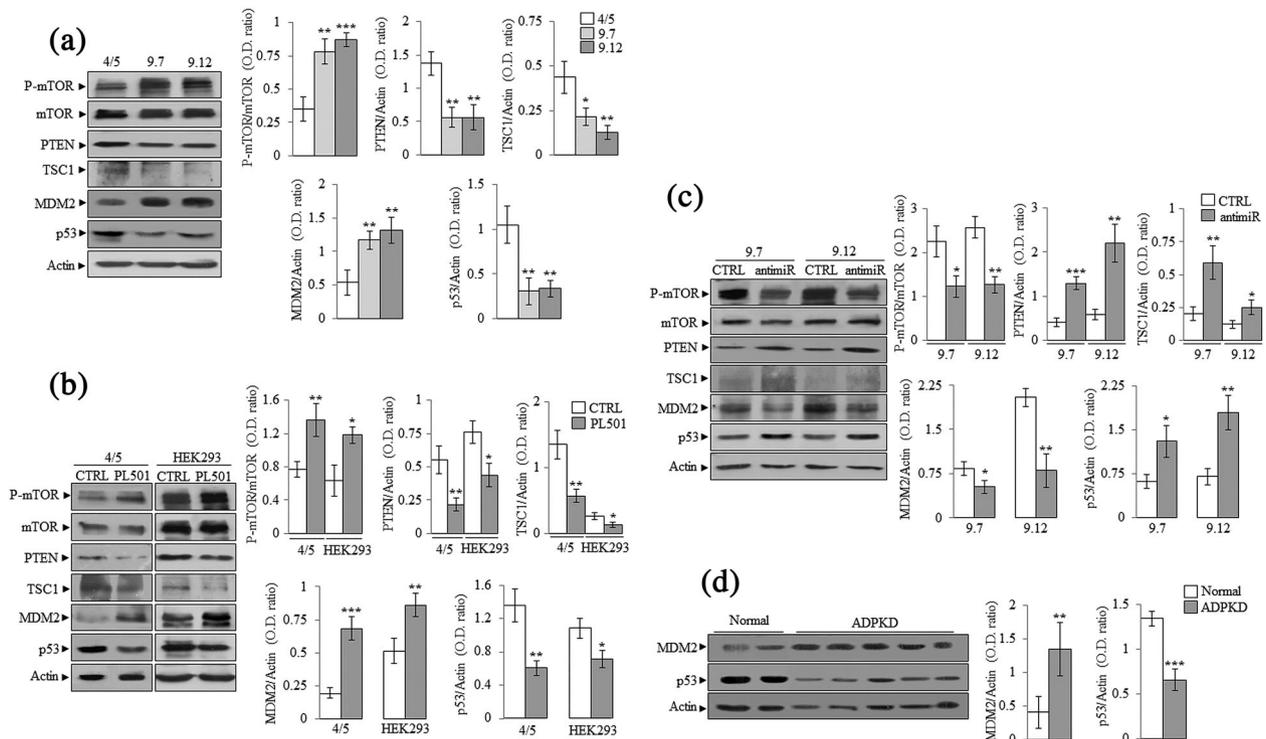


FIGURE 2 MiR501-5p leads to p53 downregulation enhancing mTOR phosphorylation and MDM2 expression by targeting PTEN and TSC1 genes. (a) The phosphorylation degree of mTOR is higher in ADPKD compared with control cells (** $p < 0.01$ and *** $p < 0.001$). PTEN and TSC1 protein levels are lower in 9.7 and 9.12 ADPKD than in 4/5 control cells (** $p < 0.01$ for PTEN; * $p < 0.05$ and ** $p < 0.01$ for TSC1). The expression of MDM2 is increased while the levels of p53 are reduced in 9.7 and 9.12 ADPKD compared with 4/5 control cells (** $p < 0.01$ for both MDM2 and p53). (b) The activity of mTOR as well as the expression of PTEN, TSC1, MDM2, and p53 were evaluated in 4/5 and HEK293 non-ADPKD kidney cells transfected with a vector expressing miR501-5p sequences (PL501) or with an irrelevant plasmid (CTRL). The overexpression of miR501-5p causes increased activity of mTOR (** $p < 0.01$ and * $p < 0.05$). Conversely, the levels of PTEN and TSC1 are decreased in 4/5 and HEK293 cells transfected with PL501 compared with control cells (** $p < 0.01$ and * $p < 0.05$ for PTEN and TSC1 in 4/5 and HEK293, respectively). The expression of MDM2 is increased, while p53 protein content is reduced in 4/5 and HEK293 cells transfected with PL501 compared with control cells (** $p < 0.001$ and ** $p < 0.01$ for MDM2; ** $p < 0.01$ and * $p < 0.05$ for p53). (c) The phosphorylation of mTOR and the expression of PTEN, TSC1, MDM2, and p53 were analyzed in 9.7 and 9.12 ADPKD cells transfected with anti-miR501-5p (anti-miR) or scramble (CTRL) sequences. The treatment with anti-miR causes a reduction of mTOR phosphorylation while increases the levels of PTEN and TSC1 compared with control cells (* $p < 0.05$ and ** $p < 0.01$ for mTOR; *** $p < 0.001$ and ** $p < 0.01$ for PTEN; ** $p < 0.01$ and * $p < 0.05$ for TSC1). The reduction of miR501-5p decreases MDM2 expression and enhances p53 protein levels (* $p < 0.05$ and ** $p < 0.01$ in both MDM2 and p53). (d) In ADPKD tissues, increased MDM2 expression and reduced p53 protein levels compared with normal kidney parenchyma were observed (** $p < 0.01$ for MDM2 and *** $p < 0.001$ for p53). Protein content was analyzed by Western blotting

transfected with both PL501 and p53-GFP plasmids exhibited a greater number of cytoplasmic aggregates that co-localized with proteasome structures than control cells (Figure 4c). Consistently, the inhibition of proteasome machinery by the treatment of 9.7 and 9.12 cells with MG132 reduced the number of p53 aggregates and induced the translocation of this protein to the nucleus (Supplementary Figure S2a). The silencing of p53 by siRNA dramatically reduced fluorescence intensity, confirming that p53 is trapped in proteasomal structures in ADPKD cells (Supplementary Figure S2a). Furthermore, the analysis of apoptotic nuclei in cells treated with MG132 showed that the inhibition of proteasome stimulated cell death in 9.7 and 9.12 cells, while this effect was moderate in 4/5 control cells (Figures 5b and 5d). The reduction of p53 expression by siRNA treatment (Supplementary Figure S2b) abrogated apoptotic nuclei formation in 9.7 and 9.12 cells treated with

MG132 (Figures 5c and 5d), suggesting that the activation of apoptosis is driven by p53.

These findings show that the overexpression of miR501-5p that activates mTOR/MDM2 pathway leads to the degradation of p53 by promoting its ubiquitination and translocation into proteasome machinery. This p53 inhibition mechanism was already observed in kidney cancer cells (Mangolini et al., 2014) as well as in other tumor cells (Meek, 2015), indicating that p53 may be commonly degraded by this process. The inhibition of proteasome machinery restores p53 function and promotes apoptosis in ADPKD cells, therefore the proteasome inhibition could be taken into account as a possible pharmacological tool for ADPKD treatment. In this regard, the proteasomal system is already a target for the treatment of several inherited and neoplastic diseases (Kaplan, Torcun, Grune, Ozer, & Karademir, 2016). However, the possible use of anti-proteasome

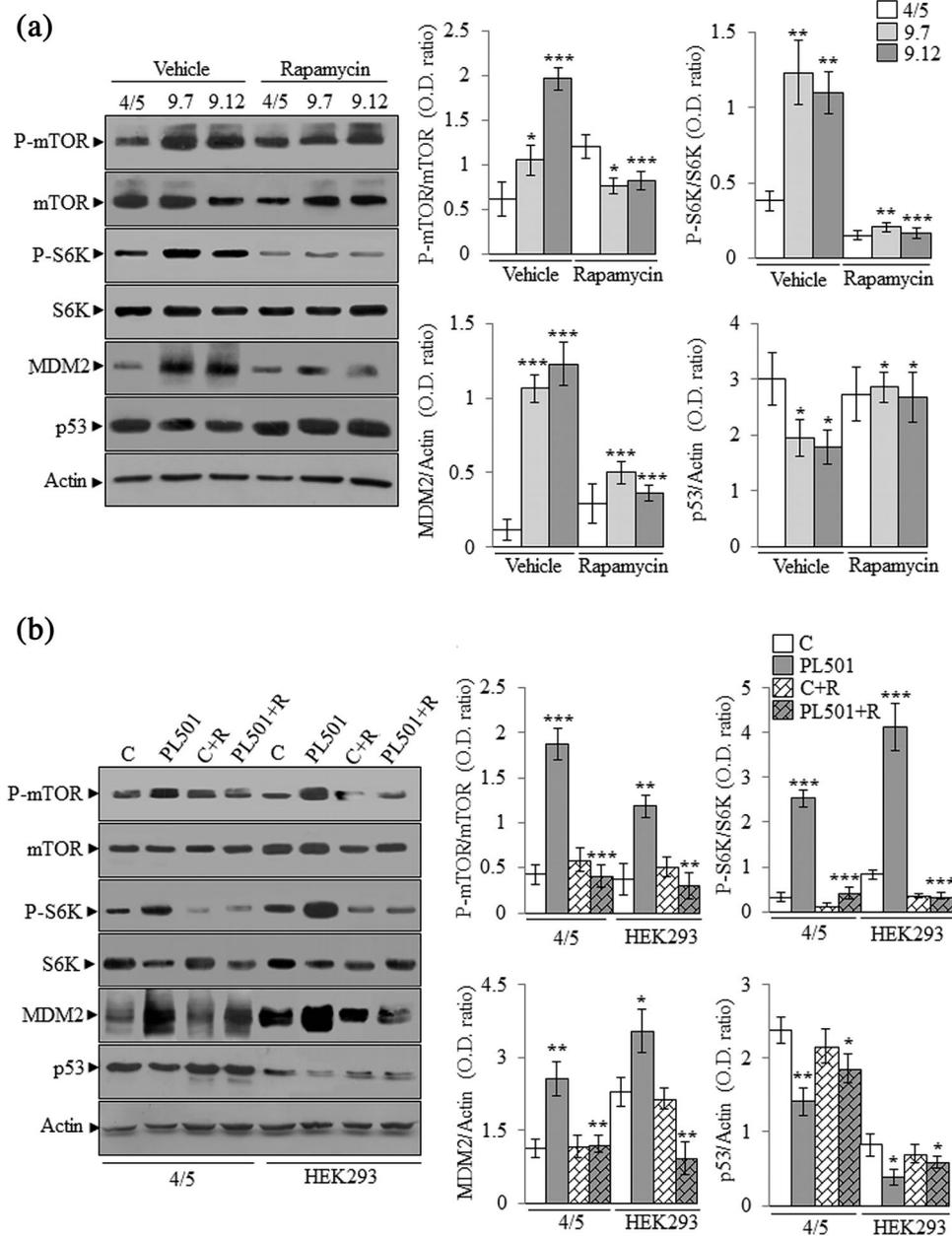


FIGURE 3 Western blot analysis shows that the inhibition of mTOR reduces p70S6K phosphorylation, MDM2 protein levels, and restores p53 expression. (a) In basal conditions (vehicle), mTOR and p70S6K phosphorylation as well as MDM2 expression are higher in ADPKD than in control cells ($*p < 0.05$ and $***p < 0.001$ for mTOR; $**p < 0.01$ for p70S6K; $***p < 0.001$ for MDM2). Conversely, p53 expression was lower in ADPKD compared with normal cells ($*p < 0.05$). The treatment with 500 nM rapamycin for 24 hr reduces mTOR and p70S6K activity, MDM2 expression and increases p53 protein levels in 9.7 and 9.12 ADPKD cells compared to untreated cells ($*p < 0.05$ and $***p < 0.001$ for mTOR; $**p < 0.01$ and $***p < 0.001$ for p70S6K; $***p < 0.001$ for MDM2; $*p < 0.05$ for p53). (b) The transfection of 4/5 and HEK293 cells with PL501 increases mTOR and p70S6K phosphorylation, MDM2 expression and reduces p53 levels compared with cells transfected with an irrelevant construct ($***p < 0.001$ and $**p < 0.01$ for mTOR in 4/5 and HEK293, respectively; $***p < 0.001$ for p70S6K; $**p < 0.01$ and $*p < 0.05$ for MDM2 and p53 in 4/5 and HEK293, respectively). The treatment with 500 nM rapamycin for 24 hr in 4/5 and HEK293 cells transfected with PL501 inhibits mTOR and p70S6K phosphorylation, reduces MDM2 protein content and increases p53 expression compared with the same cells cultured without rapamycin ($***p < 0.001$ and $**p < 0.01$ for mTOR in 4/5 and HEK293, respectively; $***p < 0.001$ for p70S6K, $**p < 0.01$ for MDM2 and $*p < 0.05$ for p53 in both 4/5 and HEK293). C, cells transfected with an irrelevant plasmid; PL501, cells transfected with a plasmid expressing miR501 sequences; R, cells treated with rapamycin

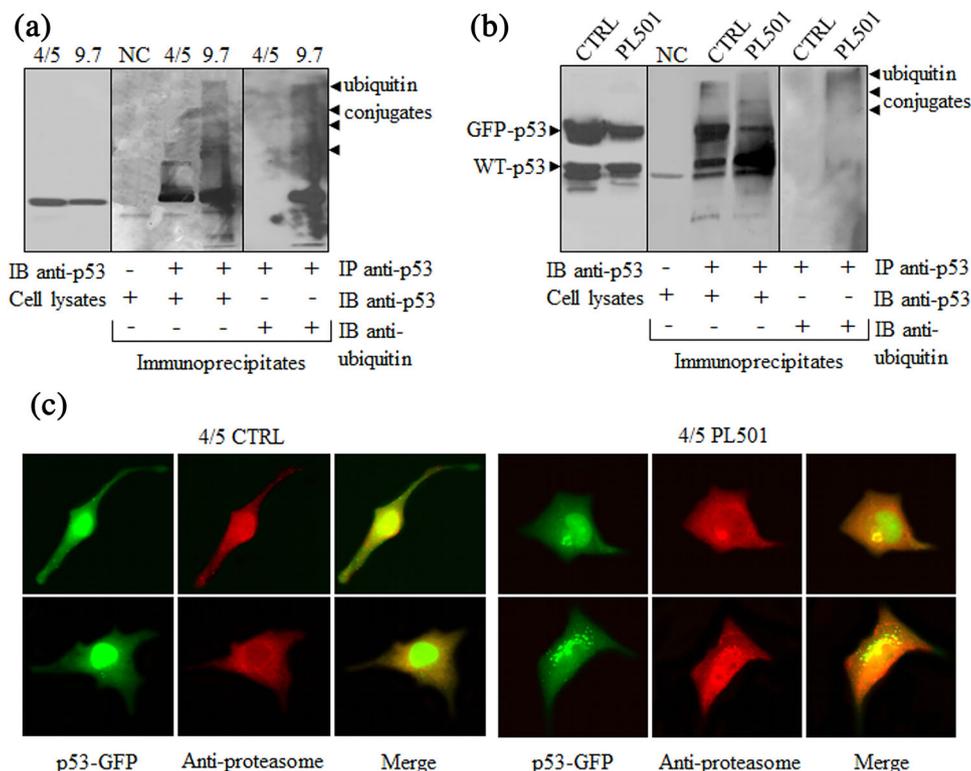


FIGURE 4 The upregulation of miR501-5p promotes p53 ubiquitination and its degradation via proteasome machinery. (a) 9.7 ADPKD and 4/5 control cells were cultured for 4 hr in the presence of the proteasome inhibitor MG132 (10 μ M) before cell lysis. An aliquot of cellular lysate was used for immunoprecipitation by a monoclonal anti-p53 antibody. Cell lysate samples and immunoprecipitated proteins were analyzed by PAGE, blotted on nitrocellulose filters, and probed with anti-p53 and anti-ubiquitin antibodies. (IB, immunoblot; IP, immunoprecipitation; NC, negative control). (b) To evaluate the effect of miR501-5p upregulation on p53 ubiquitination, 4/5 control cells were co-transfected with a p53-GFP construct and either PL501 or irrelevant plasmid (CTRL). The analysis of p53 ubiquitinated peptides was performed as described in the point (a). (c) The co-localization of p53 and proteasome vesicles were analyzed by fluorescence microscopy. A total of 4/5 control cells cultured on 24 mm glass coverslips were co-transfected with p53-GFP and an irrelevant plasmid (CTRL) or with the PL501 recombinant vector. After transfection, cells were fixed, permeabilized, treated with an anti-proteasome antibody, and finally placed in a solution containing a secondary antibody conjugated with rhodamine. After washing, cells were analyzed by a fluorescence microscope equipped with CCD camera at 40 \times magnification and images were processed by ImageJ software

inhibitors for the treatment of ADPKD as well as for other diseases should be carefully evaluated, because these drugs show several side effects including the development of serious peripheral neuropathies (Kaplan et al., 2016).

3.4 | Rapamycin treatment restores p53 function and promotes cell death

The dysregulation of mTOR signaling is involved in ADPKD progression (Shillingford et al., 2006), moreover our data suggest that the activation of this protein kinase plays a key role in p53 degradation. Thus, the inhibition of mTOR signaling could prevent MDM2-mediated p53 ubiquitination and restore its function. We have evaluated the function of p53 in cells treated in the presence/absence of rapamycin by the luciferase assay, using a construct containing the p53 binding site of the p21 promoter (Xu et al., 2008). In basal conditions, the luciferase activity was higher in 4/5 than in 9.7 and 9.12 cell lines (Figure 6a), supporting the reduced function of p53 in ADPKD cells which express higher levels of miR501-5p than

control kidney cells. Consistently, the transfection of 4/5 control kidney cells with the PL501 plasmid that increased the expression of miR501-5p (Supplementary Figure S1d), also caused the reduction of p53 activity compared with same cells transfected with an irrelevant construct (Figure 6b), confirming that miR501-5p upregulation negatively regulates p53 function. As expected, the transfection of 4/5 cells with p53 siRNA sequences strongly reduced p53 protein content (Supplementary Figure S2c) and inhibited the activity of p21 promoter (Figure 6b). The co-transfection with p53 siRNA and PL501 plasmid further reduced p53 expression as well as luciferase activity compared with same cells transfected with siRNA or PL501 alone (Figures 6b and S2c). Furthermore, the treatment with rapamycin enhanced p21 promoter activity in 4/5 cells transfected with PL501 alone or in combination with p53 siRNA, but did not affect cells treated with p53 siRNA only (Figure 6b). Finally, the treatment of 9.7 and 9.12 ADPKD cells with anti-miR501-5p oligonucleotides (antimiR) caused a significant reduction of miR501-5p expression (Supplementary Figure S1g) as well as the increase of p53 function (Figure 6c).

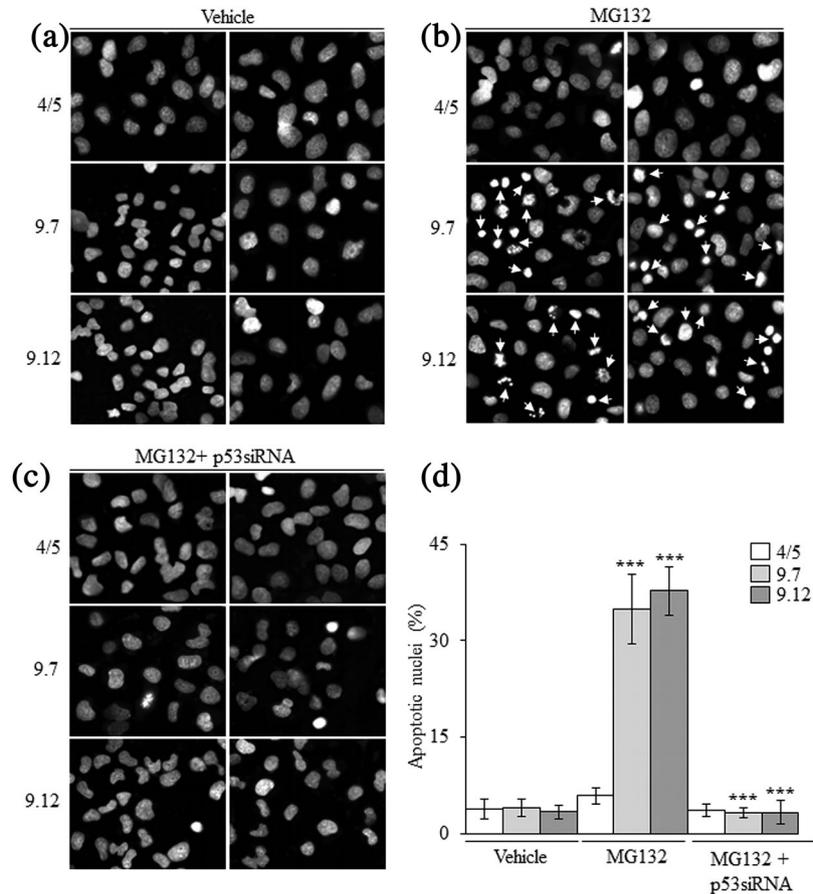


FIGURE 5 The inhibition of the proteasome promotes apoptosis in ADPKD cells. A total of 4/5, 9.7, and 9.12 cells were cultured on coverslips in DMEM/F12 0.4% BSA for 48 hr in absence (a) and in presence of 10 nM MG132 alone (b) or in combination with p53 siRNA oligonucleotides (c). Next, cells were fixed, permeabilized, and stained with Hoechst 33342. Cell images were acquired at 40 \times magnification by a fluorescence microscope and processed through the ImageJ software. Arrows indicate the apoptotic nuclei. (d) Bar graph shows the increase of apoptotic nuclei percentage in 9.7 and 9.12 cells treated with MG132 compared with untreated cells (** $p < 0.001$). The silencing of p53 blocks the formation of apoptotic nuclei in 9.7 and 9.12 cells treated with MG132 (** $p < 0.001$)

These data confirm that the expression/function of p53 is modulated by the abundance of miR501-5p through the activation of mTOR signaling.

In order to define the molecular link between mTOR and MDM2, the involvement of p70S6K was investigated. Therefore, normal (4/5) and ADPKD (9.7 and 9.12) cells were cultured with PF47086701, an inhibitor of p70S6 kinase. The treatment with PF47086701 strongly decreased p70S6K phosphorylation in all cell types (Figure 6d), but caused the reduction of MDM2 expression as well as the increase of p53 levels in 9.7 and 9.12 cells only (Figure 6e). Thus, the activation of p70S6 kinase, a downstream effector of mTOR, contributes to MDM2-dependent p53 degradation in ADPKD cells, as observed in other cells (Ding et al., 2013). Finally, the upregulation of miR501-5p (Supplementary Figure S1e) also caused an increased cell growth as compared to control cells (Figure 6f, gray and black solid lines). The treatment of these cells with rapamycin for 48 hr slowed cell proliferation compared with untreated cells (Figure 6f, solid and dashed gray lines), suggesting that the activation of mTOR promotes cell growth as already observed in other cystic kidney cells (Shillingford, Piontek, Germino, & Weimbs, 2010). Consistently, the inhibition of mTOR by rapamycin treatment

induced the formation of apoptotic nuclei in 9.7 and 9.12 ADPKD cells, but not in 4/5 control cells (Figures 7b and 7d). The silencing of p53 abolished the rapamycin-induced apoptotic nuclei formation (Figures 7c and 7d), confirming that the activation of apoptosis by mTOR inhibition in ADPKD cells occurs in a p53-dependent manner. Similar results were observed treating 9.7 and 9.12 cell lines with anti-miR501-5p sequences. In fact, the reduction of miR501-5p expression decreased cell growth and promoted the formation of apoptotic nuclei (Supplementary Figure S3a and S3b).

These findings support previous observations where the treatment with rapamycin inhibited cell proliferation in different human and mouse ADPKD cells (de Stephanis et al., 2017; Shillingford et al., 2010). The reduction of MDM2 expression after mTOR inhibition detected in ADPKD cells was also observed in embryonic liver cells. In fact, the administration of rapamycin in these cells negatively modulates MDM2 expression and enhances the activity of p53 (Moumen, Patané, Porras, Dono, & Maina, 2007). Moreover, inhibition of mTOR promotes apoptosis in cyst-lining epithelial cells of different ADPKD mouse models (Shillingford et al., 2006, 2010), as observed by us in 9.7 and 9.12 ADPKD cell lines.

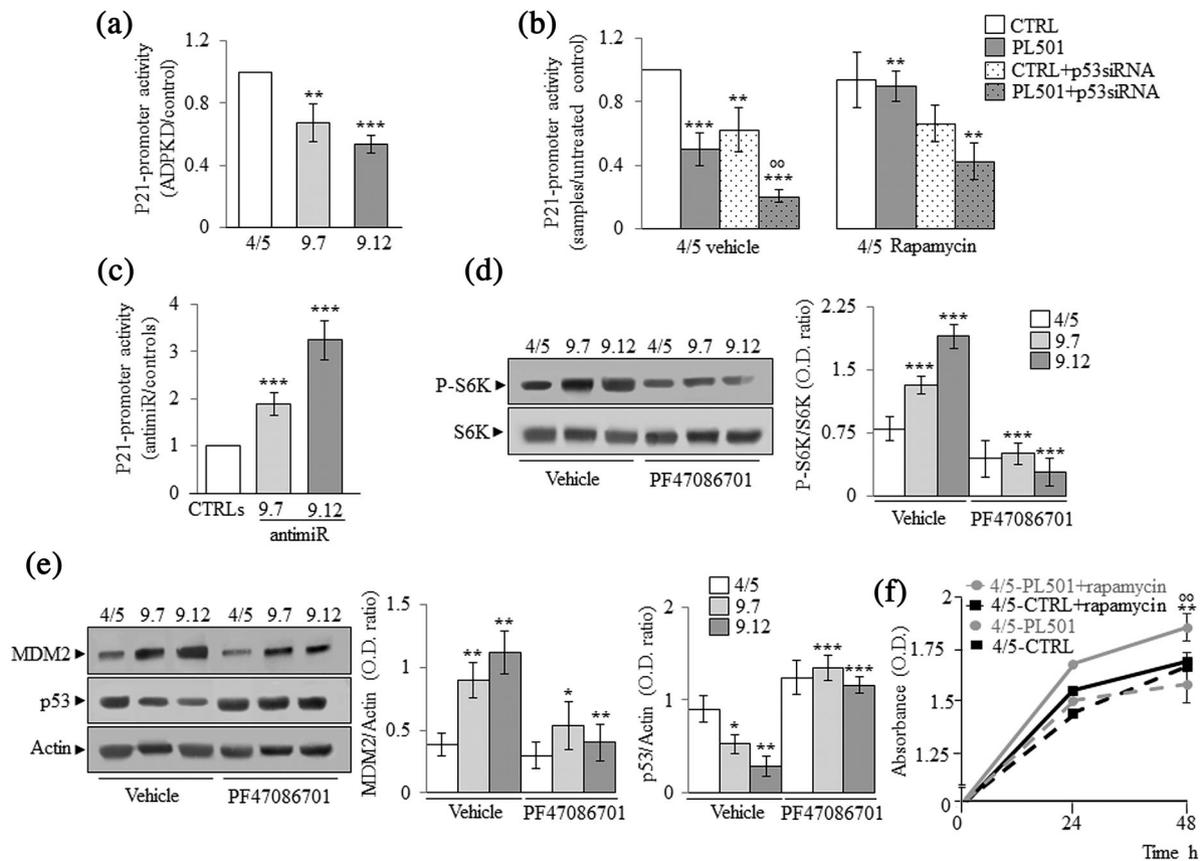


FIGURE 6 The protein kinase mTOR is able to modulate p53 function and cell proliferation in a mechanism involving p70S6K. The activity of p53 was evaluated by luciferase assay transfecting control and ADPKD cells with the p21-Luc plasmid constructed inserting the p53 binding site of p21 promoter upstream of luciferase gene (Xu et al., 2008). After cell lysis and substrate addition, luciferase activity of samples was detected by a luminometer. (a) 9.7 and 9.12 ADPKD cells show a reduced luciferase activity compared with 4/5 control cells (** $p < 0.01$ and *** $p < 0.001$). (b) The transfection of 4/5 cells with PL501 or p53 siRNA decreases the activity of p21 luciferase compared with cells transfected with an irrelevant plasmid or scramble sequences (*** $p < 0.001$ and ** $p < 0.01$, respectively). The combined treatment with PL501 and p53 siRNA further decreases luciferase promoter activity as compared to control cells or transfected with PL501 alone (*** $p < 0.001$ and ° $p < 0.01$, respectively). The treatment with 500 nM rapamycin enhances luciferase activity in both 4/5 cells transfected with PL501 alone or in combination with p53 siRNA compared with paired untreated cells (** $p < 0.01$). (c) The downregulation of miR501-5p by anti-miR treatment in 9.7 and 9.12 cells increases p21 promoter activity (*** $p < 0.001$). (d) In basal conditions the activity of p70S6K, analyzed by Western blotting, is higher in 9.7 and 9.12 cells than in 4/5 cells (*** $p < 0.001$). The treatment of cells with PF47086701 (10 μ M) decreases p70S6K phosphorylation compared with untreated cells (*** $p < 0.001$). (e) In absence of PF47086701 (vehicle), the expression of MDM2 is higher while the levels of p53 are lower in 9.7 and 9.12 cells than in 4/5 ones (** $p < 0.01$ for MDM2; * $p < 0.05$ and ** $p < 0.01$ for p53). Conversely, the inhibition of p70S6K significantly reduces MDM2 protein content and increases the expression of p53 in 9.7 and 9.12 cells (* $p < 0.05$ and ** $p < 0.01$ for MDM2; *** $p < 0.001$ for p53). (f) Rapamycin administration decreases cell growth in 4/5 cells transfected with PL501. Cells were cultured in DMEM/F12 medium with 1% FBS and treated for 24 and 48 hr with or without rapamycin. Next, cell growth was analyzed by the CellTiter method. After 48 hr of culture, 4/5 cells transfected with PL501 exhibit greater proliferation than 4/5 control cells (** $p < 0.01$). The treatment of 4/5 PL501 transfected cells with 500 nM rapamycin for 48 hr reduces cell growth compared with untreated cells (° $p < 0.01$)

Taken together our data indicate that in ADPKD cells, the inhibition of mTOR signaling or miR501-5p downregulation causes the reduction of cell growth by a process that prevents p53 degradation and promotes the activation of apoptosis.

4 | CONCLUSIONS

MiRs are associated with different kidney diseases including various forms of chronic kidney disease (CKD), von Hippel-Lindau syndrome, Wilms tumor, and other renal carcinomas (Aguari, 2015; Phua & Ho, 2015;

Trionfini, Benigni, & Remuzzi, 2015). Despite how some miRNAs were found dysregulated in different ADPKD models, their role in kidney cyst development and expansion is still debated. It is known that the upregulation of the miR-17~92 cluster in mouse models of PKD may lead to kidney cysts development by the repression of PKD1, PKD2 and hepatocyte nuclear factor-1 β gene expression as well as through the inhibition of the oxidative phosphorylation (Hajarnis et al., 2017; Patel et al., 2013). Moreover, ADPKD cysts show increased levels of miR-21, also observed in PKD mouse models. The upregulation of this miR inhibits apoptosis in cyst epithelial cells and promotes disease progression (Lakhia et al., 2016). Finally, the downregulation of miR-193b-3p was observed in

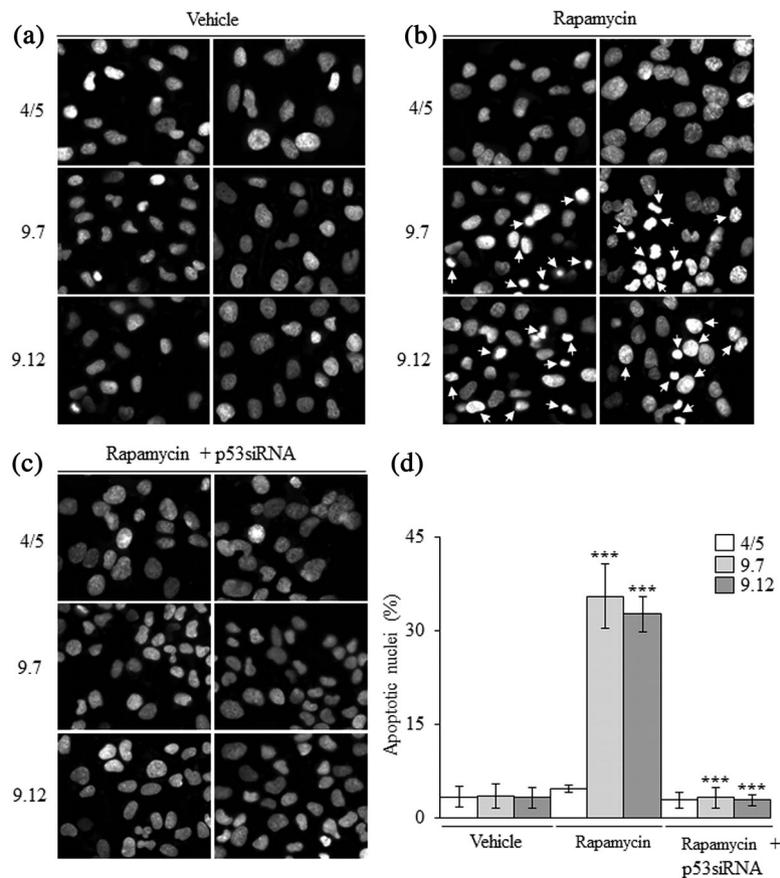


FIGURE 7 A total of 4/5, 9.7, and 9.12 cells were seeded on coverslips and cultured for 48 hr in DMEM/F12 0.4% BSA in presence of vehicle (a), with 500 nM rapamycin alone (b), or combined with p53 siRNA (c). After treatments, cells were fixed, permeabilized, and stained with Hoechst 33342. Nuclei were acquired at 40 \times magnification by a fluorescence microscope and processed through the ImageJ software. Arrows indicate the apoptotic nuclei. (d) Bars indicate the increase of apoptotic nuclei percentage in 9.7 and 9.12 cells treated with rapamycin compared with untreated cells (** $p < 0.001$). The transfection of cells with p53 siRNA arrests the formation of apoptotic nuclei in 9.7 and 9.12 cells treated with rapamycin (** $p < 0.001$)

ADPKD derived cell lines compared with control cells and causes an increased expression of ErbB4 that is a factor driving cyst growth in ADPKD (Streets et al., 2017).

In this work, we report that miR501-5p is upregulated in human ADPKD cells and tissues. The overexpression of this miR affects the activity of mTOR reducing the levels of PTEN and TSC1 proteins. Therefore, we speculate that in ADPKD cells the activation of mTOR pathway may also occur through the knockdown of PTEN and TSC1 expression by the upregulation of miR501-5p. Moreover, we describe a novel role for mTOR in ADPKD molecular pathogenesis linked to MDM2-mediated p53 ubiquitination through a network that involves the activation of p70S6 kinase. The downregulation of p53 by proteasomal degradation could be involved in abnormal proliferation of ADPKD cells.

So far, the role of p53 in ADPKD pathogenesis has been poorly considered. However, in the Pkd1 gene knockout mice, the increased proliferation of renal tubular epithelial cystic cells occurs through the downregulation of p53 (Nishio et al., 2005). Moreover, in PKD1 deficient cells the upregulation of the macrophage migration inhibitory factor promotes cell proliferation and negatively regulates apoptosis by the inhibition of p53-dependent signaling (Chen et al., 2015). We

have consistently observed that the treatment of ADPKD cells with rapamycin, PF47086701, MG132, and anti-miR501-5p sequences prevented p53 degradation and stimulated apoptosis.

These findings still support the use of mTOR inhibitors for the treatment of ADPKD, but unfortunately the treatment of ADPKD patients with rapamycin and its derivatives failed clinical trials. The reasons of this failure could be due to the selection of ADPKD patients with advanced CKD and irreversible kidney injury or to the administration of mTOR inhibitor dosages not sufficient to achieve a biological efficacy (Yu, El-Ters, & Winklhofer, 2015). However, the administration of higher doses of these compounds could enhance drug-related side effects. Thus, in order to overcome this problem, a combined use of mTOR and other PKD-related signaling blockers as well as the inhibition of new possible therapeutic targets such as MDM2 and p70S6K could be an attractive option for ADPKD treatment (de Stephanis et al., 2017; Liu, Li, et al., 2016; Mekahli et al., 2014).

Finally, our data provide evidence that PTEN, mTOR, p70S6K, MDM2, and p53 form a molecular network modulated by miR501-5p expression that promotes cell proliferation in ADPKD cells.

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CONFLICTS OF INTEREST

Authors have no conflicts of interest to disclose.

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SUPPORTING INFORMATION

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