

## SUPPLEMENTARY SECTION

### Ultrasound-Guided Therapeutic Modulation of Hepatocellular Carcinoma using Complementary microRNAs

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## SUPPLEMENTARY MATERIALS

### Synthesis of PLGA-*b*-PEG block copolymer:

PLGA-*b*-PEG copolymer was synthesized as described previously with slight modifications (1,2). In Brief, PLGA (1 g, 0.083 mmol) was dissolved in 15 mL of dry dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>), to this N-hydroxysuccinimide (NHS) (160 mg, 0.83 mmol) and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) (96 mg, 0.83 mmol) were added at room temperature and stirred for 4 h. Then, the resulted PLGA-NHS was precipitated by dropwise addition to cold diethylether/MeOH (1:1) (35 mL), vortexed, centrifuged (5000 rpm for 5 min), and the supernatant was decanted, and the resulted pellet was further washed (three times). The pellet of PLGA-NHS was dried at high vacuum for 2 h. The PLGA-NHS (980 mg, 0.081 mmol) and NH<sub>2</sub>-PEG-COOH (275 mg, 0.081 mmol) were dissolved in dry chloroform (10 mL), and diisopropylethylamine (DIPEA) (104 mg, 0.81, mmol) was added at room temperature and stirred for 24 h. The resulted PLGA-*b*-PEG copolymer was precipitated by dropwise addition to cold diethylether/MeOH (1:1) (35 mL), centrifuged (5000 rpm for 5 min), the supernatant was decanted, and the pellet was further washed (three times) and dried under high vacuum afforded the PLGA-*b*-PEG-COOH (810 mg, 0.0526 mmol). The PEG conjugation to PLGA was confirmed by <sup>1</sup>H NMR spectroscopy (Agilent 400 MHz NMR, CDCl<sub>3</sub>).

### Formulation and characterization of antimiR-21 and miRNA-122 loaded PLGA-*b*-PEG NPs:

PLGA-*b*-PEG copolymer NPs was prepared as described previously with slight modifications (3). PLGA-*b*-PEG NPs encapsulated with antimiR-21 were formulated by water-in-oil-in-water (w-o-w) double emulsion solvent evaporation technique (Table S1). To stabilize the highly anionic antimiR-21 (10 nmol), it was complexed with spermidine in an N/P ratio of 15:1 at room temperature for 15 min in DNase/RNAase free water (0.20 mL) (Invitrogen, USA). The antimiR-21-spermidine complex (w1 phase) was then added dropwise to the stirred solution of PLGA-*b*-PEG polymer (10 mg) in CH<sub>2</sub>Cl<sub>2</sub> (1 mL) containing 3 % Span-80 (oil phase), followed by sonication for 1 min at 40% amplitude in ice bath to form the first emulsion. The first emulsion was then added dropwise to 5 ml of 1% Tween-80 (w/v) in DNase/RNAase free water (w2 phase) and sonicated at 40% amplitude in ice bath for 1 min to form second emulsion, followed by stirring

for 3 h to allow the  $\text{CH}_2\text{Cl}_2$  to evaporate and harden the NPs. The NPs were sterile filtered using 0.45  $\mu\text{m}$  syringe filter (Whatman PURADISC 25 AS, PES, GE Healthcare), and excess surfactants and free anti-miR-21 were removed by an ultracentrifuge filter device with 100 kDa MWCO Membrane (Millipore, USA) by centrifuging at 3000 rpm. The concentrated NPs were further washed and centrifuged several times with DNase/RNase free water to remove the free anti-miR-21. The concentrated NPs were diluted to a known volume, estimated for their particle size, zeta potential and the loaded miRNAs level, prior to use in therapeutic studies. The miRNA-122 loaded NPs were prepared under same condition using miRNA-122. Control NPs were prepared using plain DNase, RNAs free water instead of miR-spermidine complex under same conditions.

### **Particle Size and Zeta-Potential Measurements**

Particle size and zeta potential measurement of NPs were performed using a Zetasizer-90 (Malvern Instruments, U.K.). Size measurement was performed at 25°C at a 90° scattering angle. The mean hydrodynamic diameter was determined by cumulant analysis. Zeta-potential measurements were executed using the Smoluchowski model and using an aqueous dip cell in an automatic mode. The shape, surface morphology of NPs were examined by using a FEI TITAN 80-300 kV ETEM (environmental transmission electron microscope) at Stanford's Nano Shard Facilities by operating at 80 kV using negative-stained (1% phosphotungstic acid (PTA) NPs (4).

### **Entrapment efficiency determination for anti-miR-21 in PLGA-*b*-PEG-NPs**

The loading percentage and encapsulation efficiency of anti-miR-21 in NPs was determined by using organic/aqueous extraction method (3). Briefly, known volume of lyophilized NPs was dissolved in 0.5 mL of dichloromethane ( $\text{CH}_2\text{Cl}_2$ ) with brief vortexing, and keeping at RT for 30 min. The anti-miR-21 were extracted from the organic ( $\text{CH}_2\text{Cl}_2$ ) phase using 0.5 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) by vortexing for 1 min, and centrifuging at 12,000 rpm for 5 min at 4°C; aqueous phase was separated, and this procedure was repeated twice to totally extract all the RNAs present in the sample. All three aqueous phases were combined and the amounts of anti-miR-21 in the aqueous fraction were measured

using the Quant-iT RNA-quantitation kit (Invitrogen, Carlsbad, CA), according to the manufacturer's guidelines.

The Entrapment efficiency from the NPs was calculated using the following formula:

$$\text{Entrapment efficiency (\%)} = [\text{Mass of total miRNAs or antimiRs quantified in freeze dried NPs (W)} / \text{Mass of total miRNAs or antimiRs used (M) for encapsulation}] \times 100$$

The Entrapment efficiency based on wash solution was calculated using the following formula:

$$\text{Entrapment efficiency (\%)} = [\text{Mass of total miRNAs or antimiRs used (M)} - \text{Mass of free miRNAs or antimiRs in the wash solution (W)}] / \text{M}] \times 100$$

### **Endogenous expression of miRNA-122 and miRNA-21**

The endogenous expression levels of miRNA-122 and miRNA-21 in doxorubicin-resistant and non-resistant HCC cells were analyzed using quantitative real-time polymerase chain reaction (qRT-PCR) as described(5). In brief,  $10^5$  cells were plated in 6 well plates and grown for 24h. The cells were incubated in 300  $\mu$ l lysis buffer for 3min and total RNA (mRNA and miRNA) was isolated from the lysate using miRVana RNA extraction Kit (Life technologies, Grand Island, NY) according to the manufacturer's protocol. For quantifying miRNA, 50 ng of total RNA was reverse transcribed using RT-primers (Life technologies) of miRNA-122 and antimiR-21 using TaqMan® kit and microRNA Reverse Transcription Kit (Life technologies) to produce corresponding cDNA. The cDNA synthesis was carried out in a 15  $\mu$ l reaction volume. qRT-PCR was performed using cDNA (5 ng of RNA equivalent) combined with TaqMan RT-PCR reagents (primer and probe mix) of miRNA-122 and antimiR-21. qRT-PCR was performed by 2 min incubation at 50 °C, followed by activation of the DNA polymerase at 95 °C for 10 min, 60 cycles of 95 °C for 15 s, and 60 °C for 60 s in an Eppendorf Realplex qRT-PCR system (Eppendorf AG, Hamburg, Germany). The qRT-PCR reaction was carried out in a 20  $\mu$ l reaction volume. The expression of miRNA-122 and antimiR-21 was calculated using the  $2^{-\Delta\text{CT}}$  method (6), in which the miRNA-122 and antimiR-21 levels were normalized to the endogenous levels of miR-10b (a miRNA not dysregulated in HCC) in resistant and non-resistant cell lines.

## **Assessment of Multi Drug Resistance (MDR) protein activity and expression**

MDR protein activity and cell membrane expression in resistant and non-resistant HCC cell lines before and after miRNA treatment was assessed using fluorescence microscopy and western blot analysis using standard protocols.

### Fluorescence microscopy to assess:

#### a) Effect of miRNA treatment on doxorubicin uptake in resistant and non-resistant HCC cells

Since the MDR proteins induce resistance by actively pumping drugs out of cells, the amount of drug present intracellularly is a direct indication of MDR protein activity.  $10^5$  doxorubicin-resistant and non-resistant HepG2 HCC cells were plated on cover slips and grown for 12h. After 12h, the media was removed and the cells were either left untreated (controls) or treated with 10 picomoles each of either miRNA-122 or anti-miR-21 or 5 picomoles each of a combination of miRNA-122 and anti-miR-21 loaded PLGA-NP for 48h in fresh media. The PLGA-NP containing media was removed and the cells were exposed to media containing 75 ng/ml of doxorubicin for 12h. The drug containing media was then removed, the cells were washed twice with PBS, and fresh growth medium (no phenol red) was added to the cells. Cover slips were then imaged to detect doxorubicin fluorescence using an Olympus BX 51 fluorescence microscope (Shinjuku, Tokyo, Japan). Image J was used to quantify fluorescence in the images obtained. Cells treated with empty PLGA-NP were used as negative control. Data were represented as % of fluorescence signal obtained in untreated control cells.

#### b) Effect of miRNA treatment on p-glycoprotein activity in resistant and non-resistant HepG2 cells

P-glycoprotein (a MDR protein) expression has been established as a determinant for decreased chemotherapy response in HCC(7,8). To establish downregulation of p-glycoprotein expression after complimentary miRNA/anti-miR-21 treatment we evaluated the uptake of p-glycoprotein substrate Rhodamine-123 (Rho-123)(9,10) into treated and untreated resistant and non-resistant HepG2 cells.  $10^5$  doxorubicin-resistant and non-resistant cells were plated on cover slips and grown for 12h. After 12h, the media was removed and the cells were either left untreated or treated with 10 picomoles each of either miRNA-122 or anti-miR-21 or 5 picomoles each of a combination of miRNA-122 and anti-miR-21-loaded PLGA-NP for 48h in fresh media. The PLGA-NP containing media was removed and the cells were exposed

to media containing 200 ng/ml of Rho-123 for 2h. The drug containing media was then removed and the cells were washed twice with PBS and fresh growth medium (no phenol red) was added to the cells. The cover slips were then imaged to detect Rho-123 fluorescence as a quantitative marker of p-glycoprotein activity using an Olympus BX 51 fluorescence microscope (Shinjuku, Tokyo, Japan). Image J was used to quantify fluorescence in the images obtained. Cells treated with empty PLGA-NP were used as negative control. Data were represented as percentage of fluorescence signal obtained in untreated control cells.

*c) Effect of miRNA treatment on MDR protein expression in resistant HCC cells*

10<sup>5</sup> HepG2 HCC cells were plated on cover slips and grown for 12h. After 12h, the media was removed and the cells were either left untreated or treated with 10 picomoles each of either miRNA-122 or anti-miR-21 or 5 picomoles each of a combination of miRNA-122 and anti-miR-21-loaded PLGA-NP for 48h in fresh media. Untreated and treated cells were fixed with 4% paraformaldehyde for 30 min. Fixed cells were then treated with mouse anti-MDR primary antibody (Santa Cruz Biotechnology, Dallas, TX) for 12h at 4°C followed by three washes with PBS and treatment with rabbit anti-mouse-Alexa 488 secondary antibody (Life Technologies, Carlsbad, CA) for 2h at room temperature. The cover slips were then washed twice and imaged using an Olympus BX 51 fluorescence microscope. DAPI was used to stain the nucleus of the cells.

*Western Blotting:*

Effect of miRNA treatments on expression levels of anti-apoptotic and pro-apoptotic proteins in HCC was assessed using western blotting. Expression of anti-apoptotic proteins including insulin like growth factor receptor-1 (IGFR-1), CD320 and pro-apoptotic protein programmed cell death 4 (PDCD-4) were assessed to evaluate the efficacy of the treatment. MDR expression was assessed to evaluate the effects of the treatment on drug resistance. Western blotting was performed according to standard protocols with a Novex ExCell Sure lock SDS-PAGE Electrophoresis System (Life Technologies, Carlsbad, CA). Briefly, 20 µg protein samples obtained from cell lines treated with different concentrations of miRNA-122 and anti-miR-21 varying from 0 picomoles (empty control PLGA-NP) to 15 picomoles were re-suspended in a reduced sample buffer, and then electrophoresed on a 7.5 to 10% Tris gel with Tris running buffer, blotted to a PVDF membrane, and probed with primary antibodies against CD320, IGFR-1, PDCD4 and MDR (Abcam,

Cambridge, UK). A horseradish peroxidase-conjugated goat anti-rabbit antibody (Abcam, Cambridge, UK) was then added as secondary antibody which was detected by using enhanced chemiluminescence (ECL Plus, GE Healthcare, Milwaukee, WI). GAPDH and Tubulin were used as a protein loading controls.

### **Intracellular uptake of miRNA-loaded PLGA-NP in human HCC cells**

Uptake of PLGA-NP into human HepG2 HCC cells was assessed by using quantitative RT-PCR and fluorescence microscopy following standard protocols.

Quantitative RT-PCR:  $10^5$  cells were plated in 6 well plates and grown for 24h. The plated cells were then treated with 5, 12.5, and 25 picomoles each of miRNA-122 and anti-miR-21 loaded PLGA-NP for 4h. Following treatment, the cells were incubated in 300  $\mu$ l lysis buffer for 3min and total RNA (mRNA and miRNA) was isolated from the lysate using miRvana RNA extraction Kit (Life technologies, Grand Island, NY) according to the manufacturer's protocol. For quantifying miRNA, 50 ng of total RNA was reverse transcribed using RT-primers (Life technologies) of miRNA-122 and anti-miR-21 using TaqMan® kit and microRNA Reverse Transcription Kit (Life technologies) to produce corresponding cDNA. The cDNA synthesis was carried out in a 15  $\mu$ l reaction volume. RT-PCR was performed using cDNA (5 ng of RNA equivalent) combined with TaqMan RT-PCR reagents (primer and probe mix) of miRNA-122 and anti-miR-21. Quantitative PCR was performed by 2 min incubation at 50 °C, followed by activation of the DNA polymerase at 95 °C for 10 min, 60 cycles of 95 °C for 15 s, and 60°C for 60 s in an Eppendorf Realplex RT-PCR system (Eppendorf AG, Barkhausenweg, Hamburg, Germany). The quantitative PCR reaction was carried out in a 20 $\mu$ l reaction volume. The expression of miRNA-122 and anti-miR-21 was calculated using the  $2^{-\Delta CT}$  method(6), in which the miRNA-122 and anti-miR-21 levels were normalized to the endogenous levels of miR-10b (a miRNA not dysregulated in HCC) in the HCC cell line. Typically, PLGA-NP are taken up into cells through endocytosis. To confirm that quantified miRNA was from internalized PLGA-NP in HCC cells and not miRNA from PLGA-NP attached non-specifically to the cellular membrane, an endocytosis inhibitor assay was performed as described (11). For the endocytosis inhibitor assay cells were kept in  $K^+$  deficient media (which prevents clathrin mediated endocytosis) for 2h containing the two miRNA's before the qRT-PCR assessment.

Fluorescence Microscopy: To further confirm PLGA-NP uptake in cells, fluorescence microscopy was performed.  $10^5$  HepG2 cells with stable green fluorescence were plated on cover slips and grown for 12 h. The media was then removed and cells were treated with 10 picomoles of Cy5-labeled anti-miR-21 loaded into PLGA-NP for 48 h in fresh media. Post incubation, the media was removed and the cells were fixed with 4% paraformaldehyde for 30 min after two washes with PBS. Cover slips were imaged using an Olympus BX 51 fluorescence microscope (Shinjuku, Tokyo, Japan). DAPI was used to stain the nucleus of the cells.

### ***In Vitro Treatment Response Assessment***

Treatment response following administration of miRNA-loaded PLGA-NP either individually or in combination was assessed in resistant and non-resistant HCC cells (resistant up to  $5 \mu\text{M}$  dox) using several standard assays, including MTT cell viability assay, live cell counting cell proliferation assay, migration assay, and invasion assay.

[3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT)] assay: Cells were washed once with PBS, and incubated with various concentrations (0-50 picomoles) of miRNA-122/anti-miR-21 loaded PLGA-NP either singly or in combination for 24-72 h. Untreated cells and cells incubated with empty PLGA-NP were used as negative controls. Cell viability was assessed by MTT assay according to the manufacturer's protocol (Invitrogen, USA). Prior to assay, the media was removed and the cells were washed with PBS. Cells were then replenished with 500  $\mu\text{l}$  of phenol-red free media with 50  $\mu\text{l}$  MTT stock (12 mM) solution. After 3 h of incubation, media was aspirated carefully without disturbing the precipitate, and 400  $\mu\text{l}$  of DMSO was added, and the precipitate was kept at  $4^\circ\text{C}$  overnight to dissolve the metabolically reduced tetrazolium MTT precipitate from the cells. The collected solutions were spun at 10,000 rpm for 5 min at  $4^\circ\text{C}$  and the absorbance of supernatant was measured at 540 nm using a multiwell plate reader (Infinite 2100, Tecan, Männedorf, Switzerland). For each condition, experiments were performed six times ( $n=6$ ). The relative cell viability (%) compared to control cells was calculated as follows: Cell viability (%) =  $[\text{Absorbance (sample)} - \text{Absorbance (blank)}] / [\text{Absorbance (control)} - \text{Absorbance (blank)}] \times 100$ .

Live cell counting assay to assess:



*a) Effect of miRNA treatments on resistant HCC cell proliferation*

$5 \times 10^4$  doxorubicin-resistant HepG2 cells were plated in 6 well plates and allowed to attach for 12 h. Post incubation, the media was removed and fresh media was added to the cells containing different concentrations of miRNA-122 and anti-miR-21 loaded PLGA-NP varying from 0 picomoles (empty control PLGA-NP) to 50 picomoles individually or in combination. Cells were incubated for 96 h after which they were trypsinized and counted using a Nexelcom Auto 2000 Cell Viability Counter (Nexelcom, Lawrence, MA, USA). The cell numbers after 96 h were represented as a % of untreated control cells. For each condition, experiments were performed six times ( $n=6$ ).

*b) Effect of miRNA and doxorubicin treatment on resistant HCC cell proliferation*

$10^4$  doxorubicin-resistant HepG2 cells were plated in 96 well plates and allowed to attach for 12 h. Post incubation, the media was removed and fresh media was added to the cells containing different concentrations of miRNA-122 and anti-miR-21 loaded PLGA-NP varying from 0 picomoles (empty control PLGA-NP) to 50 picomoles individually or in combination and incubated again for 18 h. The cells were then incubated with doxorubicin ( $1 \mu\text{M}$ ) for 24 h after which the cells were trypsinized and counted using a Nexelcom Auto 2000 Cell Viability Counter (Nexelcom, Lawrence, MA, USA). The cell numbers after 24 h were represented as % of untreated control cells. For each condition, experiments were performed six times ( $n=6$ ).

*c) Effect of miRNA treatment on doxorubicin sensitivity in resistant and non-resistant HepG2 cells*

Since the goal of our therapeutic strategy is to re-sensitize HCC tumors to doxorubicin we assessed the effect of a single miRNA-122 and anti-miR-21 combination treatment (5 picomoles each) on improvement of doxorubicin sensitivity in both resistant and non-resistant cell lines by calculating the doxorubicin dose that produces 100% cell death in the treated and untreated cell lines of each phenotype.  $10^4$  non-resistant or resistant HepG2 cells were plated in 96 well plates and allowed to attach for 12 h. Post incubation, the media was removed and fresh media was added to the cells containing 5 picomoles of miRNA-122 and anti-miR-21 incubated again for 18 h. The cells were then incubated with doxorubicin (0-200 ng/ml for non-resistant cells and 0- $8 \mu\text{M}$  for doxorubicin resistant cells) for 24 h after which the cells were trypsinized and

counted using a Nexelcom Auto 2000 Cell Viability Counter (Nexelcom, Lawrence, MA, USA). The cell numbers after 24 h were represented as number of viable cells. For each condition, experiments were performed six times (n=6).

Migration assay: The effect of the combination miRNA treatment on HepG2 cell migration was assessed using an *in vitro* cellular migration assay.  $10^5$  HepG2 cells were plated in 6 well plates and grown to 90% confluency and a 500 $\mu$ m wide scratch was drawn through the confluent cell layer. The cells were then treated with media containing either 10 picomoles miRNA-122 containing PLGA-NP, 10 picomoles anti-miR-21 containing PLGA-NP, or a combination of 5 picomoles each of miRNA-122 and anti-miR-21 containing PLGA-NP. HepG2 cells without treatment were used as controls. The width of the scratch after 48 h was measured and compared among the three treatments to determine the effects of the treatment on cellular migration. For each condition, experiments were performed three times (n=3).

Invasion assay: The effect of the combination miRNA treatment on resistant HepG2 cell invasion was assessed using an *in vitro* cellular migration assay.  $10^5$  HepG2 cells were plated in 25 cm<sup>2</sup> flasks and grown to 70% confluency. The cells were then treated with 10 picomoles miRNA-122 containing PLGA-NP, 10 picomoles anti-miR-21 containing PLGA-NP, or a combination of 5 picomoles each of miRNA-122 and anti-miR-21 containing PLGA-NP for 24 h after which they were trypsinized and resuspended in media without serum. The resuspended cells were plated on Matrigel coated transwell plates containing serum rich media below the matrigel layer. HepG2 cells without treatment were used as control. The invasive ability of treated and non-treated HCC cells was evaluated based on the number of cells that could cross the matrigel layer to reach the serum containing media after 48 h. For each condition, experiments were performed three times (n=3).

### **Microbubbles and ultrasound apparatus**

Clinical grade lipid-shelled perfluorobutane-and-nitrogen-filled microbubbles (BR38; Bracco Research, Geneva, Switzerland) were used. An ultrasound-guided drug delivery apparatus optimized by us previously for PLGA-NP delivery in cancers was used for all experiments(5). Microbubbles (BR38, Bracco) were

supplied as a lyophilized powder and stored in sealed vials with perfluorobutane and nitrogen gas in the head space. Before use, the lyophilized microbubble powder was resuspended in sterile 0.9% saline. The microbubbles had a mean diameter of  $1.6 \pm 0.4 \mu\text{m}$  and were neutrally charged (zeta potential,  $-0.3 \pm 0.3 \text{ mV}$ ).

For ultrasound-guided drug delivery, 1.8 MHz ultrasound pulses were used, generated by an array transducer (P4-1, Philips Healthcare, Andover, MA) connected to a research platform (V1, Verasonics, Redmond, WA). The pulses were calibrated in degassed water using a needle hydrophone (HNR-0500, Onda, Sunnyvale, CA). The full width at half maximum (FWHM) beam widths on the pressure profile were calibrated to be 1.4, 10.1, and 12.6 mm in the transducer's X, Y and Z directions, respectively. Successful intra-tumoral microbubble delivery following intravenous injection was confirmed using contrast mode ultrasound imaging using a 21-MHz transducer (MS250, VisualSonics, Toronto, Ontario, Canada) connected to a small animal ultrasound system (Vevo 2100, VisualSonics; lateral and axial resolution, 165 and  $75 \mu\text{m}$ , respectively).

### ***Ex vivo* assessment of successful PLGA-NP delivery in HCC xenografts and assessment of miRNA treatment effects**

Successful delivery of PLGA-NP into HCC cells in xenografts was visualized by TEM and intratumoral delivery of miRNA was quantified by qRT-PCR. Therapeutic effects in HCC xenografts were further assessed by a TUNEL apoptosis assay.

TEM: Cellular internalization following US and MB mediated PLGA-NP delivery was confirmed using TEM. In brief, 24h after treatment tumors were excised and immediately fixed in Karnovsky's fixative: 2% Glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) and 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1M sodium cacodylate (Electron Microscopy Sciences, Hatfield, PA) pH 7.4 for 1h, and chilled on ice. Tumors were then allowed to warm to room temperature in cold 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA) for 1h, washed three times with ultra-filtered water and stained en bloc overnight in 1% Uranyl Acetate at  $4^{\circ}\text{C}$  while rotating. Samples were then dehydrated with a series of ethanol washes (50%, 70%, and 95%) for 30 min each at  $4^{\circ}\text{C}$  after which they were allowed to rise to room temperature. The samples were then treated with 100% 2X propylene oxide

for 15 min and infiltrated with EMBED-812 resin (Electron Microscopy Sciences, Hatfield, PA) mixed 1:2, 1:1, and 2:1 with propylene oxide for 2 h each. The samples were left rotating overnight in 2:1 EMBED-812 resin: propylene oxide at room temperature. The samples were then placed into EMBED-812 for 2 to 4h, followed by placement into molds fresh resin, orientated and placed into 65°C oven overnight. Sections were taken between 75 and 90 nm, picked up on formvar/Carbon coated slot Cu grids, stained for 30 s in 3.5% uranyl acetate in 50% acetone, followed by staining in 0.2% lead citrate for 3 min. The tumor sections were imaged using a JEOL JEM-1400 (JEOL, Pleasanton, CA) operating at 120kV and photos were taken using a Gatan Orius 4k X 4k digital camera (Gatan, Pleasanton, CA). Three mice with a total of 6 non-resistant HepG2 xenografts (one on each hindlimb each) were used for this evaluation. Tumors on one side were treated with US-guided sonoporation with the contralateral tumors serving as intra-animal no US treated controls.

Quantitative RT-PCR: Four and 24 h after US and MB mediated PLGA-NP delivery, tumors were excised and 5 mg tumor tissue was incubated in 300 µl lysis buffer for 3 min and homogenized using a PRO 250 tissue homogenizer (Pro Scientific, Oxford, CT). qRT-PCR was carried out as described above for *in vitro* experiments. The expression of miRNA-122 and anti-miR-21 was calculated using the  $2^{-\Delta CT}$  method (6), for which the miRNA-122 and anti-miR-21 levels were normalized to the endogenous levels of miRNA-122 and anti-miR-21 in control tumors with neither PLGA-NP administration nor US and MB mediated treatments. Twenty-three mice with 46 non-resistant tumors were used for these experiments. The mice were left either untreated (n=3 mice with 6 tumors) or were treated with miRNA-122 and anti-miR-21 loaded PLGA-NP (n=10 mice with 20 tumors) or control empty PLGA-NP (n=10 mice with 20 tumors). In each mouse, one tumor was treated with US with the tumor on the contralateral hindlimb left untreated as intra-animal control.

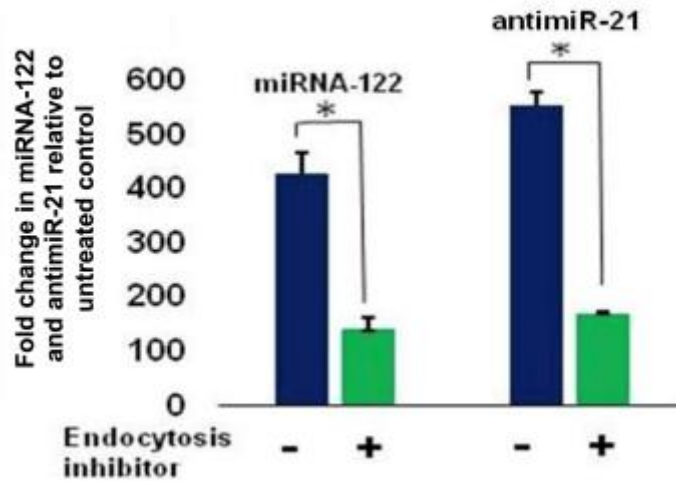
Apoptosis TUNEL assay: To confirm therapeutic effects associated with miRNA-loaded PLGA-NP delivery in non-resistant and doxorubicin-resistant HCC, resistant and non-resistant HepG2 xenografts were treated with a single combined dose of PLGA-NP loaded with either miRNA-122 or anti-miR-21 (14nmol/kg body weight of each miRNA in total 25mg/kg body weight PLGA-NP), followed by a single intravenous doxorubicin treatment (2 mg/kg; 5 times lower than recommended human dose) at 7 days after miRNA

treatment (n=10 mice with 20 tumors). The following three controls were used: 1) Mice (n=10 with 20 tumors) treated with miRNA-loaded PLGA-NP and with and without US but no doxorubicin; 2) mice (n=10 mice with 20 tumors) treated with doxorubicin only with and without US; 3) mice (n=6 with 12 tumors) treated only with US but no miRNA-loaded PLGA or doxorubicin. Furthermore, untreated tumors (n=6 mice with 12 tumors) were used for assessing baseline apoptosis values in HCC xenografts. 48 h after the single doxorubicin injection, tumors were excised, cryo-frozen, and sectioned using a Leica CM1850 cryostat (Leica Biosystems, Buffalo grove, IL) for analysis of apoptosis using TUNEL assay. Tumors not treated with doxorubicin were allowed to grow for 9 (7+2) days after treatment before excision. TUNEL staining was done on tumor sections with an *in situ* apoptosis detection kit (TACS; Trevigen, Gaithersburg, MD), according to the manufacturer's instructions. In brief, immobilized tissue samples were washed with PBS and incubated with 50  $\mu$ l Cytonin solution for 30 min. The slides were then washed two times in deionized water, 2 min each, followed by washing in immerse sample in Quenching Solution for 5 min. The samples were then washed in 1X PBS for 1 min and immersed in 1X Terminal deoxynucleotidyl transferase (TdT) Labeling Buffer for 5 min. The samples were then covered with 50  $\mu$ l of Labeling Reaction Mix and incubated for 60 min at 37 °C in a humidity chamber. The samples were then immersed in 1X TdT Stop Buffer for 5 min, and washed two times in deionized H<sub>2</sub>O, 5 min each. The samples were then covered with 50  $\mu$ l of Strep-HRP Solution, and incubated for 10 min at 37°C in a Humidity Chamber to avoid evaporation following which they were washed two times in 1X PBS, 2 min each immersed in DAB Solution for 2 min. The slides were then washed two times in deionized H<sub>2</sub>O, 2 min each and immersed in 1% Methyl Green for 30 s and gradually dehydrated by dipping slides ten times each in 2 changes of deionized H<sub>2</sub>O, 70 % 95%, and 100% ethanol. Tissue samples were covered with a glass cover slip and mounting media and imaged using Nanozoomer 2.0 digital slide scanner (Hamamatsu Photonics, Japan). TUNEL-positive cells in four different fields per sample were counted, and results were expressed as % of apoptosis area per mm<sup>2</sup> of tissue section. 12 sections per tumor were used for analysis.

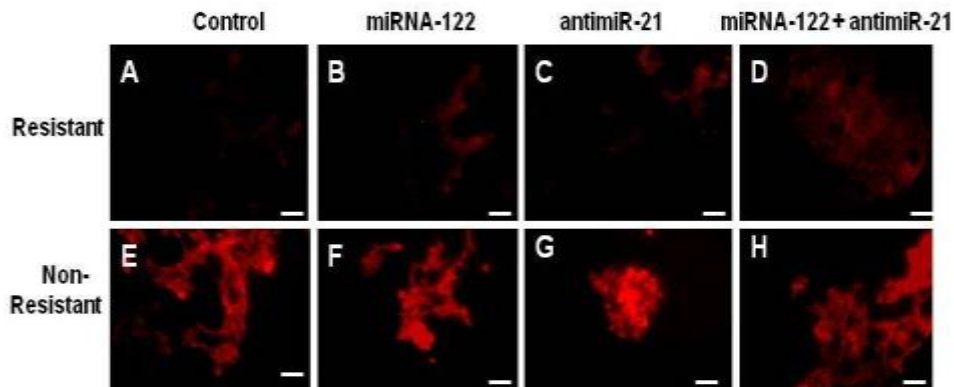
**Table S1:** Size, surface charge, anti-miR/miRNA loading percentage, and number of miRNA molecules loaded per PLGA-NP.

miRNA-PLGA- NP	Mean Size (nm)	Poly dispersity index (PDI)	Zeta ( $\zeta$ )-potential (mV)	Encapsulation efficiency (%)	miRNA-loading % (pmols/mg of PLGA-NPs)	miRNA Molecules /NP
anti-miR-21	146.5 ± 28	0.19 ± 0.03	-34.6 ± 3.4	68.4 ± 4.8	0.392 (684)	870 ± 64
miRNA-122	138.6 ± 26	0.18 ± 0.03	-32.4 ± 4.1	67.2 ± 5.8	0.385 (672)	724 ± 58
Control PLGA- NP	120.4 ± 23	0.18 ± 0.03	-23.7 ± 3.8	-	-	-

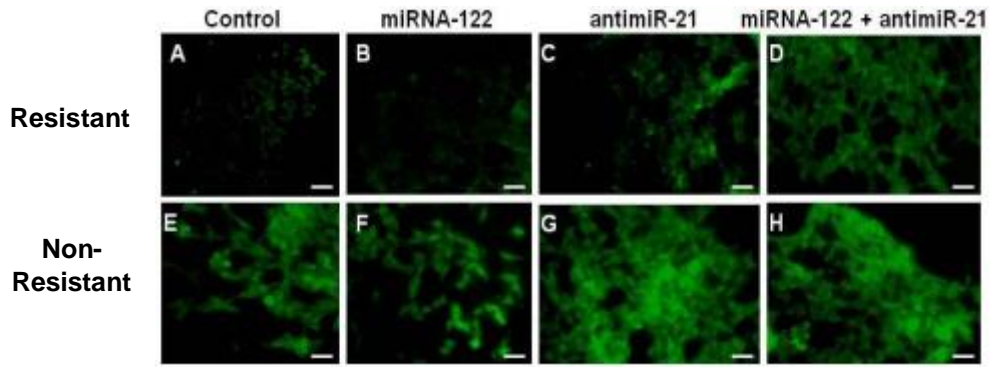
## SUPPLEMENTARY FIGURES



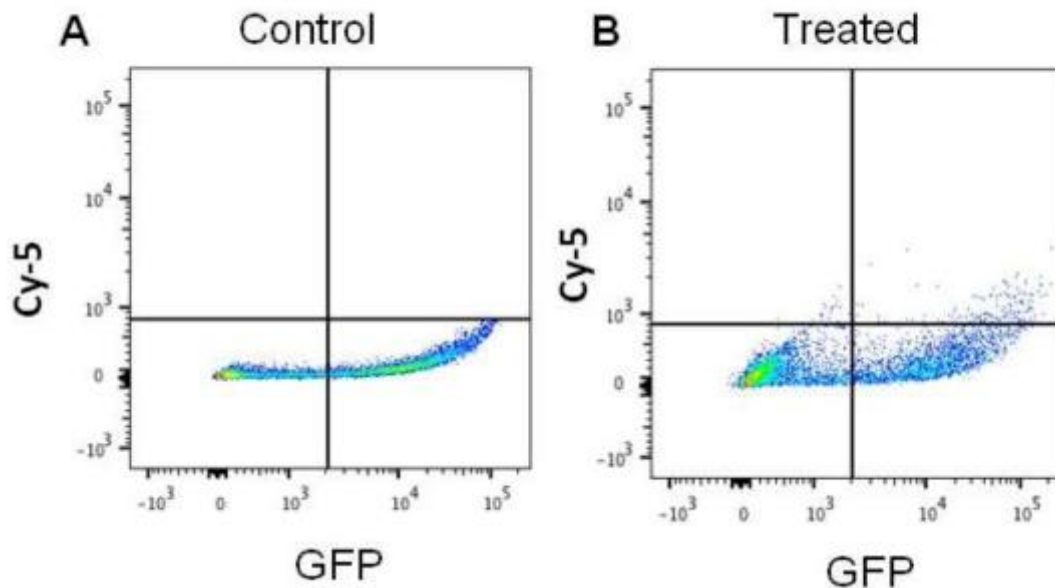
**Figure S1** Endocytosis inhibition assay using  $K^+$  depletion (an inhibitor of clathrin) shows that clathrin mediated endocytosis can be blocked in HCC cells, preventing miRNA-loaded PLGA-NP from cellular uptake.



**Figure S2.** Doxorubicin uptake in treated and non-treated (control) doxorubicin-resistant and non-resistant HCC cells assessed by fluorescence imaging. Representative images show substantially increased doxorubicin uptake in HCC cells ( $p < 0.001$ ;  $n = 12$ ) treated with the miRNA combination therapy compared to single treatments. Non-resistant HCC cells did not show significantly different ( $P > 0.7$ ;  $n = 12$ ) doxorubicin uptake with any of the four treatment conditions. Scale bar = 20  $\mu\text{m}$ .



**Figure S3.** Rho-123 uptake in treated and non-treated (control) doxorubicin-resistant (5 $\mu$ M) and non-resistant HCC cells assessed by fluorescence imaging. Representative images show substantially increased Rho-123 uptake (green color) in HCC cells ( $P < 0.007$ ;  $n = 12$ ) treated with the miRNA combination therapy compared to single treatments. Non-resistant HCC cells did not show significantly different ( $P > 0.3$ ;  $n = 12$ ). Rho-123 uptake with any of the four treatment conditions. Scale bar = 20  $\mu$ m.



**Figure S4.** Flow cytometry based quantification of Cy-5-labelled anti-miR-21-loaded PLGA-NP delivery into stably GFP expressing human HCC cells after ultrasound and microbubble mediated drug delivery. **(A)** Control HCC cells isolated from untreated human HCC xenografts show background Cy5 fluorescence. **(B)**



Number of Cy5-labelled anti-miR-21 containing human HCC cells substantially increased following treatment.

## REFERENCES

1. Bhargava-Shah A, Foygel K, Devulapally R, Paulmurugan R. Orlistat and antisense-miRNA-loaded PLGA-PEG nanoparticles for enhanced triple negative breast cancer therapy. *Nanomedicine (Lond)* 2016;11(3):235-47.
2. Devulapally R, Sekar TV, Paulmurugan R. Formulation of Anti-miR-21 and 4-Hydroxytamoxifen Co-loaded Biodegradable Polymer Nanoparticles and Their Antiproliferative Effect on Breast Cancer Cells. *Mol Pharm* 2015;12(6):2080-92.
3. Devulapally R, Sekar NM, Sekar TV, Foygel K, Massoud TF, Willmann JK, et al. Polymer Nanoparticles Mediated Codelivery of AntimiR-10b and AntimiR-21 for Achieving Triple Negative Breast Cancer Therapy. *ACS Nano* 2015;9(3):2290-302.
4. Paulmurugan R, Bhethanabotla R, Mishra K, Devulapally R, Foygel K, Sekar TV, et al. Folate Receptor-Targeted Polymeric Micellar Nanocarriers for Delivery of Orlistat as a Repurposed Drug against Triple-Negative Breast Cancer. *Mol Cancer Ther* 2016;15(2):221-31.
5. Wang T-Y, Choe JW, Pu K, Devulapally R, Bachawal S, Machtaler S, et al. Ultrasound-guided delivery of microRNA loaded nanoparticles into cancer. *Journal of controlled release* 2015;203:99-108.
6. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ CT method. *methods* 2001;25(4):402-08.
7. Soini Y, Virkajärvi N, Raunio H, Pääkkö P. Expression of P-glycoprotein in hepatocellular carcinoma: a potential marker of prognosis. *Journal of Clinical Pathology* 1996;49(6):470-73.
8. Ng IO, Liu CL, Fan ST, Ng M. Expression of P-Glycoprotein in Hepatocellular Carcinoma. *American journal of clinical pathology* 2000;113(3):355-63.
9. Yumoto R, Murakami T, Nakamoto Y, Hasegawa R, Nagai J, Takano M. Transport of rhodamine 123, a P-glycoprotein substrate, across rat intestine and Caco-2 cell monolayers in the presence of cytochrome P-450 3A-related compounds. *Journal of Pharmacology and Experimental Therapeutics* 1999;289(1):149-55.
10. Troutman MD, Thakker DR. Rhodamine 123 requires carrier-mediated influx for its activity as a P-glycoprotein substrate in Caco-2 cells. *Pharmaceutical research* 2003;20(8):1192-99.
11. Vercauteren D, Vandenbroucke RE, Jones AT, Rejman J, Demeester J, De Smedt SC, et al. The use of inhibitors to study endocytic pathways of gene carriers: optimization and pitfalls. *Molecular Therapy* 2010;18(3):561-69.