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Autophagy-mediated reduction of miR-345 contributes to hepatic cystogenesis in polycystic liver disease

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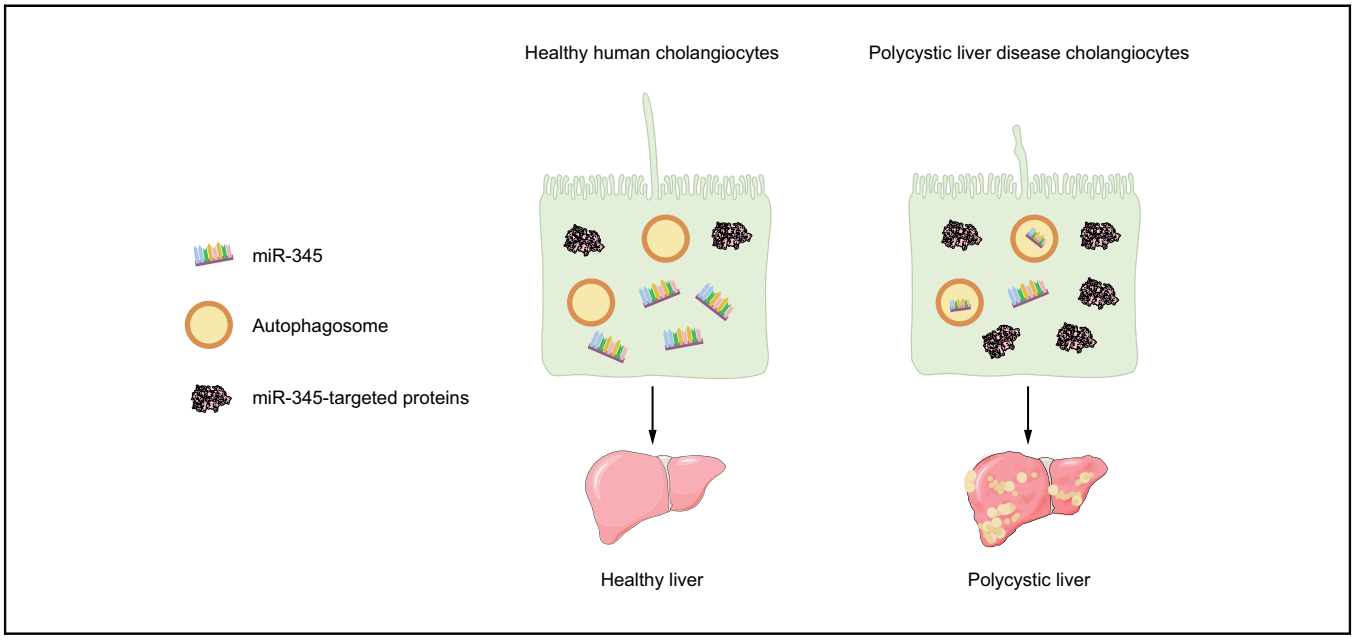
Graphical abstract



Highlights

- The miRNA profile is altered in PLD.
- MiR-345 is the most-reduced miRNA in PLDCs.
- The reduction of miR-345 increases PLDC proliferation and hepatic cystogenesis.
- MiR-345 in PLDCs is regulated by autophagy, termed 'miRNAutophagy'.
- Restoration of miR-345 in PLDC is beneficial for PLD.

Lay summary

Polycystic liver disease (PLD) is an incurable genetic disorder characterised by the progressive growth of hepatic cysts. We found that hepatic cystogenesis is increased when the levels of miR-345 in PLD cholangiocytes (PLDCs) are reduced by autophagy. Restoration of miR-345 in PLDCs via inhibition of autophagy decreases hepatic cystogenesis and thus, is beneficial for PLD.

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Research article

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Autophagy-mediated reduction of miR-345 contributes to hepatic cystogenesis in polycystic liver disease

Tatyana Masyuk,[1] Anatoliy Masyuk,[1] Christy Trussoni,[1] Brynn Howard,[1] Jingyi (Francess) Ding,[1] Bing Huang,[1] Nicholas LaRusso[1,*]

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Background & Aims: Polycystic liver disease (PLD) is characterised by increased autophagy and reduced miRNA levels in cholangiocytes. Given that autophagy has been implicated in miRNA regulation, we tested the hypothesis that increased autophagy accounts for miRNA reduction in PLD cholangiocytes (PLDCs) and accelerated hepatic cystogenesis.
Methods: We assessed miRNA levels in cultured normal human cholangiocytes (NHCs), PLDCs, and isolated PLDC autophagosomes by miRNA-sequencing (miRNA-seq), and miRNA targets by mRNA-seq. Levels of miR-345 and miR-345-targeted proteins in livers of animals and humans with PLD, in NHCs and PLDCs, and in PLDCs transfected with pre-miR-345 were assessed by *in situ* hybridisation (ISH), quantitative PCR, western blotting, and fluorescence confocal microscopy. We also assessed cell proliferation and cyst growth *in vitro*, and hepatic cystogenesis *in vivo*.
Results: In total, 81% of miRNAs were decreased in PLDCs, with levels of 10 miRNAs reduced by more than 10 times; miR-345 was the most-reduced miRNA. *In silico* analysis and luciferase reporter assays showed that miR-345 targets included cell-cycle and cell-proliferation-related genes [*i.e.* cell division cycle 25A (*CDC25A*), cyclin-dependent kinase 6 (*CDK6*), *E2F2*, and proliferating cell nuclear antigen (*PCNA*)]; levels of 4 studied miR-345 targets were increased in PLDCs at both the mRNA and protein levels. Transfection of PLDCs with pre-miR-345 increased miR-345 and decreased the expression of miR-345-targeted proteins, cell proliferation, and cyst growth *in vitro*. MiR-345 accumulated in autophagosomes in PLDCs but not NHCs. Inhibition of autophagy increased miR-345 levels, decreased the expression of miR-345-targeted proteins, and reduced hepatic cystogenesis *in vitro* and *in vivo*.
Conclusion: Autophagy-mediated reduction of miR-345 in PLDCs (*i.e.* miRNAutophagy) accelerates hepatic cystogenesis. Inhibition of autophagy restores miR-345 levels, decreases cyst growth, and is beneficial for PLD.
Lay summary: Polycystic liver disease (PLD) is an incurable genetic disorder characterised by the progressive growth of hepatic cysts. We found that hepatic cystogenesis is increased when the levels of miR-345 in PLD cholangiocytes (PLDCs) are reduced by autophagy. Restoration of miR-345 in PLDCs via inhibition of autophagy decreases hepatic cystogenesis and thus, is beneficial for PLD.

Introduction

Polycystic liver disease (PLD) is a group of genetic disorders characterised by the presence of multiple cholangiocyte-derived cysts. The most common form of PLD, caused by mutations in 6 genes [(*i.e.* encoding polycystic kidney disease 1/2 (*PKD1/2*) glucosidase II alpha subunit (*GANAB*), low-density lipoprotein receptor-related protein 5 (*LRP5*), DnaJ heat shock protein family (Hsp40) member B11 (*DNAJB11*), and alpha-1,2-mannosyltransferase (*ALG9*)], coexists with autosomal dominant polycystic kidney disease (ADPKD). Mutations in 2 genes [*i.e.* polycystic kidney and hepatic disease 1 (*PKHD1*) and DAZ interacting zinc finger protein 1 like (*DZIP1L*)] lead to PLD associated with autosomal recessive PKD. Isolated autosomal dominant polycystic liver disease (ADPLD) is linked to mutations in 7 genes (*i.e.* protein kinase C substrate 80K-H (*PRKCSH*), SEC63 homolog, protein translocation regulator (*SEC63*), *LRP5*, *GANAB*, alpha-1,3-glucosyltransferase (*ALG8*), SEC61 translocon subunit beta (*SEC61B*), and *PKHD1*).[1,2]

Mutations in PLD-causative genes initiate the formation of hepatic cysts, the progressive growth of which involves multiple mechanisms. In PLD cholangiocytes (PLDCs), the mRNA profile is dramatically changed, with up to 60% of transcripts being up- or downregulated.[3] Clustering of these dysregulated transcripts into biological pathways revealed ~30 disturbed pathways in PLDCs, among which autophagy, cell proliferation, cell cycle, and cAMP signalling are the most altered.[3] Genetic elimination of the cell cycle protein, cell division cycle 25A (CDC25A), or inhibition of autophagy in polycystic kidney (PCK) rats reduces hepatic cystogenesis, further emphasising the contributing role of these pathways to PLD progression.[1,4–8]

Keywords: Cholangiocytes; PLD treatment; Genetic liver diseases; Cell cycle-related proteins; Cholangiocyte proliferation.
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Low levels of multiple miRNAs have been reported in cholangiocytes of an animal model of PLD, the PCK rat.[9] However, the mechanisms that account for miRNA reduction remain unclear. Autophagy is also increased in PLDCs and its inhibition attenuates PLD progression in PCK rats.[3] Importantly limited research have implicated autophagy in miRNA regulation,[10–13] a process that we suggest to term 'miRNAutophagy'. However, it remains unknown whether miRNAutophagy contributes to miRNA regulation and hepatic cystogenesis in PLD.

In this study, we performed miRNA-sequencing (miRNA-seq) analysis of cholangiocytes isolated from healthy humans (NHCs) and patients with ADPKD-associated PLD, and observed that, in PLDCs, the levels of most miRNAs were decreased, with miR-345 being the most-reduced miRNA. A decrease in miR-345 levels in PLDCs resulted in overexpression of several miR-345-targeted proteins, cholangiocyte hyperproliferation, and enhanced cystogenesis. Consistent with the concept of miRNAutophagy, miR-345 was detected in autophagosomes of PLDCs but not of NHCs. We also found that miR-345 levels in PLDCs were autophagy regulated because inhibition of autophagy increased miR-345 and expression of miR-345-targeted proteins. Subsequently, cholangiocyte proliferation and hepatic cyst growth were decreased. Thus, our results provide a mechanistic link between increased autophagy and decreased miR-345 in PLDCs and show that miRNAutophagy accelerates hepatic cystogenesis. Inhibition of autophagy restores miR-345 levels, decreases cyst growth, and is beneficial for PLD.

Materials and Methods

Human and rodent liver tissue, cell cultures, reagents, and animals

Paraffin sections of liver tissue were used from healthy humans and patients with PLD, wild type (WT) and PCK rats, and WT, *Pkd2*$^{WS25/-}$, and *Pkhd1*$^{del2/del2}$ mice. Spontaneously immortalised cholangiocytes derived from normal humans (NHCs), patients with ADPKD-associated PLD (PLDCs), normal rat (NRCs) and PCK rat (PCKCs) were maintained as previously described.[14] Given that long-term cultured cholangiocytes might lose some morphological and functional features, we validated them by short tandem repeat profiling to ensure that they were not misidentified and then tested for contamination. For autophagy inhibition studies, cultured cholangiocytes were grown for 24 h and then treated with 100 nM DMSO (control) or 100 nM HCQ (Sigma, St Louis, MO, USA) for an additional 24 h. The study was approved by the Mayo Clinic Institutional Review Board and abides by the Declaration of Helsinki principles. The Mayo Clinic Institutional Animal Care and Use Committee approved the animal studies.

miRNA and mRNA sequencing, and pathway enrichment analysis

miRNA sequencing of NHCs and PLDCs (both n = 3) was performed by the Mayo Clinic Medical Genome Facility as previously described.[15] Differentially expressed miRNAs were selected based on p <0.05, log2 fold change ≥1.5 or ≤1.5. mRNA-seq of NHCs and PLDCs (both n = 3) was performed as previously described.[3] False discovery rate (FDR) 0.05 and log2 fold change ≥1 or ≤−1 were considered as the cutoff for up- and down-regulated genes. DAVID (https://david.ncifcrf.gov/) was used to perform functional and pathway enrichment analysis. Gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were carried out for transcripts dysregulated in PLDCs.[16] TargetScan algorithms were used to search for miR-345 targets.

miRNA extraction and quantification

Total RNA was extracted by using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Mature miR-345 and U6 small nuclear RNA (snRNA) were detected according to the manufacturer's instructions using the TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and analysed with the Rotor-gene quantitative PCR instrument (Qiagen, Germantown, MD, USA). The expression of miR-345 was normalised to snU6 using the change-in-threshold 2-ΔCT method. PLDCs were transfected with an hsa-miR-345 precursor and pre-miR

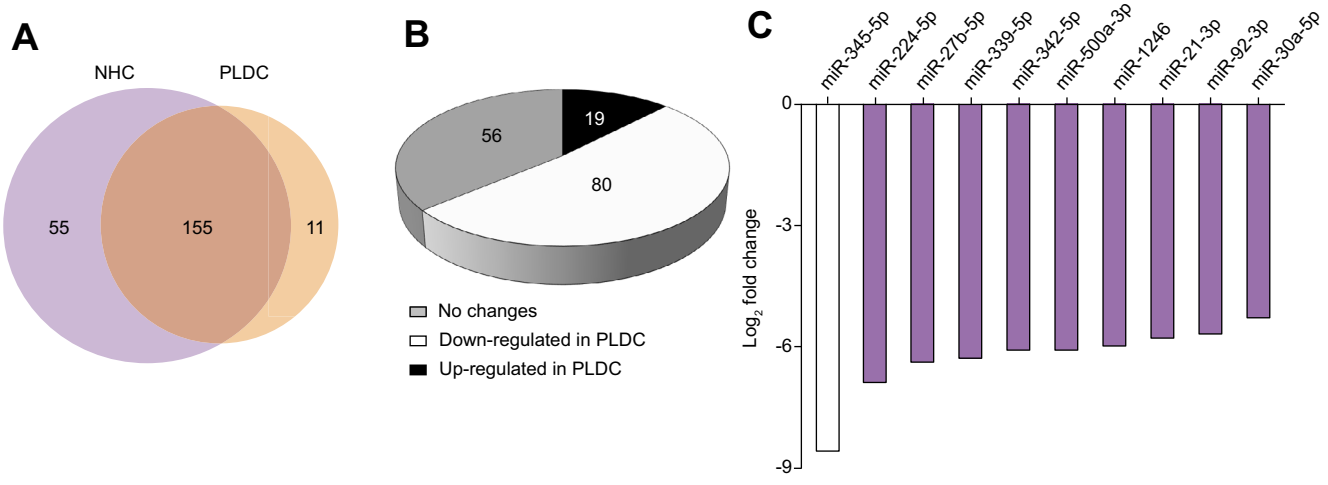

Fig. 1. Most miRNAs are decreased, and miR-345 is the most reduced miRNA, in PLDCs. (A) In total, 210 miRNAs were detected in NHCs and 166 in PLDCs; 55 miRNAs were uniquely expressed in NHCs and 11 in PLDCs; 155 miRNAs were expressed in both NHCs and PLDCs. (B) The levels of 56 miRNAs were comparable between NHCs and PLDCs. The levels of 80 miRNAs were reduced and the levels of 19 were increased. n = 3 for each cell line. (C) Of the 10 most-downregulated miRNAs in PLDCs, miR-345 was the most reduced. NHC, normal human cholangiocyte; PLDC, polycystic liver disease cholangiocyte.

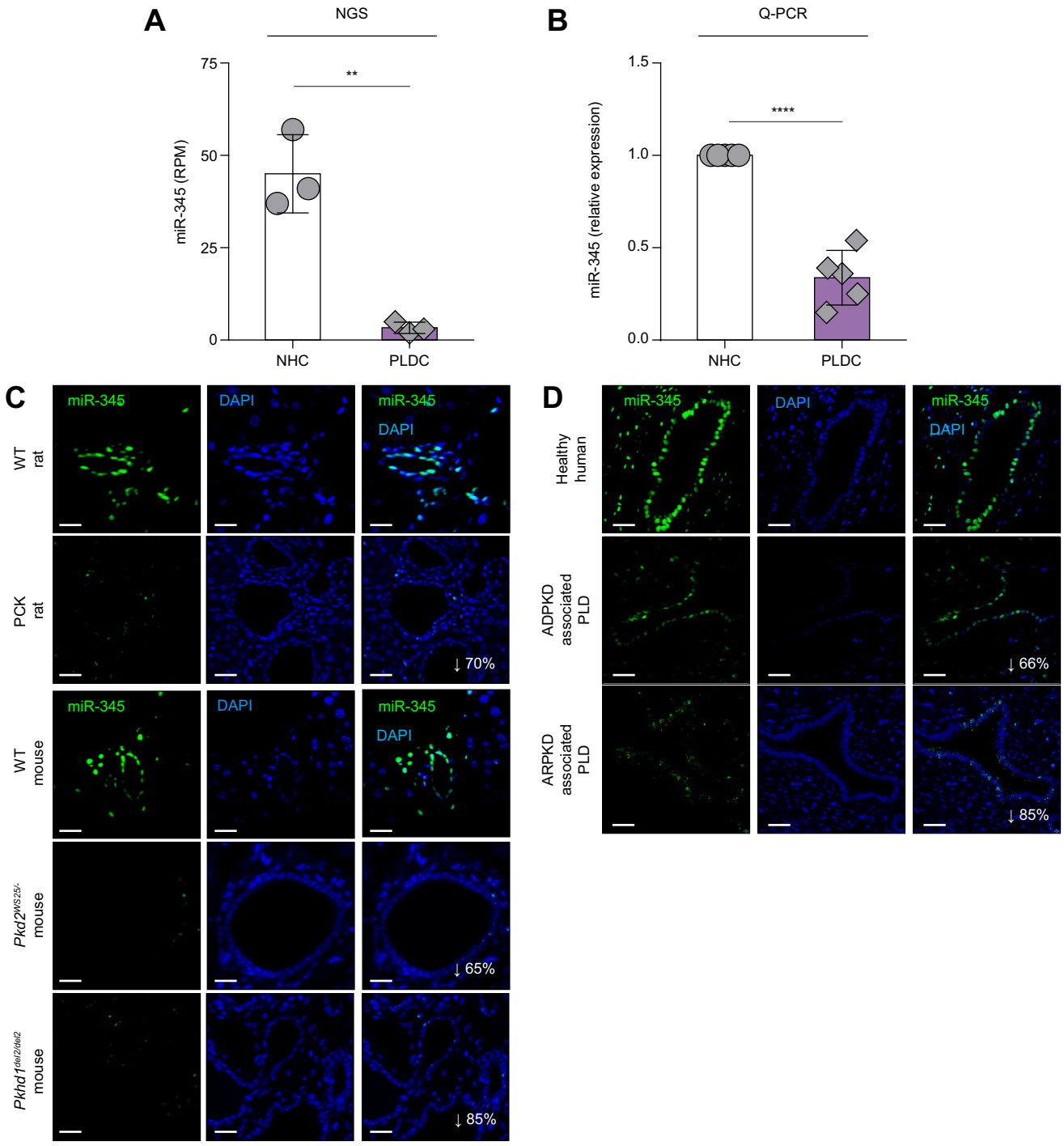

Fig. 2. miR-345 is decreased in PLDCs. (A) miR-345 levels (RPM) are lower in PLDCs compared with NHCs. n = 3 for each cell line. (B) Reduced miR-345 levels in PLDCs were confirmed by quantitative PCR. n = 5 for each cell line. (C,D) ISH showed decreased immunoreactivity of miR-345 (green) in livers of rodents and patients with PLD compared with their respective controls. n = 5 patients or rodents per group. Nuclei (blue) are stained with DAPI. Data are mean ± SD. $^{**}p$ <0.01, $^{****}p$ <0.0001. Scale bars = 50 μm. ISH, *in situ* hybridisation; NHC, normal human cholangiocytes; PLD, polycystic liver disease; PLDC, polycystic liver disease cholangiocyte; RPM, reads per million.

precursor control (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

In situ hybridisation

In situ hybridisation (ISH) was performed using sections of liver tissue from WT and PCK rats; WT, *Pkd2*$^{WS25/-}$, and *Pkhd1*$^{del2/del2}$ mice; healthy humans and patients with ADPKD- and autosomal recessive polycystic kidney disease (ARPKD)-associated PLD; and cultured NHCs, PLDCs, NRCs, and PCKCs according to published protocols[17] and visualised with Zeiss LSM 510 confocal microscope (Thornwood, NY, USA).

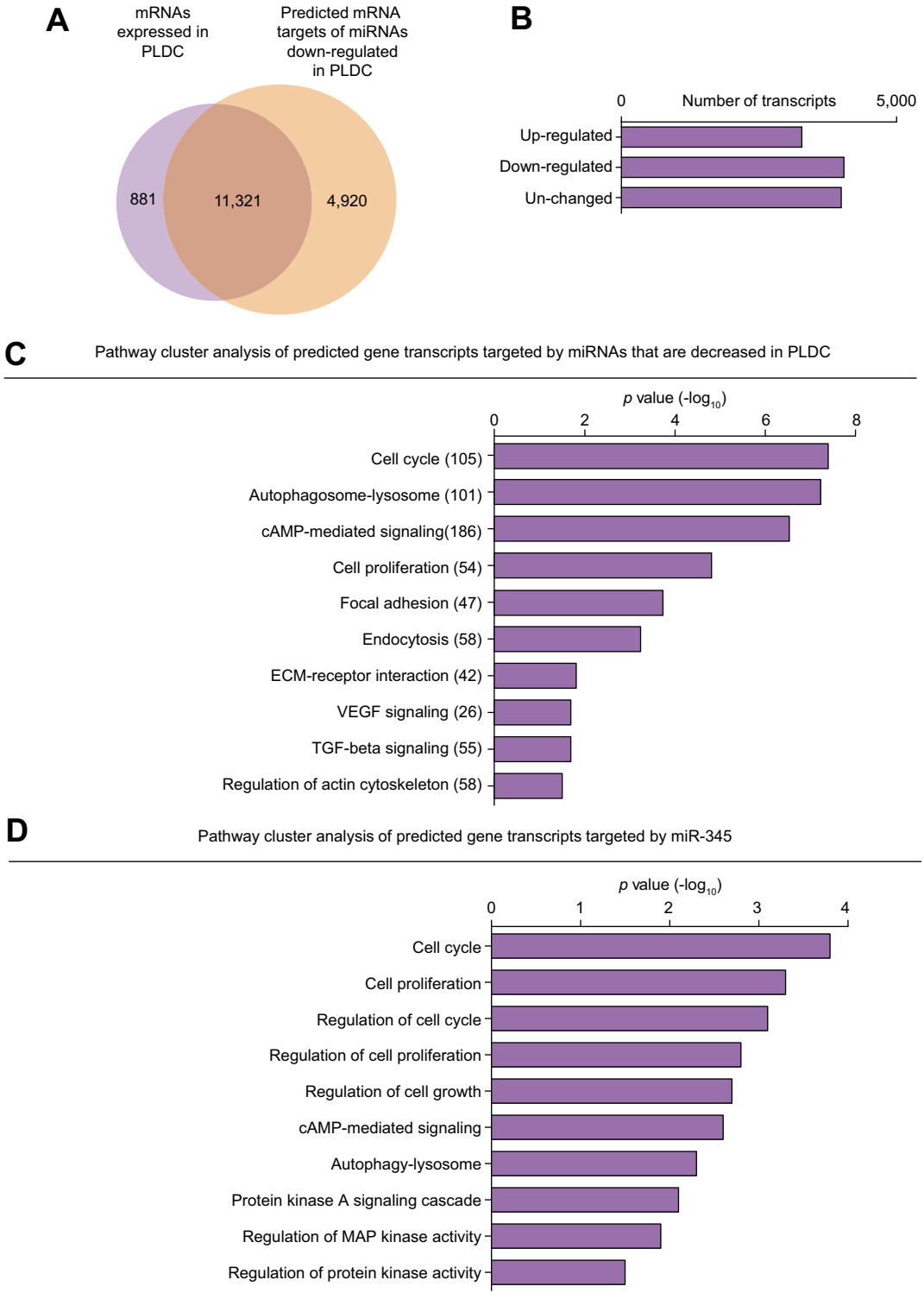

Fig. 3. Cell cycle and cell proliferation are the most-dysregulated pathways in PLDCs. (A) Based on *in silico* analysis, 16,241 potential mRNA targets were identified of the 80 miRNAs downregulated in PLDCs. mRNA-seq showed that, out of 12,202 mRNAs expressed in PLDCs, 11,321 represent potential targets of reduced miRNAs. (B) In PLDCs, 3,281 transcripts were upregulated, 4,044 were downregulated, and 3,996 were unchanged compared with NHCs. (C) Pathway cluster analysis revealed the most-altered pathways in PLDCs. The number of transcripts dysregulated in each pathway is shown in parentheses. (D) Pathway cluster analysis of 2,523 transcripts targeted by miR-345 revealed the cell cycle, cell proliferation, and cAMP-mediated signalling as the major pathways affected in PLDCs. NHC, normal human cholangiocyte; PLDC, polycystic liver disease cholangiocyte.

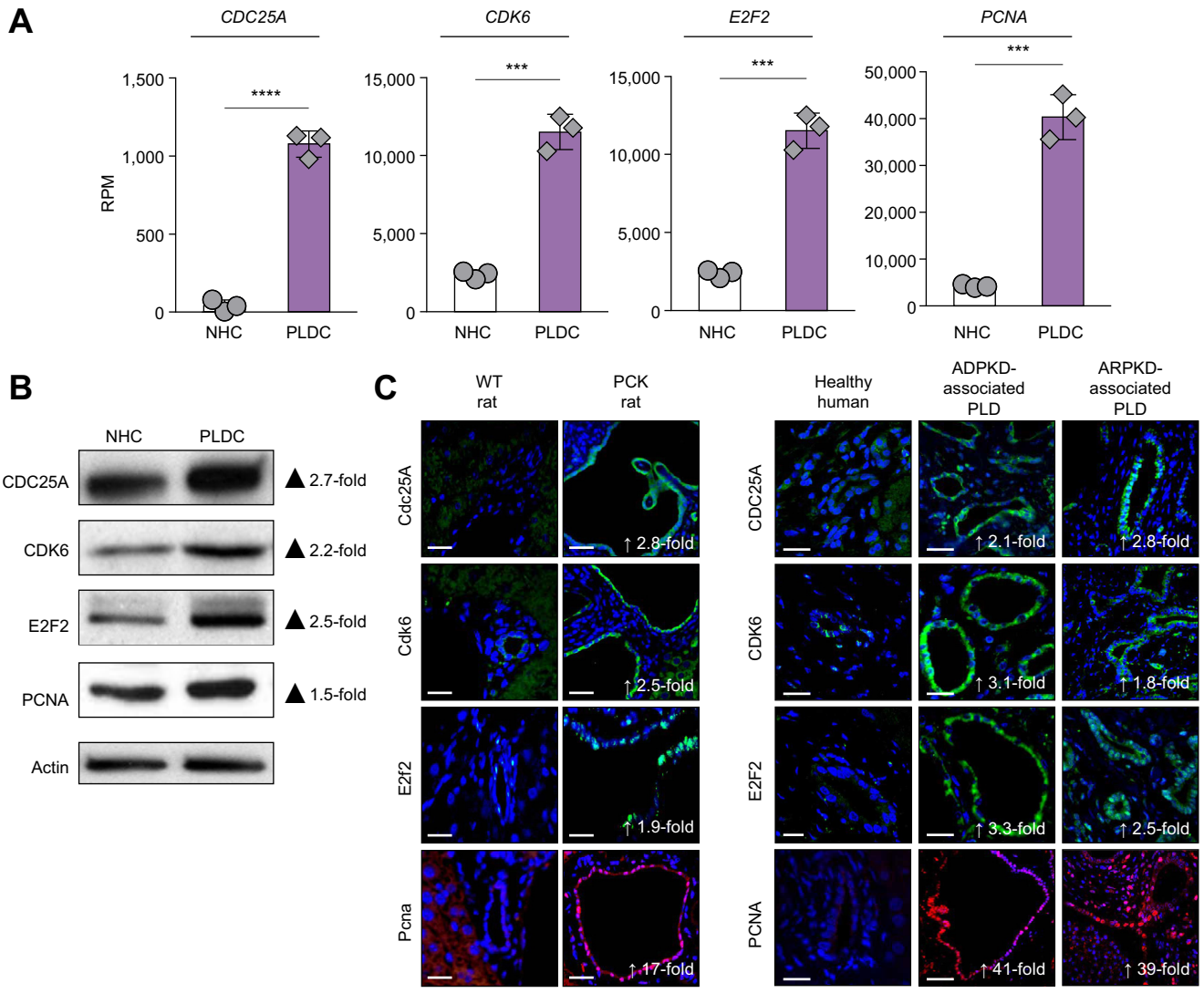

Fig. 4. miR-345-targeted proteins are overexpressed in PLDCs. (A) CDC25A, CDK6, E2F2, and PCNA were increased in PLDCs at both the mRNA (RPM) and (B) protein levels. (C) Confocal microscopic images demonstrated overexpression of CDC25A, CDK6, and E2F2 (all green) and an increased number of PCNA-positive nuclei (red) in cholangiocytes of PCK rats and humans with PLD compared with their respective controls. Nuclei (blue) are stained with DAPI. n = 3 patients or rodents per group. Data are mean ± SD. Scale bars = 50 μm. ***p <0.001, ****p <0.0001. CDC25A, cell division cycle 25A; CDK6, cyclin-dependent kinase 6; PCNA, proliferating cell nuclear antigen; PLDC, polycystic liver disease cholangiocyte; RPM, reads per million.

Cell proliferation

Cell proliferation was determined by using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) and cells were counted by using the Cellometer Auto4 (Nexcelom Bioscience, Lawrence, Massachusetts, USA) cell counter. Cholangiocytes (2500 cells/well) were grown for 24 h before the assay. Alterations in the proliferation of cultured cholangiocytes after treatment were expressed as the percentage change compared with untreated cholangiocytes in which cell proliferation was considered to be equal to 100%. Proliferation of PLD cholangiocytes *in vivo* was evaluated by the number of proliferating cell nuclear antigen (PCNA)-positive nuclei, as described previously.[3]

3D cultures

Cholangiocytes were seeded and grown in 3D matrices as previously described.[18] Images were taken at days 1 (24 h after seeding) and 3. The circumference of any cystic structures was measured by ImageJ as previously described.[18]

Treatment protocol

PCK rats (4–6-weeks old, n = 3 female, n = 3 male) were injected intraperitoneally with hydroxychloroquine (HCQ; 15 mg/kg body weight) dissolved in DMSO every other day for 6 weeks; doses were adjusted to the weight of each rat every week. The untreated group (4–6-weeks old, n = 3 female, n = 3 male) received equal doses of DMSO via intraperitoneal injection. Rats were sacrificed and the livers removed, fixed, and paraffin embedded for histology. Cystic hepatic areas were analysed as described elsewhere.[3,14,19] Based on previous experience, that the percentage of liver parenchyma occupied by hepatic cysts does not differ between male and female PCK rats,[14,19] these samples were combined for estimation of cystic and fibrotic areas. A sample

size of 6 animals per group was considered the minimum required to guarantee sufficient statistical power.

Statistical analysis

The data are expressed as mean ± SD or as the fold change in mean. Data were analysed with a 1-way ANOVA using Prism software, which was also used for the generation of all bar graphs. Results were considered statistically significant at p <0.05.

A detailed description of all methods is provided in the supplementary materials.

Results

miRNA profile is altered in PLDCs

We performed miRNA profiling in NHCs and PLDCs. In total, 210 miRNAs were detected in NHCs and 166 in PLDCs. Of these miRNAs, 55 were uniquely present in NHCs and 11 in PLDCs; 155 miRNAs were common to both cell lines (Fig. 1A).

Most miRNAs are decreased and miR-345 is the most-reduced miRNA in PLDCs

We further analysed the expression of 155 miRNAs common to both NHCs and PLDCs. The levels of 56 miRNAs (*i.e.* 34%) were comparable in NHCs and PLDCs. Out of 99 miRNAs (*i.e.* 66%) differentially expressed in PLDC, the levels of 19 miRNAs were increased and the levels of 80 miRNAs were reduced (Fig. 1B). The 10 most-decreased miRNAs in PLDC are detailed in Fig. 1C; all differentially expressed miRNAs are listed in Supplementary Tables S1 and S2. Notably, miR-345 was the most decreased miRNA in PLDCs (Fig. 1C).

The levels of miR-345 are decreased in PLDCs

The reduced expression of miR-345 in PLDCs was demonstrated by miRNA-seq (Fig. 2A), quantitative PCR (Fig. 2B) and ISH in cholangiocytes lining liver cysts in animal models and patients with PLD compared with the respective controls (Fig. 2C,D).

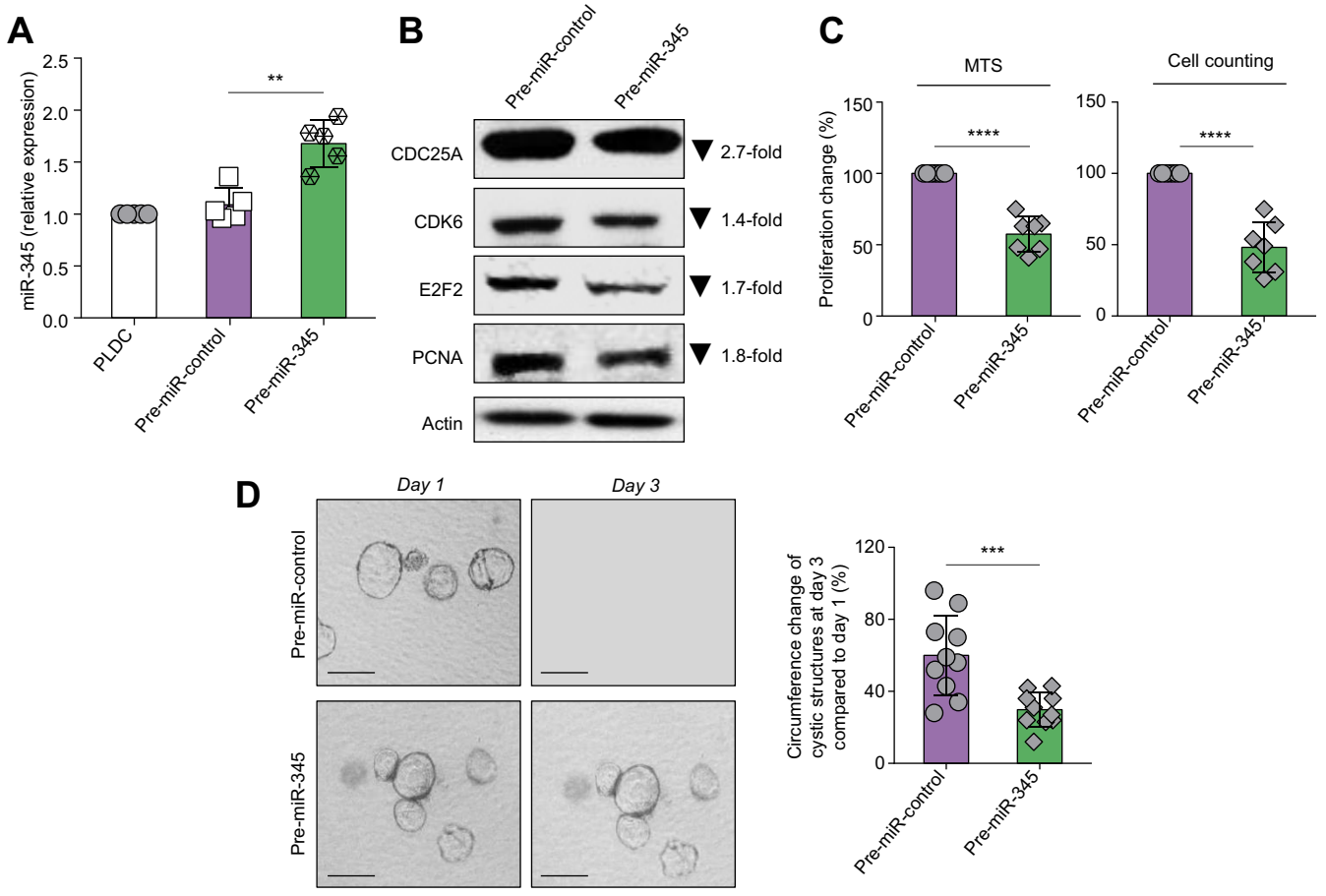

Fig. 5. Transfection of PLDCs with pre-miR-345 increases miR-345 and reduces expression of miR-345-targeted proteins, cell proliferation, and cyst growth in 3D cultures. (A) Increased miR-345 levels were observed by quantitative PCR in PLDCs transfected with pre-miR-345 but not a pre-miR control. n = 5 for each cell line. (B) Expression of CDC25A, CDK6, E2F2, and PCNA in PLDCs transfected with pre-miR-345 was decreased compared with controls. n = 3 for each data set. (C) Proliferation of PLDCs transfected with pre-miR-345 was decreased. n = 7 for each data set. (D) Growth of cystic structures formed by PLDCs transfected with pre-miR-345 was inhibited. n = 10 individual cysts for each data set. Scale bars = 100 μm. Data are mean ± SD. $^{**}p$ <0.01, $^{***}p$ <0.001, $^{****}p$ <0.0001. CDC25A, cell division cycle 25A; CDK6, cyclin-dependent kinase 6; PCNA, proliferating cell nuclear antigen; PLDC, polycystic liver disease cholangiocyte.

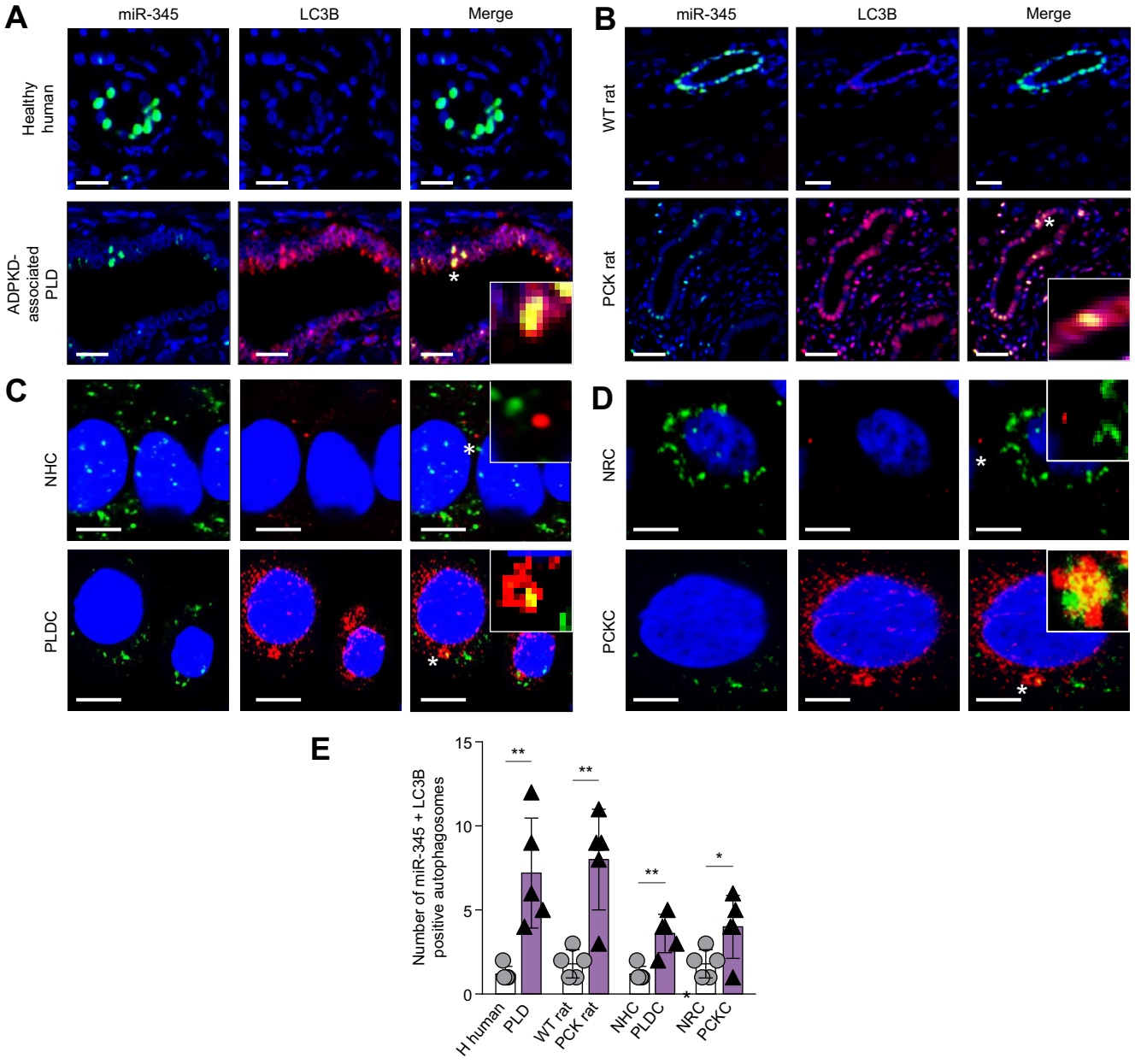

Fig. 6. miR-345 colocalises with autophagosomes in PLDCs. (A) Combined ISH for miR-345 (green) and confocal microscopy for the autophagy marker, LC3B (red) shows the increased number of miR-345-LC3-positive autophagosomes in cholangiocytes of patients with PLD (n = 5 patients per group; scale bars = 50 μm), (B) PCK rats (n = 5 rodents per group; scale bars = 50 μm), and cultured (C) PLDCs, and (D) PCKCs (n = 5 for cell line; scale bars = 5 μm) compared with their respective controls. (E) Quantitative assessment of miR-345-LC3-positive autophagosomes. Insets show higher magnification miR-345 and LC3B merged images (yellow) indicated by asterisks. Data are mean ± SD. $^{*}p$ <0.05, $^{**}p$ <0.01. ISH, *in situ* hybridisation; H, healthy human; NHC, normal human cholangiocyte; NRC, normal rat cholangiocyte; PCK, polycystic kidney; PCKC, polycystic kidney cholangiocyte; PLD, polycystic liver disease; PLDC, polycystic liver disease cholangiocyte.

Cell cycle and cell proliferation are the most-dysregulated pathways in PLDCs

Out of 16,241 mRNAs detected by an *in silico* search as potential targets of 80 downregulated miRNAs, 11,321 transcripts were present in PLDCs (Fig. 3A). Further analysis revealed that 3,996 transcripts were unchanged, 3,281 were upregulated, and 4,044 transcripts were downregulated (Fig. 3B). Importantly, these 11,321 mRNAs belonged to several functional pathways, including cell cycle, autophagy, cAMP-mediated signalling, and cell proliferation, which are crucial for PLD progression (Fig. 3C).

Next, 2,523 out of 3,371 mRNAs predicted to be targeted by miR-345 were detected in PLDCs. Clustering of these 2,523 mRNAs revealed the top-10 affected pathways in PLDCs, including cell cycle, cell proliferation and cAMP-mediated signalling (Fig. 3D). Notably, miR-345 had more mRNA targets compared with the other 9 most-decreased miRNAs

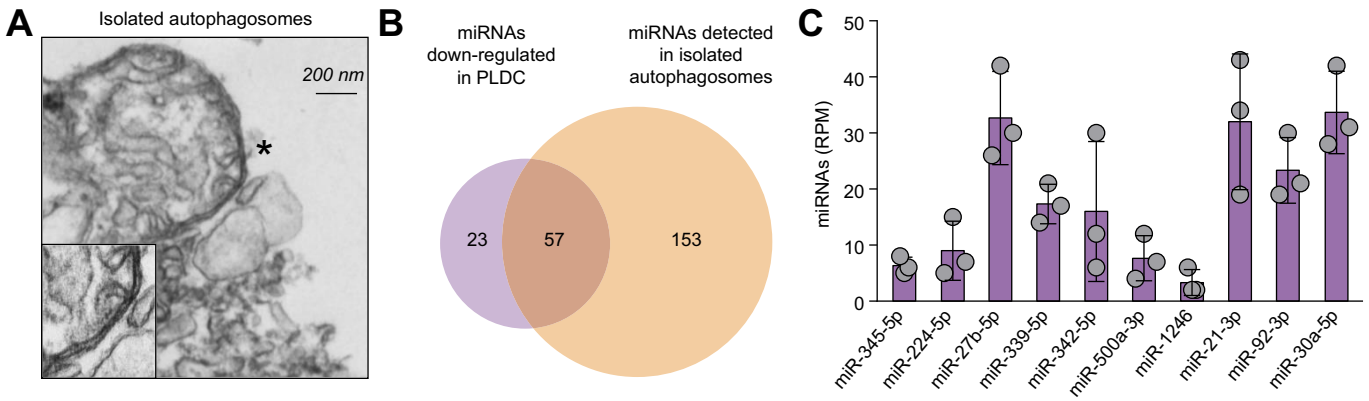

Fig. 7. Multiple miRNAs, including miR-345, are present in autophagosomes isolated from PLDCs. (A) Transmission electron microscopy of autophagosomes isolated from PLDCs. Insets (area indicated by asterisks) show that the isolated organelles have a double membrane, a feature of autophagosomes. (B) Based on miRNA-seq, 210 miRNAs were identified in isolated PLDC autophagosomes. Out of a total of 80 miRNAs downregulated in PLDCs, 57 were identified in isolated autophagosomes. (C) The 10 most-reduced miRNAs, including miR-345, were detected in PLDC autophagosomes. n = 3 for each data set. Data are mean ± SD. miRNA-seq, miRNA sequencing; PLDC, polycystic liver disease cholangiocyte.

(Supplementary Table S3 and Fig. S1). CDC25A, cyclin-dependent kinase 6 (CDK6), transcription factor E2F2, and proliferating cell nuclear antigen (PCNA) were among the miR-345-targeted mRNAs predicted by an *in silico* search. Importantly, only miR-345 targets all the assessed cell cycle and cell proliferation-related genes (Supplementary Table S4). To confirm the binding of miR-345 to mRNAs of interest, we performed *in vitro* functional assays using the luciferase reporter containing the miR-345 recognition sequences for CDC25A, CDK6, E2F2, and PCNA (Supplementary Fig. S2A,B). A significant decrease in relative luciferase expression was observed in NHCs treated with the miR-345-mimic compared with untreated controls, indicating the binding of miR-345 to the 3′-untranslated region (UTR) of corresponding mRNAs (Supplementary Fig. S2C).

As expected, CDC25A, CDK6, E2F2, and PCNA were increased in PLDCs at both the mRNA (Fig. 4A) and protein levels (Fig. 4B) in cholangiocytes lining liver cysts in animal models and patients with PLD (Fig. 4C and Supplementary Fig. S3).

Transfection of PLDC with pre-miR-345 increases miR-345 levels and decreases expression of miR-345-targeted proteins, cell proliferation, and cyst growth in 3D cultures

PLDCs were transfected with pre-miR-345 and control pre-miR. The levels of miR-345 in PLDCs transfected with control miRNAs were comparable to those of untransfected PLDC, whereas the level of miR-345 was increased by ∼70% after transfection with pre-miR-345 (Fig. 5A). In PLDCs transfected with pre-miR-345, the expression of miR-345-targeted proteins was inhibited ∼2-fold (Fig. 5B), and proliferation was reduced by 55–60% (Fig. 5C). Finally, *in vitro* growth of cystic structures formed by PLDCs transfected with pre-miR-345 was decreased by 50% (Fig. 5D).

In PLDC, miR-345 is localised to autophagosomes

We performed combined ISH for miR-345 and confocal microscopy for the autophagosome marker, LC3B, in healthy and PLDCs *in vitro* and *in vivo*. An increased number of miR-345-LC3-positive structures was observed in cholangiocytes lining liver cysts in rodents and patients with PLD, and in cultured NHCs, PLDCs, NRCs and PCKCs compared with the corresponding controls (Fig. 6A–E).

MiR-345 is present in autophagosomes isolated from PLDCs

We isolated autophagosomes from PLDCs (Fig. 7A) and analysed the miRNA profiles by miRNA-seq. In total, 210 miRNAs were detected in isolated PLDC autophagosomes (Fig. 7B). We then compared the profiles of miRNAs reduced in PLDCs with profiles of miRNAs detected in isolated PLDC autophagosomes. In total, 57 miRNAs (*i.e.* 71%) were detected in isolated PLDC autophagosomes out of a total of 80 miRNAs that were reduced in PLDCs (Fig. 7B). Importantly, all 10 of the most-reduced miRNAs, including miR-345, were present in isolated PLDC autophagosomes (Fig. 7C).

Inhibition of autophagy increases miR-345 levels and decreases expression of miR-345-targeted proteins and hepatic cystogenesis

Treatment of cultured PCKCs with the autophagy inhibitor, HCQ, increased miR-345 levels (Fig. 8A,B), inhibited proliferation (Fig. 8C), and reduced expression of CDC25A, CDK6, E2F2, and PCNA (Fig. 8D). Consistent with this observation, the level of miR-345 was also increased in cholangiocytes of PCK rats treated with HCQ, whereas the levels of miR-345-targeted proteins and hepatic cystogenesis were reduced (Fig. 8E,F).

Discussion

The key findings of this study are that, in PLD: (i) the levels of most cholangiocyte miRNAs were decreased and the levels of 10 miRNAs were reduced by more than 10 times; (ii) miR-345 was the most reduced miRNA; (iii) CDC25A, CDK6, E2F2, and PCNA targeted by miR-345 were overexpressed at both the mRNA and protein levels; (iv) re-introduction of miR-345 decreased the expression of miR-345-targeted proteins, proliferation, and cyst growth; (v) miR-345 accumulated in autophagosomes; and (vi) inhibition of autophagy increased miR-345 levels and inhibited expression of miR-345-targeted proteins and hepatic cystogenesis. Thus, our data demonstrate that autophagy-mediated reduction of miR-345 in PLDC contributes to hepatic cystogenesis.

We analysed the profile of miRNAs in cholangiocytes isolated from patients with ADPKD-associated PLD and found that 66% of miRNAs were differentially expressed, with most of them (*i.e.* 81%) being decreased. This finding is consistent with previous

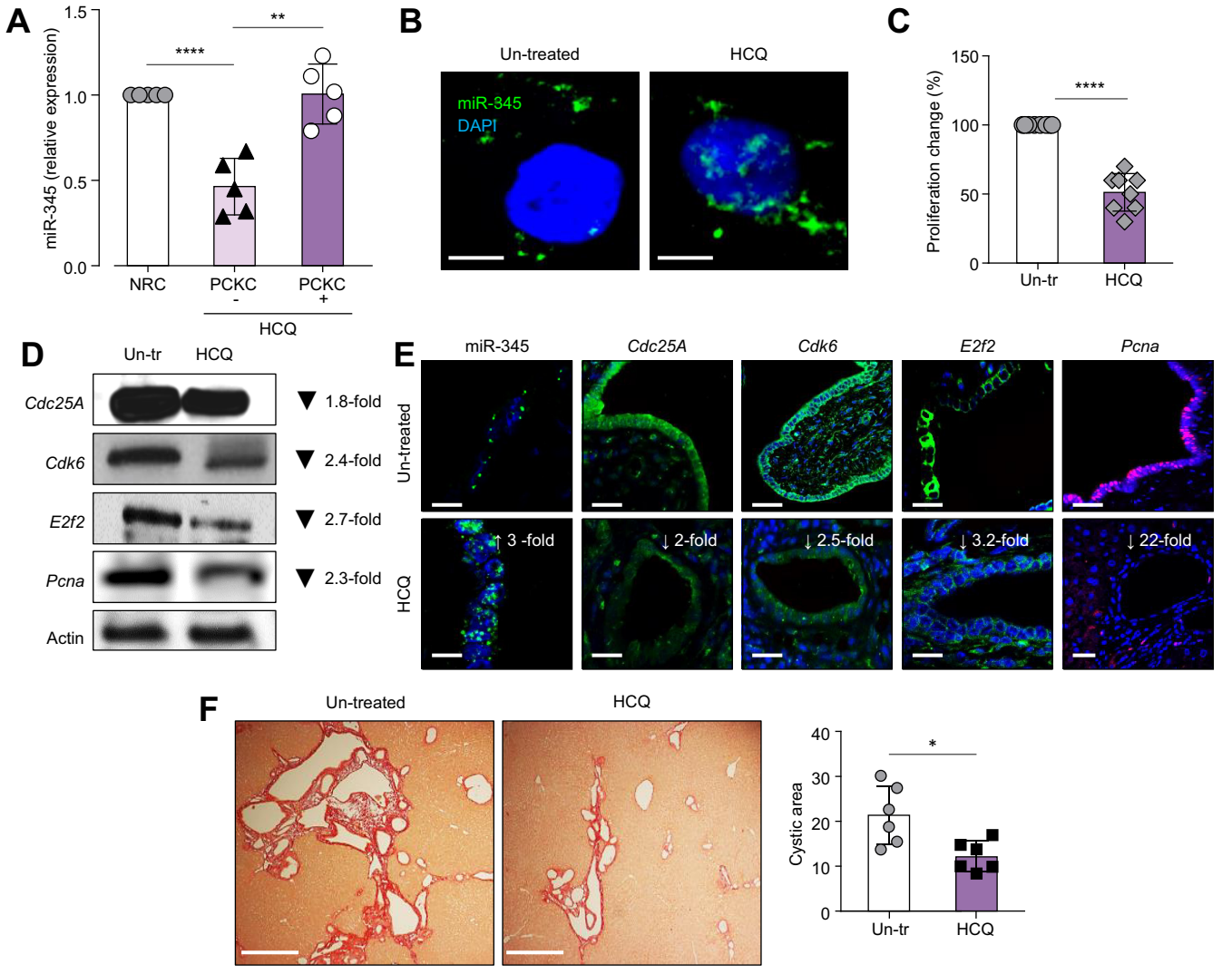

Fig. 8. HCQ increases miR-345, reduces expression of miR-345-targeted proteins, and attenuates hepatic cystogenesis. (A) miR-345 levels were decreased in PCKCs compared with NRCs but increased after treatment with HCQ. n = 5 for each data set. (B) Increased miR-345 levels in HCQ-treated PCKCs were confirmed by ISH. n = 10 microscopic fields for each data set. Scale bars = 5 μm. (C) Proliferation (assessed by cell counting) of PCKCs was decreased in response to HCQ. n = 8 for each data set. (D) Western blots showing decreased levels of CDC25A, CDK6, E2F2, and PCNA in HCQ-treated PCKCs. n = 3 for each data set. (E) miR-345 (green) was increased, whereas expression of miR-345 targets (all green) and number of PCNA-positive nuclei (red) were decreased in HCQ-treated PCK rats. Nuclei are stained with DAPI. Scale bars = 50 μm. (F) Light microscopic images of liver sections stained with Picro Sirius Red and bar graphs demonstrate that HCQ decreased cystic areas in PCK rats compared with untreated (Un-tr) controls. n = 6 rats per group. Data are mean ± SD. *p <0.05, **p <0.01, ****p <0.0001. CDC25A, cell division cycle 25A; CDK6, cyclin-dependent kinase 6; HCQ, hydroxychloroquine; ISH, *in situ* hybridisation; NRC, normal rat cholangiocyte; PCK, polycystic kidney PCKC, polycystic kidney rat cholangiocyte; PCNA, proliferating cell nuclear antigen; PLDC, polycystic liver disease cholangiocyte.

observations demonstrating a global reduction of miRNAs in cholangiocytes from PCK rats.[9,20] Our data are in line with other reports showing altered miRNA profiles in multiple pathological conditions, with most miRNAs being decreased.[21–24]

Combined miRNA-seq–mRNA-seq analysis for targets of downregulated miRNAs in PLDCs identified the cell cycle, autophagy, cAMP-mediated signalling, and cell proliferation as the 4 most-affected pathways. All 4 pathways have a crucial role in hepatic cystogenesis and have been considered for therapeutic interventions in PLD.[3,5,6,19,25] The benefit of cAMP targeting in attenuation of hepatic cyst growth has been demonstrated in multiple preclinical and clinical studies.[1,6,7,14,19,25,26] Inhibition of autophagy has also been reported to decrease hepatic cystogenesis in animal models of PLD.[3] Our current study substantially refines and extends previously published data by showing the linkage between increased autophagy, altered miR-345–miR-345-targeted protein networks, cholangiocyte hyperproliferation, and hepatic cystogenesis.

Our data also provide experimental evidence that autophagy-mediated decrease of miR-345 contributes to PLD pathogenesis. Reduced miR-345 was observed in patients with PLD and in animal models of this disease. We also found that miR-345 targets more mRNAs expressed in PLDCs (*i.e.* 21%) compared with the other 9 most-decreased miRNAs. Targets of miR-345 include genes that encode proteins related to cell cycle and cell proliferation (*i.e.* 2 cellular processes that are crucial for hepatic cystogenesis[26–28]). In line with our observation, decreased miR-345 has also been reported in cancers and, in several instances,

miR-345 was the most decreased miRNA.[29,30] Finally, similarly to PLD, reduced expression of miR-345 in cancer cells was linked to dysregulated cell cycle and increased cell proliferation.[31–33]

We found by *in silico* analysis that miR-345 has multiple predicted mRNA targets, many of which are expressed in PLDCs, as detected by mRNA-seq. In this study, we focussed on: (i) CDC25A, a master cell-cycle phosphatase; (ii) CDK6, a cell cycle-related protein; (iii) E2F2, a transcriptional factor involved in the regulation of cell cycle-related proteins; and (iv) PCNA, a cell proliferation-related protein. These targets were chosen because: (i) the pathway cluster analysis of miR-345-targeted genes showed the cell cycle and cell proliferation to be the top most-affected pathways in PLDCs; (ii) the cell cycle has previously been reported to be dysregulated in PLDCs and cell cycle-related proteins are overexpressed;[8,9] and (iii) cell proliferation is a marker of PLD progression/regression.[8,19]

Most miRNAs are known to negatively regulate their targets and miRNA–mRNA pairing inversely correlates with their levels (*i.e.* if expression of a given miRNA is decreased, the levels of targeted genes are increased and vice versa. Indeed, miR-345-targeted proteins (*i.e.* CDC25A, CDK6, E2F2, and PCNA) are overexpressed in PLDCs at both the mRNA and protein levels. As expected, when we experimentally increased miR-345 in PLDCs, the level of miR-345-targeted proteins was reduced, cell proliferation decreased, and cyst growth inhibited. These data suggest that miR-345 contributes to cyst expansion and, therefore, might represent a novel molecular target in PLD.

The mechanisms that account for miRNA dysregulation in disease are poorly understood, but several have been proposed, including genomic defects in miRNA coding regions, repression by transcriptional factors, or epigenetic modifications.[34] Limited research suggests that miRNA levels are regulated by autophagy. For example, in *Caenorhabditis elegans*, autophagy modulates miRNA expression by removing a component of the miRNA processing machinery, the miRNA RNA-induced silencing complex (miRISC).[10] In mammalian cells, the components of miRNA biogenesis, DICER and Argonaute 2 (AGO2), are targeted for autophagic degradation, decreasing miRNA expression.[11] Finally, autophagy-mediated degradation of mature miR-224 was observed in hepatocellular carcinoma.[12]

Therefore, we examined the role of autophagy in miR-345 degradation. We found that, in PLDCs, miR-345 is localised to autophagosomes. In addition, the other 9 miRNAs most decreased in PLDCs were detected in isolated autophagosomes. Although autophagy was initially considered as a bulk degradation pathway, it is now evident that it is also selective (*i.e.* mitophagy, ribophagy, lipophagy, etc.).[35] Our data show that miRNAs are degraded by autophagy, and we propose to term this selective type of autophagy, 'miRNAutophagy'.

The upstream regulator of miRNAutophagy in PLDCs is unknown. In general, regulation of autophagy occurs by multiple intracellular signalling pathways including, but not limited to, mammalian target of rapamycin (mTOR) signalling.[36] Consistent with this, it was previously demonstrated that increased autophagy in PLD is linked to the cAMP-protein kinase A (PKA)-cAMP-response element binding protein (CREB) pathway.[3] Given that molecular and cellular events underlying hepatic cystogenesis are interconnected, we speculate that the activated PLDC cAMP pathway might be the upstream regulator of miRNAutophagy.

We found that accumulation of miR-345 in PLDC autophagosomes is associated with overexpression of miR-345-targeted proteins. HCQ, a widely accepted autophagy inhibitor,[37,38] increased miR-345, reduced the expression of miR-345 targets, and inhibited cholangiocyte proliferation and hepatic cystogenesis. Although our data provide a mechanistic link between increased autophagy and decreased miR-345 in PLD, we recognise that effects of bulk autophagy inhibitors on hepatic cystogenesis might also occur via miR-345-independent mechanisms.

In addition to miR-345, by *in silico* search, we found other miRNAs with binding sites to *CDC25A*, *CDK6*, *E2F2*, and *PCNA*. We observed that many of them are downregulated in PLDCs and present in isolated autophagosomes (Supplementary Table S5). Therefore, we cannot exclude the possibility that multiple miRNAs target the genes of interest, acting in concert and contributing to the levels of CDC25A, CDK6, E2F2, and PCNA in PLDCs under basal conditions or in response to HCQ.

Direct pharmacological modulation of miRNA expression in PLD/PKD remains an undeveloped area. The anti-miR-17A antisense oligonucleotide drug, RGLS4326, decreases the level of miR-17A overexpressed in kidney, subsequently inhibiting renal cystogenesis in an animal model of PKD.[39,40] Another drug that re-establishes the levels of downregulated miR-29 in fibrotic diseases, but not in PKD, is remlarsen.[41,42] However, these drugs might not be feasible for PLD treatment because our miRNA-seq data show that the levels of miR-17A and miR-29 are comparable in healthy and PLD cholangiocytes. Thus, inhibition of autophagy is currently the only option to restore miRNA levels in PLDCs.

In summary, our data demonstrate that autophagy-mediated reduction of miR-345 levels in PLDCs is associated with increased expression of miR-345-targeted proteins and enhanced hepatic cystogenesis, whereas restoration of miRNAs in PLDCs is beneficial for disease progression.

Abbreviations

ADPKD, autosomal dominant polycystic kidney disease; ADPLD, autosomal dominant polycystic liver disease; AGO2, Argonaute 2; *ALG8*, alpha-1,3-glucosyltransferase; *ALG9*, alpha-1,2-mannosyltransferase; ARPKD, autosomal recessive polycystic kidney disease; CDC25A, cell division cycle 25A; CDK6, cyclin-dependent kinase 6; GANAB, glucosidase II alpha subunit; DNAJB11, DnaJ heat shock protein family (Hsp40) member B11; DZIP1L, DAZ interacting zinc finger protein 1 like; FDR, false discovery rate; GO, Gene Ontology; HCQ, hydroxychloroquine; ISH, *in situ* hybridisation; KEGG, Kyoto Encyclopedia of Genes and Genomes; LRP5, low-density lipoprotein receptor-related protein 5; miRISC, RNA-induced silencing complex; miRNA-seq, miRNA-sequencing; mTOR, mammalian target of rapamycin; NHC, normal human cholangiocyte; NRC, normal rat cholangiocyte; PCK, polycystic kidney; PCKC, polycystic kidney rat cholangiocyte; PCNA, proliferating cell nuclear antigen; PKD1/2, polycystic kidney disease 1/2; PKHD1, polycystic kidney and hepatic disease 1; PLD, polycystic liver disease; PLDC, polycystic liver disease cholangiocyte; PRKCSH, protein kinase C substrate 80K-H; RPM, reads per million; SEC61B, SEC61 translocon subunit beta; SEC63, SEC63 homolog, protein translocation regulator; snRNA, small nuclear RNA; WT, wild type.

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Conflicts of interest

The authors declare no conflicts of interest that pertain to this work. Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Substantial contributions to study conception and design: T.M., A.M., and N.L.; execution of experiments: T.M., A.M., C.T., B.H., J.D., and B.H.; data analysis and interpretation: T.M., A.M., and N.L.; drafting of article: T.M., A.M., and N.L.

Data availability statement

Where possible, experimental data can be shared via contact with the corresponding author.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2021.100345.

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Author names in bold designate shared co-first authorship

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