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RESEARCH ARTICLE

The inhibition of MDM2 slows cell proliferation and activates apoptosis in ADPKD cell lines

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Abstract

Introduction: Autosomal dominant polycystic kidney disease (ADPKD) is characterised by progressive cysts formation and renal enlargement that in most of cases leads to end stage of renal disease (ESRD). This pathology is caused by mutations of either *PKD1* or *PKD2* genes that encode for polycystin-1 (PC1) and polycystin-2 (PC2), respectively. These proteins function as receptor-channel complex able to regulate calcium homeostasis. *PKD1/2* loss of function impairs different signalling pathways including cAMP and mTOR that are considered therapeutic targets for this disease. In fact, Tolvaptan, a vasopressin-2 antagonist that reduces cAMP levels, is the only drug approved for ADPKD treatment. Nevertheless, some ADPKD patients developed side effects in response to Tolvaptan including liver damage. Conversely, mTOR inhibitors that induced disease regression in ADPKD animal models failed the clinical trials.

Results: Here, we show that the inhibition of mTOR causes the activation of autophagy in ADPKD cells that could reduce therapy effectiveness by drug degradation through the autophagic vesicles. Consistently, the combined treatment with rapamycin and chloroquine, an autophagy inhibitor, potentiates the decrease of cell proliferation induced by rapamycin. To overcome the dangerous activation of autophagy by mTOR inhibition, we targeted MDM2 (a downstream effector of mTOR signalling) that is involved in TP53 degradation by using RG7112, a small-molecule MDM2 inhibitor used for the treatment of haematologic malignancies. The inhibition of MDM2 by RG7112 prevents TP53 degradation and increases p21 expression leading to the decrease of cell proliferation and the activation of apoptosis.

Conclusion: The targeting of MDM2 by RG7112 might represent a new therapeutic option for the treatment of ADPKD.

KEYWORDS
ADPKD, apoptosis, autophagy, cell growth, RG7112

Abbreviations: ATG7, autophagy related 7 protein; BSA, bovine serumalbumin; cAMP, cyclic adenosinemonophosphate; CASP-3, caspase-3; DMEM, Dulbecco'sModified Eagle Medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphatedehydrogenase; GFP, green fluorescent protein; LC3, microtubule-associatedproteins 1A/1B light chain 3B; MDM2, mouse double minute 2 homolog; mTOR, mammalian target of rapamycin; PARP-1, poly[ADP-ribose] polymerase 1; PBS, phosphate-buffered saline; PKD, polycystic kidney disease; SQSTM1, sequestosome-1 protein; TP53, tumor protein p53.

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is the most common inherited kidney disorder characterised by the formation of fluid-filled cysts in the kidneys (Vasileva et al., 2021). ADPKD causes the formation and enlargement of cysts in the kidney and other organs leading to end-stage renal disease (ESRD) (Nobakht et al., 2020). This pathology is mainly associated with mutations of *PKD1* or *PKD2* genes encoding for polycystin-1 and -2 (PC1 and PC2), respectively (Vasileva et al., 2021). PC1 and PC2 interacting by each other form the polycystic complex that is localised in the primary cilium of kidney epithelial cells and regulate calcium influx in response to mechanical stimuli (Nobakht et al., 2020). PC1 and PC2 loss of function causes the dysfunction of several signalling pathways including those regulated by cAMP and mTOR (de Stephanis et al., 2017, 2018; Nobakht et al., 2020). The main treatment for ADPKD patients remains the dialysis; however, recent findings have allowed a better understanding of the molecular mechanisms linked to cyst formation leading to the discovery of new therapeutic drugs based on the molecular targeting. In particular, the treatment with Tolvaptan, a selective vasopressin receptor 2 (V2R) antagonist, is actually able to slow ADPKD progression (Vasileva et al., 2021). Tolvaptan is first drug approved for ADPKD treatment; nevertheless, ADPKD patients treated with Tolvaptan developed several side effects including thirst, polyuria and liver injury (Riella et al., 2014; Vasileva et al., 2021). Other treatments using inhibitors of the mechanistic target of rapamycin (mTOR), such as Sirolimous and Everolimus, have failed clinical trials even if they were able to arrest cyst growth in ADPKD pre-clinical models (Riella et al., 2014; Shillingford et al., 2010). The inhibition of mTOR, a negative regulator of autophagy, causes the activation of autophagy that is used by the cell to destroy old and malfunctioning proteins to generate energy available for cell life (Feng et al., 2014). The role of this biological process in ADPKD remains obscure and debated. Some observations report that the induction of autophagy causes disease progression (Lee et al., 2020), but other studies suggest that the activation of autophagy suppresses cystogenesis (Zhu et al., 2017). Recently, we demonstrated that the abnormal activation of mTOR in ADPKD causes the degradation of TP53 protein by proteasome machinery in a mechanism involving MDM2. Moreover, the inhibition of mTOR by rapamycin treatment reactivates TP53 expression (de Stephanis et al., 2018). Importantly, the induction of autophagy could be used by ADPKD cell to remove and destroy the pharmacological compound used as therapeutic treatment contributing to drug resistance as observed in cancer (Li et al., 2017).

We purpose to bypass the activation of autophagy due to mTOR inhibition by the direct targeting of MDM2 using the compound RG7112, a small-molecule MDM2 inhibitor already used for the treatment of haematologic malignancies (Andreeff et al., 2016). The treatment of ADPKD cells with RG7112 restores TP53 expression, reduces cell growth, increases apoptosis and causes the block of autophagic flux.

MATERIAL AND METHODS

Materials

Media and plastic material for cell culture were obtained from Euroclone (Milan, Italy). Antibodies anti-mTOR, anti-P-mTOR, anti-MDM2, anti-ATG7, anti-TP53, anti-p21, anti-C-Parp1, anti-C-Casp3, anti-GAPDH and anti-Actin were purchased from Cell Signalling Technologies (Euroclone). Antibodies anti-LC3 and anti-p62/SQSTM1 were purchased from Sigma-Aldrich. Enhanced chemiluminescent substrates for Western blotting, HRP-conjugated goat anti-rabbit and anti-mouse antibodies were obtained from Cell Signalling Technologies (Euroclone). Rapamycin, chloroquine and the MDM2 inhibitor RG7112 were purchased from Sigma-Aldrich (Milan, Italy), while the TurboFect transfecting reagent was obtained from Thermo Fisher Scientific (Milan, Italy). Vectors expressing shRNAs for *ATG7* gene silencing were purchased from Sigma-Aldrich. The recombinant p21-Luc construct was kindly gift from Dr. Schimmer (University Health Network, Toronto, Canada), while the GFP-LC3 plasmid was a gift of Prof. Guido Kroemer (Université de Paris, Hôpital Européen George Pompidou, Paris, France). This work was in line with the ethical guidelines of the 1975 Declaration of Helsinki.

Cell lines, proliferation, transfection and transduction

SW40-transformed epithelial kidney control cells (4/5) as well as ADPKD cystic cells (9.7 and 9.12) (Loghman-Adham et al., 2003) were kindly provided by Prof. Harris (Mayo Clinic College of Medicine, Rochester, MN). These cell lines express similar levels of large T-antigen (Figure S1A). HEK293 clones stably transfected with a recombinant plasmid expressing silencing sequences for the *PKD1* gene (HEK^{PKD1a} and HEK^{PKD1b}) and with the empty vector (HEK^{pS}) used as control were generated in our laboratory (Aguiari et al., 2008). The effective silencing of *PKD1* gene was checked in all clones (Figure S1B). Heterozygous (PH2) and homozygous (PN24) *Pkd1*-inactivated mouse cell lines were produced by others (Wei et al., 2008). All cell lines were cultured at 37°C in DMEM/F12 (1:1) medium supplemented with 10% FBS in a humidified incubator (95% air and 5% CO_2). Control and *PKD1* silenced HEK293

clones were continuously cultured in presence of the antibiotic G-418. Cell lines were periodically screened for mycoplasma contamination. Cell proliferation was analysed by using the CellTiter method (Promega, Milan, Italy). Cell proliferation analysis was performed seeding 5000 cells for well in 96-well plates in DMEM/F12 10% FBS and starved overnight in DMEM/F12 0.4% BSA. Next, cells were cultured in DMEM/F12 1% FBS for 24 h in presence of 500 nM rapamycin and 25 μM of the autophagy inhibitor chloroquine (CQ) alone or in combination. Cells were also treated with RG7112 at different concentrations (1, 2 and 5 μM). As control, cells were cultured in presence of DMSO or ethanol as appropriate. At the end of incubation, cells were cultured at 37°C for 5 h with a solution containing tetrazolium salts that were converted by living cells in formazan, a purple compound. Finally, the culture medium was transferred in a new 96-well plate and colour medium was analysed by a plate reader at 490 nm. The intensity of colour is directly proportional to the number of living cells (Bonon et al., 2013).

Cell transfection was performed using the non-immunogenic transfection reagent TurboFect (Thermo Fisher Scientific). 200,000 cells for well were seeded in six well plates and cultured overnight in DMEM/F12 medium supplemented with 10% FBS. Next, the medium was replaced with DMEM/F12 0.4% BSA and cells were transfected for 6 h with GFP-LC3, mCherry-GFP-LC3 or p21-Luc plasmids as appropriate following the manufacturer's instructions.

The silencing of *ATG7* gene was performed by cell transduction of 4/5 CTRL and 9.12 ADPKD cell lines using lentiviruses expressing specific ATG7 shRNAs (shATG7) or the empty vector (pLKO), used as control. 4/5 and 9.12 cells were cultured overnight in DMEM/F12 10% FBS. Next, medium was replaced with DMEM/F12 1% FBS and cells were transduced adding medium containing shATG7 and pLKO lentivirus particles (dilution 1:10) for 24 h (Patergnani et al., 2020). Finally, 4/5 and 9.12 cells were cultured in DMEM/F12 1% FBS for further 24 h.

Cell imaging and luciferase assay

200,000 cells were seeded on 24 mm coverslips placed in six-well plates and cultured overnight in DMEM/12 10% FBS. Next, cells were transfected with the GFP-LC3 plasmid for 6 h using the Turbofect reagent (Thermo Fisher Scientific) in DMEM/F12 0.4% BSA and cultured for further 24 h in DMEM/F12 1% FBS added with ethanol (vehicle) or 500 nM rapamycin. After treatments, cells were washed twice in PBS buffer, fixed at RT in 4% paraformaldehyde for 15 min, permeabilised in 100% methanol for 10 min at –20°C and washed twice with PBS buffer. Finally, images were acquired at 60× magnification using an Olympus FW3000 confocal microscope and processed by ImageJ software as follows. Process: Subtract Background → Image: Adjust: Threshold → Process: Binary: Fill Holes → Process: Binary: Convert To Mask → Analyse: Analyse Particles. For the analysis of the autophagic flux, immunoblot and live cell imaging techniques were used. For the immunoblot approach, 9.7 and 9.12 cells were seeded in six-well plates, treated with vehicle or 5 μM RG7112 for 24 h in duplicate and cultured for further 2 h with or without 25 μM NH_4Cl. Cells were then collected, lysed and immunoblotted for the autophagy marker LC3. For a quantitative analysis of the autophagy flux by imaging technique, cells were cultured on 24 mm glass coverslips and then transfected with the tandem plasmid mCherry-GFP-LC3, as previously reported (Danese et al., 2022; Patergnani et al., 2013), by using the Lipofectamine LTX reagent (Thermo Fisher Scientific). After 36 h of expression, cell images were acquired by using an Olympus FW3000 confocal microscope at 60× magnification. Cells were processed and analysed at the ImageJ software. For co-localisation analysis, the background of both channels was removed by using the 'Subtract Background' command. Next, co-localisation was quantified by using the 'JACOP' plugin.

Apoptosis was evaluated by Hoechst staining (apoptotic nuclei) and Western blotting (C-Parp1 and C-Casp3 cleavage) in control cells and ADPKD cellular models cultured in DMEM/F12 0.4% BSA and treated for 24 h in the presence/absence of RG7112 (5 μM).

After treatment, cells were fixed, permeabilised, and stained with Hoechst 33258 (10 mg/ml) in the dark. Images were acquired at 40× magnification using a Zeiss Axiovert 200 fluorescence microscope equipped with a back-illuminated CCD camera (Roper Scientific, Tucson, AZ) and processed by ImageJ program.

Luciferase assay was performed in cells treated with DMSO (vehicle) or RG7112 (5 μM) seeding 200,000 cells for well in a six-well plate. Briefly, cell were cultured overnight in DMEM/F12 medium supplemented with 10% FBS. Next, cells were transfected with the p21-Luc vector in combination with β-galactosidase construct (ratio 3:1) for 6 h using the Turbofect reagent (Thermo Fisher Scientific) in DMEM/F12 0.4% BSA. After transfection, cells were washed and cultured for further 24 h in DMEM/F12 medium supplemented with 1% FBS with or without rapamycin. Afterwards, cells were lysed, and a part of protein solution was mixed with luciferase and β-galactosidase substrates in separated tubes following the manufacturer's protocol (Promega). Luciferase and β-galactosidase activity was measured as count number using a 20/20n luminometer (Turner Biosystems, Sunnyvale, CA). Values of luciferase activity were calculated as ratio between counts of luciferase and those of β-galactosidase used for sample normalisation (Aguiari et al., 2012).

Western blotting

After treatments, cells were washed twice in PBS buffer containing a cocktail of protease inhibitors (Sigma-Aldrich), detached by a cell scraper and collected by centrifugation. Next, cell pellets were lysed in TBS 1% Triton X-100 buffer supplemented with a cocktail of protease and phosphatase inhibitors. Twenty-five micrograms of total protein lysate were electrophoresed using 4%–15% SDS-polyacrylamide gel gradients and transferred onto nitrocellulose filters (Euroclone). 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 three washing in PBS-T at room temperature, filters were incubated for 1 h at room temperature in PBS-T with the appropriate horseradish peroxidase-conjugated secondary antibody and washed again three times in PBS-T. Finally, protein bands were visualised by using Super Signal Femto or Pico chemiluminescence systems (Euroclone) by using photographic films or digitally acquired with the imaging system ImageQuant LAS 4000 (GE Healthcare). Protein band intensity was processed using the ImageJ software. Phosphorylation levels of mTOR were calculated as the ratio between the phosphorylated form and the band intensity of total protein. LC3 protein content were obtained as ratio between the short (LC3II) and the long (LC3I) chain of the protein. Relative abundance of the other investigated proteins was quantified as the ratio between the protein of interest and β-Actin or GAPDH as appropriate.

Statistical analysis

Statistical analysis was performed by the GraphPad Prism software using the Student's *t*-test or ANOVA, as appropriate. Data are reported as mean ± Standard Deviation of at least three independent experiments and differences are considered significant at $p < 0.05$. Data for statistical analysis are inserted in Table S1.

RESULTS

Analysis of autophagy in kidney control and ADPKD-related cells cultured in basal conditions and treated with rapamycin

ADPKD is associated with the abnormal activation of mTOR that is a negative regulator of autophagy. We analysed autophagy by the expression of LC3I/II and p62/SQSTM1 proteins in control kidney cells (4/5) and in two different ADPKD cell lines (9.7 and 9.12). Notably, when autophagy is activated, the cytoplasmic form of LC3 (LC3I) is cleaved to form LC3-phosphatidylethanolamine conjugate (LC3II) that associates with the membranes of autophagosomes and correlates with the number of autophagosomes (Patergnani et al., 2021). Conversely, p62/SQSTM1 expression levels are inversely correlated with the levels of autophagy (Klionsky et al., 2021). As shown in Figure 1a, the levels of LC3II in 9.7 and 9.12 are lower than in 4/5 control cells, while those of p62/SQSTM1 are higher. These data were confirmed in two different human and mouse ADPKD cellular models (Figure 1b). Because the administration of rapamycin reduces mTOR activity, this treatment should stimulate autophagy. Actually, the treatment with rapamycin causes the reduction of mTOR phosphorylation as well as the increase in LC3II protein content in 4/5 control as well as in 9.7 and 9.12 ADPKD cells compared with untreated (Figure 1c). Data found by Western blotting were confirmed transfecting cells with a plasmid expressing LC3 protein linked to GFP (Missiroli et al., 2016). As shown in Figure 2a,b, 4/5 CTRL cells transfected with GFP-LC3 show a greater number of green puncta that represent the autophagic vesicles than 9.7 and 9.12 ADPKD cells. Consistently, the number of autophagosomes is higher in cell lines transfected with GFP-LC3 and treated with rapamycin than in those cultured in presence of vehicle (Figure 2a,c). These findings indicate that ADPKD cells exhibit reduced autophagy compared with control cells and the inhibition of mTOR activates autophagy all cell types.

Evaluation of TP53 expression and cell proliferation in cells treated with rapamycin and chloroquine alone and in combination

Since the inhibition of mTOR causes the increase of autophagy, it is reasonable to block this process to prevent the possible degradation of drugs by the autophagic machinery. We evaluated cell proliferation in control and ADPKD cells treated with rapamycin and the potent autophagy inhibitor CQ, alone or in combination with each other. As expected 9.7 and 9.12 ADPKD cells cultured in basal conditions grow faster than 4/5 control cells (Figure 3a). The application of rapamycin and CQ alone reduces cell proliferation in both 9.7 and 9.12 but not in 4/5 cells, while the combined treatment slows cell growth in all cell types (Figure 3a). Interestingly, the double treatment with rapamycin and CQ further decreases cell proliferation especially in 9.12 ADPKD cells (Figure 3a). Consistent with our previous data (de Stephanis et al., 2018), the expression of TP53 is lower in 9.7 and 9.12 ADPKD than in 4/5 CTRL cells (Figure 3b). The treatment with rapamycin significantly enhances the levels of TP53 protein in ADPKD cells but not in control ones (Figure 3b). No significant changes in TP53 protein content after treatment with CQ were observed. In addition, the combined

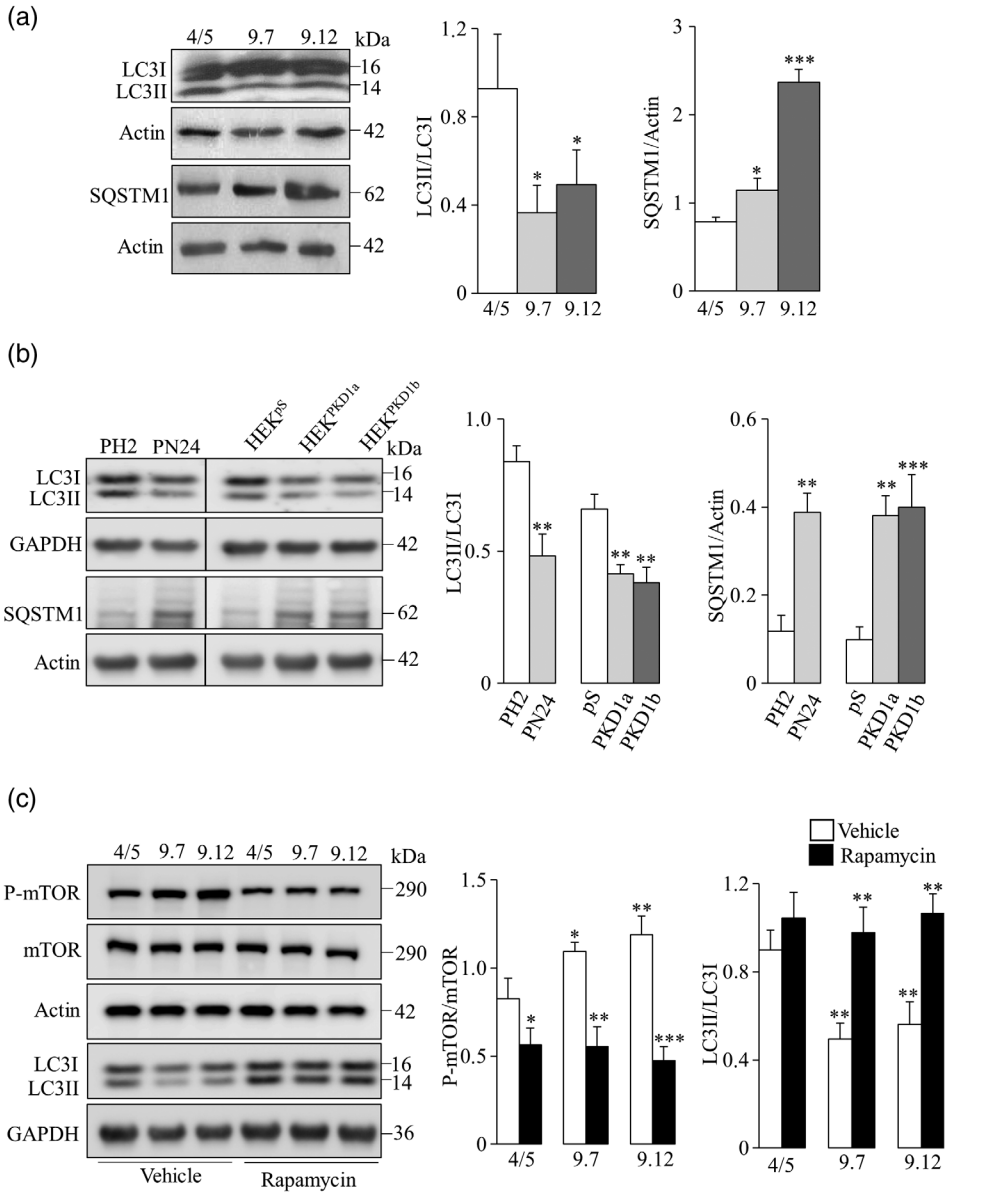

FIGURE 1 Analysis of autophagy in different ADPKD cellular models cultured in basal conditions. Autophagy was also evaluated in ADPKD cystic cells treated with rapamycin. (a) Autophagy was analysed using anti-LC3 and anti-p62/SQSTM1 antibodies in kidney control (4/5) and ADPKD (9.7 and 9.12) cells. The levels of LC3II are lower and those of p62/SQSTM1 are higher in 9.7 and 9.12 cells compared with 4/5 (for LC3: $*p < 0.05$; for p62/SQSTM1: $*p < 0.05$ and $***p < 0.001$). (b) The analysis of autophagy was performed in mouse heterozygous (PH2) and homozygous (PN24) *Pkd1*-inactivated cell lines as well as in human control HEK293 cells (pS) and *PKD1* silenced clones (PKD1a and PKD1b). LC3II protein content in both human and mouse PKD1-deficient cells is lower than in controls ($**p < 0.01$), while the expression of p62/SQSTM1 is higher ($**p < 0.01$ and $***p < 0.001$). (c) Control and ADPKD cystic cells were cultured in DMEM/F12 1% FBS in presence/absence of 500 nM rapamycin for 24 h. In absence of rapamycin, phosphorylation levels of ADPKD cells are greater than in CTRL cells, while values of LC3II expression are lower (for mTOR: $*p < 0.05$ and $**p < 0.01$, while for LC3: $**p < 0.01$). The treatment with rapamycin reduces mTOR activity in in all cell types and increases the expression of the autophagic marker LC3II in 9.7 and 9.12 cells (for mTOR: $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$; for LC3: $**p < 0.01$)

treatment with CQ and rapamycin does not enhance the expression of TP53 as compared to rapamycin alone (Figure 3b). These findings indicate that in ADPKD cells the degradation of TP53 by autophagy could be irrelevant. To confirm this speculation, we have inhibited autophagy by the silencing of *ATG7* gene. The delivery of *ATG7* shRNAs by using lentiviruses in 4/5 and 9.12 cells reduces the expression of ATG7 and enhances the levels of p62/SQSTM1 confirming the occurred autophagy inhibition (Figure 3c). As shown in Figure 3c, the inhibition of autophagy by *ATG7* silencing does not affect TP53 expression in both CTRL and ADPKD cells confirming that the amount of TP53 protein removed by autophagy in these cells seems to be poor.

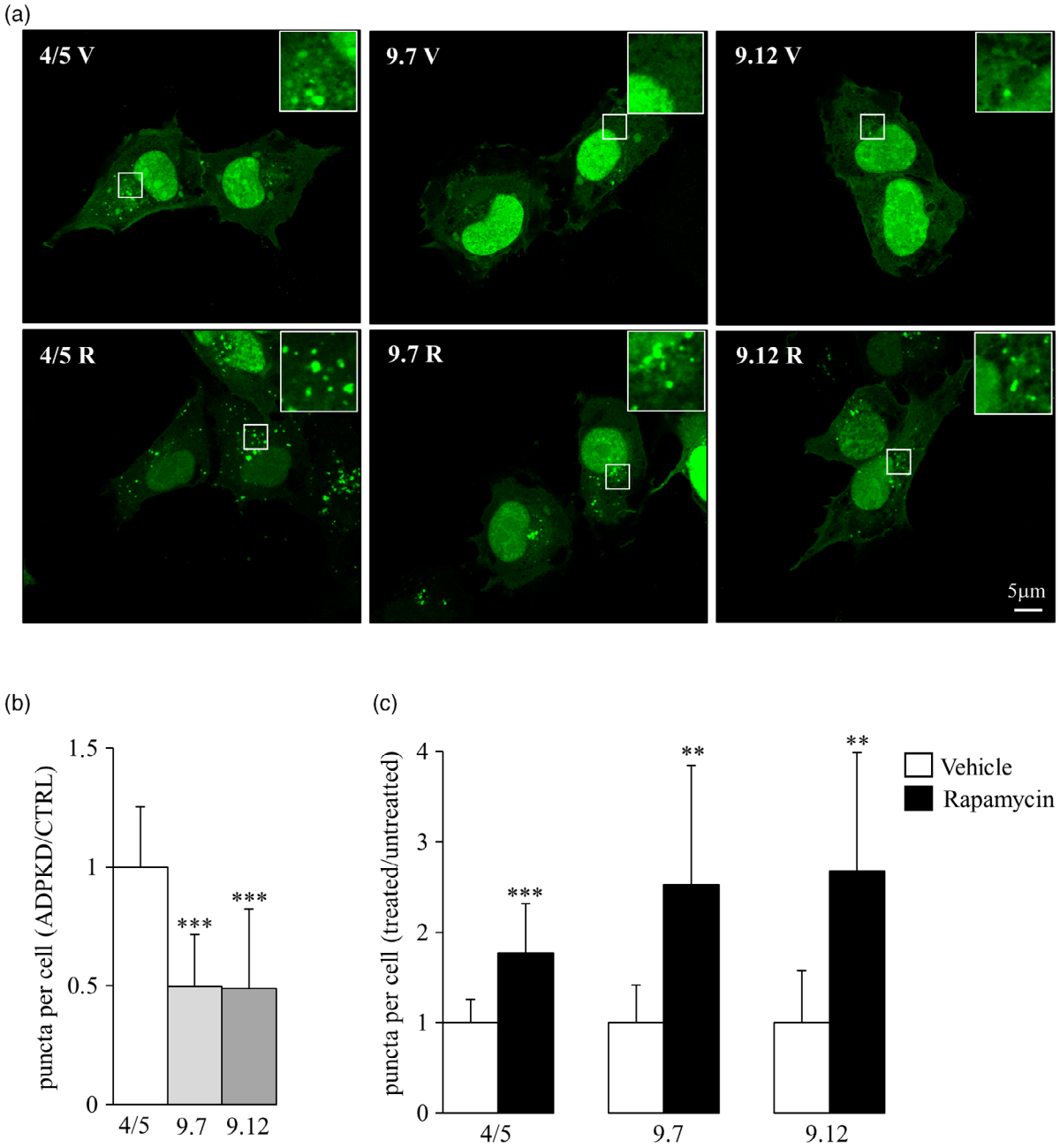

FIGURE 2 Analysis of autophagy by fluorescence microscopy in cells transfected with a plasmid expressing GFP-LC3. (a) Autophagic vesicles were visualised by using a fluorescence confocal microscope at 60× magnification in 4/5 CTRL, 9.7 and 9.12 ADPKD cells treated with rapamycin (R) or in presence of vehicle (V). For each cell, four planes of 0.40 μm each were acquired to collect cell autophagosomes. Acquired images were processed by ImageJ software as described in Material and Methods section. Representative images shown are z-projection of the collected pictures. (b) 4/5 CTRL cells show more cytoplasmic dots than 9.7 and 9.12 ADPKD cells (***$p < 0.001$). (c) The treatment with rapamycin increases the autophagic vesicles in all cell types (***$p < 0.001$ and **$p < 0.01$). Data are expressed as mean ± standard deviation calculated as ratio between the number of puncta detected in ADPKD and control cells or between treated and untreated cells ($N = 10$ for each cell type)

Analysis of MDM2, TP53 and p21 expression as well as cell proliferation and apoptosis in cells treated with RG7112

The treatment with mTOR inhibitors leads to the increase of autophagy, which may affect different biological processes including drug resistance. Therefore, the use of drugs targeting downstream effectors of mTOR could improve ADPKD treatment without increasing the autophagic activity. In this regard, we treated 9.7 and 9.12 ADPKD cells with the MDM2 inhibitor RG7112 since in ADPKD cells TP53 is removed through the

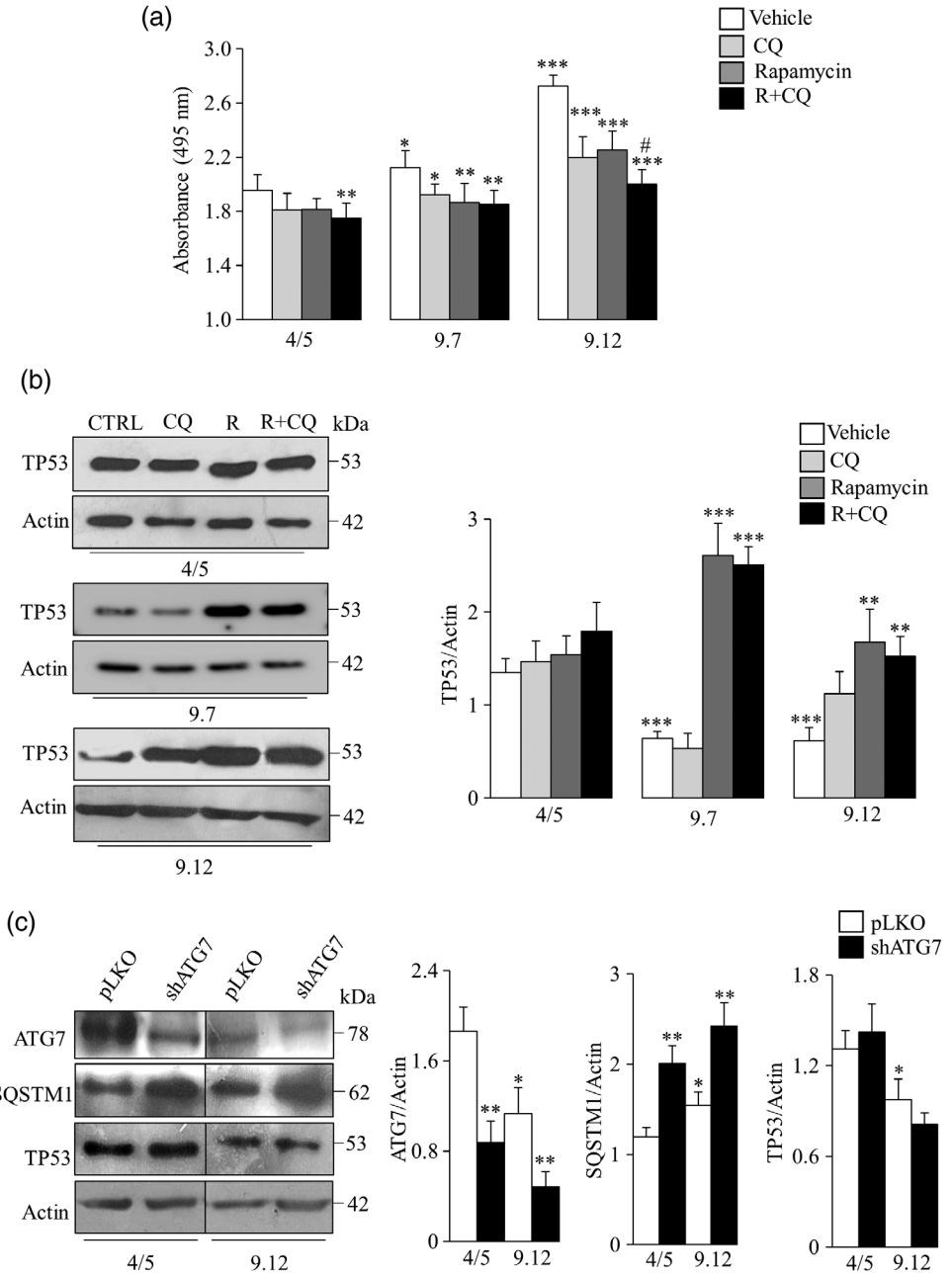

FIGURE 3 Analysis of cell proliferation and TP53 expression in cells treated with rapamycin and chloroquine. TP53 expression was also evaluated after autophagy inhibition by *ATG7* silencing. (a) 9.7 and 9.12 cells cultured for 24 h in DMEMF12 1% FBS in presence of vehicle (DMSO) exhibit a greater cell proliferation than 4/5cells (*$p < 0.05$ and ***$p < 0.001$). Cells were also cultured in presence of rapamycin (R) and chloroquine (CQ) alone or in combination (R+CQ). In 4/5 CTRL cells only the double treatment with R+CQ reduces cells growth compared with untreated cells (**$p < 0.01$). In 9.7 and 9.12 cells, the treatments with R and CQ alone or in combination decreases cell proliferation (for 9.7: *$p <$ 0.05 and **$p < 0.01$; for 9.12: ***$p < 0.001$). In 9.12 cells the combined treatment with R+CQ potentiates the inhibition of proliferation as compared to same cells treated with R alone (#$p < 0.05$). (b) In basal conditions, the expression of TP53 is higher in 4/5 CTRL compared with 9.7 and 9.12 ADPKD cells (***$p < 0.001$). The treatment with rapamycin alone or in combination with chloroquine increases TP53 expression in both 9.7 and 9.12 cells as compared to untreated cells (***$p < 0.01$ for 9.7; **$p < 0.01$ for 9.12). (c) 9.12 cells cultured in basal conditions express lower levels of ATG7 than 4/5 cells (*p<0.05). The treatment with shRNAs for the silencing of *ATG7* gene (shATG7) reduces ATG7 expression in both control (4/5) and ADPKD (9.12) cell lines compared with cells transduced with the empty vector pLKO (**$p < 0.01$). As previously observed, the expression of p62/SQSTM1 is higher while the levels of TP53 are lower in 9.12 ADPKD cells than in 4/5 CTRL cells cultured in DMEM/F12 1% FBS (*$p < 0.05$). The silencing of ATG7 causes the increase of p62/SQSTM1 protein in both cell lines (**$p < 0.01$), but no significant changes in TP53 expression were detected

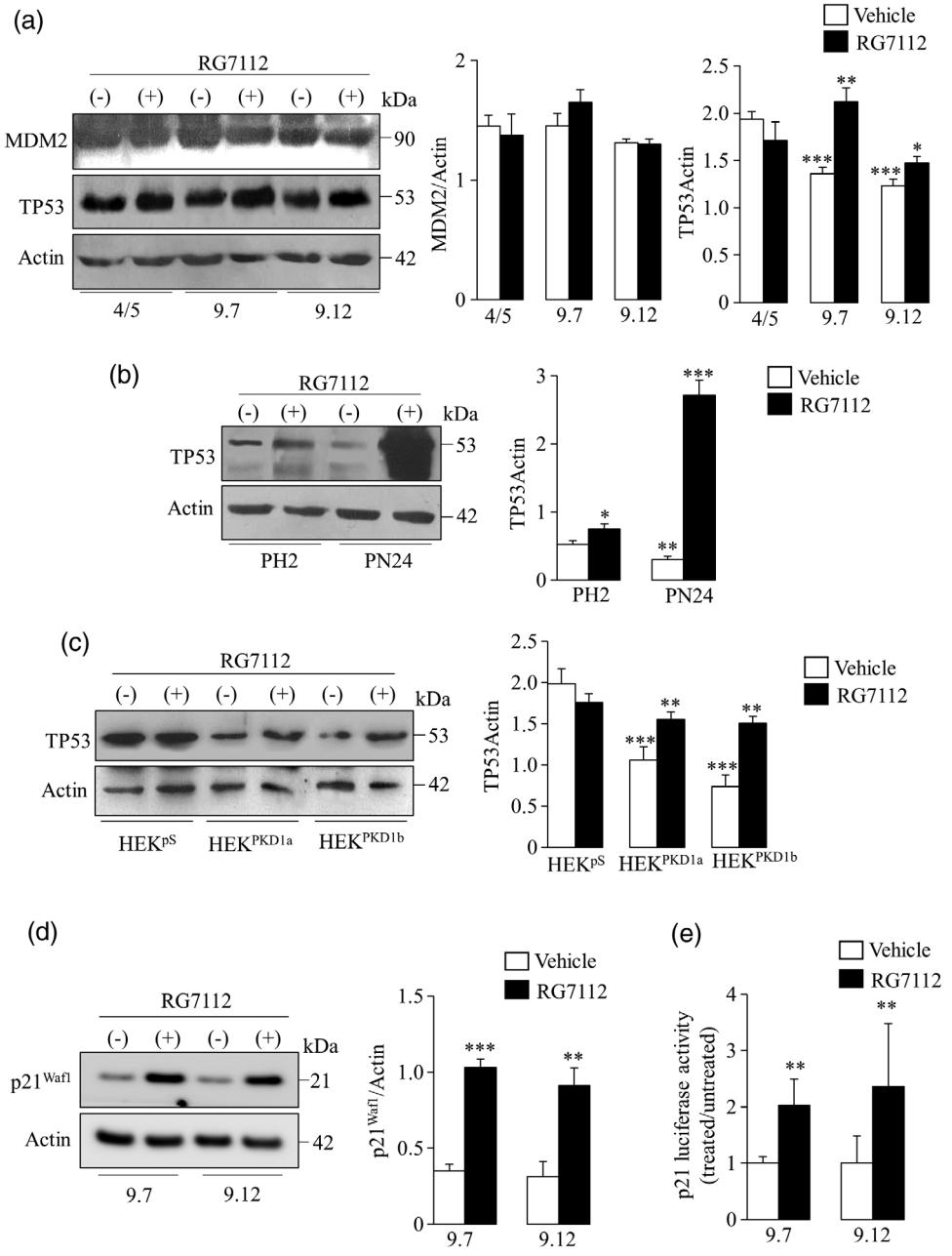

FIGURE 4 Evaluation of TP53-related signalling in cell treated with RG7112. (a) The treatment of CTRL and ADPKD cells with RG7112 (5 μM) does not cause significant changes in MDM2 expression. As expected, 9.7 and 9.12 cells cultured in basal conditions show decreased levels of TP53 as compared to 4/5 cells (***$p < 0.001$). The treatment with RG7112 increases the levels of TP53 in 9.7 and 9.12 cells compared with those untreated (**$p < 0.01$ and *$p < 0.05$). As observed in 9.7 and 9.12 cells, TP53 expression is lower in mouse homozygous *Pkd1*-inacivated (PN24) cells (b) as well as in *PKD1* silenced (HEK^{PKD1a} and HEK^{PKD1b}) clones (c) compared with control (PH2 and HEK^{pS}) cells (**$p < 0.01$ and ***$p < 0.001$). The application of RG7112 increases the levels of TP53 in PN24 cells as well as in HEK^{PKD1a} and HEK^{PKD1b} clones compared with untreated cells (***$p < 0.001$ and **$p < 0.01$). This treatment weakly affects TP53 expression also in mouse heterozygous *Pkd1*-inacivated (PH2) cells (*$p < 0.05$). Consistently, the inhibition of MDM2 by RG7112 enhances p21 protein levels (d) and p21-luciferase activity (e) in both 9.7 and 9.12 cells (***$p < 0.001$ and **$p < 0.01$ for p21 protein levels and **$p < 0.01$ for luciferase activity). Values of luciferase activity are expressed as fold increase (treated/untreated).

proteasome in a mechanism involving the ubiquitin ligase MDM2 (de Stephanis et al., 2018). The treatment with RG7112 does not change MDM2 expression in both 9.7 and 9.12 cells, but significantly increases the levels of TP53 (Figure 4a). The increase of p53 expression induced by RG7112 was also observed in mouse *Pkd1*-inactivated kidney cells as well as in two different *PKD1* silenced HEK293 cell lines (Figure 4b,c). Consistently, the application of RG7112 in 9.7 and 9.12 cells enhances the expression of the cyclin-dependent kinase

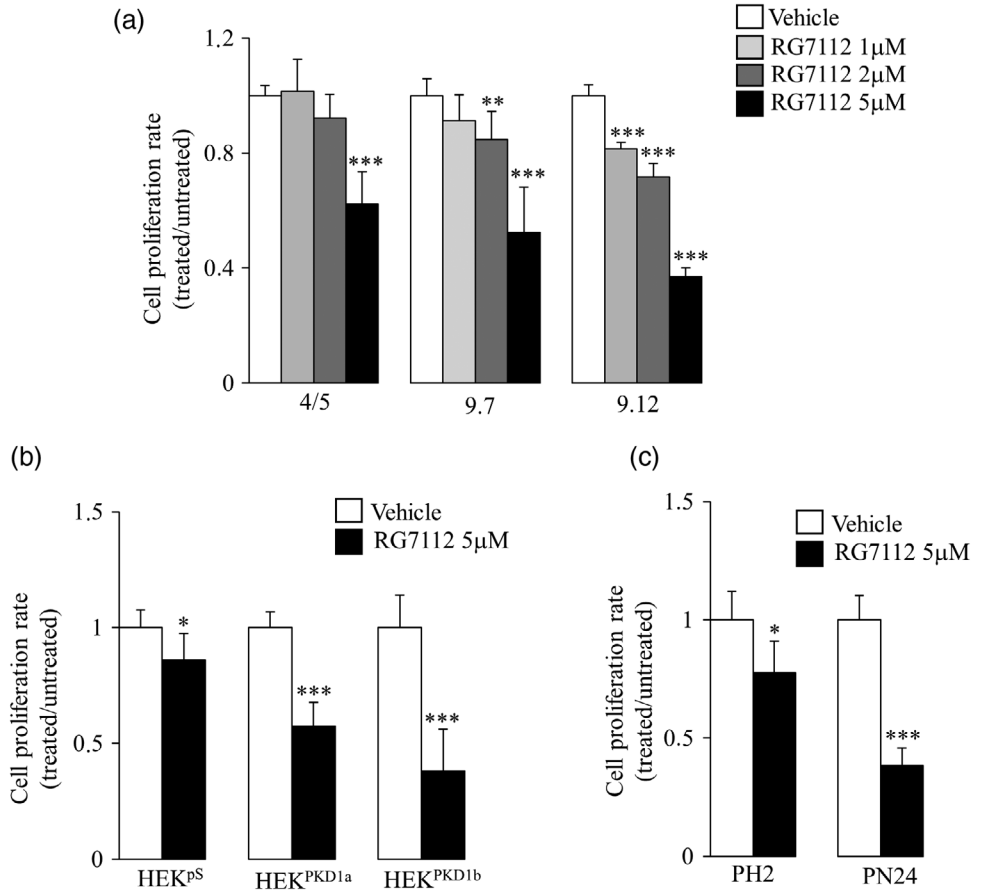

FIGURE 5 Analysis of cell proliferation in cells treated with RG7112. (a) Control and ADPKD cells were cultured in DMEM/F12 1% FBS with different concentrations of RG7112 (1, 2 and 5 μM) for 24 h. The application of RG7112 in 4/5 cells decreases cell growth only at the highest concentration (***$p < 0.001$). In 9.7 cells, the treatment with RG7112 at concentration of 2 and 5 μM slows cell proliferation (**$p<0.01$ and ***$p < 0.001$). RG7112 strongly reduces cell proliferation at all concentrations used in 9.12 cells (***$p < 0.001$). Consistently, the treatment with RG7112 (5μM) decreases cell proliferation mostly in human (HEK^{PKD1a} and HEK^{PKD1b}) *PKD*1 stably silenced clones (b) and in mouse *Pkd1*-deficient (PN24) cells (c) (***$p < 0.001$). Data of cell proliferation are expressed as ratio between cells treated with RG7112 and those untreated.

inhibitor p21 compared with untreated cells (Figure 4d). In addition, this compound potentiates luciferase activity in 9.7 and 9.12 cells transfected with a recombinant plasmid expressing the luciferase gene fused with TP53-responsive elements of the p21 promoter (Figure 4e). As shown in Figure 5a, the treatment with 5 μM RG7112 inhibit cell proliferation in 4/5 control as well as in 9.7 and 9.12 ADPKD cells. Interestingly, the effectiveness of RG7112 in 9.7 and 9.12 cells was observed also at lower doses. In particular, this drug significantly reduces cell growth already at 1μM concentration in 9.12 ADPKD cells (Figure 5a). Similar results were obtained in human and mouse *PKD1*-deficient cells treated with 5mM RG7112 (Figure 5b,c). Finally, the administration of RG7112 dramatically enhances apoptosis in all cellular models for ADPKD compared with untreated cells, while no significant effects in control cells were observed (Figure 6a, Figure S2). Consistently, the treatment with RG7112 induces the activation of C-Parp-1 and Caspase-3 in both 9.7 and 9.12 cells, but not in 4/5 ones confirming that this compound causes the induction of apoptosis in ADPKD cells (Figure 6b). Taken together, these findings suggest that the inhibition of MDM2 reduces cell growth and increases apoptosis by TP53 restoration.

Study of autophagy in cells treated with RG7112

The inhibition of MDM2, a downstream element of mTOR, should prevent the activation of autophagy observed after mTOR inactivation by rapamycin treatment. Surprisingly, as shown in Figure 7a and Figure S3, the treatment with RG7112 causes the increase of LC3II expression in all cell types, thereby suggesting that this compound might activate autophagy. Conversely, the expression levels of p62/SQSTM1 were also increased after RG7112 application indicating that the autophagy activity is decreased. The discrepancy of these data

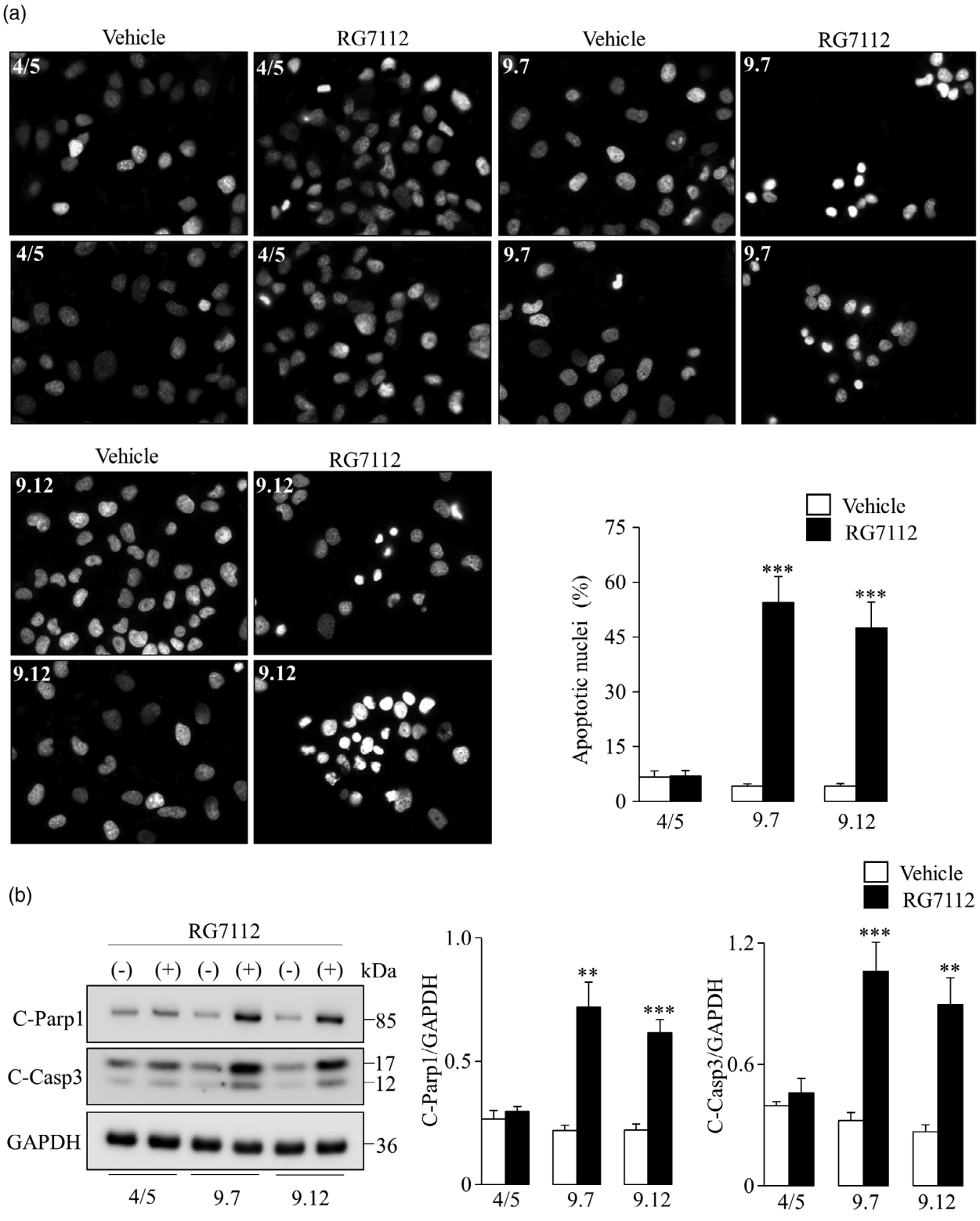

FIGURE 6 Evaluation of apoptosis in control and ADPKD cells treated with RG7112. (a) Apoptosis was analyzed by nuclear staining with Hoechst 33342. Briefly, control (4/5) and ADPKD (9.7 and 9.12) cells were seeded at density of 200,000 for well in six well plates containing coverslips and cultured for 24 h in DMEM/F12 0.4% BSA in presence/absence of 5 μM RG7112. Next, cells were fixed, permeabilised, and stained with Hoechst 33342. After two washing with PBS buffer, images of nuclei were acquired using a fluorescence microscope at 40× magnification and processed by ImageJ software. The treatment with RG7112 strongly increases the percentage of apoptotic nuclei in 9.7 and 9.12 cells (***$p < 0.001$). (b) Apoptosis was also studied by Western blotting evaluating the activation of C-Parp-1 and C-Casp3 in cells treated in presence/absence of RG7112. This compound increases the cleaved form of both C-Parp-1 and C-Casp3 in 9.7 and 9.12 ADPKD cells but not in 4/5 control cells (**$p < 0.01$ and ***$p < 0.001$ for C-Parp1; ***$p < 0.001$ and **$p < 0.01$ for C-Casp3). The percentage of apoptotic nuclei was calculated from at least six images for each sample.

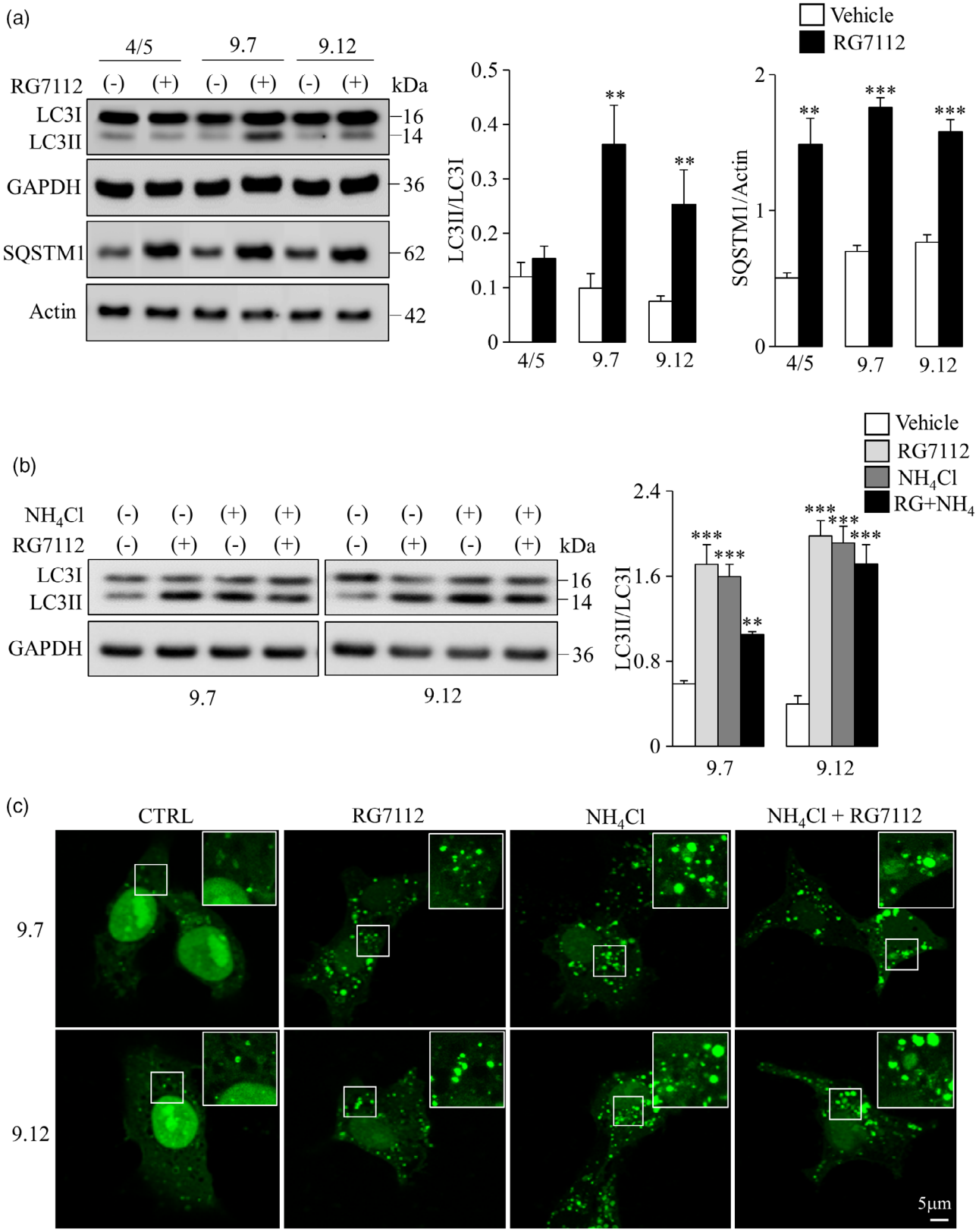

FIGURE 7 Study of autophagy in control and ADPKD cells treated with RG7112. (a) The expression of autophagic markers LC3 and p62/SQSTM1 was evaluated in 4/5, 9.7 and 9.12 cells cultured in presence/absence of 5μM RG7112. The treatment with RG7112 increases the levels of LC3II in 9.7 and 9.12 cells (**$p < 0.01$). RG7112 also enhances the expression of p62/SQSTM1 in all cell types (**$p < 0.01$ and ***$p < 0.001$). The analysis of autophagic flux was performed by Western blotting (b) and fluorescent microscopy (c) in 9.7 and 9.12 cells treated with RG7112 and NH_4Cl alone or in combination. RG7112 and NH_4Cl administrated individually or mixed together increase the levels of LC3II compared with untreated cells (***$p < 0.001$ and **$p < 0.01$ for 9.7; ***$p < 0.001$ for 9.12). Consistently, same treatments enhance the number of

could be explained with the block of autophagy flux. If this event occurs, mature autophagosomes are continuously produced, but not degraded by the lysosome system. Consequently, all autophagosomes markers accumulate. To verify the possible interference of the autophagic flux mediated by RG7112, ADPKD cells were treated with this compound and the inhibitor of autophagic flux NH_4Cl, alone and in combination. If the autophagic flux is still operational, the treatment with both compounds should dramatically increase the levels of LC3II. As expected, the individual application of RG7112 and NH_4Cl increases LC3II expression (Figure 7b). However, the combined treatment does not enhance the levels of LC3II, indicating that the autophagic flux was blocked (Figure 7b). These findings were confirmed by the transfection of ADPKD cells with a plasmid expressing LC3 protein fused with GFP. As shown in Figure 7c and Figure S4A, the single application of both RG7112 and NH_4Cl increases the number of green puncta (autophagosomes) compared with untreated cells. Conversely, no further increase of green puncta after cell treatment with a mix of these compounds was observed. To further verify these data, we have monitored the autophagic flux by using a mCherry-GFP-LC3 tandem system in 9.7 and 9.12 cells treated with RG7112. As shown in Figure 8 and Figure S4B, cells treated with RG7112 and NH_4Cl alone or in combination exhibit a greater number of yellow vesicles (autophagosomes) than red puncta (autophagosomes fused with lysosomes). No changes in yellow/red puncta ratio in ADPKD cells treated with both compounds were detected indicating that the autophagic flux is impaired.

These observations indicate that the inhibition of MDM2 by RG7112 in ADPKD cells prevents the fusion between autophagosomes and lysosomes blocking the autophagic process.

DISCUSSION

ADPKD is a severe inherited kidney disorder that leads to renal injury in approximately 70% of cases at around 70–80 years old. Despite the progresses made to discover the physiopathological alterations associated with the disease, a specific therapy able to cure this pathology is unavailable. Tolvaptan, a vasopressin-2 receptor antagonist, is the only drug approved from the US Food and Drug Administration for the treatment of ADPKD (Blair et al., 2019). Tolvaptan is able to slow disease progression but shows different side effects including liver toxicity that lead to the treatment discontinuation (Watkins et al., 2015). Another promising treatment is represented by the inhibition of mTOR signalling with rapamycin derives, but despite the excellent results obtained in pre-clinical models these drugs have failed the clinical trials (Li et al., 2017; Serra et al., 2010; Tao et al., 2005). Therefore, the research of new possible molecular targets is crucial for the future treatment of ADPKD. The serine-threonine kinase mTOR modulates different signals and processes including autophagy that is inhibited by mTOR activation and could affect cystogenesis and disease progression. We have found that ADPKD cells show a strong basal activation of mTOR and consequently the autophagic activity in these cells is very low. However, the role of autophagy in ADPKD is very controversial since some researcher reports that the activation of this process induces cystogenesis, while others have described that the induction of autophagy suppress cystogenesis in models of polycystic kidney disease (Chang et al., 2018; Lee et al., 2020; Nowak et al., 2020; Zhu et al., 2017). Our results indicate that the autophagic activity is quite low in different cellular models for ADPKD. However, the inhibition of mTOR causes the activation of autophagy, which could be used by ADPKD cells to trap and destroy drugs making these cells insensitive to pharmacological therapy, as already observed in kidney cancer cells (Jones et al., 2020). In this regard, we found that the inhibition of autophagy by CQ treatment reduces cell growth in two ADPKD cell lines, but not in control cells. Moreover, the combined treatment of CQ with rapamycin enhances the inhibition of cell proliferation compared with rapamycin alone. This data seems to confirm that autophagy might decrease drug efficacy. Thus, the use of molecules targeting mTOR that lead to the rise of autophagy should be carefully evaluated. Taken together, these observations suggest that the inactivation of autophagy in ADPKD cells might preserve drug integrity enhancing the pharmacological treatment. However, we believe that the use of different drugs targeting mTOR in combination with autophagy inhibitors could be difficult to apply taking into consideration the probable side effects. We have previously found that the activation of mTOR increased the expression of MDM2 that leads to the degradation of TP53 by proteasome machinery in ADPKD cells (de Stephanis et al., 2018). We have also described that TP53 can be degraded via autophago-lysosomal system in kidney cancer cells (Patergnani et al., 2020). Nevertheless, the inhibition of autophagy by CQ treatment or ATG7 silencing do

autophagosomes (green puncta). However, the combination of NH_4Cl and RG7112 does not enhance neither LCII expression nor the number of puncta as compared to cells treated with NH_4Cl alone, suggesting that the autophagic flux is blocked. Images were collected by using a fluorescence confocal microscope at 60× magnification. For each cell, four planes of 0.40 μm each were acquired to collect autophagosomes. Acquired images were processed by ImageJ software. Representative images shown are z-projection of the collected pictures. At least 15 fields for each cell type were acquired.

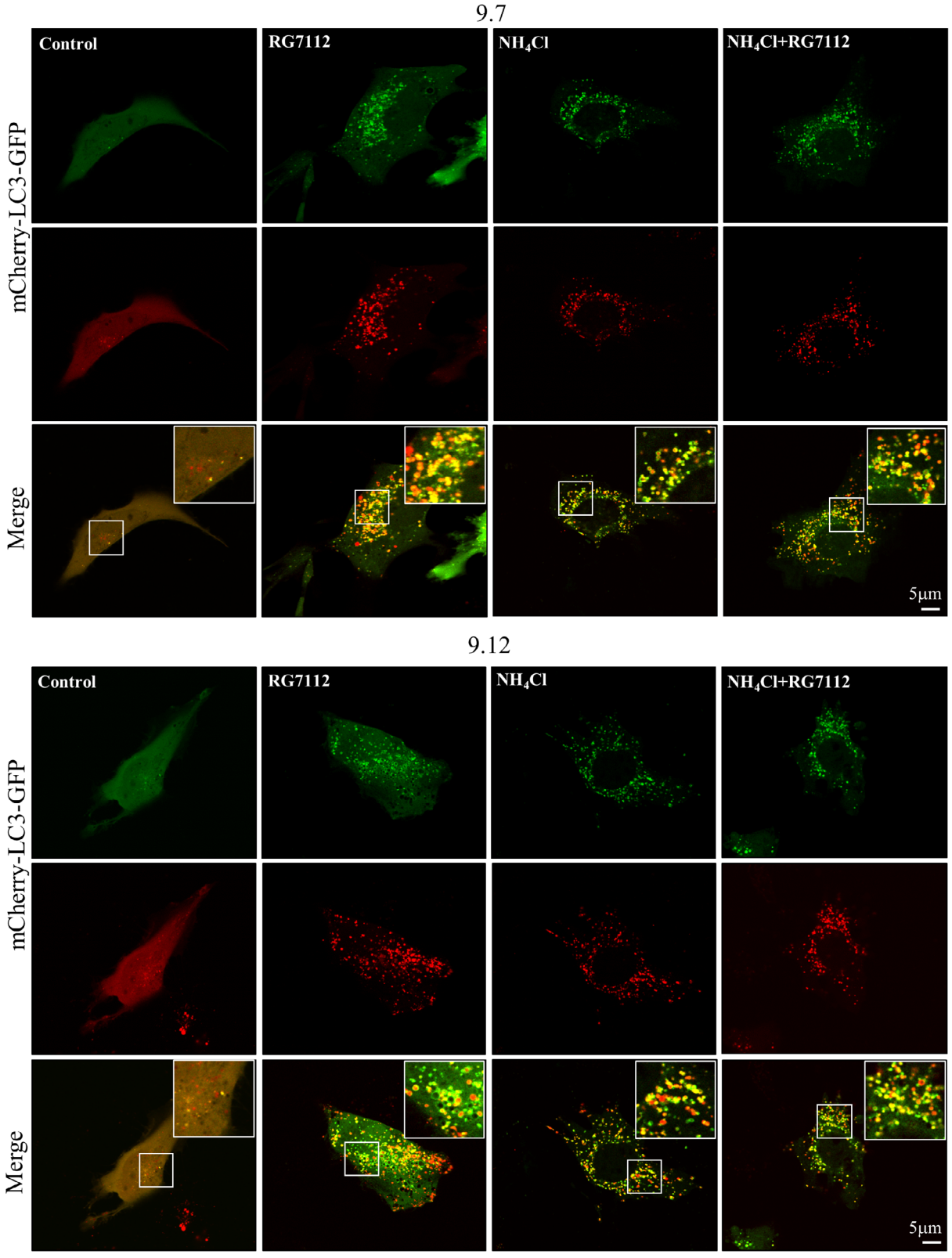

FIGURE 8 Analysis of autophagic flux by using a mCherry-GFP-LC3 tandem system in ADPKD cells. 9.7 and 9.12 cells were seeded on coverslips in six well plates and transfected with a construct expressing double tag LC3 proteins. After transfection, cells were cultured in DMEM/F12 1% FBS and treated with RG7112 (5 μM) and NH_4Cl alone or in combination for 24 h. Next, cells were washed in PBS buffer, fixed and vesicles were visualised by using a fluorescence confocal microscope at 60× magnification. For each cell, four planes of 0.40 μm each were acquired to collect autophagosomes. Acquired images were processed by ImageJ. Representative images shown are z-projection of the collected pictures. At least 10 fields for each condition were acquired. The treatment with RG7112 and NH_4Cl alone or in combination causes the accumulation of autophagosomes (greater number of yellow puncta) compared with untreated cells confirming that this MDM2 inhibitor leads to the dysfunction of autophagic flux.

not affect TP53 expression in ADPKD cells suggesting that the main route used by ADPKD cells to degrade TP53 is the MDM2-proterasome machinery. In fact, the inhibition of MDM2 by RG7112 increases TP53 expression preventing its degradation by MDM2/proteasome axis. RG7112 is a *cis*-imidazoline inhibitor that prevents p53-MDM2 interaction blocking the p53-binding site of MDM2 (Konopleva et al, 2020). In addition, the inhibition of MDM2 by treatment with RG7112 reduces cell proliferation and promotes apoptosis in different ADPKD cell lines activating the pathway of p53 that leads to the enhanced expression of the cell cycle inhibitor p21. Consistently, it was reported that the treatment with RG7112 increases TP53 expression, activates p21, causes cell cycle arrest in G1 and G2-M phase and finally induces apoptosis in cancer cells as well as in pre-clinical cancer models (Fang et al., 2020; Tovar et al., 2013). The inhibition of MDM2 is already used as therapeutic option in cancer including renal carcinoma (Kawata et al., 2020; Konopleva et al., 2020). Interestingly, we detected that in ADPKD cells the targeting of MDM2 by RG7112 blocks the autophagy flux, while the inhibition of mTOR deeply activates autophagy. Taken together, these findings make MDM2 inhibition a reasonable therapeutic target for ADPKD treatment.

CONCLUSIONS

ADPKD patients need further pharmacological treatments because current drugs cause several side effects and show little improvements in disease regression. We found that the inhibition of MDM2 by the molecule RG7112 is able to reduce cell growth and rise apoptosis in vitro models for ADPKD. This compound inhibits the MDM2-mediated TP53 degradation thorough the proteasome machinery. Moreover, RG7112 prevents possible drug degradation by autophagosomes inhibiting the autophagic flux in ADPKD cells. Currently, MDM2 inhibitors might be used in cancer therapy since have completed clinical trials in different malignancies (Konopleva et al., 2020; Wang et al., 2017) and in the future, they could be applied also for ADPKD treatment.

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CONFLICT OF INTEREST

No conflict of interests to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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