

Supporting Information for the article:

MiRNA-506 promotes primary biliary cholangitis-like features in cholangiocytes and immune activation

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Materials and Methods

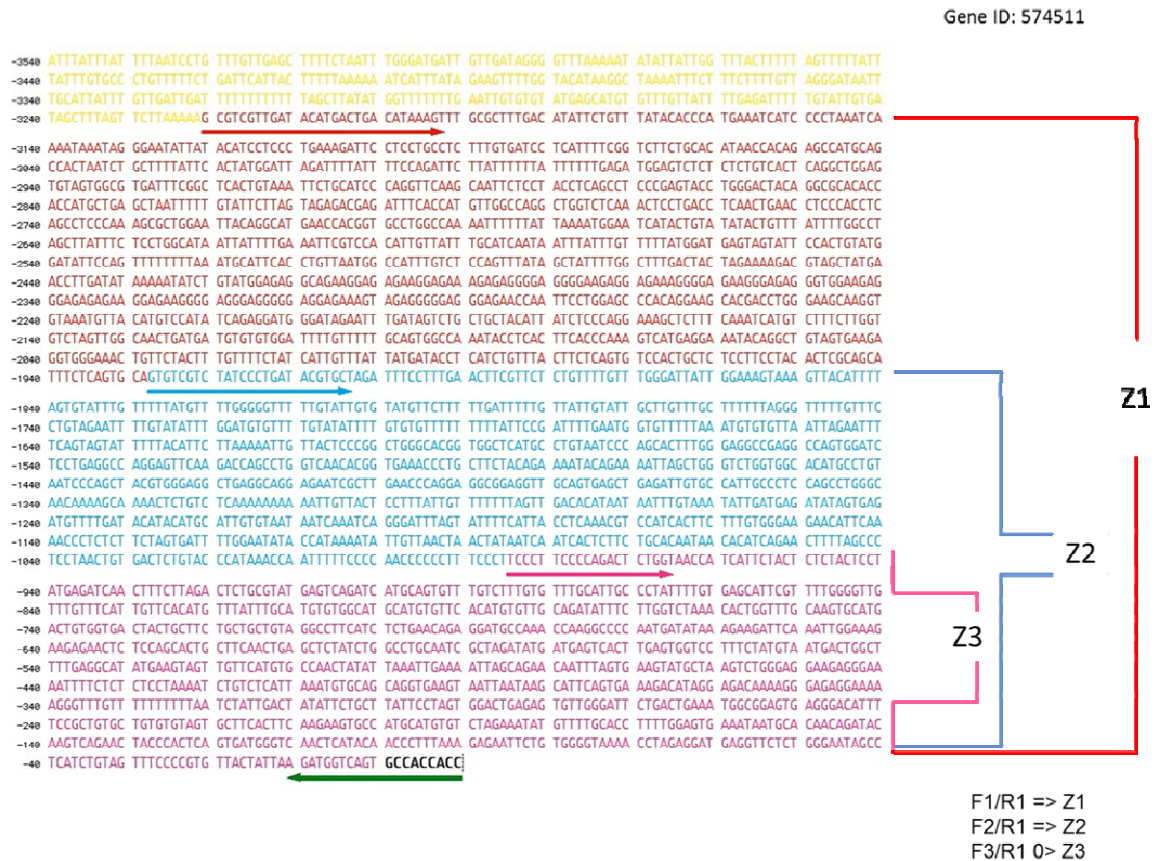
Luciferase reporter constructs and assays. Three different sizes of the human miR-506 gene (hsa-miR-506; NCBI Gene ID: 574511) promoter were cloned in a luciferase expression vector. Briefly, 3229, 1936 or 993 bp of the 5'-flanking region of hsa-miR-506 (Z1-hsa-miR-506pr, Z2-hsa-miR-506pr and Z3-hsa-miR-506pr, respectively) were cloned using genomic DNA obtained from a healthy individual (Supplementary Figures 1 A and B). First, hsa-miR-506pr constructs were amplified by high fidelity PCR using the *AccuPrime Pfx* DNA polymerase (Invitrogen). Specific oligonucleotide primers (Supplementary Table 1) with appropriate *attB* sites were added to obtain cDNA adapted for cloning using Gateway® technology. PCR products were recombined with *pDONR™221 P1-P5r* vector (Invitrogen). Next, these vectors were again recombined in a *Multisite Gateway* cloning reaction with a promoter-less destination vector (pDEST/pL) containing the coding sequence of firefly luciferase (Luc2), as previously described.⁽¹⁾

Non-tumor SV40-immortalized human cholangiocytes (i.e. H69, a gift from Dr. D. Jefferson, Tufts University, Boston, MA) were transfected with Z1, Z2 or Z3 recombinant vectors using *Lipofectamine 2000* (Life Technologies). Briefly, cells were seeded in a 24-well plate with fully supplemented DMEM/F-12 medium⁽²⁾ and incubated overnight. The following day, cells were washed twice with PBS, and then 500 µL of transfection mix (1 µg DNA, 2.5 µL lipofectamine and 3 mL Opti-MEM) was added to each well of cells. The

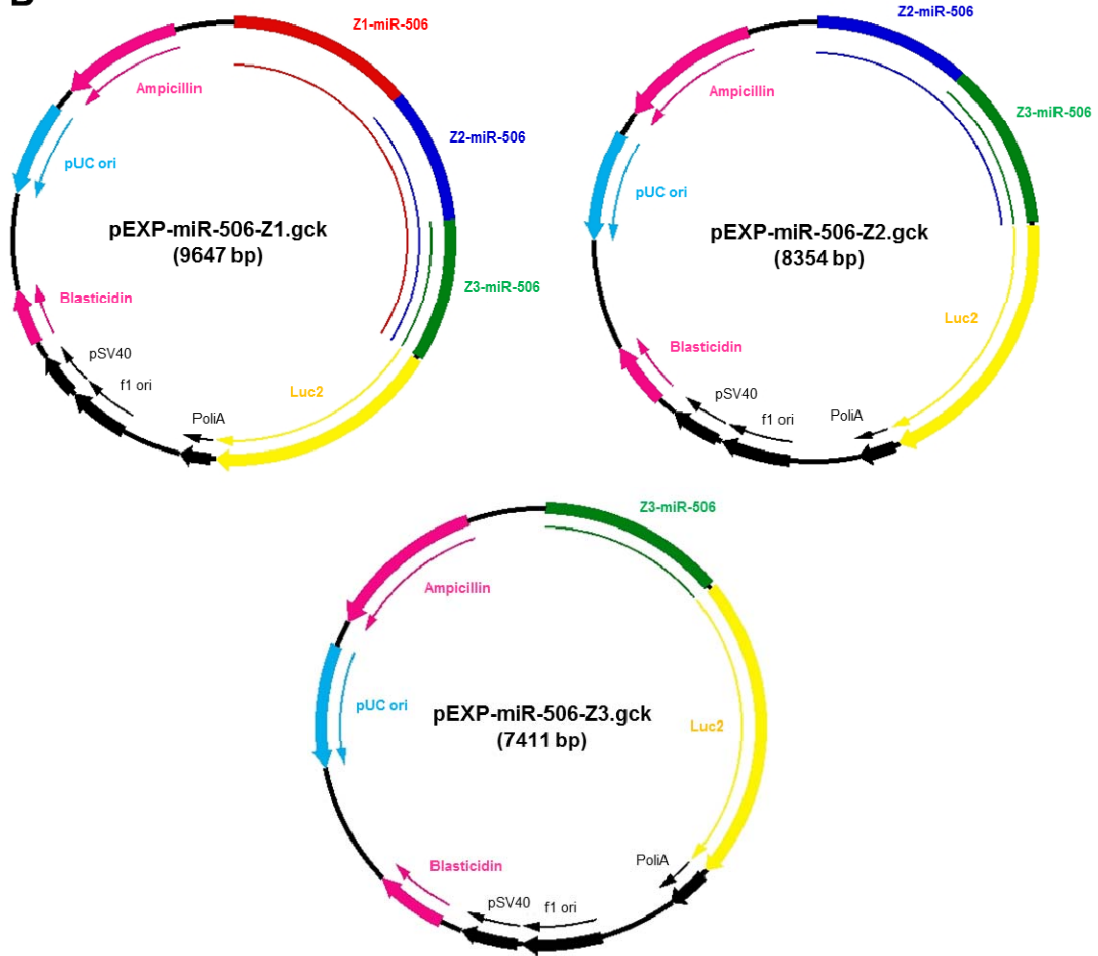
transfection mix was replaced 6 h later by DMEM+10% FBS alone or together with different pro-inflammatory cytokines [i.e. interleukins (IL) 1 β , 6, 8, 12, 17, 18, tumor necrosis factor alpha (TNF α), or interferon gamma (IFN γ)], pro-fibrotic factors [i.e. transforming growth factor beta 1 (TGF β 1)], estrogens (i.e. 17 β -estradiol), glucocorticoids [i.e. dexamethasone (DEX)], growth factors [i.e. epidermal growth factor (EGF)] and bile acids [i.e. cholic acid (CA), ursodeoxycholic (UDCA) or tauroursodeoxycholic (TUDCA)] (Supplementary Table 2). Next, 24 h after transfection, 50 μ L of Lysis Buffer was added into each well of cells and the luciferase activity was assessed (in 20 μ L of cell homogenate) using the *Luciferase Assay Kit E151A* (Promega). Luciferase activity was measured in a NOVOstar Apparatus (BMG LABTECH) and the resultant values were normalized to the total protein concentration.

Promoter cloning. Different miR-506 promoter constructs (Supplementary Figure 1) were cloned in the pEXP-gck expression vector containing the luciferase gene. These constructs were amplified by high fidelity PCR using specific oligonucleotide primers shown in Supplementary Table 1.

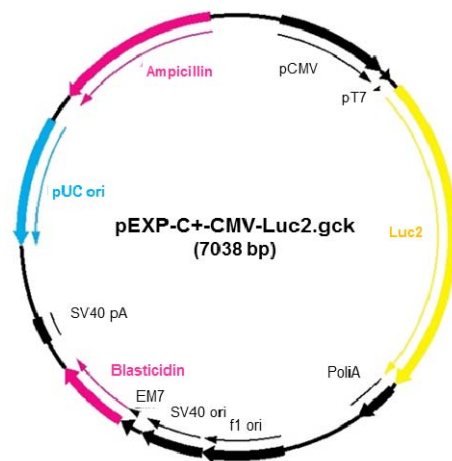
A



B



C



Supplementary Figure 1. Promoter cloning. (A) Three fragments of different length of the sequence located immediately 5'-upstream of miR-506 were cloned [i.e. Z1 (3229 bp), Z2 (1936 bp) and Z3 (993 bp)]. (B) Cloning of promoter regions were performed in an expression vector pEXP-gck containing the luciferase gene. (C) The cytomegalovirus (CMV) promoter was used as a positive control of luciferase gene expression.

Cells treatment. The luciferase activity of Z1, Z2 or Z3 recombinant vectors was evaluated under different stimuli (Supplementary Table 2) in H69 cells.

Gene expression. Quantitative gene expression was performed as previously described.⁽³⁾ Briefly, RNA was isolated from cultured cholangiocytes using *TRI-Reagent* (Sigma) followed by reverse-transcription polymerase chain reaction (RT-PCR) of 1 µg RNA using *iScript™ cDNA Synthesis Kit* (BIO-RAD) in a Veriti™ Thermal Cycler (Applied Biosystems). Gene expression was analyzed by real-time quantitative PCR (qPCR) in a LightCycler96 apparatus (Roche) with *iQ™ SYBR® Green Supermix* (BIO-RAD) following manufacturer's instructions under the following conditions: 95°C for 10 min, 40 cycles of 3 steps (95°C for 15 seconds, 60°C for 30 seconds and 72°C for 45 seconds) and 95°C for 15 seconds followed by 1 minute at 60°C. *GAPDH* was used as normalizing control. Specific primer sequences are shown in Supplementary Table 3. On the other hand, the quantification of miR-506 expression was performed using the *TaqMan MicroRNA Reverse Transcription Kit* and a commercial miR-specific primer (miR-506 Mature miRNA sequence UAAGGCACCCUUCUGAGUAGA; Applied Biosystems); data were normalized with the expression of Z-30 small nuclear RNA control (Z30 Control sequence TGGTATTGCCATTGCTTCACTGTTGGCTTTGACCAGGGTATGATCTCTTAATCTTCTCTCTGAGCTG; Applied Biosystems). TaqMan Universal PCR Master Mix, no AmpErase, was used for the qPCR. The control group was related to 100% of expression.

Mass spectrometry. Total cell proteomes were compared using iTRAQ technology⁽⁴⁾ as previously described.⁽⁵⁾ Samples were prepared and peptides were labeled as follows: cell pellets were resuspended in lysis buffer containing 7 M urea, 2 M thiourea, 4% (v/v) CHAPS, 50 mM DTT. Homogenates were spinned down at 14,000 rpm for 1 h at 15°C. Protein concentration was measured in the supernatants with the Bradford assay kit (Bio-rad). Protein extracts (160 µg) were precipitated with methanol/chloroform and pellets dissolved in 7 M urea, 2 M thiourea, 4% (v/v) CHAPS. Protein quantitation was performed

with the Bradford assay kit (Bio-Rad). iTRAQ labeling of each sample was performed according to the manufacturer's protocol (Sciex). Briefly, a total of 100 µg of protein from each cellular condition was reduced with 50 mM tris (2-carboxyethyl) phosphine (TCEP) at 60°C for 1 h, and cysteine residues were alkylated with 200 mM methylmethanethiosulfonate (MMTS) at room temperature for 15 min. Protein enzymatic cleavage was carried out with trypsin (Promega; 1:20, w/w) at 37°C for 16 h. Each tryptic digest derived from each biological replicate was labelled according to the manufacturer's instructions with one isobaric amine-reactive tags. After 1 h incubation, each set of labelled samples were independently pooled and evaporated until < 40 µL in a vacuum centrifuge. To increase the proteome coverage, the peptide pool was injected to an Ettan LC system with a X-Terra RP18 pre-column (2.1 x 20mm) and a high pH stable X-Terra RP18 column (C18; 2.1 mm x 150mm; 3.5µm) (Waters) at a flow rate of 40 µL/min. Peptides were eluted with a mobile phase B of 5–65% linear gradient over 35 min (A, 5 mM ammonium bicarbonate in water at pH 9.8; B, 5 mM ammonium bicarbonate in acetonitrile at pH 9.8). 11 fractions were collected, evaporated under vacuum and reconstituted into 20 µL of 2% acetonitrile, 0.1% formic acid, 98% MilliQ-H₂O prior to mass spectrometric analysis.

For Mass Spectrometry Analysis, peptides mixtures were separated by reverse phase chromatography using an Eksigent nanoLC ultra 2D pump fitted with a 75 µm ID column (Eksigent 0.075 x 150). Samples were first loaded for desalting and concentration into a 0.5 cm length 300 µm ID pre-column packed with the same chemistry as the separating column. Mobile phases were 100% water 0.1% formic acid (FA) (buffer A) and 100% Acetonitrile 0.1% FA (buffer B). Column gradient was developed in a 210 min two step gradient from 5% B to 25% B in 180 min and 25%B to 40% B in 30 min. Column was equilibrated in 95% B for 5 min and 5% B for 15 min. During the entire process, pre-column was in line with column and flow was maintained all along the gradient at 300 nl/min. Eluted peptides from the column were analyzed using an Sciex 5600 TripleTOF™ system. Information data acquisition was acquired upon a survey scan performed in a mass range from 350 m/z up to 1250 m/z in a scan time of 250 ms. Top 35 peaks were selected for fragmentation. Minimum accumulation time for MS/MS was set to 75 ms giving a total cycle time of 3,8 s. Product ions were scanned in a mass range from 100 m/z up to 1700 m/z and excluded for further fragmentation during 15 s. After MS/MS analysis, data files were processed using ProteinPilot™ 4.5 software from AB Sciex which uses the algorithm Paragon™ (v.4.0.0.0) ⁽⁶⁾ for database search and Progroup™ for data grouping

and searched against Uniprot reference human database. False discovery rate was performed using a non-linear fitting method and displayed results were those reporting a 1% Global False Discovery Rate (FDR) or better. Relative quantification and protein identification were performed with the ProteinPilot™ software (version 4.5; Sciex) using the Paragon™ algorithm as the search engine. Each MS/MS spectrum was searched against a database of human protein sequences (Uniprot). The search parameters allowed for cysteine modification by MMTS and biological modifications program in the algorithm (i.e. phosphorylations, amidations, semitryptic fragments, etc.). Reporter ion intensities were bias corrected for the overlapping isotope contributions from the iTRAQ tags according to the certificate of analysis provided by the reagent manufacturer (Sciex). The peptide and protein selection criteria for relative quantitation were performed as followed below. Only peptides unique for a given protein were considered for relative quantitation, excluding those common to other isoforms or proteins of the same family. Proteins were identified on the basis of having at least one peptide with an ion score above 99% confidence. Among the identified peptides, some of them were excluded from the quantitative analysis for one of the following reasons: (i) The peaks corresponding to the iTRAQ labels were not detected; (ii) the peptides were identified with low identification confidence (<1.0%); (iii) the sum of the signal-to-noise ratio for all of the peak pairs was <6 for the peptide ratios. The protein sequence coverage (95% conf.) was estimated for specific proteins by the percentage of matching amino acids from the identified peptides having confidence greater than or equal to 95% divided by the total number of amino acids in the sequence. Several quantitative estimates provided for each protein by ProteinPilot were utilized: the fold change ratios of differential expression between labelled protein extracts; the p-value, representing the probability that the observed ratio is different than 1 by chance. A decoy database search strategy was also used to estimate the false discovery rate (FDR), defined as the percentage of decoy proteins identified against the total protein identification. The FDR was calculated by searching the spectra against the decoy database generated from the target database. The results were then exported into Excel for manual data interpretation. Although relative quantification and statistical analysis were provided by the ProteinPilot software, an additional 1.3-fold change cutoff for all iTRAQ ratios (ratio <0.77 or >1.3) and a p-value lower than 0.05 were selected to classify proteins as up- or down-regulated (at least in two of three biological replicates). Proteins with iTRAQ ratios below the low range (0.77) were considered to be

underexpressed, whereas those above the high range (1.3) were considered to be overexpressed.

Immunoblotting. Changes in protein expression were detected through immunoblotting using 40 µg of cell extracts. Samples were electrophoresed in 7.5% (AE2) or 12.5% (all the other proteins) SDS-PAGE, and electrotransferred to a nitrocellulose membrane (BioRad). Membranes were blocked and incubated overnight. Horseradish peroxidase (HRP)-conjugated secondary antibodies were incubated for 1 h at room temperature, and *Novex ECL HRP Chemiluminiscent Substrate Reagent Kit* (Invitrogen) was used for further band visualization. Image J software (National Institutes of Health) was used for quantitation. β-actin staining was carried out to normalize protein loading. The antibodies used are shown in Supplementary Table 4.

Cell proliferation, adhesion and migration. The evaluation of cell proliferation, adhesion and migration was carried out in H69, H69-miR-506 or H69-miR-neg human cholangiocytes. Cell Proliferation Dye eFluor® 670 (eBioscience) was used following manufacturer's instructions. Briefly, cells were harvested, washed twice with PBS and labeled with a final 5 µM eFluor dye solution. Samples were incubated for 10 min at 37°C in darkness. Next, 5 volumes of cold DMEM 10% FBS/1% P/S were added and tubes were placed on ice for 5 min. Cells were washed three times with fully-supplemented DMEM/F-12 medium and finally 3.5x10⁴ cells per well were seeded in 12-well plates in fully-supplemented DMEM/F-12 medium. After 72 h in culture, cells were trypsinized, centrifuged and resuspended in PBS for fluorescence measurement by flow cytometry in a *Guava EasyCyte 8HT* flow cytometer.

For the analysis of cell adhesion, 5x10⁴ cells were seeded in collagen-coated 12-well plates and cultured at 37°C for 3 h. Cells were then fixed and stained with 4% formaldehyde and 0.5% crystal violet in PBS for 20 min and washed with water. Once dried, cell staining was dissolved with 10% acetic acid in PBS and the absorbance was measured at 595 nm in a *Multiskan Ascent*® (Thermo) spectrophotometer.

On the other hand, cell migration was tested using transwell migration chambers (Costar). 2x10⁵ cells were plated in 6-well plates in fully-supplemented DMEM/F-12 medium and, when reached ~80% confluence, cells were washed twice with PBS and starved for 24 h in serum-free medium (DMEM supplemented with 1% P/S). Next, 1.75x10⁵ cells were seeded in 200 µL DMEM supplemented with 1% P/S in the top side of

previously hydrated chambers. 600 μ L DMEM supplemented with 10% FBS and 1% P/S were placed outside the chamber as chemo-attractant. Cells were incubated at 37°C for 48 h. Chambers were washed with PBS and fixed and stained with 4% formaldehyde and 0.5% crystal violet in PBS for 20 min. After washing the chambers with H₂O, non-migrating cells on the top of the chamber were removed using a cotton tipped swab, keeping the cells able to migrate towards the other side of the chamber. Pictures of the migrated cells were taken on random fields of the bottom side of the chamber and staining was dissolved for measuring absorbance at 550 nm in a *Multiskan Ascent*® (Thermo) spectrophotometer.

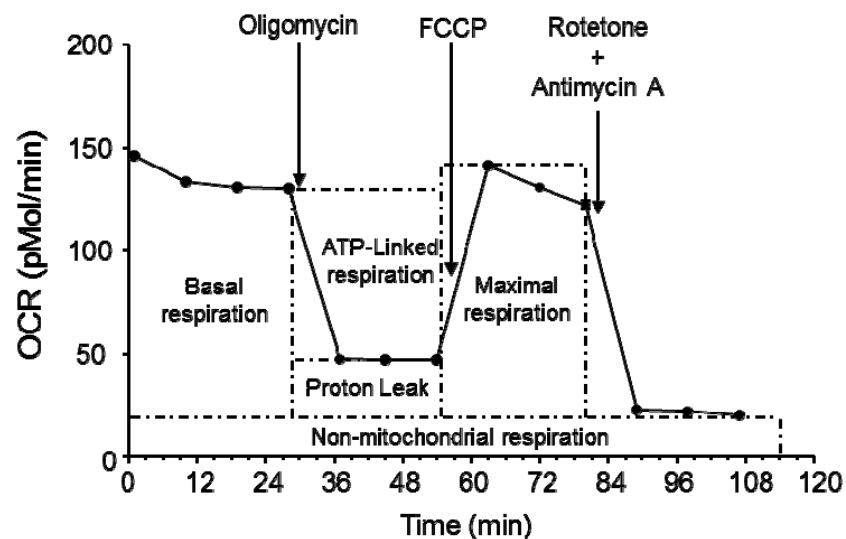
Cell viability and apoptosis

The cytotoxicity of the hydrophobic bile acids chenodeoxycholic acid (CDCA: 200 μ M, Sigma) and glycochenodeoxycholic (GCDCA: 750 μ M, Sigma) was evaluated in H69, H69-miR-neg and H69-miR-506 cholangiocytes. Additionally, caspase-mediated apoptosis and necroptosis were investigated under the presence of bile acids and with or without specific inhibitors (zVAD-fmk and necrostatin-1, respectively; both from Calbiochem, at 20 μ M) incubated for 2 h before and during bile acids exposure (48 h).

Cell viability was evaluated by using both the luminescence-based CellTiter-Glo® (Promega) and the colorimetric WST-1 (Roche) assays, following manufacturer's instructions. For this purpose, 5x10³ cells per well were seeded in 96-well plates with fully-supplemented DMEM/F-12 medium.⁽²⁾ Media was replaced by DMEM/F-12 supplemented with 3% FBS/1% penicillin/streptomycin (P/S), and the day after cells were treated as convenient and cultured for 48 h.

Cell apoptosis was evaluated by flow-cytometry. Briefly, 2.5x10⁴ cells per well were seeded in 24-well plates with fully-supplemented DMEM/F-12 medium.⁽²⁾ The day after, cells were washed with PBS1X and then incubated with DMEM/3% FBS/1% P/S treated as aforementioned for 48 h. *Puromycin dihydrochloride* (1.5 μ g/mL, from Sigma) was used as positive control of apoptosis. Apoptotic rates of cholangiocytes were measured by flow cytometry using *Annexin V Alexa Fluor 594 conjugate* (Thermo Fisher Scientific) and *Propidium Iodide* (Life Technologies) in a Guava EasyCyte 8HT apparatus.

Mitochondrial metabolic activity. A XF96 Extracellular Flux Analyzer (Seahorse Bioscience) was used with the *XF Cell Mito Stress Test Kit* for evaluating the mitochondrial metabolic parameters of H69, H69-miR-neg and H69-miR-506 cells. Briefly, 1×10^4 cholangiocytes were seeded in each well of a collagen-coated 96-well Seahorse microplate and cultured for 48 h in fully supplemented DMEM/F-12 medium.⁽²⁾ Next, cell culture medium was replaced by “Assay Medium” containing minimum essential medium (MEM; Gibco) supplemented with glucose, L-glutamine and sodium pyruvate (Sigma) at pH 7.4 and incubated for 1 h at 37°C without CO₂. Four basal OCR and ECAR measurements were obtained and then three measurements were carried out after the sequential injection of each mitochondrial inhibitor [i.e. 1 μM Oligomycin, 1.2 μM FCCP and 0.5 μM both Rotenone-Antimycin A (all from Sigma)]. Metabolic parameters were calculated as indicated by Seahorse Bioscience, calculating the rates upon different mitochondrial inhibitors, as shown in supplementary figure 2.



Supplementary Figure 2. Functional evaluation of the mitochondrial energetic metabolism. Oxygen Consumption Rate (OCR) is measured along the experiment under the presence or absence of different mitochondrial inhibitors. Adapted from Seahorse Bioscience.

PDC-E2 expression by immunofluorescent microscopy. H69, H69-miR-506 and H69-miR-neg cholangiocytes were cultured on glass coverslips overnight. Next, cells were fixed with cold methanol for 10 min at -20°C and permeabilized with a 0.5% Tween in PBS solution for 20 min at room temperature. PDC-E2 (Santa Cruz) primary antibody at 1:50

dilution was incubated for 1 h at room temperature. A fluorescent red-conjugated secondary antibody (Life Technologies, 1:1000 dilution) was incubated for 1 h and 30 min at room temperature and *Vectra System* (Vector Laboratories) was used for nuclei staining and cell mounting. PDC-E2 cellular expression was observed in a fluorescence microscope (Leica DM IRB).

Co-culture of human cholangiocytes with peripheral blood mononuclear cells (PBMCs). H69, H69-miR-506 or H69-miR-neg human cholangiocytes were co-cultured with PBMCs isolated from either a PBC patient or a normal human donor. Briefly, H69, H69-miR-506 or H69-miR-neg cholangiocytes (5×10^3 cells per well) were plated in collagen-coated 96-well plates in fully supplemented DMEM/F-12 medium. After overnight attachment, culture media was changed to RPMI (Gibco) supplemented with 10% FBS/1% P/S. After 48 h, PBMCs were isolated from a middle-aged female PBC patient using a density gradient media. Briefly, peripheral blood was diluted to 50% with physiological serum and carefully added to Lymphoprep™ (Fresenius Kabi Norge) (3/4 parts of Lymphoprep regarding the total peripheral blood volume). Samples were centrifuged at 2500 rpm for 30 min at room temperature. The PBMC fraction was carefully harvested using a Pasteur pipet and placed in a new tube containing physiological serum. Samples were centrifuged at 1500 rpm for 10 min and supernatant was carefully removed. Pellet was washed with physiological serum and centrifuged at 1500 rpm for 10 min. Supernatant was carefully removed. If erythrocytes were not present, the pellet was resuspended in RPMI supplemented with 10% FBS/1% P/S and counted as usual. In the presence of erythrocytes, an intermediate step for erythrocyte lysis was performed, incubating samples for 10 min with RBC Lysis Solution (QIAGEN) and doing a subsequent washing. Once PBMCs were isolated, cells were stained with *CellTrace™ CFSE Cell Proliferation Kit* (Invitrogen) following manufacturer's instructions and then 1.5×10^5 PBMCs in RPMI supplemented with 10% FBS/1% P/S were added to H69, H69-miR-506 or H69-miR-neg human cholangiocytes for 96 h. Afterwards, PBMCs were harvested and stained with the lymphocyte activation marker CD25 (BD Biosciences) and the cell death marker 7AAD (Life technologies) for 20 min and analyzed in a Guava EasyCyte 8HT flow cytometer. For the analysis, PBMCs was gated and the 7AAD-positive cells were discarded for testing both CFSE and CD25. Phytohaemagglutinin M form (PHA-M) (Gibco) and dimethyl sulfoxide (DMSO) (Sigma) were used as positive and negative controls for lymphocyte

activation and proliferation, respectively. The research protocol was approved by the *Ethical Committee for Clinical Research* of the Donostia Hospital, and the patients signed a written consent for the use of their blood samples for biomedical research.

Supplementary Table 1. Primers used for high fidelity PCR

Primer code	Sequences	
miR-506-Z1	<i>Forward</i>	5'- GCGTCGTTGATACATGACTGACATAAAGT-3'
	<i>Reverse</i>	5'- GGTGGTGGCACTGACCATCT-3'
miR-506-Z2	<i>Forward</i>	5'- GTGTCGTCTATCCCTGATACGTGCT-3'
	<i>Reverse</i>	5'- GGTGGTGGCACTGACCATCT-3'
miR-506-Z3	<i>Forward</i>	5'- TCCCTTCCCCAGACTCTGGT-3'
	<i>Reverse</i>	5'- GGTGGTGGCACTGACCATCT-3'

Supplementary Table 2.

Stimuli	Concentration	Company
IL18	100 ng/ml	MBL
IL17	100 ng/ml	Sigma
IL8	50 ng/ml	R&D
IL12	50 ng/ml	R&D
IL6	50 ng/ml	Sigma
IL1 β	10 ng/ml	R&D
TGF β 1	100 ng/ml	R&D
TNF α	100 ng/ml	Sigma
IFN γ	100 ng/ml	Abcam
CA	100 μ M	Sigma

UDCA	100 μ M	ACROS Organics
TUDCA	100 μ M	Calbiochem
DEX	100 μ M	Sigma
EGF	40 ng/ml	Sigma
17 β -est	100 μ M	Sigma

Supplementary Table 3. Primers used for RT-PCR of Human mRNAs

Primers		Sequences
<i>UCP1</i>	<i>Forward</i>	5'-CTCACCGCAGGGAAAGAA-3'
	<i>Reverse</i>	5'-GGTTGCCCAATGAATACTGC-3'
<i>UCP2</i>	<i>Forward</i>	5'-GTTCTACACCAAGGGCTCTGA-3'
	<i>Reverse</i>	5'-AATCGGACCTTTACCACATCC-3'
<i>Cytokeratin 7</i>	<i>Forward</i>	5'-ATCTTTGAGGCCAGATTGC-3'
	<i>Reverse</i>	5'-TTGATCTCATCATTGAGGGC-3'
<i>ZO1</i>	<i>Forward</i>	5'-CGGTCCTCTGAGCCTGTAAG-3'
	<i>Reverse</i>	5'-GGATCTACATGCGACGACAA-3'
<i>N-cadherin</i>	<i>Forward</i>	5'-TCCTGCTTATCCTTGTGCTGA-3'
	<i>Reverse</i>	5'-CGGATTCCCACAGGCTTGAT-3'
<i>IL8</i>	<i>Forward</i>	5'-GTGCAGTTTTGCCAAGGAGT-3'
	<i>Reverse</i>	5'-ACTTGTCCACAACCCTCTGC-3'
<i>PDGFB</i>	<i>Forward</i>	5'-CTCGATCCGCTCCTTTGATGA-3'
	<i>Reverse</i>	5'-CGTTGGTGCGGTCTATGAG-3'
<i>TGFβ2</i>	<i>Forward</i>	5'-AGCGACGAAGAGTACTACGC-3'
	<i>Reverse</i>	5'-TTCTCCATTGCTGAGACGTC-3'
<i>αSMA</i>	<i>Forward</i>	5'-CGGGACTAAGACGGGAATCCT-3'
	<i>Reverse</i>	5'-GTCACCCACGTAGCTGTCTT-3'
<i>VEGF</i>	<i>Forward</i>	5'-AGGGCAGAATCATCACGAAGT-3'
	<i>Reverse</i>	5'-AGGGTCTCGATTGGATGGCA-3'
<i>p21</i>	<i>Forward</i>	5'-CGATGGAACTTCGACTTTGTCA-3'
	<i>Reverse</i>	5'-GCACAAGGGTACAAGACAGTG-3'
<i>IRE1</i>	<i>Forward</i>	5'-AGGGACAGGAGGGAATCGTA-3'
	<i>Reverse</i>	5'-CAGTCCCTAATGCCACACCT-3'
<i>CHOP</i>	<i>Forward</i>	5'-TCTTCATACATCACCACACC-3'
	<i>Reverse</i>	5'-CTTGTGACCTCTGCTGGTTC-3'
<i>ATF6</i>	<i>Forward</i>	5'-GCTGGATGAAGTTGTGTCAGAG-3'

	<i>Reverse</i>	5'-TGTTCCAACATGCTCATAGGTC-3'
<i>PERK</i>	<i>Forward</i>	5'-CAGGCAAAGGAAGGAGTCTG-3'
	<i>Reverse</i>	5'-AACAACTCCAAAGCCACCAC-3'
<i>XBP-1</i>	<i>Forward</i>	5'-GCAGGTGCAGGCCAGTTGTCAC-3'
	<i>Reverse</i>	5'-CCCCACTGACAGAGAAAGGGAGG-3'
<i>GAPDH</i>	<i>Forward</i>	5'-CCAAGGTCATCCATGACAAC-3'
	<i>Reverse</i>	5'-TGTCATACCAGGAAATGAGC-3'

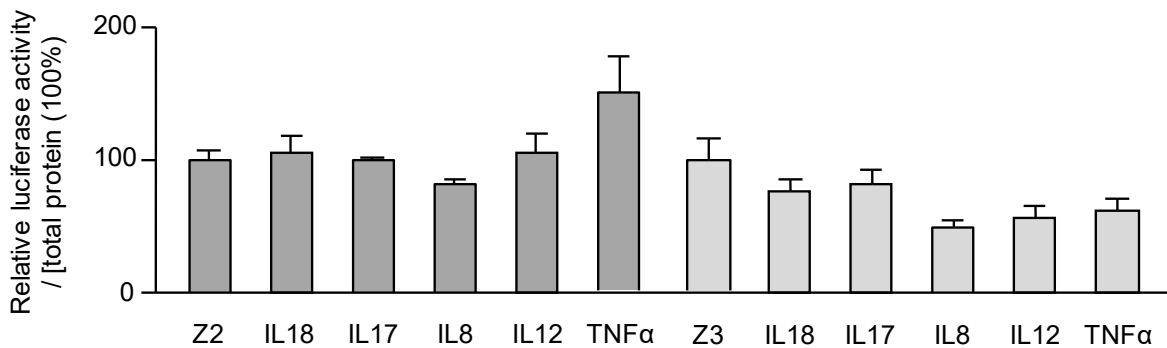
Supplementary Table 4. Antibodies used for western blot and/or immunofluorescence.

Antibody	Company	Reference	Use
Goat polyclonal anti-AE2	Santa Cruz	sc-46710	WB (1:250; room temperature)
Mouse monoclonal anti-OPA1	Novusbio	1E8-1D9	WB (1:1000; 4°C)
Rabbit polyclonal anti-Calpain 1	Abcam	ab28258	WB (1:2000; 4°C)
Mouse monoclonal anti-ATP5H	Abcam	ab110275	WB (1:1000; 4°C)
Goat polyclonal anti-PDC-E2	Santa Cruz	sc-16890	WB (1:250; 4°C) IF (1:50)
Rabbit polyclonal anti-PDC-E2	Santa Cruz	sc-32925	WB (1:250; 4°C)
Rabbit polyclonal anti-p21	Abcam	ab7960	WB (1:1,000; 4°C)
Rabbit monoclonal anti-Cleaved Caspase-3	Cell signaling	9664	WB (1:500; 4°C)
Rabbit monoclonal anti-pH2AX	Cell signaling	9718	WB (1:500 4°C)
Mouse monoclonal anti- β -actin	Sigma	A5316	WB (1:1,000; 4°C)
Anti-rabbit IgG, HRP-linked Antibody	Cell signaling	7074	WB (1:5,000 Room temperature)
Anti-mouse IgG, HRP-linked Antibody	Cell signaling	7076	WB (1:5,000; room temperature)
Donkey anti-goat IgG, HRP-linked Antibody	Santa Cruz	sc-2020	WB (1:5,000; room temperature)
Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568	LifeTechnologies	A11057	IF (1:1,000; room temperature)

Abbreviations: WB: western blot; IF: immunofluorescence

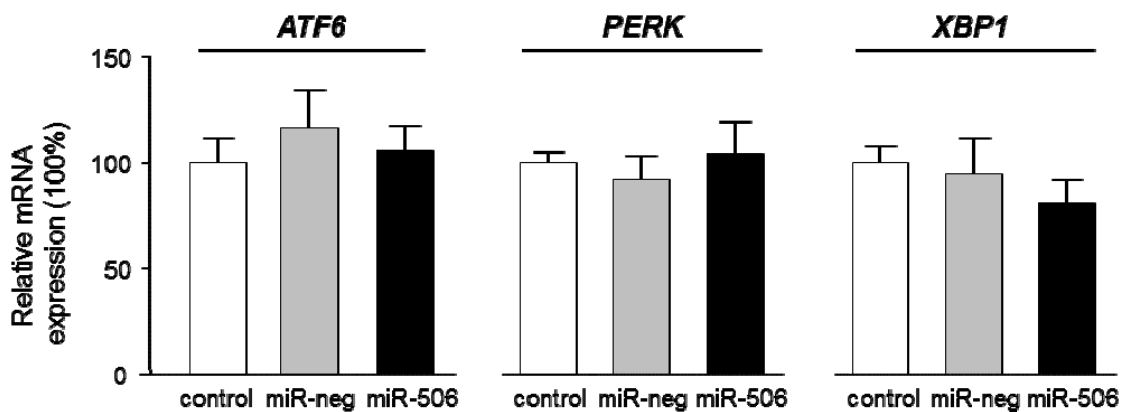
Results

Regulation of miR-506 promoter activity in human cholangiocytes. The pro-inflammatory cytokines IL8, IL12, IL17, IL18 or TNF α have no effects on the Z2 or Z3 luciferase activities in human cholangiocytes (Supplementary Figure 3).



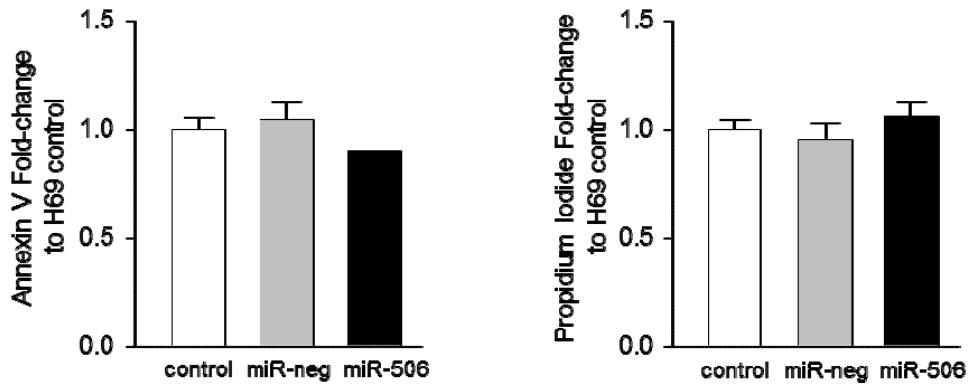
Supplementary Figure 3. Luciferase activity of human cholangiocytes transfected with the Z2 or Z3 promoter sequences, in the presence or absence of pro-inflammatory cytokines (n=10).

Role of miR-506 in cholangiocyte stress. MiR-506 did not alter the expression of *ATF6*, *PERK* and *XBP1* in cholangiocytes (Supplementary Figure 4).



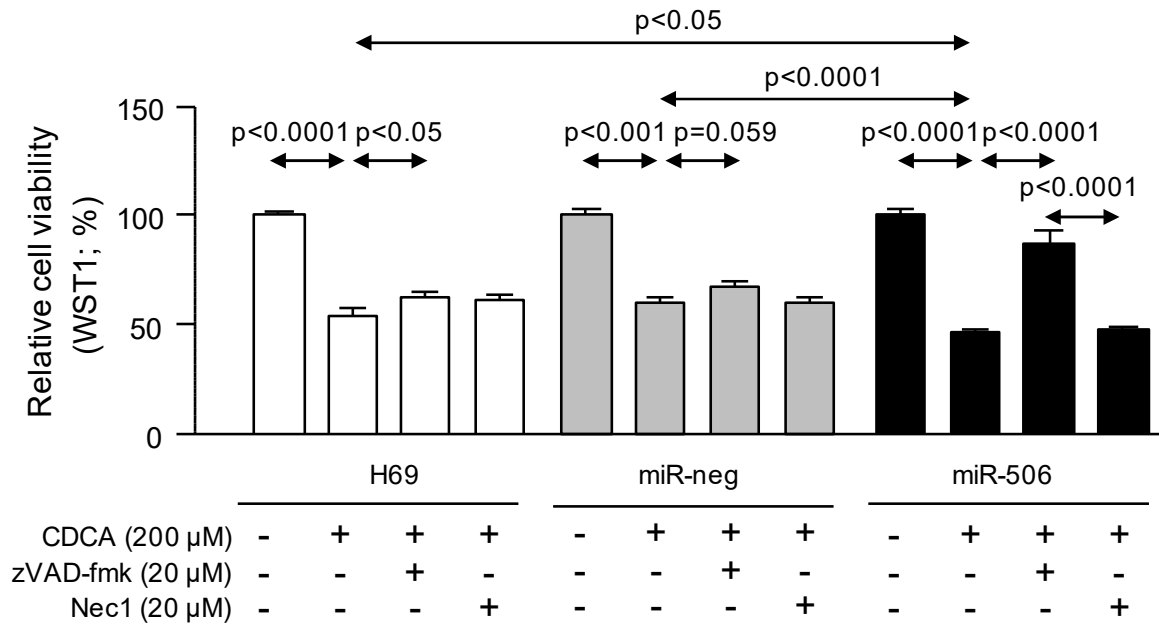
Supplementary Figure 4. Expression levels of *ATF6*, *PERK* and *XBP1* in cholangiocytes under the presence or absence of miR-506.

Effect of miR-506 on cholangiocyte baseline apoptosis. MiR-506 had no effect on basal apoptosis of H69 human cholangiocytes. (Supplementary Figure 5).



Supplementary Figure 5. Basal apoptotic rate of the cells by flow cytometry using Annexin V and Propidium Iodide staining (total n=6 of two independent experiments).

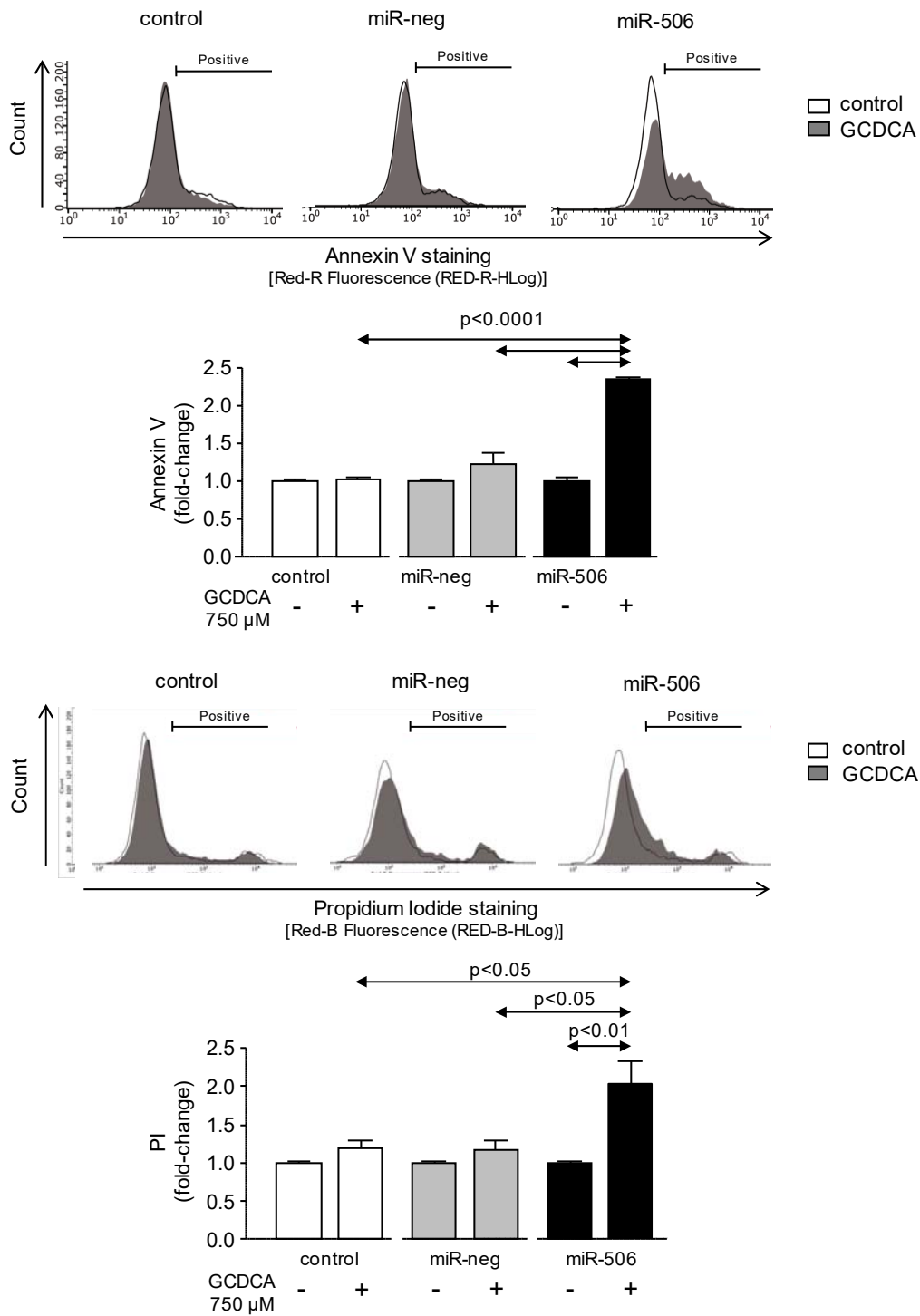
Effect of miR-506 on the cytotoxicity induced by CDCA. MiR-506 sensitized H69 human cholangiocytes to CDCA treatment (i.e. WST-1; Supplementary Figure 6) in a caspase-dependent manner, as zVAD-fmk conferred protection against CDCA-induced adverse effects (Supplementary Figure 6).



Supplementary Figure 6. Cell viability determination (WST1 assay) under CDCA treatment alone or in the presence of a caspase inhibitor (zVAD-fmk) or a necroptosis inhibitor (Nec-1) (n=9-10).

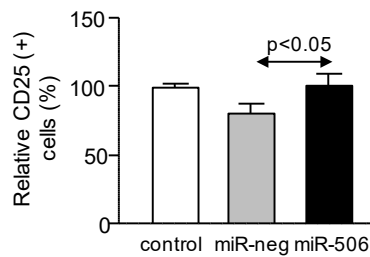
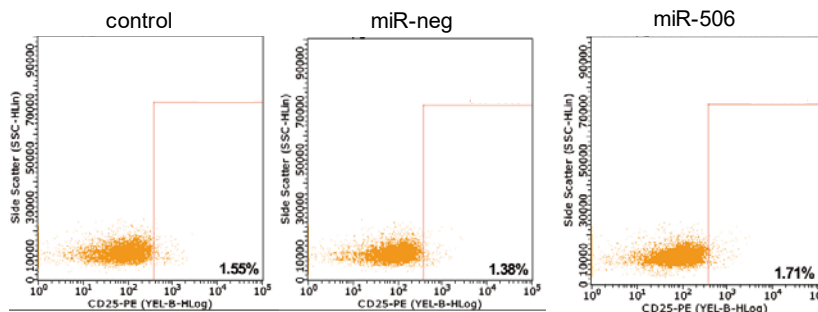
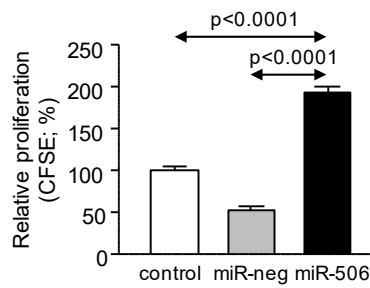
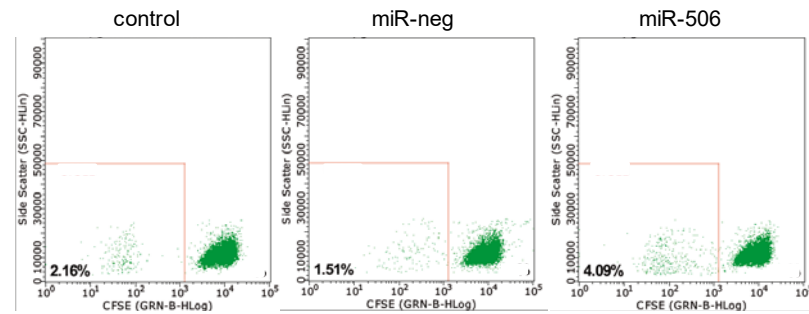
Effect of miR-506 on the cholangiocyte sensitivity to GCDCA-induced apoptosis.

Similar to CDCA treatment, flow cytometry-based apoptosis using Annexin V and Propidium Iodide staining showed that H69-miR-506 cells are more sensitive to apoptosis under 750 μM GCDCA treatment (Supplementary Figure 7).



Supplementary Figure 7. Role of miR-506 on cholangiocyte sensitivity to GCDCA-induced apoptosis. Flow cytometry-based apoptosis images and quantification of Annexin V and Propidium iodide staining under the presence of GDCA. (n=6 in each group).

Effect of miR-506 overexpressing cholangiocytes on normal PBMCs. PBMCs from a normal human donor were co-cultured together with H69, H69-miR-neg and H69-miR-506 cholangiocytes. Similar to PBMCs isolated from a PBC patient, H69-miR-506 cholangiocytes also increased the proliferation and activation of normal PBMCs compared to control conditions (Supplementary Figure 8).



Supplementary Figure 8. Role of miR-506 overexpressing cholangiocytes on the proliferation and activation of PBMCs isolated from a normal donor, measured by CFSE and CD25 staining, respectively. Representative dot-blots and histograms are shown for each cell type co-cultured. (n=5 per group)

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