Tumor-triggered personalized microRNA cocktail therapy for hepatocellular carcinoma

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Supplementary Materials and Methods

Materials

Branched polyethyleneimine (PEI) with average molecular weight (MW) of 600 Da, β-cyclodextrins, 1,1'-carbonyldiimidazole (CDI), triethylamine (TEA), oxalyl chloride, pyridine, tetrahydrofuran (THF), ethylenediamine, 1-adamantanecarboxylic acid, and 1-adamantylamine were purchased from Sigma-Aldrich (St. Louis, MO). Methoxy polyethylene glycol (PEG) with average MW of 5000 Da, N,N-dimethylformamide (DMF), dichloromethane (CH_2Cl_2), dimethyl sulfoxide (DMSO) were purchased from Aladdin Technology (Shanghai, China). 2-propionic-3-methylmaleic anhydride (CDM) was were purchased from TCI (Shanghai, China). 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI), Alexa Fluor® 647 conjugate of wheat germ agglutinin (WGA), and Pierce™ dialysis membranes were purchased from Thermo Fisher Scientific (Waltham, MA). Carboxyfluorescein (FAM)-labeled negative control miRNA ($miRNA^{FAM+}$), Cyanine5 (Cy5)-labeled negative control miRNA ($miRNA^{Cy5+}$), miR-199a/b-3p mimics, miR-10b inhibitors (specific sequence in **Table S1**) were synthesized by GenePharma (Shanghai, China).

Cell Culture

Huh-7 (human hepatocellular cancer cells) and HEK 293 (human embryonic kidney 293 cells) cell lines were obtained from Shanghai Institute for Biological Science (Shanghai, China). Both cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Thermo Scientific, Waltham, MA) and 1% penicillin/streptomycin (p/s) (Sigma Aldrich, St. Louis, MO) in a humidified incubator containing 5% CO_2 at 37 ℃.

Animal models

BALB/c nude mice (male, 4-6 weeks old) were purchased from Shanghai Model Organisms (Shanghai, China). All animals received were bred under standard pathogen-free conditions. All experiments protocols and procedures were authorized by Animal Care Committee of Zhejiang University School of Medicine.

The Huh-7 xenograft model was generated by injection of 1.5×10^6 Huh-7 cells suspended in 100 μL PBS per mouse subcutaneously to the subaxillary site.

The patient derived xenograft (PDX) models were established by HCC tumors acquired from the operation rooms. The tumor samples were cut into similar pieces (3*3*3 mm) within 1 h after removal from patients. Tissue fragments were then transplanted into the right flanks subcutaneously with a No. 20 trocar.

Synthetic procedures and characterization of PCACP and PCAP

Polyethyleneimine (PEI)-crosslinked β-cyclodextrin (βCD) was synthesized **(Figure S1A)** according to a previously reported method[1]. βCD (1.34 g, 1.18 mmol) and 1,1'-carbonyldiimidazole (CDI) (0.61 g, 3.76 mmol) were dissolved in dimethyl sulfoxide (DMSO), and then mixed with (TEA) (200 μL). The reaction mixture was stirred at

room temperature for 3 h under nitrogen, followed by further addition of TEA (200 μL). Then a DMSO solution containing certain amount of PEI (2.17 g, 3.62 mmol) was added dropwise by a syringe pump over 4 h with stirring. The mixture was stirred at room temperature overnight for reaction, followed by extensive dialysis (Thermo, MWCO 14000 Da) against double-stilled water for 12 h. Product was lyophilized and characterized by proton nuclear magnetic resonance (^{1}H-NMR). The spectra were recorded on a Varian 400 MHz NMR spectrometer at 22 °C. Chemical shifts were reported in parts per million (ppm) on the δ scale, and were referenced to residual protonated solvent peaks (δ = 4.70 ppm for residual peak of D_2O). All the spectra were evaluated with MestReNova 6.2 (Mestrelab Research, Santiago de Compostela, Spain). The characterization of lyophilized PEI-βCD by ^{1}H-NMR was shown in **Figure 1A (Figure S1E).**

Triblock copolymer (Ad-CDM-PEG) of adamantyl moieties (Ad) and PEG segments bridged by 2-propionic-3-methylmaleic anhydride (CDM) was synthesized **(Figure S1)** in two steps as following, referring to method previously discribed[2]. First, CDM (0.276 g, 1.5 mmol) was dissolved in anhydrous dichloromethane (CH_2Cl_2), followed by addition of N, N-dimethylformamide/oxalyl chloride (DMF 40 μL/$[COCl]_2$ 0.378 g, 3 mmol) reagent system. The mixture was stirred at 0 ℃ for 15 mins and room temperature for another 1 h to obtain chloride acetylated CDM. The vacuum-dried product was further reacted with PEG (0.2 mmol) in anhydrous CH_2Cl_2 (10 mL) containing pyridine (30 μL) at room temperature for 3 h, terminated by adding the same volume of saturated ammonium chloride aqueous solution. The organic phase was

separated, dried, and precipitated in anhydrous diethyl ether at 0 °C twice to obtain CDM-PEG, characterized by ^{1}H-NMR **(Figure 1A, Figure S1C).** Ad-CDM-PEG was synthesized by a ring-open reaction. Then adamantylamine (226.9 mg, 1.5 mmol) and synthesized compound CDM-PEG (5.0 g, 1.0 mmol) were dissolved in DMSO (10 mL), followed by stirring at room temperature for 2 h in dark. The mixture was then transferred into ultrapure water and dialyzed (Thermo, MWCO 2000 Da) against double-stilled water for 12 h. The product was lyophilized and characterized by ^{1}H-NMR (400 MHz, D_2O) (**Figure 1A, Figure S1D**). Synthesis of Ad-PEG has also been described[1]. PEG (1.00 g, 0.2 mmol) and CDI (0.2g, 1.23 mmol) were dissolved in DMSO prior to stirring under nitrogen for 3 h, and precipitated in ether/tetrahydrofuran solution (Volume ratio = 4:1) at 0 ℃ for 2 h. Product dissolved in DMSO (10 mL) was added dropwise to 5 mL of 1-adamantylamine (151.2 mg, 1.00 mmol) solution in the presence of triethylamine (1.5 mL), the mixture was stirred for another 10 h prior to extensive dialysis (MWCO 2000) against ultrapure water for 24 h and lyophilization to yield Ad-PEG.

PEI-βCD@Ad-CDM-PEG (PCACP) was simply self-assembled by host-guest interaction between PEI-βCD and Ad-CDM-PEG. PEI-βCD and Ad-CDM-PEG were dissolved in distilled water with a molar ratio of 4:1 as previously reported[1], the mixed solution was further stirred at room temperature for 12 h and lyophilized to obtain uniform white powder - the final product PCACP complex **(Figure 1A, Figure S1F).** Synthesis of PCAP was similar by using Ad-PEG instead of Ad-CDM-PEG (**Figure S1G**).

Determination of the miRNA loading capacity of PCACP

Gel electrophoresis was performed to detect the electrophoretic mobility of PCACP/miR199 polyplexes with various weight ratios. The amount of miR199 is 1 μg per well, the nanoparticles were prepared by mixing 1 μg miR199 with 0 μg, 1 μg, 2 μg, 3 μg, 4 μg, 5 μg and 6 μg PCACP solution and diluted into equivalent volume, followed by vortex and incubation at room temperature for 30 mins before application. Gels were prepared by 0.9% agarose gel containing 0.5 mg/ml ethidium bromide. Gel electrophoresis was carried out in TAE running buffer (40×10^{-3} M Tris-acetate, 1×10^{-3} M EDTA) with a voltage of 80 mV and 200 mA for 20 mins in a Sub-Cell system (Bio-Rad Lab, Hercules, CA). The bands of miRNA were visualized by an UV transilluminator and BioDco-It imaging system (UVP, Upland, CA). Complex ability of PCACP to miRNA was decided by the minimum weight ratio to inhibit nucleotide migration, and a weight ratio tenfold greater was applied to further experiments, as conventionally used in previous cationic polymer studies[3].

Determination of loaded miRNA release behavior

PCACP /miR199 or PCAP /miR199 solutions were packed in sealed dialysis membrane (8000 Da) bags. The entire bag was incubated in 50 ml DEPC H_2O with pH 6.5 or pH 7.4 at 37 °C with gentle shaking (87 rpm). 1 ml external solutions were collected and replenished with pure DEPC H_2O at different time points in 36 h to monitor the cumulative release of miRNA. The incremental released amount of miRNA in the supernatant was measured using the Quant-iT™ RiboGreen kit (Invitrogen, Carlsbad,

CA). The best-fit curve of time-dependent cumulative release (R%) was performed by GraphPad according to the following formula:

$$R\% = R_{max} \times (1 - 2^{-\frac{t}{t_{1/2}}})$$

where R_{max} is the maximum cumulative release amount, t is the time post treatment, and $t_{1/2}$ is the half-life of first-order release.

Preparation of nanoparticle solutions for in vitro experiments

The working nanomolar concentration of miRNA is 37.5 nM. Take 6-wells plate for example, we adopted 1 μg miRNA per well (2 mL culturing medium in total) as typical dosage. Nanoparticle (30 μg/μL) and miRNA (1 μg/μL) with weight ratio of 30:1 were mixed by vortex and condensed at room temperature for 30 mins before use. Then the nanoparticle/miRNA polyplexes (2 μL in total) were incubated with PBS at pH 6.5 or pH 7.4 (20-30 μL) for 4 h, followed by applying to cells. The volume of nanoparticle solution (20-30 μL) is pretty small relative to cell culture medium (2 mL/well), and the medium contains acid-base buffer, thus its effect on the overall pH of cell culture medium is negligible.

In vitro therapeutic effect by cell viability assays

Cell viability assays were conducted by cell counting kit-8 assay (CCK8; Dojindo, Kumamoto, Japan). Specifically, Huh-7 cells were seeded in 96-well plates with density of 8000 cells per well in 200 μL DMEM with 10% FBS and 1% penicillin-streptomycin for 18 h, then replaced with culture medium containing indicated formulations in dose series and cultured for another 72 h. At the end of treatment course, 10 μL of CCK8

solution was added per well and cells were incubated for 4 h. The relative cell viability (V%) to control group was calculated by absorbance detected at 450 nm using Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Waltham, MA). The half-maximal inhibitory concentration (IC_{50}) was determined by best-fit curve to the following equation:

$$V\% = \frac{100}{1 + ([miR199]/IC_{50})^{p}}$$

In the equation, V% is the relative viability and [miR199] is the dose (μg/mL) of miR-199a/b-3p mimics.

Evaluation of cells migration and invasion by Transwell assay

The invasion and migration activity of cells could be evaluated by their ability to pass through the 8 μm pores polycarbonate filters (6.5mm in diameter) in Transwell chambers (Corning, NY) with or without gel matrix Matrigel (Becton Dickinson, Franklin Lakes, NJ). The Matrigel was diluted with serum-free medium with a ratio of 1:5, and 40 mL Matrigel working solutions were coated on Transwell chambers per well, which were incubated at 37 ℃ for 1 h to solidify. Huh-7 cells treated with indicated formulations for 24 h in serum-free medium were harvested and seeded in upper compartments in Transwell with density of 5×10^4. Meanwhile, the lower compartments were filled with DMEM containing 10% FBS. After 48 hours, non-invasive cells on the upper surface of the filters were removed completely by wiping the filter surface with a cotton swab. Viable invasive cells adhering to the lower surface of the filter were directly fixed and stained with 3.5% crystal violet methanol solutions.

The invasive cells were finally counted in at least 5 visual fields per well under microscope (Leica, Germany).

Evaluation of cells migration activity by wound healing assay

Cells were seeded in 6-well plate (1×10^6 per plate) for 18 h in advance. The confluent cell monolayers were then wounded with a sterile pipette tip to make a straight scratch, then transfected with indicated formulations. After transfection for 6 h, the reagents were replaced with serum-free medium. Cell migration was tracked by observing wound areas using microscopy (Leica, Germany) in 72 h.

In vivo distribution study

Huh-7 cells derived tumor-bearing BALB/c nude mice were treated with PBS, naked $miRNA^{Cy5+}$, PCAP/$miRNA^{Cy5+}$ and PCACP/$miRNA^{Cy5+}$ intravenously with the dose of 1mg/kg $miRNA^{Cy5+}$. At the certain time points post administration, the mice were anesthetized with 2.5% isoflurane and visualized by a Xenogen IVIS Lumina system (Caliper Life Sciences, Alameda, CA). At the end of the tracking period, mice were sacrificed and tumors were excised to directly imaged. Results were analyzed using Living Image 3.1 software (Caliper Life Sciences, Alameda, CA).

PCACP was labeled with fluorescence by coupling reacting isocyanato of FITC fluorescence dye with the amino groups in PEI. BALB/c nude mice were injected with the fluorescent PCACP/miRNA system (60 μg PCACP with 2 μg miRNA in total) via tail vein. Blood was collected from retrobulbar sinus by capillary glass tubes at different time points in 24 h (nearly 10 μL each time). The blood concentration of PCACP was calculated by absorbance (FITC, Ex 480 nm, Em 520 nm) detected with SpectraMax

i3x (Molecular Devices; San Francisco, CA). Logarithm time diagram of plasma concentration was plotted to calculate the first order elimination rate constant k and t1/2 (k = 0.143, t1/2 = 2.947 h) according to following equation:

$$lgC = -\frac{k}{2.303}t + lgC_0$$

$$t_{1/2} = \frac{0.693}{k}$$

In the equations, C is the blood concentration of PCACP (ng/mL), k is the first order elimination rate constant, t is the time post administration, C_0 is the initial blood concentration of nanoparticle, while $t_{1/2}$ refers to the half-life of the nanocarrier in blood.

In vivo treatment and assessment

After xenograft tumor volume reached 70-90 mm^3, the tumor-bearing mice were randomly divided into groups with indicated regimen via tail-vein intravenous injection. Nanoparticle (30 μg/μL) and miRNA (1 μg/μL) with weight ratio of 30:1 were mixed by vortex and condensed at room temperature for 30 mins before use. The dosage of miRNA for each mouse is 1 mg/kg every single time. The treatment was conducted every 3 days for 5-6 times, and tumor growth was monitored by electric vernier caliper every 3 days by a designated researcher, so was mice body weight. The tumor volume (mm3) was calculated by $0.5ab^2$ where a and b are the length and width (mm) of the tumor, respectively. At the end of the treatment course, all mice were sacrificed and tumors were dissected, weighed and imaged. Half of each tumor was kept in liquid nitrogen, the other half was fixed in 4% formaldehyde for further

experiments. We also harvested main organs, heart, liver, spleen, lung and kidney, which were fixed in 4% formaldehyde.

RNA extraction and quantitative real-time PCR (qRT-PCR)

For total RNA extraction, cells cultured in six-well plates and treated with different formulations were washed with ice-cold PBS for three times before reacting with 1mL TRIzol Reagent (TaKaRa, Shiga, Japan), while the tumor tissues were ground with 1mL TRIzol and liquid nitrogen in mortars. The cell or tissue samples after lysis were transferred to a new tube. Add 200 μL chloroform into each tube, cap the tubes securely and vortex samples vigorously for 15 seconds, followed by incubation at room temperature for 2-3 minutes. Centrifuge the samples at 12,000 g for 15 minutes at 4 ℃, and carefully transfer the upper aqueous phase without disturbing the interphase into a new tube (typically 400 μL). Use 500 μL isopropanol to precipitate the RNA from the aqueous phase and incubated at room temperature for 10 minutes. Centrifuge at 12,000 g for 10 minutes at 4 ℃. Discard the supernatant, add 1 mL 75% ethanol into each tube to wash the RNA precipitation. Centrifuge at 10,000 g for 5 minutes at 4℃, remove all leftover ethanol and air-dry the RNA pellet for 10 minutes. Dissolve RNA in DEPC water and determine the RNA concentration by Nanodrop (Thermo Fisher, Waltham, MA).

qRT-PCR could be done in two steps, first is reverse transcription and then the qPCR. Step one, one microgram of total RNA was used as template to generate the genomic cDNA with the PrimeScript™ RT reagent Kit and gDNA Eraser (TaKaRa) according to the manufacturer's instructions. For miRNA cDNA production, the RT primers mixI

in cDNA kit were replaced with stem-loop RT primer for certain miRNA (GenePharma). Step two was performed by SYBR Green I chimeric fluorescence method to amplify cDNA. The target forward and reverse PCR primers of miRNA were designed and synthesized by GenePharma (Shanghai, China), and PCR primers of mTOR, PAK4, RHOC, c-raf, MEK, ERK, AKT, E-cadherin and vimentin were synthesized by Sunya (Shanghai, China), as listed in **Table S1**. The relative miRNA or RNA levels were quantified by $2^{-\Delta\Delta CT}$ method followed by normalization to the housekeeping transcript U6 as the endogenous reference.

Western Blotting

For cell samples, Huh-7 cells were seeded into 6-well plates with a density of 2×10^5 cells per well and incubated for 18h before treated with designated formulations for 48 h. Cells were washed with ice-cold PBS twice and harvested in 100 μL cell lysis buffer (Cell Signaling, Danvers, MA) containing protease inhibitors (Cell Signaling). While tumor tissue samples were preserved in liquid nitrogen after excising from mice. We then ground and lysed tissues in ice-cold lysis buffer with protease inhibitors. The obtained solutions were centrifuged at 12000 rpm for 30 mins to collect supernatant. The protein concentration of lysates was determined using the bicinchoninic acid method kit (Beyotime Biotechnology, Shanghai, China). Cell lysates (40 μg per lane) were separated using 10% SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked with Tris-buffered saline/0.1% Tween 20 (TBS/T) containing 5% bovine serum albumin (BSA) and incubated overnight at 4 °C with specific antibodies.

Antibodies to phospho-mTOR (Ser2481; 2974), phosphor-AKT (Ser473; 4060), PAK4 (3242), phospho-Raf1 (9427), phosphor-MEK (2338), phospho-ERK (9101), E-cadherin (3195), GAPDH (5174) were purchased from Cell Signaling Technology (Danvers, MA). Anti-RHOC (ab180785), anti-vimentin (ab137321) were from Abcam (Cambridge, MA). Membranes were washed three times with TBS/T and incubated for 4 h at 4 °C with the appropriate secondary antibody conjugated to goat anti-mouse or anti-rabbit horseradish peroxidase (1:2,000; GE Healthcare, Piscataway, NJ). The membranes were washed and immunoreactive bands were developed using an enhanced chemiluminescence reagent (GE Healthcare) and visualized by chemiluminescence imaging system ChemiScope (CliNX, Shanghai, China).

Histology and Immunohistochemistry Studies

The fixed samples were dehydrated with gradient ethanol, embedded in paraffin and cut into 4-μm sections. Tumor and organ tissue sections were first processed for hematoxylin and eosin (H&E) stain. For immunohistochemistry (IHC), paraffin-embedded tumor sections were incubated in citrate antigen repair buffer, 3% hydrogen peroxide solution and 3% BSA sequentially. The samples were then incubated with specific antibodies at 4 ℃ overnight and then with Herseradish Peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies (Servicebio Technology, Wuhan, China) for 50 mins at room temperature. Anti-phosphor-MEK (2338) were purchased from Cell Signaling Technology (Danvers, MA). Anti-mTOR (phosphor S2448; ab109268), anti-PAK4 (ab62509) and anti-RHOC (ab180785) were from Abcam (Cambridge, MA). Anti-Ki67 (GB111141), anti-PCNA were obtained from Servicebio

Technology (Wuhan, China). The slides were then stained by Diaminobenzidine (DAB) kit (DAKO, Denmark). The apoptotic status of tumor cells was detected using TUNEL Apoptosis Assay kit (Roche, Switzerland), then observed and photographed under fluorescence microscope (Nikon, Japan) with following excitation (Ex) and emission (Em) wavelength: DAPI (Ex 350 nm, Em 420 nm; blue), FITC (Ex 490nm, Em 525nm; green), Cy3 (Ex 525nm, Em 590 nm; red).

Detection of serum alpha fetoprotein (AFP) concentrations

The whole blood of BALB/c nude mice after treatment was obtained from retrobulbar sinus by capillary glass tubes, placed at room temperature for 1 h and centrifuged at 3000 rpm for 10 mins to collect blood serum. The concentration of serum AFP was evaluated by AFP Elisa Kit (Solarbio; Beijing, China) according to the manufacturer's instructions. Optical Density (OD) values were determined at 450 nm by Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Waltham, MA).

miRNA sequencing of PDX tumors

PDX tumor samples (original patient's HCC tumor, Tumor; patient's nonneoplastic tissue, Normal; PDX models treated with different formulations) kept in liquid nitrogen were isolated to collect total RNA. During which, RNA degradation and contamination was monitored on 1% agarose gel; purity was evaluated by NanoPhotometer® spectrophotometer (IMPLEN, CA, USA); RNA concentration was measured by Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA); RNA integrity was assessed using the RNA Nano 6000 Assay Kit of Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

3 μg total RNA per sample was used as input material to generate the small RNA library by NEBNext® Small RNA library Prep Set for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. The library quality was assessed by DNA High Sensitivity Chips on Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq SR Cluster Kit v3-cBot-HS (Illumina, NEB, USA) according to manufacturer's instructions. All alignments and annotations were summarized. In miRNA editing assays, position 2-8 of a mature miRNA were highly conserved called seed region, miRNA which might have base edit could be detected by aligning all the sRNA tags to mature miRNA. Then the library preparations were sequenced on an Illumina Hiseq 2500/2000 platform and 50 bp single-end reads were generated. miRNA expression levels were estimated by TPM (transcript per million) through following criteria[4]: Normalized expression = mapped readcount/Total reads * 100,000,0. Differential expression analysis of two samples was performed by DEGSeq R package (2010) [5]. P-value was adjusted using qvalue [6], qvalue<0.01 and |log2(foldchange)|>1 was set as the threshold for significantly differential expression by default.

Supplementary Figures

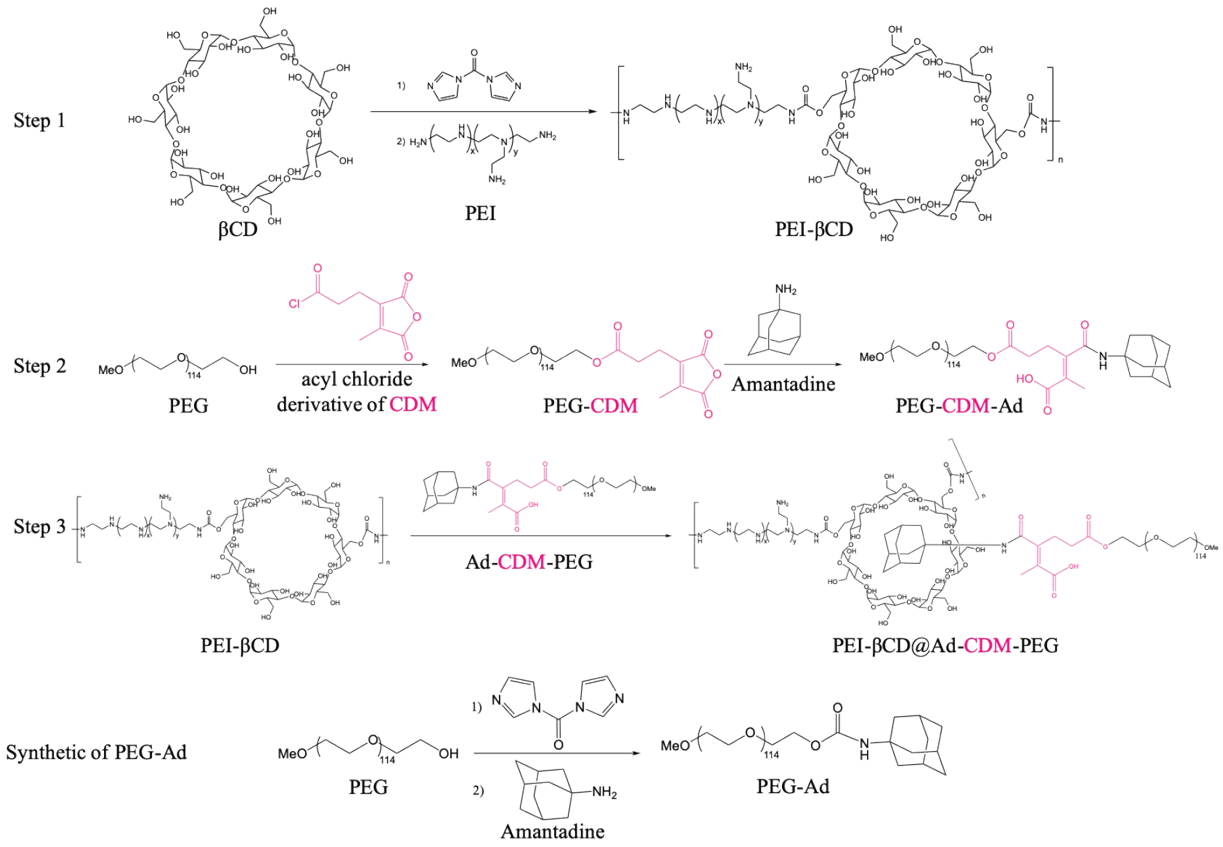

Figure S1A. Synthetic procedure of PEI-βCD, CDM-PEG, Ad-CDM-PEG, Ad-PEG, and PCACP, accordingly.

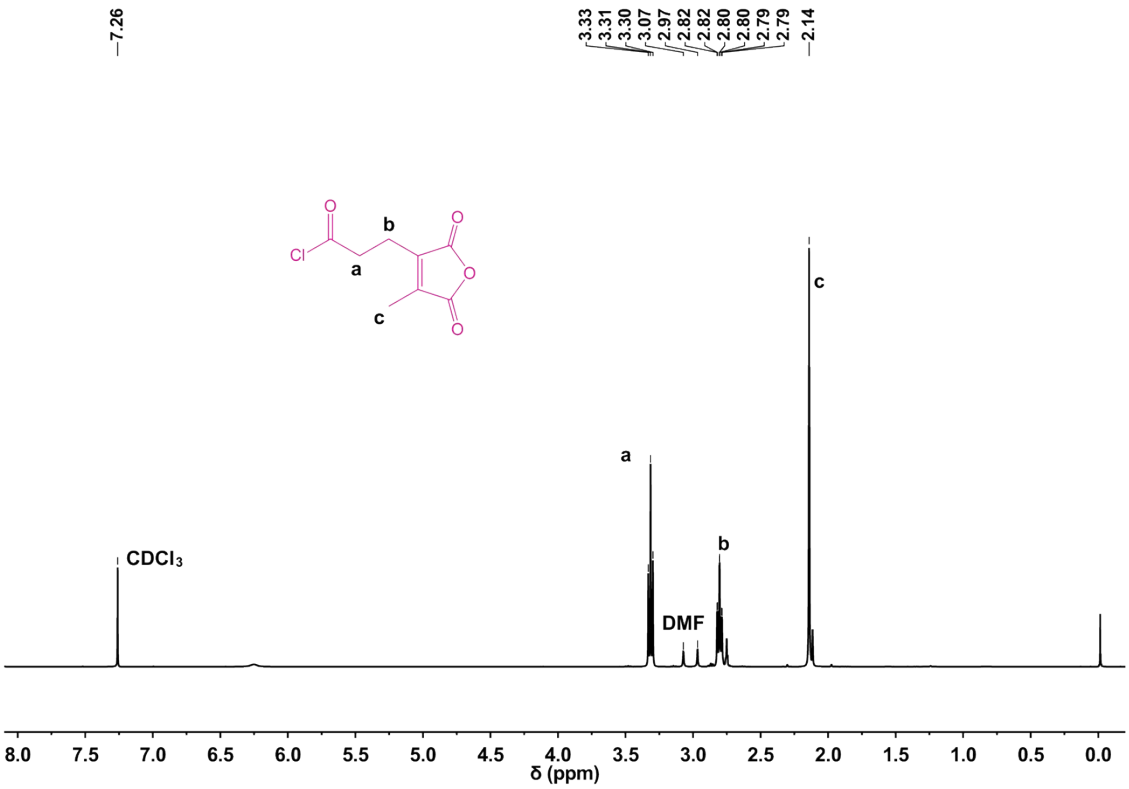

Figure S1B. ^{1}H-NMR of acyl chloride derivative of CDM in D_2O measured by an ANANCE III 400 MHz spectrometer: δ 3.40-3.25 ppm, (m, -**CH_2**CH_2COO-); 2.85-2.68 ppm, (m, -CH_2**CH_2**COO-); 2.10-1.95 ppm, (m, -**CH_3** of CDM).

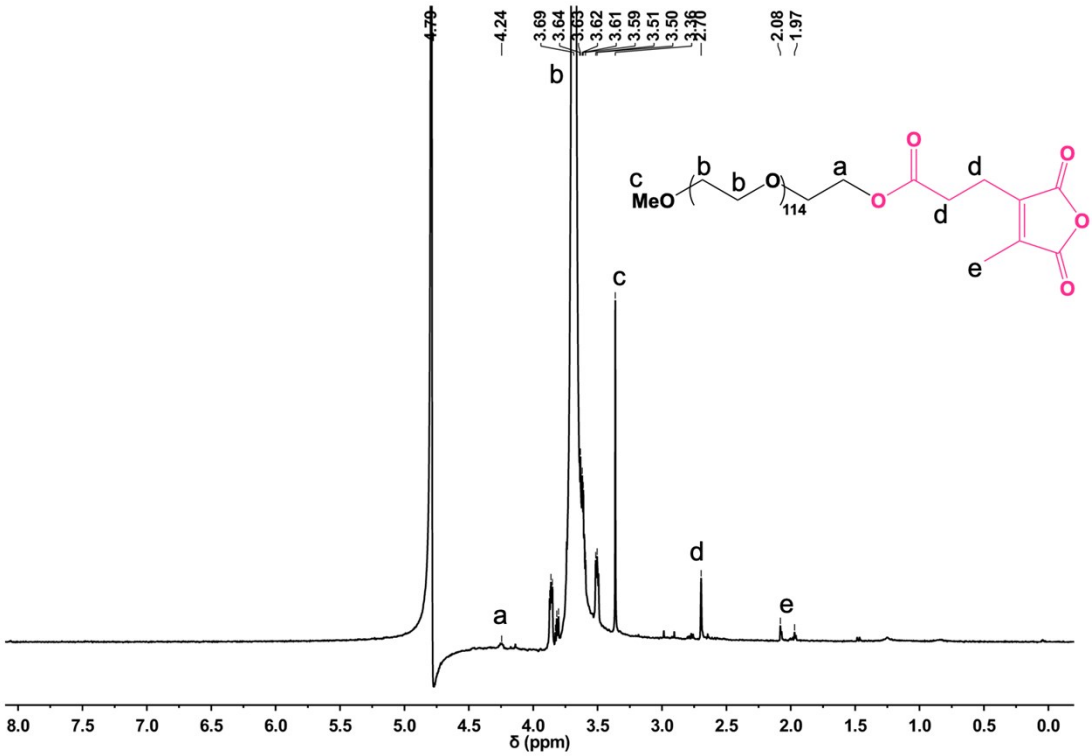

Figure S1C. ^{1}H-NMR of PEG-CDM in D_2O measured by an ANANCE III 400 MHz spectrometer: δ 4.30-4.15 ppm, (m, -COO**CH_2**-); 3.92-3.45 ppm, (m, -C**H_2CH_2**O- of PEG); 3.38 ppm, (s, -OMe of PEG); 2.85-2.68 ppm, (m, -**CH_2CH_2**COO-); 2.10-1.95 ppm, (m, -**CH_3** of CDM).

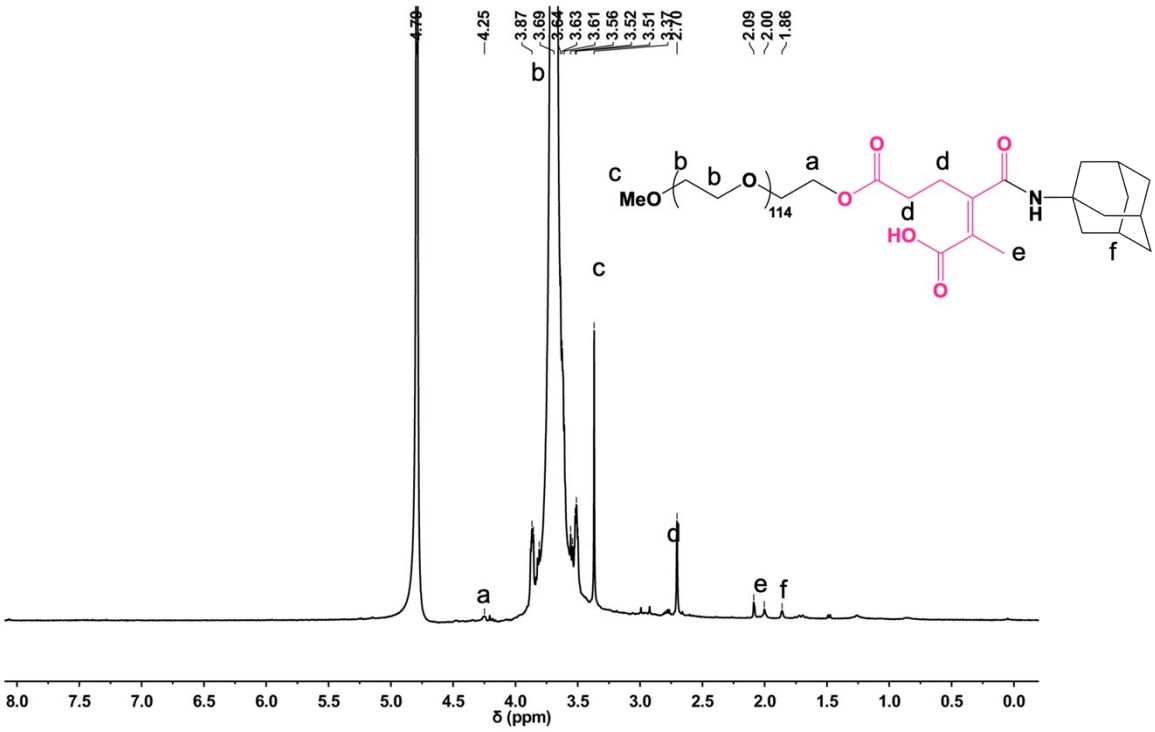

Figure S1D. ^{1}H-NMR of PEG-CDM-Ad in D_2O measured by an ANANCE III 400 MHz spectrometer: δ 4.30-4.15 ppm, (m, -COO**C**$\mathbf{H_2}$-); 3.92-3.45 ppm, (m, -C$\mathbf{H_2}$C$\mathbf{H_2}$O- of PEG); 3.38 ppm, (s, -OMe of PEG); 2.85-2.68 ppm, (m, -**C**$\mathbf{H_2}$**C**$\mathbf{H_2}$COO-); 2.10-1.95 ppm, (m, -$\mathbf{CH_3}$ of CDM); 1.79-1.76 ppm, (m, from Ad).

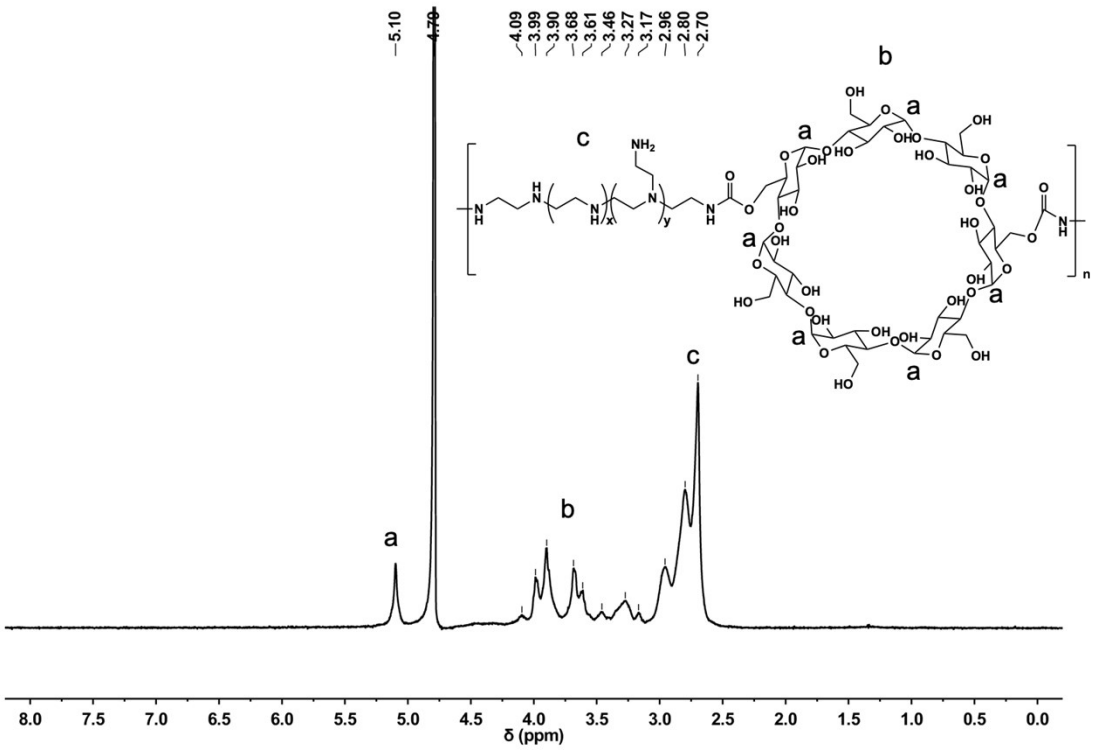

Figure S1E. ^{1}H-NMR of PEI- β CD in D_2O measured by an ANANCE III 400 MHz spectrometer: δ 5.10 ppm, (s, αH of βCD); 4.20-3.15 ppm, (m, from βCD except αH); 3.10-2.52 ppm, (m, from PEI).

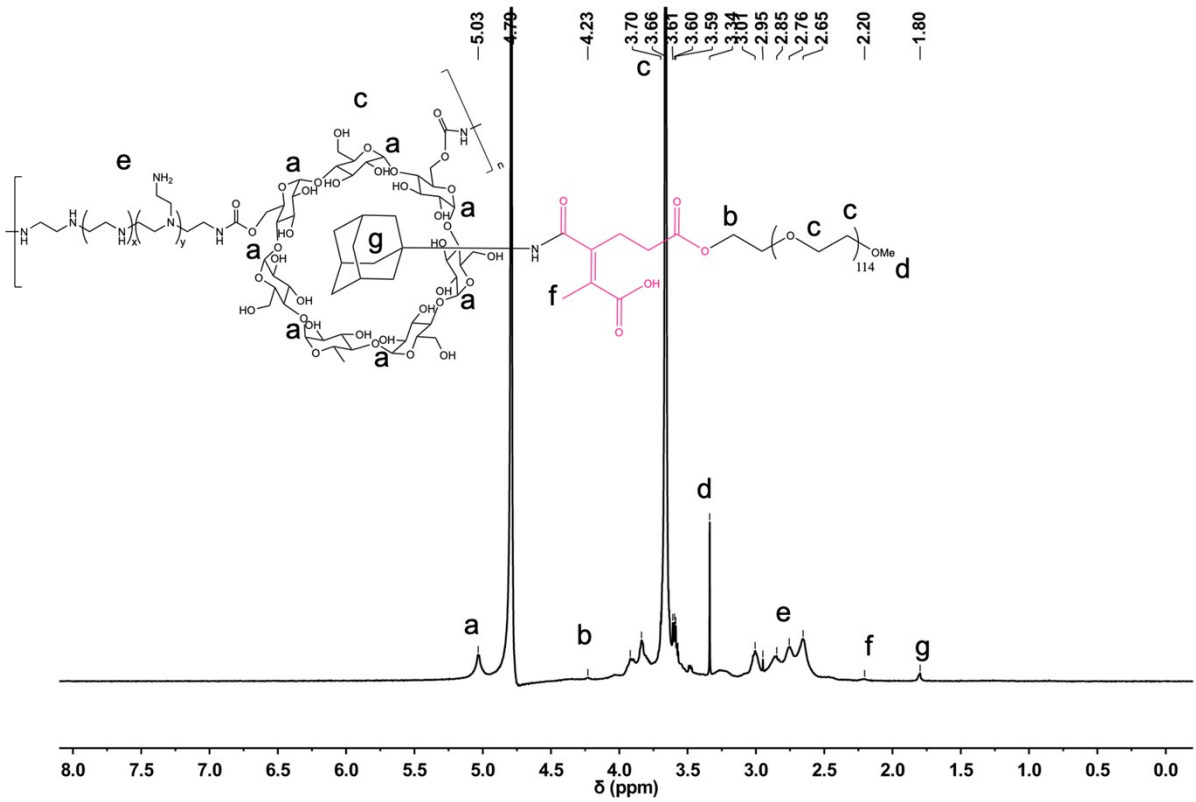

Figure S1F. ^{1}H-NMR of PEI-βCD@Ad-CDM-PEG (PCACP) in D_2O measured by an ANANCE III 400 MHz spectrometer: δ 5.03 ppm, (s, αH of βCD); 4.25-4.20 ppm, (m, -COO**C**$\mathbf{H_2}$-); 4.18-3.12 ppm, (m, from βCD except for αH, overlapped with -O**C**$\mathbf{H_3}$ and -C$\mathbf{H_2}$C$\mathbf{H_2}$O- of PEG); 3.10-2.52 ppm, (m, from PEI); 2.22-2.18 ppm, (m, -$\mathbf{CH_3}$ of CDM); 1.82-1.78 ppm, (m, from Ad).

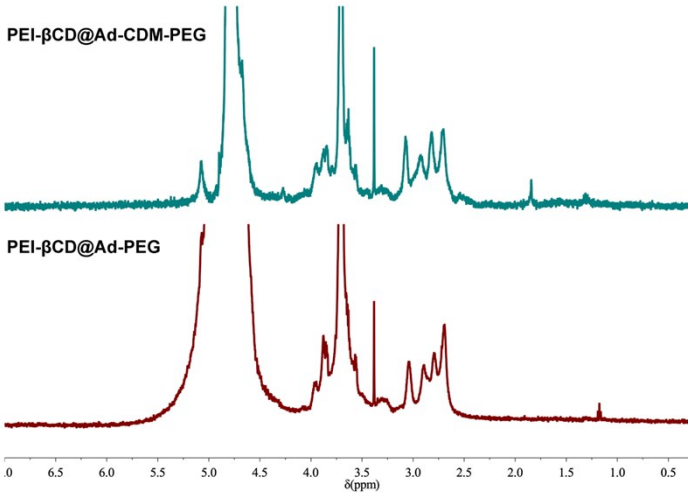

Figure S1G. ^{1}H-NMR of PCAP and PCACP in D_2O measured by an ANANCE III 400 MHz spectrometer at 22 ℃. The peak at around δ 2.0 ppm in PCACP was from methyl groups in CDM linkage.

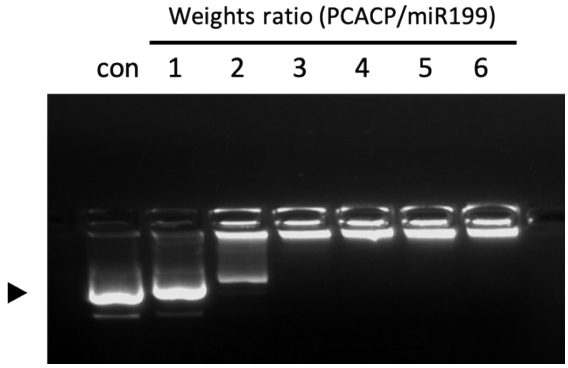

Figure S2A. Agarose gel electrophoresis retardation of miR-199a/b-3p mimics with PCACP at various weight ratios. The bands of uncondensed miRNA are marked by black triangle. PCACP showed complete miRNA complexation at the minimum weight ratio of 3:1 (5:1 in nitrogen to phosphate [N/P] ratio).

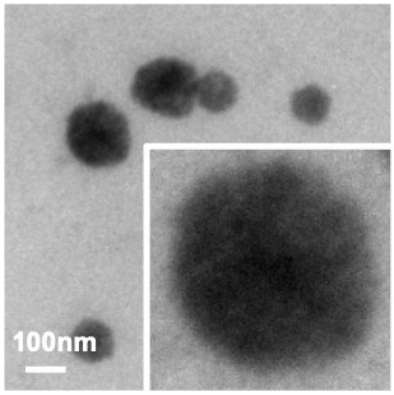

Figure S2B. Visualization of PCACP after condensation with miR-199a/b-3p by transmission electron microscopy. Enlarged images of the nanoparticles are shown for detailed morphological view. The bar represents 100 nm.

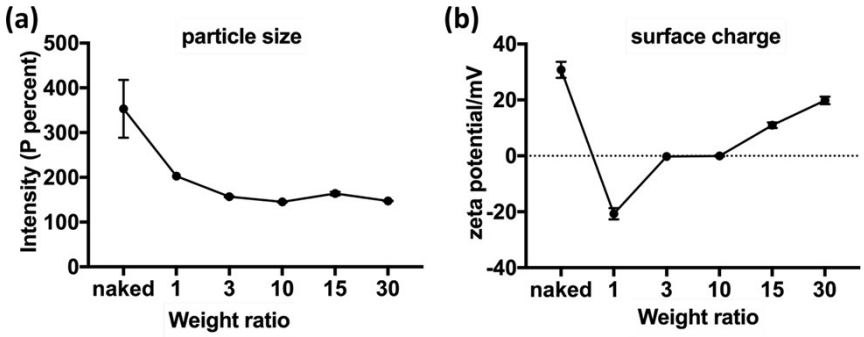

Figure S2C. (a) Particle size and (b) zeta-potential of PCACP/miR199 with weight ratios from 0 to 30:1. Data are shown as Mean ± SD (n=3).

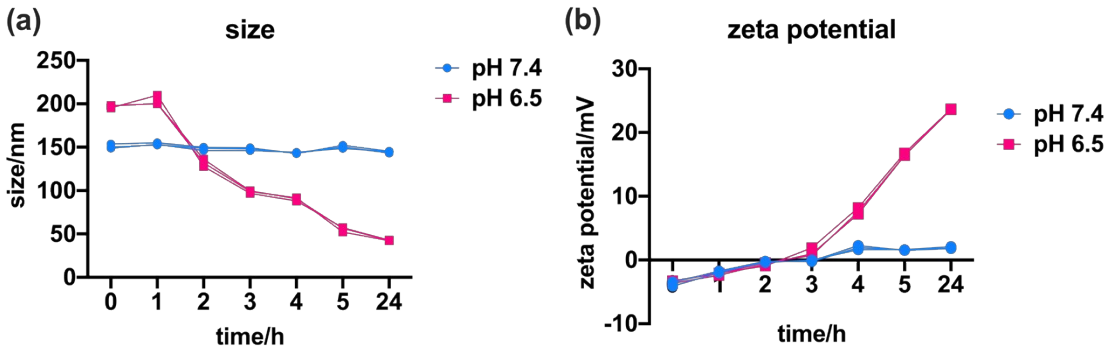

Figure S2D. Dynamic change of size and zeta potential of PCACP/miR199 treated with pH 6.5 or pH 7.4 solution in 24 h (weight ratio = 3:1). Data are shown as Mean ± SD (n=3).

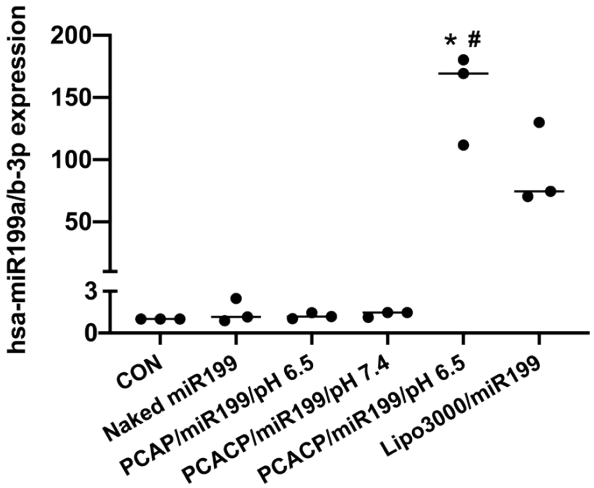

Figure S3A. Uptake efficiency of PCACP/miR199 system by conducting qRT-PCR of intracellular miR-199a/b-3p after incubation with various formulations for 2 h. * $p <$ 0.0001, vs. Con, naked miR199, PCAP/miR199/pH 6.5 and PCACP/miR199/pH 7.4; # $p < 0.05$, vs. Lipo3000/miR199; One-way ANOVA followed by Tukey's test. Data are shown as Mean ± SD (n=3).

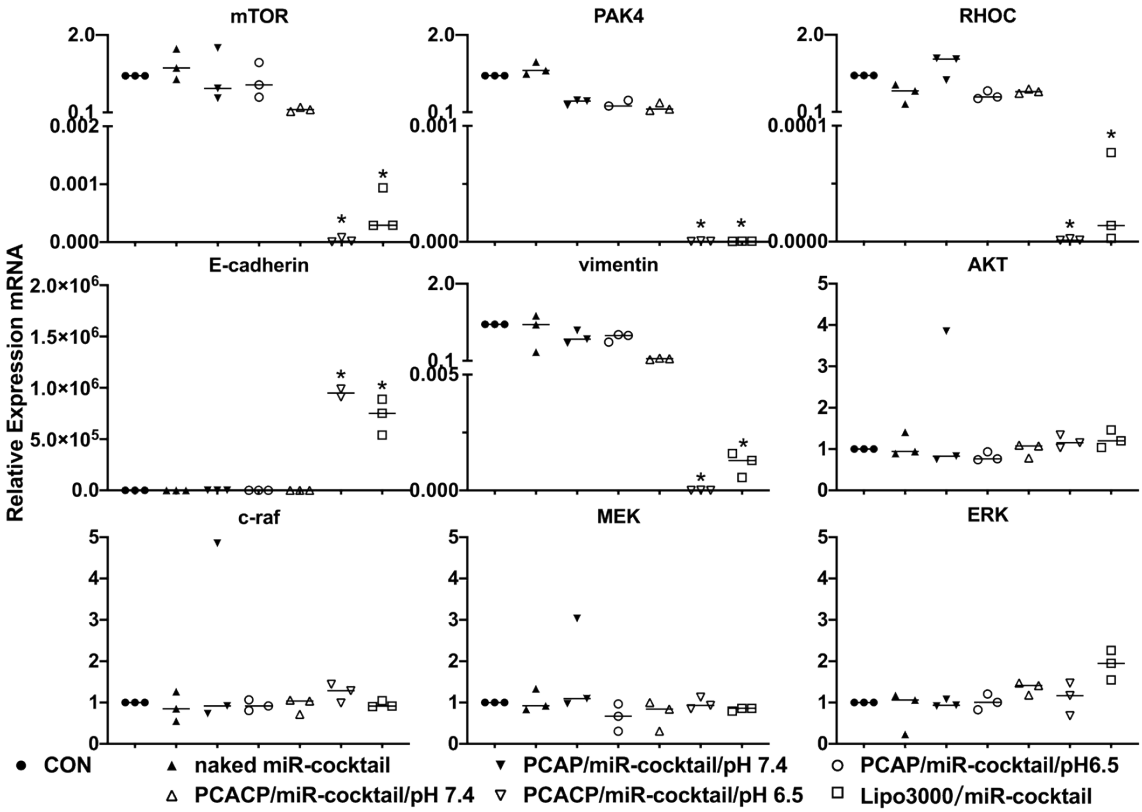

Figure S3B. Relative expression of mTOR, PAK4, RHOC, E-cadherin, vimentin, AKT, c-raf, MEK and ERK in Huh-7 after 48 h transfection with various formulations by qRT-PCR. * $p < 0.0001$, vs. Con; One-way ANOVA followed by Tukey's test. GAPDH as the endogenous reference. Data are shown as Mean ± SD (n = 3).

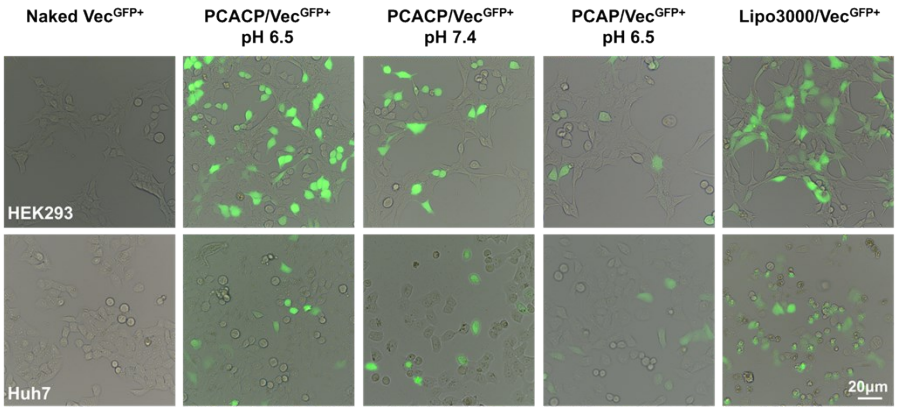

Figure S3C. Overlapped images of bright field and fluorescent photo of HEK 293 and Huh 7 cells after transfection with naked plasmids, and plasmids loaded PCACP (pH 6.5 and pH 7.4), PCAP and Lipo3000 for 48 h. The plasmid vectors encoded GFP fluorescence (Vec^{GFP+}). The bar represents 20 μm. Representative images in 3 replicates.

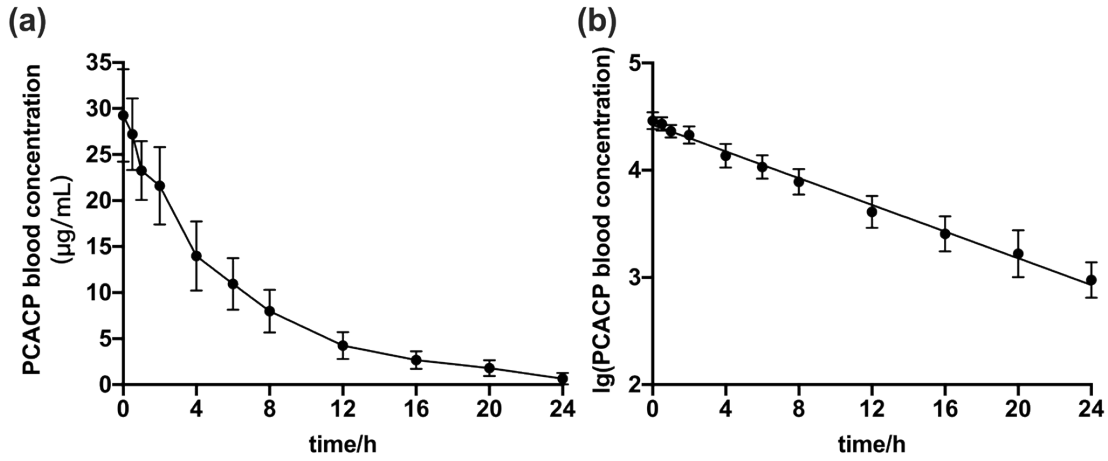

Figure S3D. (a) Blood concentration of FITC labeling PCACP and (b) corresponding logarithm time diagram of plasma concentration in BALB/c nude mice (n = 3) after tail vein injection. Blood was captured from retrobulbar sinus at different time points in 24 h.

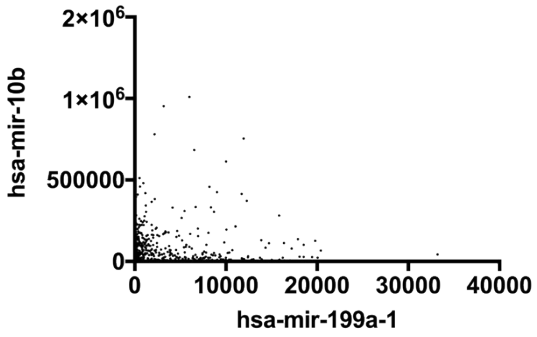

Figure S4. The correlation between miR-199a/b-3p and miR-10b according to miRNA expression profiling in tumor tissues of 425 HCC cases. r = -0.2123, $p < 0.0001$, Correlation analysis. Data were derived from the online database The Cancer Genome Atlas (TCGA).

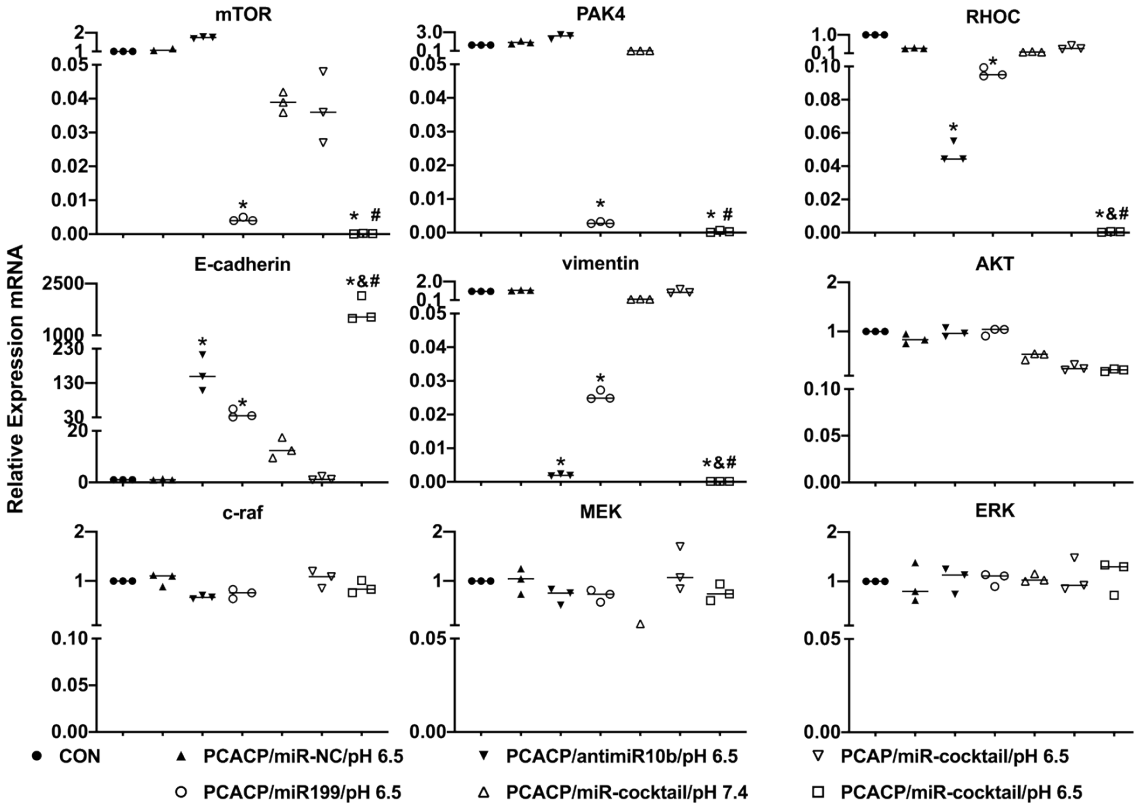

Figure S5A. Relative expression of mTOR, PAK4, RHOC, E-cadherin, vimentin, AKT, c-raf, MEK and ERK in Huh-7 after 48 h transfection with various formulations by qRT-PCR. * $p < 0.0001$, vs. Con; & $p < 0.001$, vs. PCACP/antimiR10b/pH 6.5; # $p < 0.001$, vs. PCACP/miR199/pH 6.5; One-way ANOVA followed by Tukey's test. GAPDH as the endogenous reference. Data are shown as Mean ± SD (n = 3).

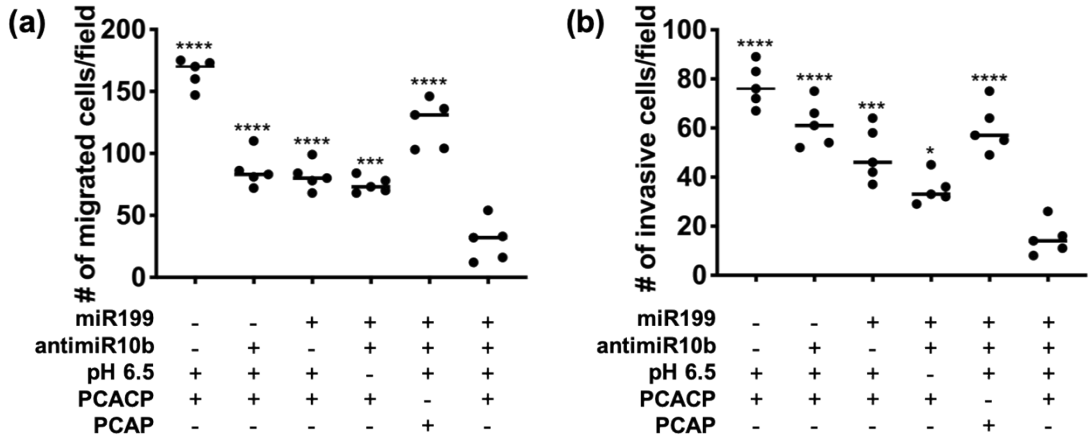

Figure S5B. Quantitative results of cells migration and invasion results by Transwell assay. The number of stained cells in five vision fields was counted to quantify migration rate (a) and invasion rate (b). *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$, vs. PCACP/miR-cocktail group; One-way ANOVA followed by Tukey's test. Scale bar represents 10 μm.

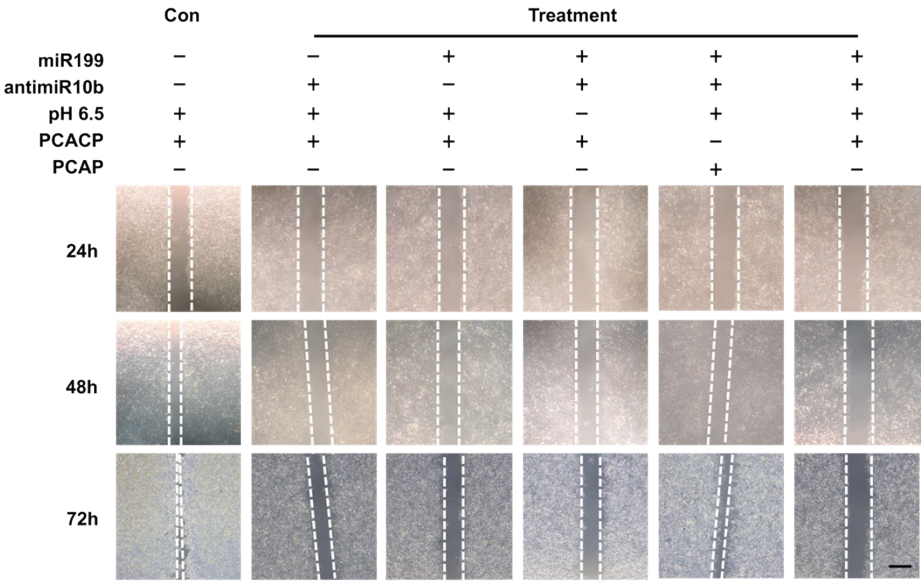

Figure S5C. Representative field photos of wound areas at different points of time after treatment with designated formulations in Huh 7 cells, showing dynamic wound closure

from 24 h to 72 h. Borderlines are marked with white dashed lines. The bar represents 100 μm.

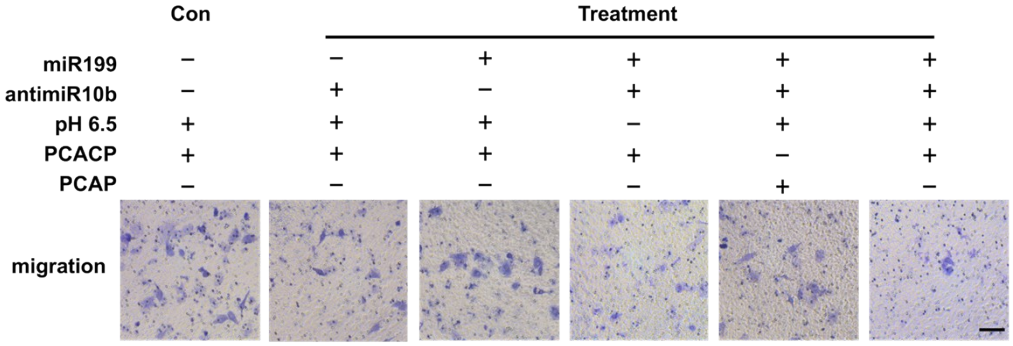

Figure S5D. Representative field photos of stained cells in Transwell migration assays of LM3 cells treated with different formulations for 72 h. The bar represents 20 μm.

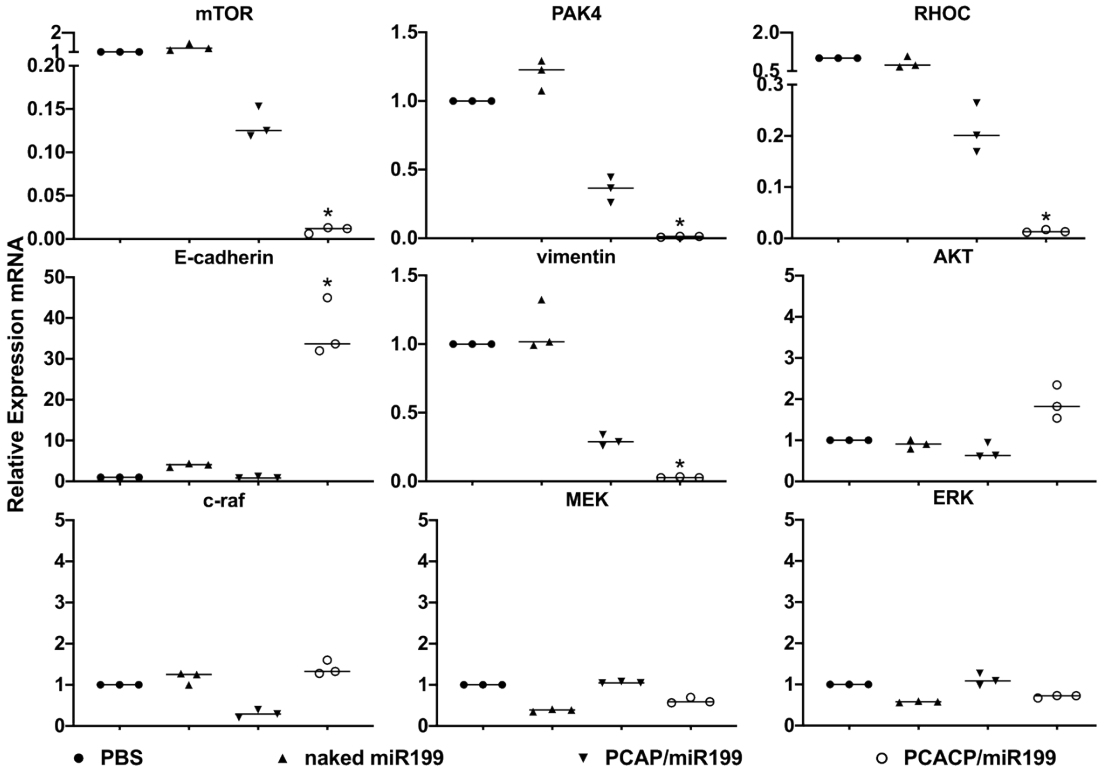

Figure 6A. Intratumoral relative expression level of mTOR, PAK4, RHOC, E-cadherin, vimentin, AKT, c-raf, MEK and ERK by qRT-PCR after treated with PBS, naked miR199, PCAP/miR199 or PCACP/miR199. * $p < 0.0001$, vs. PBS, naked

miR199, and PCAP/miR199; One-way ANOVA followed by Tukey's test. GAPDH as the endogenous reference. Data are shown as Mean ± SD (n = 3)

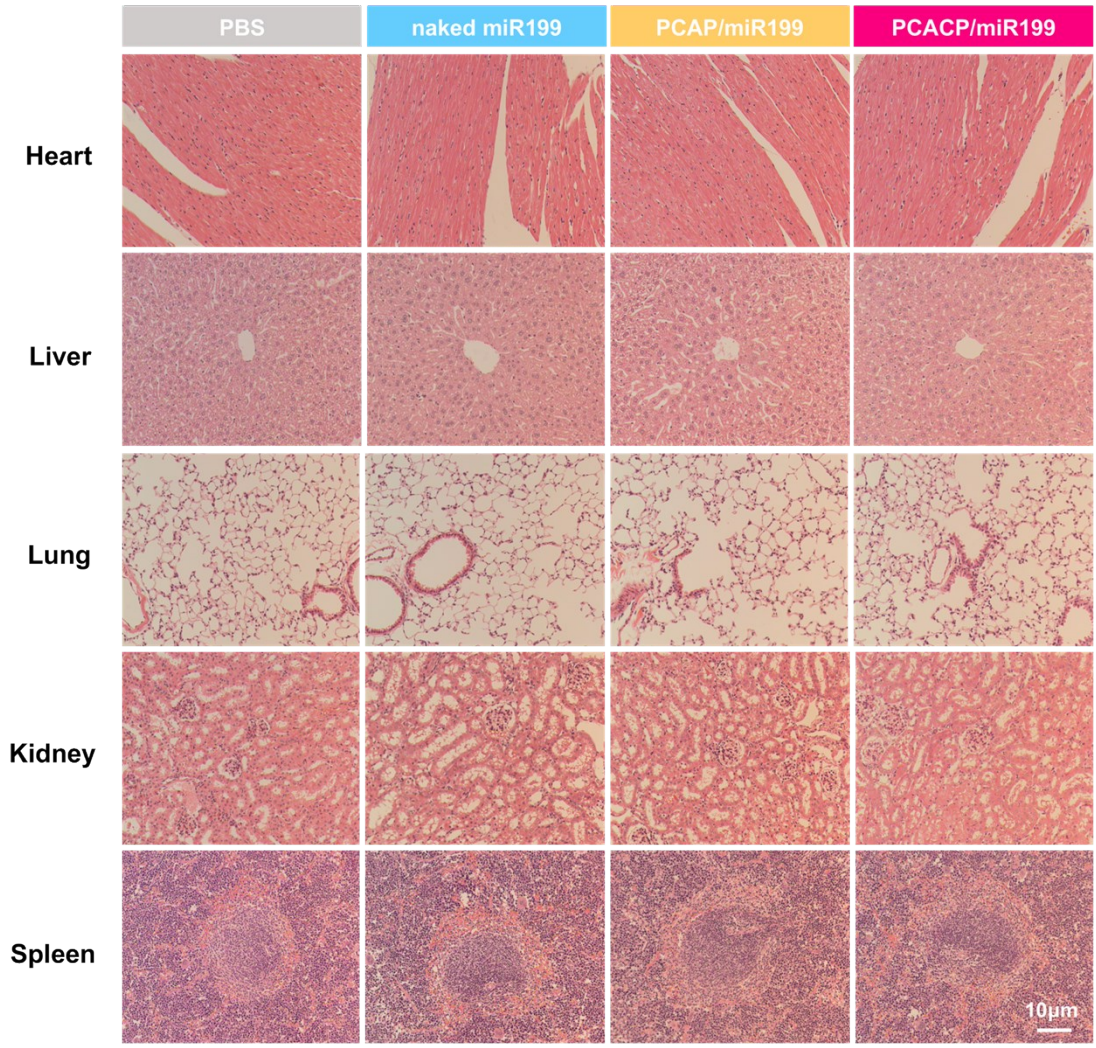

Figure S6B. Representative photos of H&E analysis of major organs, including liver, heart, lung, kidney and spleen, excised from BALB/c nude mice treated with PBS, naked miR199, PCAP/miR199 or PCACP/miR199 for 18 days. The bar represents 10 μm.

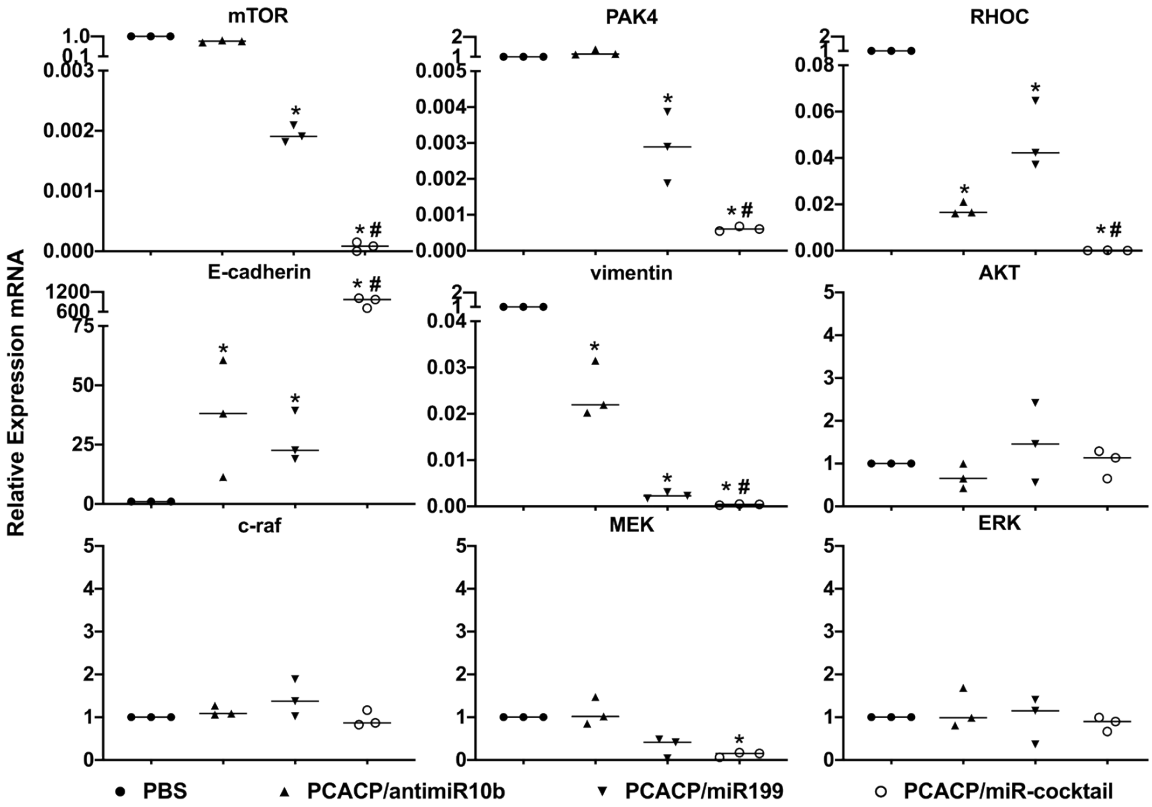

Figure S7. Intratumoral relative expression level of mTOR, PAK4, RHOC, E-cadherin, vimentin, AKT, c-raf, MEK and ERK by qRT-PCR after treated with PBS, PCACP/antimiR10b, PCACP/miR199 or PCACP/miR-cocktail. * $p < 0.0001$, vs. PBS; # $p < 0.001$, vs. PCACP/antimiR10b and PCACP/miR199; One-way ANOVA followed by Tukey's test. GAPDH as the endogenous reference. Data are shown as Mean ± SD (n = 3).

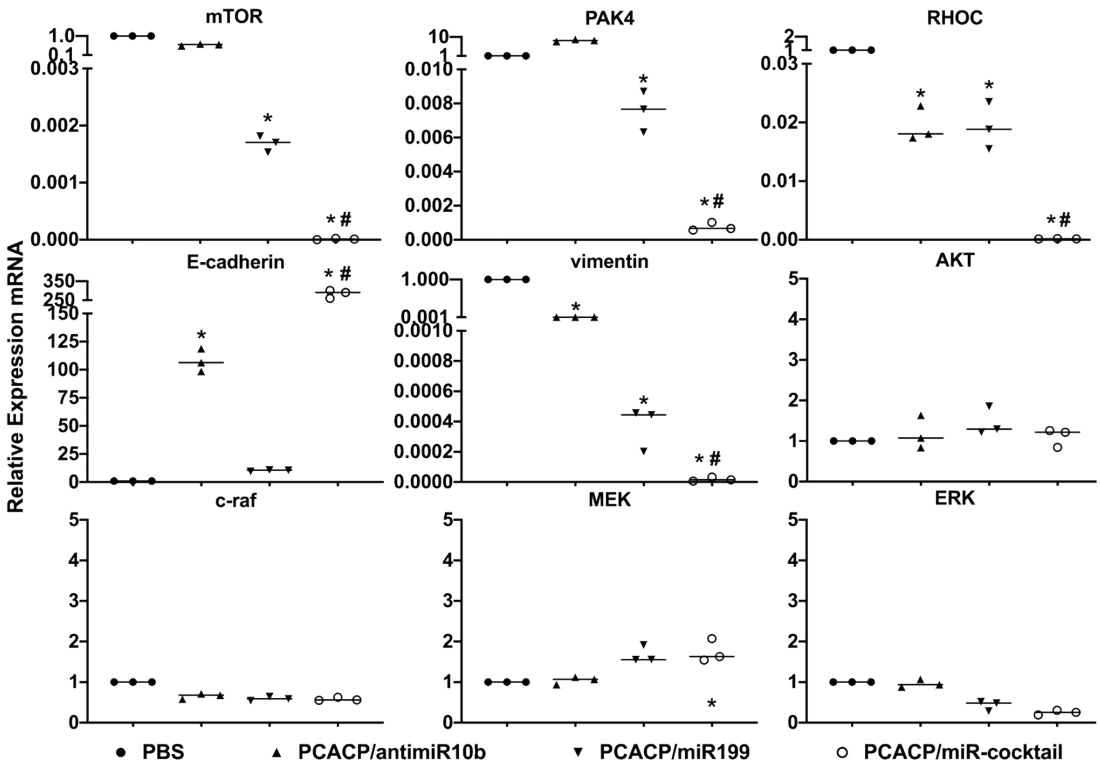

Figure S8. Relative expression of mTOR, PAK4, RHOC, E-cadherin, vimentin, AKT, c-raf, MEK and ERK by qRT-PCR in patient derived xenograft (PDX) tumors treated with PBS, PCACP/antimiR10b, PCACP/miR199 or PCACP/miR-cocktail. * $p <$ 0.0001, vs. PBS; # $p < 0.001$, vs. PCACP/antimiR10b and PCACP/miR199; One-way ANOVA followed by Tukey's test. GAPDH as the endogenous reference. Data are shown as Mean ± SD (n = 3).

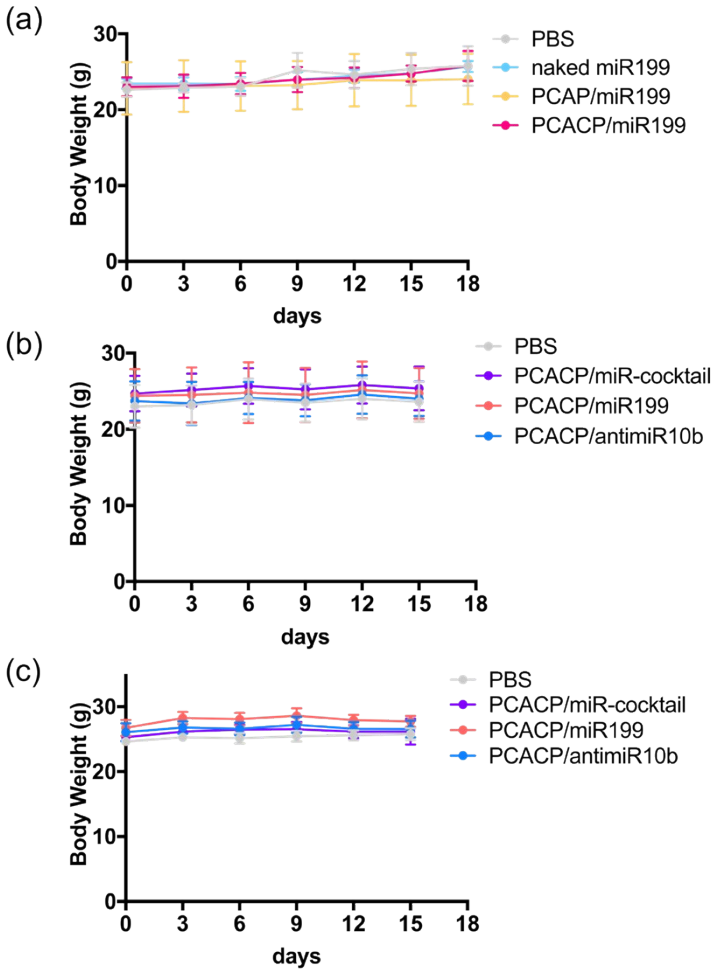

Figure S9. Average body weight changes of BALB/c mice over time from the initial administration to the end of the treatment course in 3 independent in vivo experiments, which were treated with different nanoparticle formulations, respectively.

Table S1. The sequence of nucleocides used in experiments.

miRNA	Sequence
has-miR199a/b-3p mimics	sense (5'-3'): ACAGUAGUCUGCACAUUGGUUA antisense (5'-3'): ACCAAUGUGCAGACUACUGUUU
has-miR199a/b-3p mimics NC	sense (5'-3'): UUCUCCGAACGUGUCACGUTT antisense (5'-3'): ACGUGACACGUUCGGAGAATT
hsa-miR10b-5p inhibitors	5'-3': CACAAAUUCGGUUCUACAGGGUA
hsa-miR10b-5p inhibitors NC	5'-3': CAGUACUUUUGUGUAGUACAA

has-miR199a/b-3p PCR primers	F primer: GCCACAGTAGTCTGCACAT R primer: GTGCAGGGTCCGAGGT
has-miR10b PCR primers	F primer: GGCGAAGAGTACCCTGTAGAACC R primer: TATGGTTGTTCACGACTCCTTCAC
U6 PCR primers	F primer: CAGCACATATACTAAAATTGGAACG R primer: ACGAATTTGCGTGTCATCC
GAPDH PCR primers	F primer: ACATCGCTCAGACACCATG R primer: CATGGGTGGAATCATATTGGAAC
mTOR PCR primers	F primer: TCCGAGAGATGAGTCAAGAGG R primer: CACCTTCCACTCCTATGAGGC
PAK4 PCR primers	F primer: TCCCCCTGAGCCATTGTG R primer: TGACCTGTCTCCCCATCCA
c-raf1 PCR primers	F primer: GGGAGCTTGGAAGACGATCAG R primer: ACACGGATAGTGTTGCTTGTC
ERK PCR primers	F primer: TACACCAACCTCTCGTACATCG R primer: CATGTCTGAAGCGCAGTAAGATT
MEK PCR primers	F primer: CAATGGCGGTGTGGTGTTC R primer: GATTGCGGGTTTGATCTCCAG
AKT PCR primers	F primer: AGCGACGTGGCTATTGTGAAG R primer: GCCATCATTCTTGAGGAGGAAGT
E-cadherin PCR primers	F primer: TGCAGTTGAGGATCCAATGG R primer: CTGAGGATGGTGTAAGCGATG
Vimentin PCR primers	F primer: ACGGTTGAAACTAGAGATGGAC R primer: GCAGAAAGGCACTTGAAAGC

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