**ORIGINAL RESEARCH ARTICLE**

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

**Running head:** mTOR and p53 modulation by miR501-5p in ADPKD

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## ABSTRACT

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 signalling pathways. In addition, microRNAs (miRs) may also regulate the ADPKD related signalling 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 signalling. 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 signalling and the restoring of p53 function. The data presented here confirm that the impaired mTOR signalling plays an important role in ADPKD.



**Key words**: ADPKD; mTOR signalling; MDM2 and proteasome; p53; miR501-5p.

 

## 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 [1]. 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 [2]. ADPKD cyst formation is driven by abnormal proliferation of renal tubular epithelial cells, increased apoptosis and extracellular matrix alterations [3]. In ADPKD cells, these processes are caused by the dysregulation of different signalling pathways including mTOR-related signalling [4]. 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 [5]. Cell cycle and apoptosis in ADPKD cells are also regulated by the tumour suppressor p53, which is downregulated in both human and mice ADPKD kidney cysts [6]. 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 [6]. The tumour 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 [7]. 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 [8-9]. The silencing of miR-17 attenuates cyst growth in PKD mouse models and suppresses proliferation and cyst growth of primary ADPKD cells [9].

We have described that the putative oncogenic microRNA501-5p stimulates cell proliferation in different kidney carcinoma cell lines by the activation of mTOR signalling pathway [10].

Therefore, its differential expression could affect ADPKD molecular pathogenesis as already observed for the oncogenic miR-17~92 cluster [8]. 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 (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, Italy). 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 (Italy), while anti-TSC1 antibody (Bethyl Cat# A300-316A RRID:AB_2287978) was obtained from BETHYL Laboratories, inc. (Tema Ricerca, 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, Italy). Rapamycin and the S6K inhibitor PF4708671 were obtained from Sigma-Aldrich and Santa Cruz Technologies (Italy), respectively. AntagomiR anti-miR501-5p and scramble sequences were purchased by Ambion (Life Technologies, Italy), while p53-siRNA sequences were obtained by Santa Cruz Technologies (Italy). The recombinant plasmids (PL501) expressing hsa-miR501-5p

sequences alone or linked to GFP were generated by OriGene Technologies (Tema Ricerca, Italy). Recombinant plasmids p53-GFP and p21-Luc were produced by other laboratories [11].

*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 [12]. 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 [13]. 4/5 kidney control cells were isolated from a non-ADPKD subject. 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 [12-13]. Control and PKD1 silenced HEK293 stable clones were generated in our laboratory as previously described [12, 14]. 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 analysed 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, 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) as previously described [10]. Differential miR expression was analysed through the GeneSpring GX software

(AgilentTechnologies) 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, Italy) using specific primers for selected miRs and the small nuclear U6B RNA used as endogenous control (reference gene) [10, 15]. Quantitative PCR was carried out using the TaqMan method with the Rotor-Gene Q Real time PCR cycler (Qiagen, Italy).

MiR expression values were expressed as fold change ratio (ADPKD/control samples), calculated by the delta-delta Ct method as previously reported [15-16].

*2.4 Cell transfection*

Cells, plated overnight in DMEM/F12 medium supplemented with 10% FBS, were transiently transfected using TurboFect Transfection Reagent (Thermo Scientific, Italy). Cells were transfected with the constructs PL501 (1.5 μg/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 ($2x10^5$ cells/well and $5x10^3$ cells/well, respectively) for 6 h 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 1.

*2.5 Western blotting and immunoprecipitation*

Cells were washed twice in PBS buffer containing a cocktail of protease inhibitors (Sigma-Aldrich, Italy), 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, Italy). Filters were blocked at room temperature for 1 h 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 h 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, Italy). Protein band intensity was detected by film scanning with the GS-700 Imaging Densitometer (BIO-RAD, 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 h 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 h. After washing, immunoprecipitates were analysed 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 h 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 h and incubated with an anti-proteasome antibody solution for 2 h at room temperature. After washing, cells were treated for 1 h with a secondary rhodamine-conjugated antibody (Thermo Fisher Scientific, Italy). Finally, cells were washed three times in PBS buffer and fluorescent signals were detected by a fluorescence microscope. Images were acquired at 40X magnification using a Zeiss Axiovert 200 fluorescence microscope equipped with a back-illuminated CCD camera (Roper Scientific, Tucson, USA) and processed by ImageJ software [10].

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 h in the presence/absence of rapamycin (500 nM) or MG132 (10 nM). Apoptosis was also analysed 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 antimiR (30 nM). After treatment, cells were fixed, permeabilized, and stained with Hoechst 33258 (10 mg/mL) in the dark. Images were acquired at 40X magnification by a fluorescence microscope, equipped with CCD camera, as described above.

The activity of p53 was measured by luciferase assay by using the Lucifrase Assay System (promega, Italy) in 4/5 control cells as well as in 9.7 and 9.12 ADPKD cells. $2x10^5$ cells for well were seeded in a 6-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 h 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 h 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, Italy) and the samples were analysed using a 20/20n luminometer (Turner Biosystems, Sunnyvale, CA, USA). Data calculated as relative firefly luciferase units (RLUS) normalized by the β-galactosidase units, were expressed as the n-fold change ratio (sample/control) [17].

*2.7 Cell proliferation analysis*

Analysis of cell proliferation was performed by the CellTiter method (promega, Italy) plating 5000 cells/well in 96 well plates. Cells were starved for 24 h in DMEM/F12 0.4% BSA and transfected with PL501, antimiR and relative controls (irrelevant plasmid and scramble sequences, respectively). After transfection, cells were cultured for further 24 h and 48 h 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 h with a solution containing tetrazolium salts that were converted by living cells in formazan, a purple coloured compound. Cell medium was transferred in a new 96 well plate and colour intensity was detected at 490 nm by a plate reader. Color intensity is directly proportional to the number of living cells [18].

*2.8 Statistical analysis*

All data for statistical analysis are inserted in supplementary table 1. The statistical analysis was performed using a Student's t test, except for microarray findings, which were analysed

 

by an Anova test. Data were reported as mean ± 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 [13]. 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 has-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 (Fig. 1B). Conversely, the expression of the other miRs was not significantly changed in ADPKD tissues compared to controls (Fig. 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 (Fig. 1C). Consistently, increased levels of this miR in stably PKD1-gene silenced HEK293 clones compared with those transfected with scramble sequences were also observed (Fig. 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 [10]. 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 1β [8-9]. Moreover, the increased expression of miR199a-5p in ADPKD tissues and cell lines promoted cell proliferation by targeting CDKN1C mRNA [19]. 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 signalling and induced the inhibition of apoptosis in cyst epithelial cells [20]. 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 signalling 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 signalling 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 [21]. 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 (Fig. 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 [10, 22]. 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 (Fig. 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 Fig. 1A) also induced the downregulation of both PTEN and TSC1 genes as well as the activation of mTOR kinase (Fig. 2B). Moreover, the upregulation of this miR stimulated the expression of MDM2 as well as the reduction of p53 (Fig. 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 Fig. 1F) and consequently increased PTEN and TSC1 protein levels leading to the reduction of mTOR phosphorylation (Fig. 2C). As expected, the downregulation of miR501-5p enhanced p53 expression and reduced MDM2 protein levels (Fig. 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 (Fig. 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 signalling. Interestingly, renal cyst formation caused by the elevated mTOR activity in TSC1 knockout mice was already described [23]. 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 [24]. Despite PTEN being an upstream effector of mTOR signalling, 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 [23]. 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 signalling.

 

In order to investigate the role of mTOR signalling 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 (Fig. 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 (Fig 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 (supplementary Fig. 1B and Fig. 3B). 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 [10], confirming that this miR may affect p53 expression through the activation of mTOR signalling. 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 [25]. Taken together these findings suggest that ccRCC and ADPKD cells could share a common mechanism used to negatively regulate p53 expression.

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 [26], as well as in epithelial cells derived from renal cysts of ADPKD mice [6]. 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 [6]. 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 [27]. Actually, an increase in high molecular weight forms of p53 in 9.7 kidney cystic cells compared with 4/5 control cells was observed (Fig. 4A). The overexpression of miR501-5p in 4/5 cells transfected with PL501 plasmid (supplementary Fig. 1C) also enhanced the levels of p53-ubiquitin conjugates compared with 4/5 control cells (Fig. 4B). Moreover, 4/5 cells transfected with both PL501 and p53-GFP plasmids exhibited a greater number of cytoplasmic aggregates that co-localized with proteasome structures than control cells (Fig. 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 2A). The silencing of p53 by siRNA dramatically reduced fluorescence intensity, confirming that p53 is trapped in proteasomal structures in ADPKD cells (supplementary figure 2A). 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 (Fig. 5B, and D). The reduction of p53 expression by siRNA treatment (supplementary Fig. 2B) abrogated apoptotic nuclei formation in 9.7 and 9.12 cells treated with MG132 (Fig. 5C and D), 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 [10] as well as in other tumour cells [28], 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 [29]. However, the possible use of anti-proteasome 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 [29].

*3.4 Rapamycin treatment restores p53 function and promotes cell death*

The dysregulation of mTOR signalling is involved in ADPKD progression [30], moreover our data suggest that the activation of this protein kinase plays a key role in p53 degradation. Thus, the inhibition of mTOR signalling 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 [11]. In basal conditions, the luciferase activity was higher in 4/5 than in 9.7 and 9.12 cell lines (Fig. 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 Fig. 1D), also caused the reduction of p53 activity compared with same cells transfected with an irrelevant construct (Fig. 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 Fig. 2C) and inhibited the activity of p21 promoter (Fig. 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 (supplementary Fig. 2C and Fig. 6B). 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 (Fig. 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 Fig. 1G) as well as the increase of p53 function (Fig. 6C).

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

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 (Fig. 6D), but caused the reduction of MDM2 expression as well as the increase of p53 levels in 9.7 and 9.12 cells only (Fig. 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 [31]. Finally, the upregulation of miR501-5p (supplementary Fig. 1E) also caused an increased cell growth as compared to control cells (Fig. 6F, grey and black solid lines). The treatment of these cells with rapamycin for 48 h slowed cell proliferation compared with untreated cells (Fig. 6F, solid and dashed grey lines), suggesting that the activation of mTOR promotes cell growth as already observed in other cystic kidney cells [32]. 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 (Fig. 7B and D). The silencing of p53 abolished the rapamycin-induced apoptotic nuclei formation (Fig. 7C and D), 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 3A, and B).

These findings support previous observations where the treatment with rapamycin inhibited cell proliferation in different human and mouse ADPKD cells [5, 32]. 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 [33]. Moreover, inhibition of mTOR promotes apoptosis in cyst-lining epithelial cells of different ADPKD mouse models [30, 32], as observed by us in 9.7 and 9.12 ADPKD cell lines.

Taken together our data indicate that in ADPKD cells, the inhibition of mTOR signalling 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 tumour and other renal carcinomas [34-36]. 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-1β gene expression as well as through the inhibition of the oxidative phosphorylation [8-9]. 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 [20]. Finally, the downregulation of mir-193b-3p was observed in ADPKD derived cell lines compared with control cells and causes an increased expression of ErbB4 that is a factor driving cyst growth in ADPKD [37].

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 [6]. 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 signalling [38]. 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 [39]. 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 signalling blockers as well as the inhibition of new possible therapeutic targets such as MDM2 and p70S6K could be an attractive option for ADPKD treatment [5, 40-41].

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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## 6. CONFLICT OF INTEREST

Authors have no conflicts of interest to disclose.

 

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## 8. FIGURE LEGENDS

**Figure 1**

Expression of miRs in ADPKD cells and tissues analysed 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) [10]. (B) Real time RT-PCR analysis of six miRs 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 ΔΔCt method as previously described [15-16]. 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, analysed 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 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 analysed in 9.7 and 9.12 ADPKD cells transfected with anti-miR501-5p (antimiR) or scramble (CTRL) sequences. The treatment with antimiR 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 analysed by Western blotting.

**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 h 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 h 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 h in the presence of the proteasome inhibitor MG132 (10 μM) 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 analysed 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 analysed by fluorescence microscopy. 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, permeabilised, treated with an anti-proteasome antibody and finally placed in a solution containing a secondary antibody conjugated with rhodamine. After washing, cells were analysed by a fluorescence microscope equipped with CCD camera at 40X magnification and images were processed by ImageJ software.

**Figure 5**

The inhibition of the proteasome promotes apoptosis in ADPKD cells. 4/5, 9.7 and 9.12 cells were cultured on coverslips in DMEM/F12 0.4% BSA for 48 h in absence (A) and in presence of 10 nM MG132 alone (B) or in combination with p53 siRNA oligonucleotides (C). Next, cells were fixed, permeabilised and stained with Hoechst 33342. Cell images were acquired at 40X 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).

**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 [11]. 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 antimiR treatment in 9.7 and 9.12 cells increases p21 promoter activity (***$p$<0.001). (D) In basal conditions the activity of p70S6K, analysed 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 μM) 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 h and 48 h with or without rapamycin. Next, cell growth was analysed by the CellTiter method. After 48 h 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 h reduces cell growth compared with untreated cells (°°$p$<0.01).

**Figure 7**

4/5, 9.7 and 9.12 cells were seeded on coverslips and cultured for 48 h 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, permeabilised and stained with Hoechst 33342. Nuclei were acquired at 40X 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).



 

MicroRNA501-5p target genes related with ADPKD.

| Gene acronym | Gene Description | Target sites | Function | Signalling | References |
|---|---|---|---|---|---|
| BCL2 | B-cell CLL/lymphoma 2 | 1 | Apoptosis regulator | JNK/Bcl-2 | Yu W et al, 2010 |
| CREB1 | cAMP responsive element binding protein 1 | 3 | Transcription factor | cAMP/CREB/AR | Aguiari G et al, 2012 |
| EGFR | epidermal growth factor receptor | 1 | Growth factor receptor | EGFR/Raf1/ERK | Yamaguchi T et al, 2003 |
| HGF | hepatocyte growth factor | 1 | Growth factor | HGF/cMET/Wnt | Qin S et al, 2012 |
| ID2 | DNA-binding protein inhibitor | 1 | Cell cycle regulator | ID2/p21 | Li X et al, 2005 |
| JAK2 | Janus Kinase | 1 | Cell growth regulator | JAK2/STAT1/p21 | Bhunia AK et al, 2002 |
| JUN | jun proto-oncogene | 2 | Cell cycle regulator | JNK/AP-1 | Parnell SC et al, 2002 |
| KRAS | Kirsten rat sarcoma viral oncogene homolog | 1 | Cell cycle regulator | Ras/Raf1/ERK | Parker E et al, 2007 |
| NRAS | neuroblastoma RAS viral oncogene homolog | 1 | Cell cycle regulator | Ras/Raf1/ERK | Parker E et al, 2007 |
| NFAT | nuclear factor of activated T-cells | 1 | Transcription factor | NFAT/PKCa | Aguiari G et al, 2008 |
| P2RX7 | purinergic receptor P2X, ligand-gated ion channel 7 | 1 | Cationic permeable channel | P2RX7/ERK | Chang MY et al, 2010 |
| PDE4B/D | phosphodiesterase 4 isophorms B-D | 1 | Phospho-diesterase, cAMP-specific | cAMP level regulation | Pinto CS et al, 2015 |
| PDGF | platelet-derived growth factor beta polypeptide | 1 | Growth factor | PDGF/PDGFR | Wilson PD et al, 1993 |
| PKD2L1 | polycystic kidney disease 2-like 1 | 1 | Calcium channel | Receptor channel complex | Molland KL et al, 2010 |
| PKD2L2 | polycystic kidney disease 2-like 2 | 2 | Calcium channel | Plasma membrane channel component | Chen Y et al, 2008 |
| PKHDL1 | polycystic kidney and hepatic disease like 1 | 1 | Membrane receptor | Plasma membrane receptor | Hogan MC et al, 2003 |
| PTEN | Phosphatase and tensin homolog | 2 | Protein phosphatase | PTEN/PI3K/Akt | Zhou J et al, 2009 |
| RICTOR | RPTOR independent companion of mTOR | 2 | Cell growth regulator | mTORC2 | Ravichandran K et al, 2014 |
| SP1 | Transcription factor Sp1 | 1 | Transcription factor | Polycystin-1 expression | Jeon JO et al, 2007 |
| STAT1 | Signal transducer and activator of transcription 1 | 1 | Transcription factor | JAK/STAT1/p21 | Bhunia AK et al, 2002 |

 

| | | | | | |
|---|---|---|---|---|---|
| STIM1 | stromal interaction molecule 1 | 2 | Calcium sensor | STIM1/IP3R | Santoso NG et al, 2011 |
| TSC1 | tuberous sclerosis 1 | 3 | mTOR inhibitor component | TSC1/TSC2/mTOR | Zhou J et al, 2009 |
| TRPC3 | transient receptor potential channel, subfam. C, member 3 | 1 | Calcium channel | TRPP2/TRPC3/TRPC7 | Miyagi K et al, 2009 |
| TRPC4 | transient receptor potential channel, subfam. C, member 4 | 2 | Calcium channel | TRPP2/TRPC4 | Du J et al, 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.



 

**A**

| miRs | probes | ADPKD vs normal | P-value |
|---|---|---|---|
| hsa-miR501-5p | A_25_P00011045 | 3.20 | 0.035 |
| hsa-miR196b | A_25_P00010523 | 2.00 | 0.036 |
| hsa-miR202 | A_25_P00011128 | 1.80 | 0.02 |
| hsa-miR368 | A_25_P00010013 | 0.40 | 0.001 |
| hsa-miR601 | A_25_P00010642 | 0.34 | 0.03 |
| hsa-miR142-5p | A_25_P00010896 | 0.28 | 0.048 |

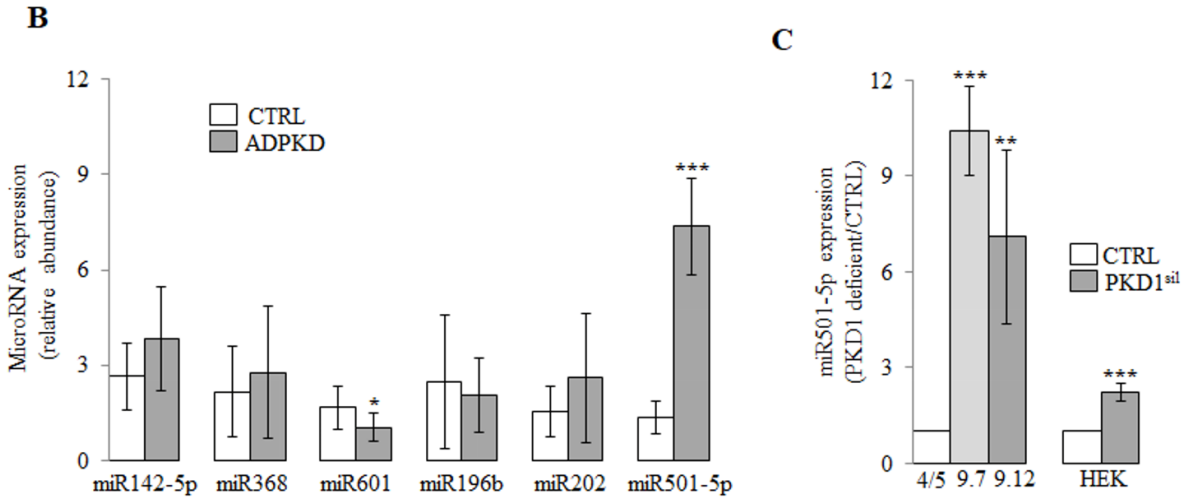


*Figure 1*

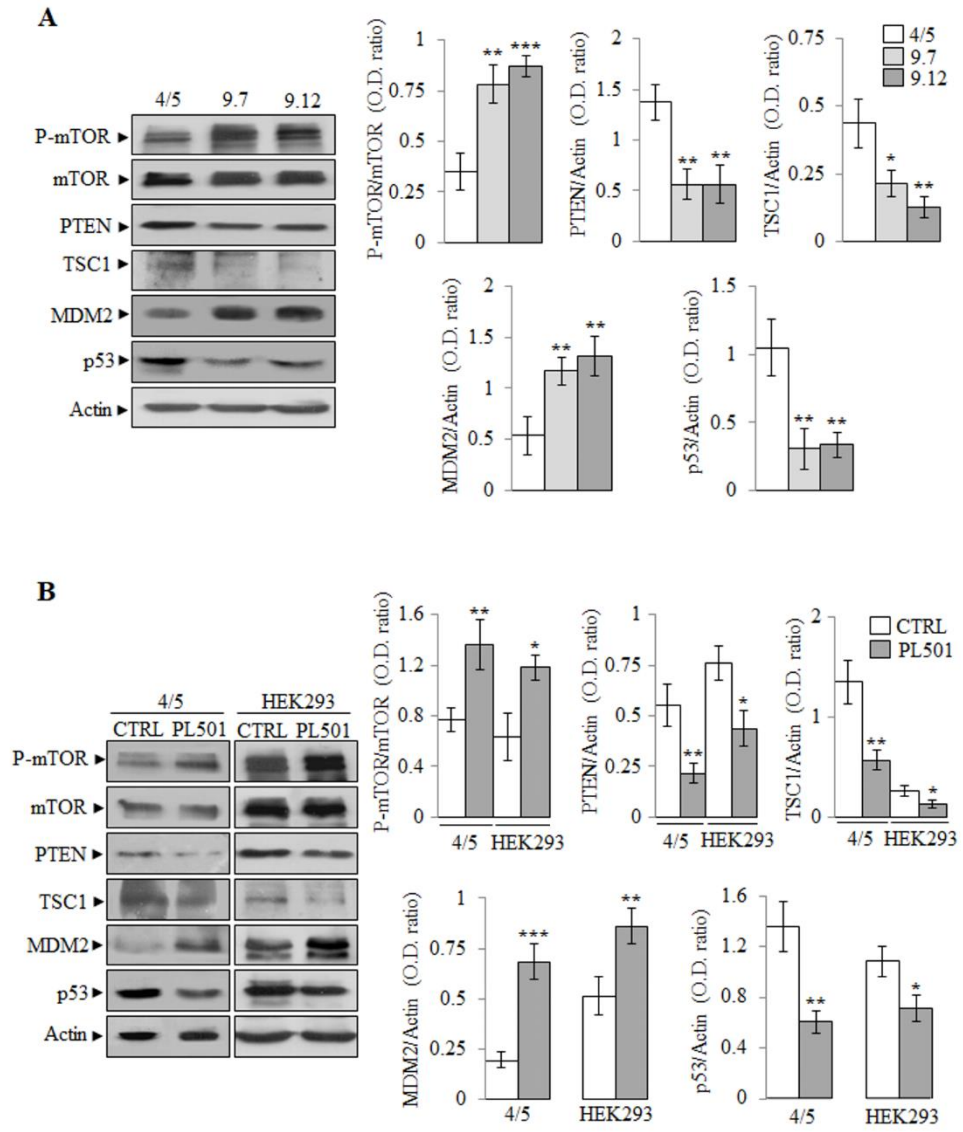


*Figure 2*

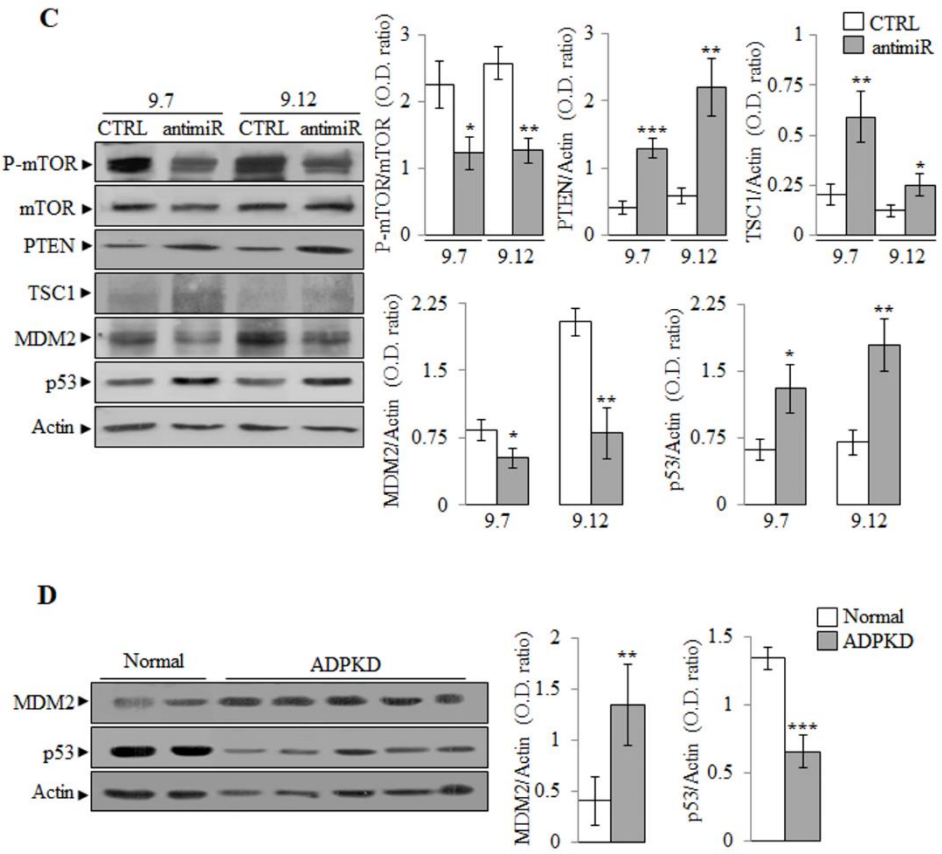


*Figure 2*

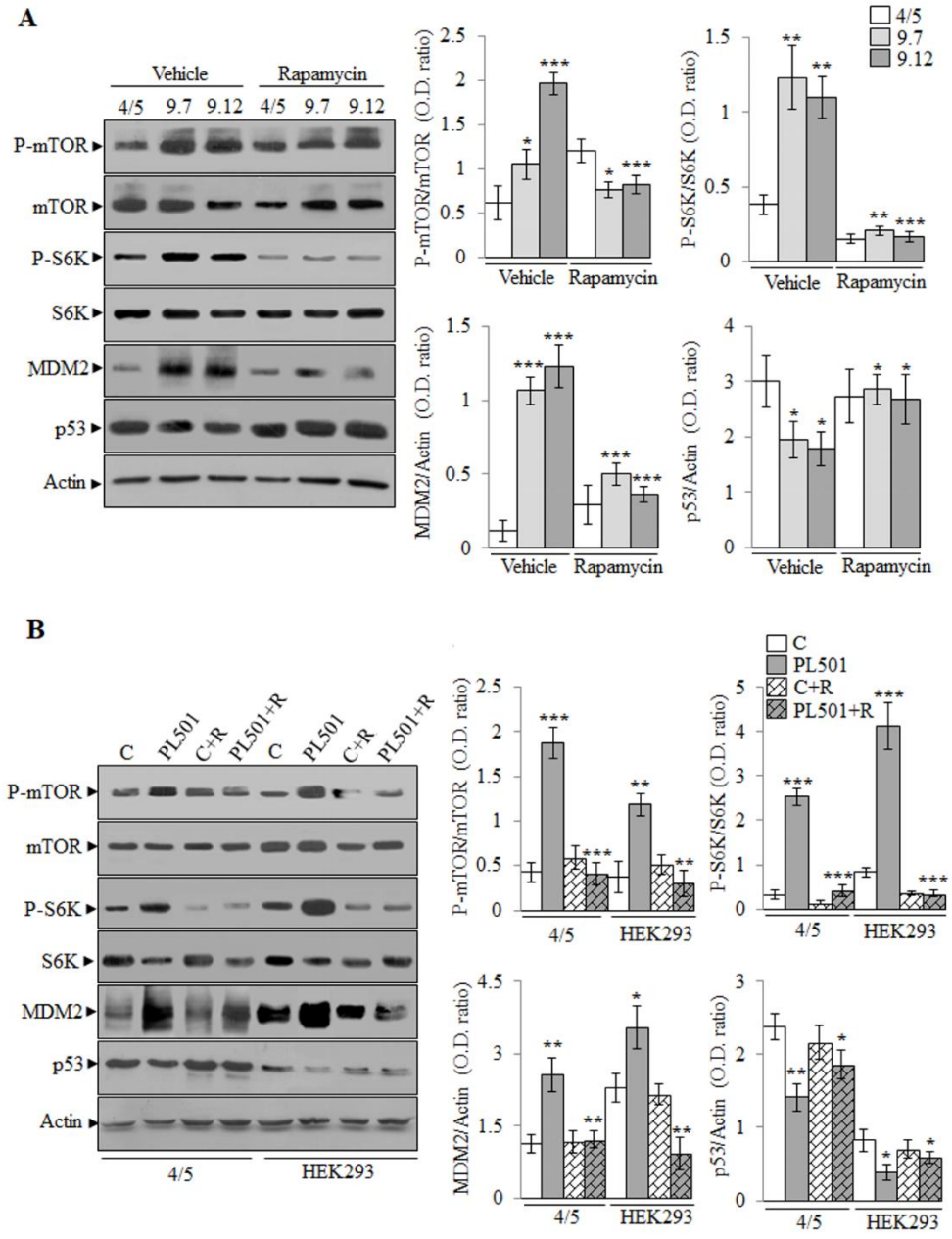


*Figure 3*

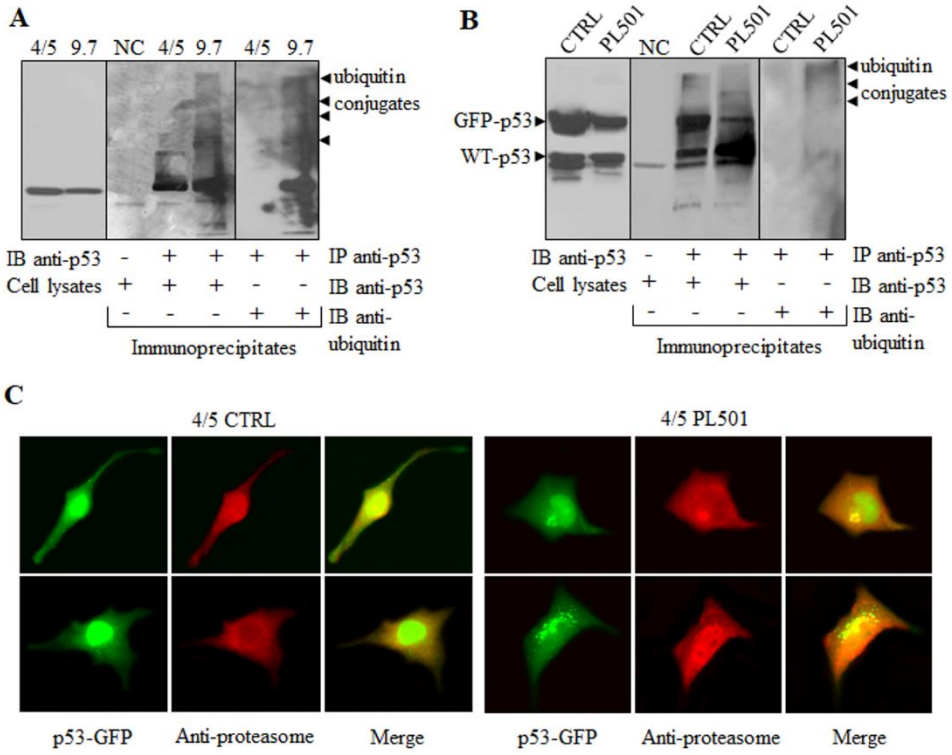


*Figure 4*

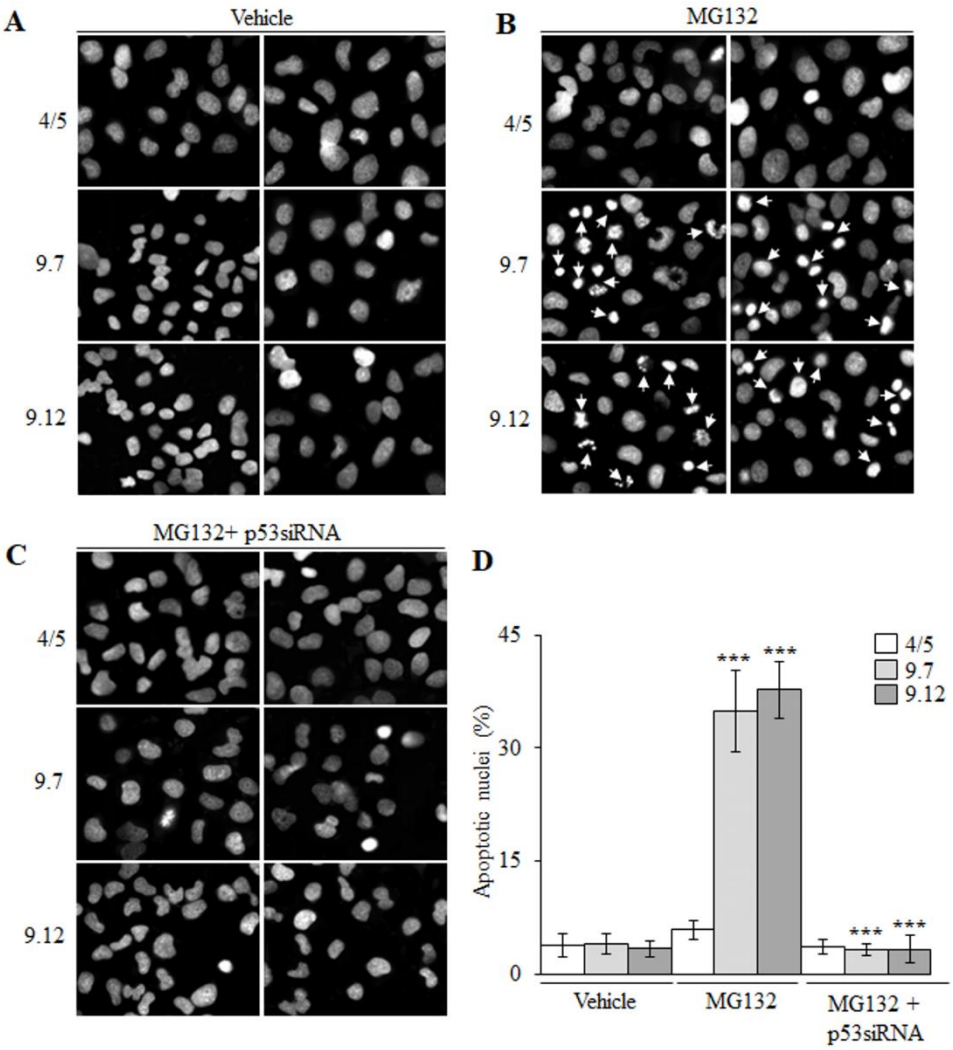


*Figure 5*

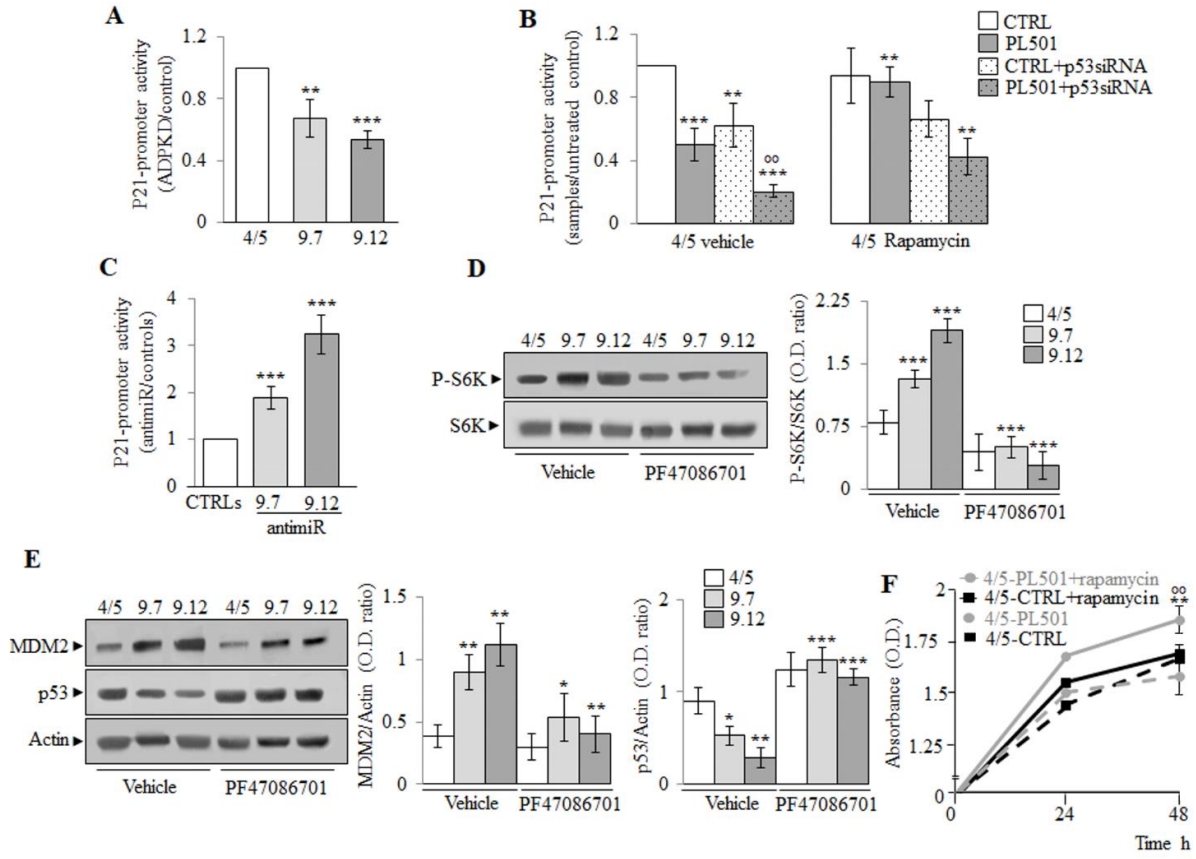


*Figure 6*

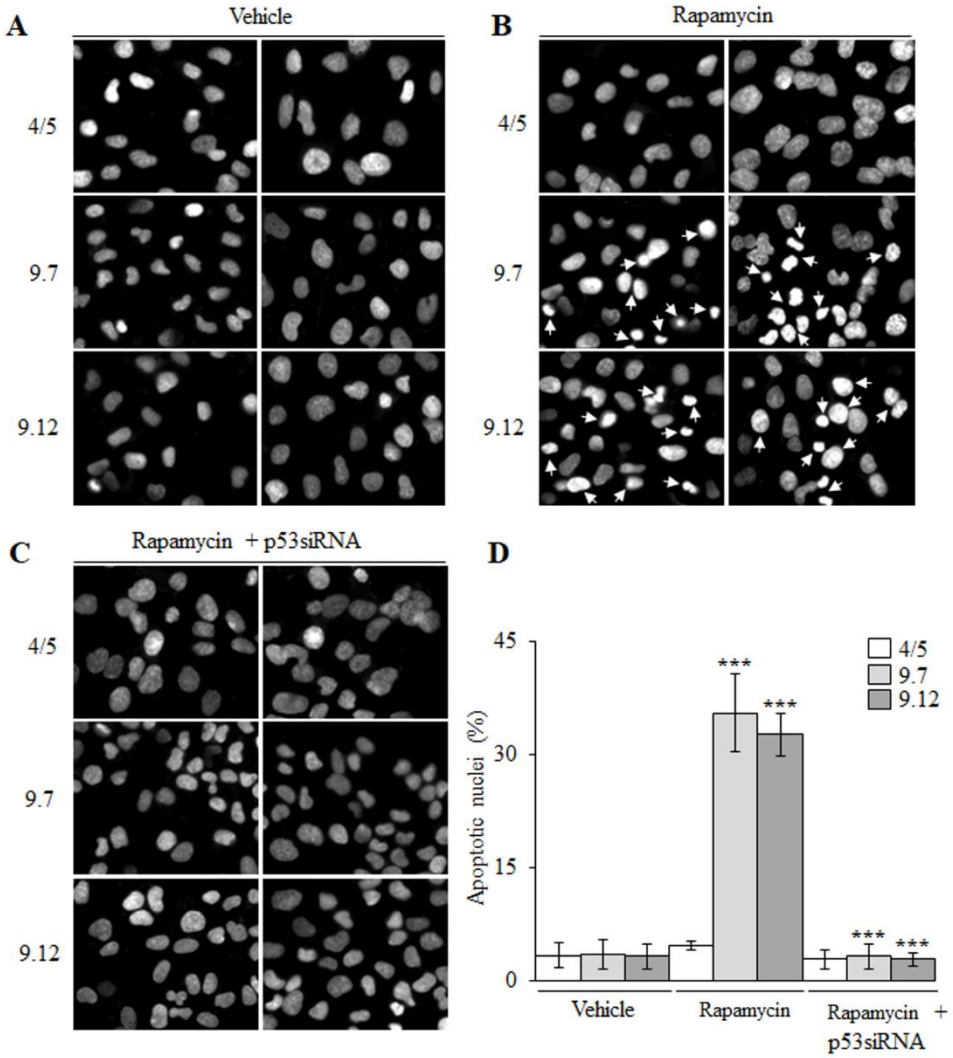


*Figure 7*