A novel method for producing functionalized vesicles that efficiently deliver oligonucleotides *in vitro* in cancer cells and *in vivo in mice*[3,4,5]

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Abstract

Nano-based delivery systems have enhanced our ability to administer and target drugs and macromolecules to their targets. Oligonucleotide drugs have great therapeutic potential but often have off-target effects and stability issues. Therefore, they are often encapsulated in vesicles with targeting ligands such as antibodies (Ab) to deliver their cargo. Herein, we describe a novel, scalable and straightforward approach to produce functionalized vesicles called the "Functionalized Lipid Insertion Method." This method differs from an older approach called the "Detergent-Dialysis Method." The older method required excess detergent and extensive dialysis over many hours to produce the functionalized vesicles. With our method, only the functionalized lipid is detergent-solubilized during the engineering of the vesicle. The approach reduces the dialysis time, keeps the vesicles intact while orienting the targeting moieties of the functionalized lipid toward the outside of the vesicle. Pilot *in vitro* and *in vivo* experiments was performed to show the feasibility of the "Functionalized Lipid Insertion Method." The dynamic light scattering (DLS) technique suggests that the original vesicular structure was unperturbed. Changes in vesicle size by our method were consistent with the lipid inserted externally into the vesicle. Our approach efficiently delivered oligonucleotides and affected the function of HepG2 cells. Functionalized vesicles achieve targeted delivery of oligonucleotides in mice without inducing a significant immune response through cytokine production or physical signs of the immune response such as inflammation. The industrial and therapeutic significance and implications of functionalized vesicles produced by our method are also discussed.

Keywords: Drug delivery, oligonucleotides, liposomes, extracellular vesicles, bioengineering

1 **Abbreviations:** Ab, antibody; C_t, cycle threshold; CMC, critical micelle concentration;
2 EMEM, Eagle's minimal essential medium (EMEM); EV, extracellular vesicle; FA, fatty
3 acid; FBS, Fetal Bovine Serum; HepG2, human liver cancer cell line; HCC, hepatocellular
4 carcinoma; LNP, liposomal nanoparticle; miR, microRNA; miRNA, microRNA; mEV,
5 modified extracellular vesicle; mLNP, modified liposomal nanoparticle; PBMCs,
6 peripheral blood nuclear cells; PBS, phosphate-buffered saline.

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Introduction

Nano-based delivery systems have been developed to deliver a wide range of molecules, including drugs, nucleotides, and proteins [1]. Several nano-based delivery systems are available, including liposomes, dendrimers, and carbon nanotubes [2]. To be effective *in vivo* carriers, they are designed to be biodegradable, biocompatible, and non-immunogenic [3–5]. Ideal nano-based delivery systems must overcome many challenges, including rapid clearance, instability, toxicity, and inefficient targeting [5–7].

Two major nano-based delivery systems recently employed are extracellular vesicles (EVs) and liposomal nanoparticles (LNPs) [e.g., 8,9]. EVs are naturally occurring vesicles with an average diameter around 200 nm excreted from various body fluids such as blood and urine [10]. Depending on their source, their sizes range from 20 nm to 10 μm in diameter [10]. In contrast, LNPs are artificially produced delivery systems and can be made into specific sizes through different techniques [e.g., 9,11]. EVs are formed by inward budding of the plasma membrane with other surface membrane invaginations from the Golgi apparatus [11]. These play an essential role in cell-to-cell communication and naturally carry RNA and proteins [11]. EVs are advantageous for drug delivery because of their inherent biocompatibility, long-circulating half-life, low toxicity, and tendency to be endocytosed into target tissues [12]. On the other hand, LNPs also have a lot of therapeutic potential because they can be designed with improved biocompatibility, stored lyophilized for relatively long periods, and produced on an industrial scale [13,14].

EVs and LNPs can be designed to have a particular function by engineering them with a fluorescent fatty acid (FA) for tracking, or a FA added with a targeting molecule such as an antibody (Ab) or a receptor ligand. They can also be loaded with

macromolecular or a small molecule as cargo. These modified EVs (mEVs) and modified LNPs (mLNPs) fit into a general category of vesicles referred to as functionalized vesicles. Functionalized vesicles have been engineered using relatively simple methods [15,16]. EVs have been covered with antibodies against the exosomal transmembrane protein CD9 and have improved delivery of miRNA to effector T-cells [15]. Since the early 1980s, an approach called the “Detergent-Dialysis Method” has been employed that uses detergent and dialysis to produce functionalized vesicles [17–24]. This approach's complete solubilization of the vesicular components leads to complete disruption of the vesicular structure [17–24]. These functionalized vesicles reassemble randomly by extensive and time-consuming dialysis to remove detergent [17–24]. Recently, a two-stage process for producing mEVs was developed where the EVs were PEGylated and covalently linked with antibodies [16]. The three-day process has a lot of potential for industrial scale-up [16,25,26]. Unfortunately, the mEVs undergo large temperature fluctuations between 4°C and 40°C, destabilizing the cellular proteins of the mEVs and the attached antibodies (Abs) of the vesicles [16,25,26].

Functionalized vesicles can be loaded with oligonucleotide drugs. Oligonucleotide drugs can be from natural sources or manufactured synthetically [27,28]. They have shown a lot of potential for treating diseases, and several have been approved by the FDA [28]. Oligonucleotide drugs can be easily modified and produced, which is a major advantage over traditional small molecule drugs [29]. Most oligonucleotide drugs have sequences that complement their DNA or RNA targets [28]. Oligonucleotide drugs include small interfering RNAs (siRNA) [30,31], antisense oligonucleotides (ASOs) [32,33] and

microRNAs (miRNAs) [34]. Unfortunately, oligonucleotide drugs suffer from immunogenicity, enzymatic degradation, and off-target effects [27].

SiRNA is generally recognized as a short 21 to 23 base pair double-stranded RNA (dsRNA) molecule with two 3' nucleotide overhangs [30]. They play a vital role in RNA interference (RNAi), which is involved in cellular growth and differentiation [35]. They can also target several disease-causing genes [30,36]. Therefore, siRNA drugs represent viable drug candidates and are in different stages of drug development [30,36]. Like other oligonucleotide drugs, siRNAs are susceptible to enzymatic degradation, off-target gene silencing, and potential activation of the immune response [31]. To overcome these challenges, these nucleotide polymers have been chemically modified and delivered through nanoparticle delivery vehicles such as liposomes [31].

ASOs are short 15-25 base pair polynucleotides that can be designed to bind to disease-related mRNA targets [32,33]. Mechanistically, they can attach to a target mRNA molecule and induce mRNA degradation [33]. Chemically modified ASOs can inhibit mRNA degradation by causing steric hindrance with their mRNA targets [33]. They ultimately affect mRNA translation and subsequent protein expression of the target mRNA molecule [33]. However, ASOs suffer from similar challenges as other oligonucleotide drugs, so they have been delivered by various means, including liposomes, cationic amphiphiles, and dendrimers [33,37].

Finally, a small polynucleotide called microRNA (miRNA) represents another potential therapeutic that can be delivered with functionalized vesicles [34]. These macromolecules are small non-coding polynucleotides that range in length between 17 and 25 nucleotides [38]. They play an essential role in physiology by modulating gene

expression through binding to mRNA [38]. MiRNAs have been associated with many diseases, including cardiovascular disorders, cancer, and allergic responses [39]. They can also elicit anticancer drug resistance in cancerous tumors [40]. Thus, miRNAs have served as biomarkers for various diseases, and they can modulate gene expression [34,39]. These miRNA-related therapeutics can be synthetic mimics of naturally occurring miRNAs or miRNA inhibitors called anti-miRs [34]. Unfortunately, these polymers suffer from similar problems as other oligonucleotide drugs [34]. To compensate for these inherent weaknesses, both miRNA mimics and anti-miRs have been chemically modified to reduce degradation and improve their therapeutic efficacy [34]. They have also been encapsulated in liposomes, dendrimers, and polymers [34]. Several miRNA therapeutics are currently undergoing different drug development stages to treat cancer and liver diseases [34]. Therefore, targeted delivery of miRNA represents an excellent opportunity to test the effectiveness of our approach to produce functionalized vesicles.

In these pilot studies, a straightforward, efficient, and non-disruptive approach for producing functionalized vesicles called the “Functionalized Lipid Insertion Method” is presented and compared to the “Dialysis-Detergent Method” [17–24]. The functionalized vesicles can be produced much more efficiently than [16] and the older method [17–24]. Our innovative approach maintains the temperature where vesicles, proteins, and Abs are stable [25,26,41]. Functionalized vesicles can be engineered with diverse targeting molecules, including receptor ligands, antibodies, and affibodies. These vesicles will give them the potential ability to target any cell, tissue, or organ. The *in vitro* targeting and functional effects of mEVs and mLNPs by our procedure are demonstrated with liver cancer HepG2 cells. In mice, mEVs and mLNPs were designed with different Abs to target

specific organs. The potential commercial advantages of targeted therapeutic delivery using our approach are discussed. In the last section, additional experiments and analysis are suggested to expand on and complete these pilot studies to make the “Functionalized Lipid Insertion Method” a powerful tool to deliver and study miRNA.

Materials and Methods

Materials

The conjugating fatty acid (FA) label, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide(polyethylene glycol)-2000] (DSPE-PEG2000-maleimide), and fluorescent 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DSPE) were purchased from Avanti Polar lipids (Alabaster, AL). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), *n*-dodecyl-β-*D*-maltoside (DDM) detergent, and Histopaque 1077 Reagent were purchased from Sigma Aldrich (St. Louis, MO). For the construction of liposomal nanoparticles (LNPs), *Escherichia (E.) coli* polar lipid extract was ordered from Avanti Polar lipids (Alabaster, AL), and chloroform was acquired from Sigma Aldrich (St. Louis, MO). Asialoglycoprotein receptor 1 (ASGPR1)/HL-1 polyclonal antibody ($ASGR1_{PAB}$) (ab49355) and recombinant ACE2 monoclonal antibody ($ACE2_{MAB}$) (ab108252) were purchased from Abcam (Cambridge, MA). NPHS2 (podocin) polyclonal antibody ($NPHS2_{PAB}$) (MBS3013144) was acquired from MyBioSource (San Diego, CA). GFP monoclonal antibody (GF28R) (GFP_{MAB}) (MA5-15256) was obtained from Thermo Fisher (Waltham, MA).

MiRNA mimics (mmu-miR-298-5p (0.38 mg/ml), hsa-miR-26a-5p (0.28 mg/ml)), and TaqMan™ MicroRNA assay were ordered from Thermo Fisher Scientific (Waltham, MA). Six ml Becton, Dickinson, and Company (BD) (Franklin Lakes, NJ) hematological tubes spray-coated with 1.8 mg/ml of dipotassium ethylene diamine tetraacetic acid (EDTA), and the blood separation agent Histopaque® 1077 Reagent, which is a solution of polysucrose and sodium diatrizoate (1.077 g/mL), was obtained from Sigma-Aldrich (St. Louis, MO). DharmaFECT™ 4 transfection reagent was bought from Horizon

Discovery (Cambridge, UK). The LegendPlex™ 8-panel Th1/Th2 Bio-plex cytokine assay kit was obtained from BioLegend (San Diego, CA).

Cell isolation and culture

Human peripheral blood mononuclear cell (PBMC) donors were enrolled for blood collection in compliance with the World Medical Association's Declaration of Helsinki and the Human Research Protection Program and Institutional Review Board (IRB) guidelines for human subject research at the University of Georgia. Enrolled healthy volunteers signed consent forms to inform them about the study. The human blood protocol (University of Georgia IRB no STUDY00006632) and the consent form were reviewed and approved by the IRB of the University of Georgia.

The human liver cancer HepG2 cells were purchased from American type culture collection (ATCC, Maryland, MD). These were grown and maintained in Eagle's minimum essential medium (EMEM) (Corning), which was supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA) and 5% penicillin/streptomycin (Thermo Fisher, Waltham, MA). These cells were incubated in a humidified atmosphere of 5% CO_2 at 37ºC in Thermo Fisher Scientific Napco Series 8000 WJ CO_2 incubator (Waltham, MA).

Isolation of extracellular vesicles (EVs)

Approximately 10 ml of human blood were put into EDTA-coated BD hematological tubes to declot them. The peripheral blood nuclear cells (PBMCs) were isolated as described with some modifications [42,43]. Five ml of EDTA-treated blood samples were layered onto an equal amount of Histopaque® 1077 Reagent in a 15 ml conical tube. The tube was centrifuged at approximately 400 g (1478 rpm) for 30 minutes

and 4°C in an Eppendorf 5810R centrifuge (Hamburg, Germany), which was separated the blood into plasma PBMCs, and erythrocyte layers. The top plasma layer was removed and discarded. The turbid middle layer of PBMCs was removed and put into a clean 15 ml conical tube. The tube was centrifuged at approximately 450 g (1917 rpm) for 10 minutes at 4°C. The supernatant was carefully removed using a transfer pipette to avoid disrupting the PBMC pellet. The pellet was then washed with 5 ml of an isotonic phosphate-buffered saline (PBS) (137 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) solution and centrifuged twice at 300 g (1278 rpm). This pellet was suspended in RPMI growth media without glutamine and phenol red (Corning, NY, USA) and added to a 1M HEPES buffer solution (pH 7.4) to a final concentration of 25 mM HEPES to provide additional buffering capacity for the media. Cells were then transferred to a sterile Cellstar T-75 culture flask with a red filter screw cap (Greiner Bio-One, Monroe, NC) containing RPMI 1640 (with glutamine and phenol red) supplemented with 10% w/v fetal bovine serum (FBS) with 100 U/ml penicillin and 100 μg/ml streptomycin. This solution was incubated for about ~12 hours (overnight) in a humidified atmosphere at 37°C and 5% CO_2 in Thermo Fisher Scientific Napco Series 8000 WJ CO_2 incubator (Waltham, MA). Afterward, the media was transferred with a sterilized transfer pipette into 2 ml microcentrifuge tubes. The microcentrifuge tubes were centrifuged at 10,000 g (~14,000 rpm) for 5 min. on a tabletop centrifuge at room temperature. About 1 ml of supernatant from each microcentrifuge tube was transferred to a new 2 ml microcentrifuge tube. Four hundred microliters of precipitation buffer B from the Qiagen (Formerly, Exiqon) miRCURY Exosome Isolation Kit (Qiagen, Germantown, MD) was added to the supernatant in each tube. The microcentrifuge tubes were inverted and vortexed and

incubated for ~12 hours (overnight) at 4ºC. The longer incubation time improved the EVs yield from the PBMCs. The remaining steps follow the manufacturer's instructions for the Qiagen miRCURY Exosome Isolation Kit (Qiagen, Germantown, MD). The EVs were resuspended in 300 µl of the resuspension buffer supplied by the kit, combined in one tube, and stored at -80ºC until needed. The total protein concentration of the EVs was measured using the Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) to estimate the concentration and the yield, which was typically around ~10 µg.

Preparation of liposomal nanoparticles (LNPs)

Unilamellar liposomal nanoparticles (LNPs) were prepared using the filter extrusion method [44]. The LNPs were composed of 80% w/v *E. coli* Avanti polar lipids and 20% w/v cholesterol as described previously [45]. Briefly, lipids and cholesterol were mixed in 10 ml of chloroform to get a final concentration of 10 mg/ml. This solution was evaporated to dryness in a Rotavapor Model R-114 (Buchi). After evaporation, the film was reconstituted in 0.1 mM EGTA and 50 mM Tris/HCl. This suspension was freeze-thawed at least ten times using liquid nitrogen and extruded 11 times through a LIPEX extruder (Northern Lipids, Burnaby, B.C., Canada) with a 400 nm cutoff Millipore filter (Millipore Sigma, Burlington, MA).

Functionalized vesicles produced by the "Functionalized Lipid Insertion Method"

A 200 µl fatty acid (FA) solution was made with 100 µM fluorescent NBD-DSPE and 100 µM DSPE-PEG2000-maleimide, and 0.1% w/v DDM detergent (10X the critical micelle concentration (CMC)) in an isotonic PBS (pH 7.4) buffer. The NBD-DSPE fluorescence was monitored at 550 nm by exciting at 445-460 nm using a SpectraMax

M2 Plate Reader (Molecular Devices, Sunnyvale, CA) to monitor EVs and LNPs assembly. The FA solution was dialyzed using 0.5 mL Slide-A-Lyzer MINI Dialysis units with a 10 K_D cut-off filter (Thermo Fisher Scientific, Waltham, MA) against 2 L isotonic PBS for 2 h at 4°C to remove excess DDM detergent. Almost identical fluorescence for the NBD-DSPE FA was measured after dialysis showing that the lipids remained in solution after dialysis (data not shown). To ensure that all DSPE-PEG2000 maleimide were conjugated, two-fold excess of the targeting antibody (~200 μM) was added to the dialyzed FA solution and incubated at room temperature for 1 hour. A 100 µl of purified 10 mg/ml EVs or LNPs was added to this 200 µl of the NBD-DSPE, and DSPE-PEG2000-Antibody solution was briefly centrifuged to remove any large aggregates. The molar ratio of vesicle lipid to NBD-DPSE and DSPE-PEG2000-Ab was approximately 75:1:1. This 300 μl solution was incubated for 1 hour at room temperature to allow slow mixing and prevent any potential disruption of the vesicles. After incubation, the supernatant was dialyzed in a 0.5 mL 10 KD cut-off Slide-A-Lyzer MINI Dialysis unit against 2 L of isotonic PBS buffer for two hours at 4°C. Dialysis slowly removes the DDM detergent that surrounds the FAs. The dialysis process exposes hydrophobic surfaces of the derivatized or functionalized FA and entropically drives the FA tail into the vesicle bilayer to minimize exposure to water to form the functionalized vesicle. The inserted lipids, particularly the DSPE-PEG2000-Ab, remain embedded on the outer leaflet of the vesicle bilayer because a large thermodynamic barrier prevents them from flip-flopping to the inner leaflet of the bilayer [46]. A similar procedure has been used for directionally inserting membrane proteins into liposomal bilayers [52,53]. The orientation was confirmed enzymatically and through atomic force microscopy (AFM) [52,53]. Afterward, the mEV solution was incubated with

100 µl of precipitation buffer B from the Qiagen miRCURY Exosome Isolation Kit (Qiagen, Germantown, MD) for ~12 hours (overnight) at 4 °C (Final volume = ~250 µl). The mLNPs solution was then centrifuged at 14,000 rpm for 30 minutes to produce a pellet. To pellet the mEVs, the solution was centrifuged at 104,000 g (30,472 rpm) in a Beckman TLA 110 rotor for one hour at 20ºC in a Beckman TLX ultracentrifuge. The supernatant was carefully removed. The functionalized vesicle pellet was then resuspended in 100 µl of isotonic PBS. The purpose of centrifuging the functionalized vesicles and removing the supernatant was to remove any remaining antibodies that were not cross-linked to the DPSE-PEG2000-Maleimide FA. The concentration of mEVs was measured by protein quantification using Pierce Bicinchoninic Acid (BCA) assay. The concentration of mLNPs was determined by tracking the amount of the lipid that was used throughout the procedure.

Functionalized vesicles produced by the "Detergent-Dialysis Method."

The production of modified vesicles by the "Detergent-Dialysis Method" was done as previously described [17–24]. All the components, the lipids, the proteins if present, and the derivatized lipids, were dissolved in detergent several times higher than the detergent's CMC [17–24]. These solutions were then extensively dialyzed over many hours to remove the detergent [17–24]. The dialysis-driven detergent removal process causes the lipids, the proteins, and the derivatized lipids to randomly coalesce into modified vesicles of indeterminate sizes [17–24]. Differences between this method and the "Functionalized Lipid Insertion Method" are described in the text and Fig. 1.

Lipids functionalized with antibodies (i.e., FA-PEG2000-Antibody) were made as described above. About a milligram of the following mixtures was dissolved in 0.03% DDM

by a similar procedure as in [17]: 1) *E. coli* Avanti polar lipids, 2) *E. coli* Avanti lipids, NBD-DSPE, and DSPE-PEG2000-Antibody with molar ratios of 75:1:1, 3) PBMC-derived EVs and 4) PBMC-derived EVs, NBD-DSPE, and DSPE-PEG2000-Antibody with molar ratios of 75:1:1. These mixtures containing 0.03% DDM were put into 200 μl microcentrifuge tubes. Then they were sonicated in a Kendal ultrasonic cleaner HB23 (Kendal) at 25°C until the solutions clarified, indicating complete dissolution. The mixtures were then extensively dialyzed as described in [17] by putting 50 μl of them into 0.2 mL Slide-A-Lyzer MINI Dialysis units with a 10 KD cut-off filter (Thermo Fisher Scientific, Waltham, MA) against 2 L isotonic PBS for at least 45 hours at room temperature.

Characterizing functionalized vesicles

Dynamic light scattering (DLS) was used to characterize the vesicle sizes by the "Detergent-Dialysis Method" and the "Functionalized Lipid Insertion Method" (Fig. 3), which is well established for this purpose [47–51]. Before the DLS experiments, all the samples were centrifuged at ~8000 g (5,000 rpm) for 30 min. using a Microfuge™ 22R (Beckman Coulter, Brea, CA) at 4°C. The purpose of the centrifugation was to remove large particulates such as dust from the samples, which can interfere with the DLS measurement. Although not visible by the naked eye, all centrifuged samples were assumed to have a pellet, so only the aqueous portion on the top was carefully removed before DLS analysis. The DLS experiments were performed on a Malvern Zetasizer Nano ZS (Malvern Panalytical, Malvern, UK) using a Malvern 45 μl ultra-micro cuvette (ZEN2112). The DLS experiments were analyzed using the Zetasizer Software Version 8 (Malvern Panalytical, Worcestershire, United Kingdom), assuming a refractive index of 1.330 and a viscosity of 0.8872, which are parameters typically used for lipid-containing

vesicles [52]. The size distribution curves in this manuscript were rendered on Igor Pro 6.3 (WaveMetrics, Portland, OR). Protein concentrations of the EVs were determined using the Pierce Bis-cinchonic assay kit (BCA), and LNPs were determined by tracking the lipid concentration throughout the experiments.

Loading functionalized vesicles with miRNA

Electroporation was used to load miRNA in the vesicles, as it is an efficient method to load oligonucleotides into vesicles [48,53]. Equal amounts of mEVs or mLNPs were added with an equal amount of miRNA mimics (Thermo Fisher, Waltham, MA) in SFM to a total volume of 400 μl. The solution was put into 0.4 cm gap Bio-Rad (Hercules, CA) electroporation cuvettes. Functionalized vesicles were electroporated with ~4 μg miRNA for the *in vitro* experiments and ~100 μg miRNA for the *in vivo* experiments. Unmodified and modified vesicles were electroporated at 150 V and 10-15 ms with an exponential wave pulse in a Bio-Rad Gene Pulser X-Cell electroporator (Hercules, CA). Afterward, the samples were incubated at room temperature and at 4°C for 30 minutes to allow the vesicles to recover.

miRNA delivery to HepG2 cells by functionalized vesicles

The HepG2 cells were treated in various ways to determine their relative uptake of miRNA. The cells were counted using a hemocytometer on a Zeiss Invertoskop 40 C inverted microscope (Zeiss, Oberkochen, Germany). The cells were plated to approximately 200,000 cells per well in a 6-well VWR tissue culture-treated plates (VWR, Suwanee, GA) and incubated ~12 hours (overnight). Afterward, the media was replenished with 2.7 mL of fresh 10% Eagle's minimal essential medium (EMEM) media. The cells were FBS-starved for ~12 hours before treatment to make proliferating HepG2

cells behave homogenously [54]. For experiments with unmodified and modified vesicles, 400 μl of them were electroporated with ~4 μg miRNA and then they were added wells in the cell-containing 6-well plates. For the DharmaFECT™ 4 transfection reagent experiments, the reagent was added with ~4 μg miRNA in a 400 μl solution, and they were added to other cell-containing wells. The final volume for all the wells after adding the solutions was approximately 3 ml. Before RNA extraction, the treated plates were incubated for 72 hours under humidifying conditions at 37ºC with 5% CO_2 in a Thermo Fisher Scientific Napco Series 8000 WJ CO_2 incubator (Thermo Fisher, Waltham, MA).

RNA extraction and quantitative RT-PCR (qRT-PCR)

TRIzol Reagent (Invitrogen, Carlsbad, CA) was used to isolate intracellular RNA as per the manufacturer's protocol. All the isolated RNA was stored at -80ºC. Complementary DNA (cDNA) was prepared from intracellular RNA (i.e., microRNA and the housekeeping U6 small nuclear (snRNA)) using the TaqMan™ microRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). The cDNA was analyzed on an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) in a 384-well microplate with the TaqMan™ Universal PCR MasterMix (Thermo Fisher Scientific, Waltham, MA). For measurements to determine miRNA transfer efficiency, TaqMan™ MicroRNA assay (Thermo Fisher Scientific, Waltham, MA) kits with specific fluorescent cDNA primers for the miRNA and fluorescent cDNA primers for the U6 snRNA reference. The DNA was quantitated on the qRT-PCR instrument using the threshold cycle (C_T) method with the U6 snRNA expression as a reference to calculate $\Delta\Delta C_T$ values, which correlates to the relative miRNA uptake [55–58]. These values were exported into Microsoft Excel format to calculate fold-difference and analyzed in

GraphPad Prism 7 (GraphPad, San Diego, CA). The $\Delta\Delta C_T$ values were normalized against the non-coding U6 snRNA expression to indicate the relative amount of miRNA delivered to the cells.

In-vitro time course for miRNA uptake

Six-well VWR culture-treated plates (Suwanee, GA) were plated with HepG2 cells to a density of 100,000 cells/well. The HepG2 cells were counted using a hemocytometer on a Zeiss Invertoskop 40 C inverted microscope (Zeiss, Oberkochen, Germany). After the cells adhered, the cells were serum-starved for one day. The next day, the cells were treated with functionalized vesicles containing 4 μg of mmu-miR-298-5p. Before extracting RNA, the cells were incubated from 12 to 72 hrs in a humid atmosphere at 37°C with 5% CO_2 in a Thermo Fisher Scientific Napco Series 8000 WJ CO2 incubator (Waltham, MA). Then, the RNA was extracted and analyzed by qRT-PCR using the procedures described above.

MiRNA effect of HepG2 cell wound healing delivered by the functionalized vesicles

For quantifying the functional effect of miRNA delivered by mEVs and mLNPs, the functionalized vesicles were loaded with a tumor suppressor miRNA, hsa-miR-26a-5p [47,48]. The HepG2 cells were plated in a 24-well plate at a cell density of 300,000 cells/well. A wound was created in each well using a 200 μl pipette tip followed by gentle washing with PBS buffer. The cells were then treated with mEVs and mLNPs containing miR-26a-5p at a dosage of 0.35 mg per well (50 ml/well) in 500 ml of fresh 10% EMEM media and incubated for 72 hours. The controls for this experiment were untreated HepG2 cells (abbreviated Cells), cells treated with empty EVs (abbreviated Cells + EVs()), and cells treated with empty mEVs with the $ASGR1_{PAB}$ (abbreviated mEVs($ASGR1_{PAB}$)) under

similar conditions. The cells were imaged from 0 to 72 hours on an Olympus IX71 inverted Microscope with a TH4-100 power source (Tokyo, Japan). The images were analyzed using the Fiji image processing software and the MRI wound healing tool in ImageJ Software (National Institutes of Health, Rockville, MD).

Mouse Studies

Animal welfare at the University of Georgia is covered by the NIH Animal Welfare Assurance #: D16-00276/A3437-01. Our animal studies were performed under two animal use protocols (AUPs): A2018 03-025-R2 and A2020 03-014-A3. These studies were carried out at the animal facilities at the David Life Sciences Building and the Paul D. Coverdell Center at the University of Georgia in Athens, GA. We greatly appreciate the generosity of Drs. Mandi M. Murph and Yao Yao for providing some mice used in the studies and sharing their spaces for our animal studies. The strains, genders, ages, treatments of mice used for these studies are listed in Table S1 of the *Supplementary Information*.

***In-vivo* Quantification of miRNA**

After 72 hours, the mice were euthanized using carbon dioxide followed by a necropsy as described [59]. About 100 mg of tissue were obtained to assess the amount of miRNA delivered in the tissue. The sections were suspended in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in a 1.5 ml microcentrifuge tube. The tissues were homogenized with a 1000 µl digital pipette and a Bel-Art™ Pro Culture Cordless Homogenizer Unit (Thermo Fisher, Waltham, MA). The samples were then centrifuged at 12,000 g for five minutes at 4ºC to remove tissue debris with a Microfuge™ 22R centrifuge (Beckman Coulter, Brea, CA). The RNA was extracted using the Invitrogen PureLink™

RNA Mini Kit (Thermo Fisher, Waltham, MA) [60]. After RNA extraction, the RNA concentration was quantified using NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The RNA concentration was determined using the extinction coefficient for single-stranded RNA in the spectrophotometer of 0.025 $(\mu g/ml)^{-1}$ cm^{-1}, and the RNA purity (>99%) was estimated by the 260 nm/280 nm ratio. The miRNA and U6 snRNA were amplified for quantification using the TaqMan™ microRNA reverse transcription and the TaqMan™ Universal PCR MasterMix (Thermo Fisher Scientific, Waltham, MA) as explained above.

Cytokine assay to probe immunogenicity

The LegendPlex™ 8-panel Th1/Th2 Bio-plex cytokine assay kit was used to quantitatively determine the immunogenic response of mice after 72 hours of functionalized vesicle treatment [61]. About a milliliter of blood was withdrawn immediately after euthanizing the animals in BD Microtainer® tubes containing serum separator (SST™) (Becton, Dickinson and Company, Franklin Lakes, NJ). The blood was allowed to clot at room temperature for 30 minutes. The tubes were then centrifuged at 1000 g for 15 minutes at 4°C using a Microfuge™ 22R (Beckman Coulter, Brea, CA), and the serum was then stored at -80°C. Before performing the assay, the serum was centrifuge at 10,000 g for 10 minutes at 4°C using a Microfuge™ 22R. The kit has immunoassays coupled to magnetic beads for detecting the following eight inflammatory factors, including the granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon γ (IFN-g), tumor necrosis factor α (TNF-a), and several interleukins (IL), IL-2, IL-4, IL-5, IL-10, IL-12 (p70). The plate was then read using a Luminex Magpix system (Luminex, Austin, TX).

Statistics

One-way analysis of variance (ANOVA) tests was used to determine statistical significance between groups comparing relative miRNA expression. A confidence interval of 95% with all p-values less than 0.05 was considered significant (*). Student's T-Test was also used to compare two groups, with a 95% confidence interval. Data were analyzed with Microsoft Excel (Microsoft, Redmond, WA) and GraphPad Prism 7 (GraphPad, San Diego, CA).

Results

Methods for producing functionalized vesicles

This work describes a novel method for producing functionalized vesicles involving detergent and dialysis that we called the “Functionalized Lipid Insertion Method.” This method is distinct from the “Detergent-Dialysis Method” used for decades and described in the literature since the early 1980s [17–24].

The two approaches are shown schematically in Fig. 1. In the “Detergent-Dialysis Method” (Figs. 1A-B), all functionalized vesicle components are solubilized at a relatively high detergent concentration (Step 1) [e.g., 17,24]. The detergent-solubilized mixture must be extensively dialyzed over many hours or days (Step 2). As dialysis removes the detergent, the vesicles randomly form functionalized vesicles from the solubilized components. The composition of the original components will influence the functionalized vesicle size. During the process, the functionalized lipids in the mixture will randomly orient toward the inside and outside the vesicle (red arrows near Step 3). However, even the long dialysis period is not enough to remove all the unintegrated components. Therefore, column chromatography is often needed in addition to dialysis to remove contaminants that interfere with the functionalized vesicle (Step 3) [e.g., 17].

Fig. 1B shows the production of an mEV by the “Detergent-Dialysis Method.” A natural vesicle such as an EV must be broken up into its components to be detergent-solubilized (Step 1) [19]. This process will disrupt the vesicular structure and possibly its natural functions. Next, the natural vesicle’s components, including lipids and proteins, and the functionalized lipid are dissolved at a high detergent concentration (Step 2). This detergent solubilization stage is followed by a long period of dialysis (Step 3). Because

the detergent concentration is so high, column chromatography is often used to remove any remaining contaminating detergent (Step 4). Because of the detergent solubilization, the original EV because its natural components randomly reassemble to form it. Both integral membrane proteins (green arrows) and functionalized lipids (red arrows) assume random orientations within the mEV. Some of the outward-facing proteins are now inward-facing proteins by this approach (green arrows). Some functionalized lipids face inside the vesicle cavity, where they cannot perform their intended function (red arrows).

A graphical representation of the "Functionalized Lipid Insertion Method" discussed in this work is shown in Figs. 1C and 1D. The only component that is detergent-solubilized by this approach is the functionalized lipid. In the diagram, the reactive lipid (i.e., DPSE-PEG2000-Maliemide) is solubilized by detergent and dialyzed for two hours to remove excess detergent (Step 1). The purpose of eliminating the excess detergent is to prevent it from dissolving the target preformed vesicle. Then the detergent-solubilized reactive lipid is incubated for 1 hour with an excess of a targeting component like an antibody (Ab) to allow cross-linking between them (Step 2). Afterward, the functionalized lipid is incubated for another hour with a preformed artificial vesicle or a natural vesicle (Step 3). This incubation period allows the components to slowly combine without external perturbation that might disrupt the vesicles like sonication. Gentle mixing can be performed during this stage. Finally, the detergent bound to the functionalized lipid is removed by 2 hours of dialysis (Step 4). A lot less detergent is present, so the dialysis period is significantly shortened. Also, the relatively low detergent concentration reduces the risk that excess detergent will disrupt the target vesicle. Detergent removal from the functionalized lipid exposes hydrophobic parts of the FA tail. The functionalized lipid

inserts itself into the target vesicle as an entropic process to reduce exposure to these hydrophobic surfaces. The FA tail of the functionalized lipid will insert into the target vesicle from the outside. Naturally, the functionalized part of the lipid on the other side of the molecule (red arrowhead) will be facing outward (Step 4). We have already experimentally demonstrated that we can orient integral membrane proteins in lipid bilayers using a similar principle [52,53].

Possible mechanisms for functionalized vesicle endocytosis and miRNA delivery

The general theory for delivering molecules to a cell is that a targeting molecule on the functionalized vesicle will bind to a surface receptor or excreted receptor ligand. The vesicular uptake can occur by fusion or by several potential endocytosis mechanisms [62]. The specific mechanism of vesicular uptake has not been entirely resolved but appears to be cell-dependent [62].

Fig. 2 shows potential mechanisms that a functionalized vesicle can be endocytosed by a target cell. The top figure shows the binding of the PEG-linked Ab on the functionalized vesicle to a surface receptor on the cell (Step 1). The functionalized vesicle can bind to the receptor and induce endocytosis. Alternatively, the proximity of the functionalized vesicle to the plasma membrane may induce endocytosis through the assistance of a PEG linker (Step 2). Endocytosis of the receptor-bound vesicle leads to an invagination of the cell surface and eventually to the formation of an endosome (Step 3). The endosome eventually disintegrates intracellularly to release the miRNA or other cargo inside the target cell (Step 4).

The bottom of Fig. 2 shows the targeting of secretory receptor ligands from cells by functionalized vesicles containing miRNA. In Step 1, soluble proteins are trafficked to

the Golgi apparatus from the endoplasmic reticulum (ER) [63]. The Golgi apparatus produces secretory vesicles containing the receptor ligands that migrate to the plasma membrane [63]. At the plasma membrane, they are secreted into the extracellular (EC) space, where they are bound by a functionalized vesicle (Step 1) [63]. The functionalized vesicle bound-receptor ligands bind to cell surface receptors on the plasma membrane forming a ternary complex with the receptor. Receptor-ligand binding to the surface receptor may lead to conformational changes that lead to endocytosis (Step 2) [e.g., 64]. The functionalized vesicle is tethered to the receptor-ligand and surface receptor by a PEG linker allowing the functionalized vesicle to interact with the plasma membrane surface. Alternatively, the interaction of the functionalized vesicle through the PEG2000 linker with the plasma membrane can lead to endocytosis. In either case, the functionalized vesicle will be engulfed by the cell to form an endosome (Step 3) [62]. The endosome containing the functionalized vesicle loaded with miRNA migrates within the cell (Step 3). Eventually, the endosome and the vesicle disintegrate, releasing the miRNA or other cargo intracellularly (Step 4).

The dynamic light scattering (DLS) technique reveals how different processes affect vesicle size

The DLS technique is a well-established technique for estimating particle sizes based on their dynamic light scattering properties [65]. The technique is well suited to estimate particle size distributions [e.g., 50,66]. The DLS technique was used to determine the effects of the “Detergent-Dialysis Method” and the “Functionalized-Lipid Insertion Method” on the vesicular size distributions. Because functionalized vesicles are

assembled all at once with the “Detergent-Dialysis Method,” these experiments will test the hypothesis that the two approaches will influence the vesicular size distribution.

Fig. 3 shows DLS measurements of functionalized vesicles produced by the two methods. The predominant particle sizes were determined in each solution by comparing the particle size distribution (PSD) to the particle diameter. Fig. 3A shows the predominate DDM micelle size (solid line) and a detergent-solubilized NBD-DSPE or DPSE-PEG2000-maleimide micelle size (dotted line) present in solution. The DDM micelle was 6.8 ± 1.6 nm in diameter, while the detergent-solubilized FAs were slightly smaller at 5.7 ± 1.4 nm, consistent with the sizes you would expect for these molecules.

Figs. 3B and 3C show vesicles produced using the “Detergent-Dialysis Method.” In Fig. 3B, the LNP (solid line) had an average diameter of 87.8 ± 34.7 nm, similar to previous observations [20]. However, the mLNP (dotted line) had about half the diameter of the LNP at 51.2 ± 19.5 nm. This size difference suggests that the size of LNPs may be sensitive to their initial mixtures, as we hypothesized. Fig. 3C shows the EVs (solid line) and mEVs (dotted line) produced using the “Detergent-Dialysis Method.” The EVs created using this method had an average size of 19.7 ± 5.6 nm. In contrast, the diameter of mEVs produced by this approach increased 6-fold larger or 131 ± 64.5 nm, showing again how the initial mixtures can influence vesicle size.

Fig. 3D and Fig. 3E show the unmodified vesicles and modified vesicles produced using the “Functionalized-Lipid Insertion Method” described in this manuscript. Fig. 3D shows the average particle size of LNPs that are unmodified (solid line). They have an average diameter of 115.6 ± 35.0 nm. LNPs modified using the “Functionalized-Lipid Insertion Method” (dotted line) increased slightly over 30% to 160.3 ± 37.4 nm. A 40 nm

diameter increase is consistent with the length of the PEG2000 linker and Ab connected to the mLNP [67,68]. The unmodified EVs shown in Fig. 3E (solid line) were 21.2 ± 5.9 nm, which is similar to the EV sizes produced with the "Detergent-Dialysis Method" (Fig. 3C, solid line). In Fig. 3E (dotted line), the EVs increased only 17% or 5 nm to 24.7 ± 6.9 nm. Thus, the size increase for mEVs was relatively modest, suggesting that their original structures were intact, but less functionalized lipid was inserted per EV than LNPs. One possibility for the size difference between mLNPs and mEVs is that the plasma membranes of EVs are crowded with many intrinsic membrane proteins that interfere with functionalized lipid insertion [e.g., 69]. The other possibility is geometrical. Because mEVs are smaller than mLNPs, their surface areas will be much larger within a given volume than mLNPs. In other words, a lot more functionalized lipids will be required to saturate the membrane surface of mEVs than mLNPs.

***In-vitro* delivery of miRNA by functionalized vesicles produced from the "Functionalized-Lipid Insertion Method"**

The human liver HepG2 cancer cell line has been the focus of many *in vitro* studies related to the functioning of liver cells and liver cancer [70–72]. The endocytotic asialoglycoprotein receptor 1 (ASGR1) is highly expressed on the surface of HepG2 cells [73]. The endocytotic process involves receptor-ligand binding and ASGR1 trimerization through receptor-mediated endocytosis [74]. This receptor has already been exploited to mediate small molecule uptake into HepG2 cells [75–77]. For example, galactosamine-linked albumin and nanoparticles loaded with pullulan and arabinogalactan delivered the anti-cancer drug doxorubicin to HepG2 cells [75,76]. In another study, pegylated liposomes modified with lactoferrin successfully delivered the fluorophore coumarin-6

through ASGR1 receptor-mediated endocytosis of HepG2 cells and HepG2 cells implanted in nude mice [77]. Finally, ASOs have been delivered to HepG2 cells by linking them to the ASGR1 receptor substrate *N*-acetylgalactosamine [78]. As far as we know, $ASGR1_{PAB}$ or any ASGR1 Abs have never been used to facilitate molecular uptake through the ASGR1 receptor with HepG2 cells. In this study, functionalized vesicles were made with $ASGR1_{PAB}$, which targets the EC domain of the ASGR1 receptor.

The focus of my laboratory is on drug transporters called P-glycoprotein (P-gp), which plays a major role in drug disposition, anti-cancer drug resistance, and biological barriers like the blood-brain barrier [79]. Despite being studied for decades, transporter measurements with P-gp and other transporters remain challenging [80]. The P-gp expression can vary between different cancer cell lines [81]. Even variants of the same mammalian cancer cell line can have significantly different properties [82]. Transporter transfection of mammalian cells can produce expression of other transporters in the transfected cell [83]. Further complicating transport measurements, control mammalian cancer cells without P-gp can express different levels of endogenous transporters than the mammalian cancer cells transfected with P-gp further [81]. Additionally, culturing conditions can significantly affect P-gp expression levels [84].

We were hopeful that oligonucleotides targeting P-gp and delivered by functionalized vesicles could ameliorate some of these challenges. Our former collaborator, who is a miRNA expert, had us investigate P-gp with mouse miR-298 based on [85,86] and to be compliant with NIH funding for this project. The miR-298 in [85,86] turned out to be human miRNA-298, which has completely different targeting properties and biological functions than mouse miRNA-298 [87]. As far as we know, mouse miRNA-

298 is not known to affect P-gp expression in any species. That revelation was one of the primary reasons that the collaboration was terminated. Despite this setback, we felt the described functionalized vesicle technology could eventually be used to investigate P-gp. The mouse miRNA-298 could be used to test the feasibility of functionalized vesicles since it would theoretically not have any physiological effects on the human HepG2 cells.

MiRNA nomenclature can be confusing for a novice. The formal names for mouse and human miR-298 are mmu-miR-298-5p and hsa-miR-298-5p, respectively [87]. The first three letters are the species [87]. Mmu is for *Mus (M.) musculus* (house mouse) and hsa is for *Homo* (*H.*) *sapiens* [87]. The number typically reflects the order of miRNA deposition into the miRNA database called miRbase and is almost always unrelated to the biological function [87]. The 5p refers to the 5' side of the precursor miR-298 stem-loop that the miRNA comes from [85]. The mouse miR-298 step loop has a second miRNA on the 3' side called mmu-miR-298-3p, which can be functionally distinct from the miRNA on the 5' side [88].

Fig. 4 are experiments to estimate the relative uptake of mmu-miR-298-5p in HepG2 cells. The experiments shown in Fig. 4A were used to gauge the uptake of 4 μg of mmu-miR-298-5p into the HepG2 cell line under different conditions. The relative uptake of mmu-miR-298-5p was normalized against HepG2 cells treated with DharmaFect 4 transfection reagent with miRNA (abbreviated DharmaFect(miRNA)). No mmu-miR-298-5p was detected in untreated HepG2 cells. HepG2 cells treated with EVs electroporated with mmu-miR-298-5p (abbreviated EVs(miRNA)) had miRNA uptake efficiency that was 5-fold higher than HepG2 cells treated with DharmaFect(miRNA). The miRNA uptake efficiency of HepG2 with EVs constructed with the $ASGR1_{PAB}$ and loaded

with mmu-miR-298-5p (abbreviated mEVs(ASGR1$_{PAB}$, miRNA) showed 8-fold greater improvement in miRNA uptake than DharmaFect(miRNA) control. HepG2 cells treated with exosomes genetically engineered with an Apolipoprotein A1 targeting ligand had more efficient miRNA uptake [48]. The Apolipoprotein A1 targeting ligand on the exosome may function better with the ASGR1 receptor than the ASGR1$_{PAB}$ that targets a different location on the receptor [74]. Also, internalization through the Apolipoprotein A1 requires binding to the scavenger receptor, class B, type I (SR-BI) to initiate endocytosis [74,89,90]. Modified LNPs with ASGR1$_{PAB}$ were also made and loaded with 4 μg mmu-miR-298 (abbreviated mLNP(ASGR1$_{PAB}$, miRNA)). Relative miRNA uptake was almost 2-fold higher in HepG2 cells treated with mLNPs (ASGR1$_{PAB}$, miRNA) than cells treated with mEVs (ASGR1$_{PAB}$, miRNA). The higher miRNA uptake by HepG2 cells treated with mLNPs may result from more functionalized lipids per LNP or more miRNA per vesicle because of their relatively large size of LNPs compared to EVs.

The miRNA uptake's temporal efficiency depends on the miRNA endocytotic mechanism, the miRNA carrier vehicle, and the miRNA type [91,92]. For example, the miRNA uptake of a human ovarian cancer cell line treated with liposomes loaded with fluorescently-labeled hsa-miR-7-5p increased linearly over 24 hours [91]. The uptake of miRNA in multiple myeloma treated with stable nucleic acid-lipid particles (SNALPs) loaded with hsa-miR-34a decreased after 48 hours [92]. In these pilot experiments, the miRNA uptake in HepG2 cells after treatment with mLNP(ASGR1$_{PAB}$, miRNA) and mEV(ASGR1$_{PAB}$, mRNA) was examined over 72 hours (Fig. 4B). The uptake efficiency of HepG2 cells to mmu-miR-298-5p increased exponentially by treatment with mLNPs or mEVs. After incubating the cells for 24 hours, the uptake efficiency doubled every ~12

hours for the mEVs and ~8 hours for the mLNPs. The uptake at 72 hours for mLNPs and mEVs was 600 and 250-fold higher, respectively, above untreated HepG2 cells versus the U6 snRNA. After 72 hours in these experiments, the relatively high miRNA uptake was deemed an appropriate time frame to test mEVs and mLNPs in mice.

Modified vesicles reduce HepG2 cell proliferation by enhancing hsa-miR-26a-5p uptake

The functional effects of treating HepG2 cells were tested with the mEVs(ASGR1$_{PAB}$), and mLNPs(ASGR1$_{PAB}$) loaded with miRNA hsa-miR-26a-5p (abbreviated mEV(ASGR1$_{PAB}$, miRNA) and mLNP(ASGR1$_{PAB}$, miRNA)). The miRNA hsa-miR-26a-5p was chosen to inhibit HepG2 cell proliferation and promote HepG2 cell apoptosis [48]. In other words, the miRNA will slow the closure gap of wounds made of a layer of HepG2 cells.

Wound healing assays with bright field microscopy has been effectively used to gauge cell proliferation and migration [93,94]. The approach was used to estimate the effect of mEVs and mLNPs treatment of HepG2 cells in Fig. 5. Fig. 5A shows the wound closures of HepG2 cells after various treatments at 0 and 72 hours. The wound closed to about half its original width for untreated cells, indicating uninhibited HepG2 cell proliferation. The HepG2 cells treated with empty EVs (abbreviated EV()), unmodified extracellular vesicles electroporated with hsa-miR-26a (abbreviated EV(hsa-miR-26a)), and empty modified extracellular vesicles with the polyclonal antibody for the ASGR1 receptor (abbreviated mEV(ASGR1$_{PAB}$)) had an almost similar extent of wound closure as the untreated cells meaning no effect on HepG2 cell proliferation. The wound area for HepG2 cells after treatment with mEVs(ASGR1$_{PAB}$, miRNA) and mLNPs(ASGR1$_{PAB}$,

miRNA) almost completely inhibited wound closure, demonstrating efficient uptake of hsa-miR-26a.

This wound closure over 72 hours was assessed quantitatively in Fig. 5B. The percent wound closure by all the treatment groups was normalized to the amount of wound healing in the untreated cells. The results show that the relative amount of wound closure in the cells treated with empty EVs(), EVs(hsa-miR-26a), and mEVs(ASGR1$_{PAB}$) was virtually identical to untreated cells. For EVs(hsa-miR-26a), the lack of effect on cell proliferation may be due to inefficient uptake of the hsa-miR-26a miRNA by the HepG2 cells. In contrast, wound closure by cells treated with mEVs(ASGR1$_{PAB}$, hsa-miR-26a) or mLNPs(ASGR1$_{PAB}$, hsa-miR-26a) inhibited cell proliferation by 50%.

Along with wound closure, the relative wound area was also analyzed temporally in Fig. 5C. The wound in untreated HepG2 cells closed at 16 ± 3 % per day (open circles). This wound closure rate is close to the 19% per day observed for HepG2 treated with extracellular vesicles containing hsa-miR-26a (calculated from Fig. 5B in [48]). After three days, the wound area was reduced to 52 ± 8% of the original area, suggesting uninhibited cell proliferation. The relative wound area in the cells treated with EVs delivering miRNA (abbreviated EVs(miRNA), open squares) was reduced at the rate of 13.9 ± 1.5 % per day, thus not differing significantly from untreated cells. Treating the HepG2 cells with mEVs(ASGR1$_{PAB}$, miRNA) decreased the rate of closure to 5 ± 4.3% per day (closed circles) and with mLNPs(ASGR1$_{PAB}$, miRNA) reduced the rate of closure to 6.7 ± 1.8% per day (closed squares). HepG2 cells treated with either our mEVs or mLNPs inhibited cell proliferation better than HepG2 cell treatment with miRNA-loaded Apo A1 targeting

ligand exosomes, which only decreased HepG2 cell proliferation to 10% per day (calculated from Fig. 5B in [48]).

Process of *in vivo* targeting of modified vesicles

The uptake of miRNA in mice after treatment with mEVs and mLNPs is shown in Fig. 6. Modified vesicles are loaded with miRNA by electroporation and injected intraperitoneally (IP) into mice (Fig. 6A). Because of their small physical size, IP administration to mice was easier to perform and more reliable than intravenous (IV) administration [95–99]. Also, vesicles administered by IP injection distribute broadly to the organs and the visceral adipose tissue because of access to the lymphatic system [95–99], which was preferred for pilot experiments with our engineered vesicles.

The treated mice were euthanized after three days using carbon dioxide followed by cervical dislocation, and their organs were harvested (Fig. 6B). The tissue was analyzed using RNA extraction protocols described in the *Materials and Methods* and Fig.6C. Then the sigmoidal amplification of miRNA and U6 snRNA in the mouse organs was measured in real-time by fluorescent primers (Fig. 6D). Next, the sigmoidal amplification curves determined a crossover threshold (C_t) for the miRNA and the U6 snRNA. Finally, the C_t values allowed us to estimate the relative-fold miRNA uptake in the mouse organs by the $\Delta\Delta C_t$ method (Fig. 6E).

In vivo studies with mmu-miR-298-5p were originally intended to modulate P-gp expression as recommended and suggested by our former collaborator, which would fit well within Specific Aim #1 of the supporting grant, but did not have that function [85,86,100–102]. As far as we are aware, no microRNAs are known to affect mouse P-gp expression or any related mouse transporters within the P-gp transporter superfamily.

Our former collaborator made miRNA sound specific, especially for P-gp transporter expression, but a single miRNA can affect several physiological functions [103]. For example, the mmu-miR-298-5p is known to target the nuclear factor (NF)-κB activator 1 (Act1) involved in cell signaling pathways and autophagy [100,104]. The miRNA is also known to regulate the expression of β-Amyloid Precursor Protein-converting Enzyme 1 [101] and regulate the expression of the insulin-like growth factor-1 β (IGF1Rβ) [102]. The miRNA also has a wide variety of theoretical targets that remain to be investigated in the TargetScanVert and miRDB databases [105,106]. Therefore, the following *in vivo* experiments with the functionalized vesicles and mice will determine the miRNA's organ specificity and immunogenicity.

Eventually, our laboratory wants to use functionalized vesicles to modulate *in vitro* and *in vivo* P-gp transporter expression. Luckily, siRNAs have already been shown to affect mouse P-gp expression [53,107]. Therefore, functionalized vesicles by this approach loaded with the P-gp targeting siRNA may be a practical way to investigate the P-gp transporter expression and effects in the future.

U6 snRNA normalization for miRNA uptake should be considered qualitative

MiRNA should be normalized against a stably expressing endogenous control RNA or RNAs to estimate the relative miRNA uptake within target cells. Ideal reference genes should have low expression variability and comparable expression levels since they can significantly impact miRNA quantitation [e.g., 108].

For these pilot studies, the relative miRNA uptake was normalized against the non-coding U6 snRNA [109]. Normalizing against U6 snRNA demonstrates the feasibility of our approach but is not ideal for the following reasons. U6 snRNA expression can vary in

mouse tissues (even within the same organ), differ significantly between genders, and be affected by a mouse's age [108,110–113]. U6 snRNA expression also likely varies greatly between mouse strains and sub-strains [114,115]. Even the miRNA of interest can theoretically affect U6 snRNA levels, although no significant changes in U6 snRNA expression were observed in these pilot studies by functionalized vesicle treatment (data not shown).

A table of mice used in these pilot studies is provided in Table S1 of the *Supplementary Infomation*. Because of potential differences in the U6 snRNA expression in the mice, this work's relative miRNA uptake in the mouse organs should be considered qualitative. To roughly compare the miRNA uptake distribution between the control in Fig. 7 and the functionalized vesicles in Figs. 8 and 9, the relative miRNA uptake from each organ was divided by the sum of the relative miRNA uptake in all the organs.

***In vivo* miRNA delivery by functionalized vesicles with a control antibody**

As a negative control for *in-vivo* targeting ability of mEVs and mLNPs, young female C57BL6/J mice were treated with functionalized vesicles loaded with mmu-miR-298-5p and GFP_{MAB} as a control antibody. Fig. 7 shows the uptake of mmu-miR-298 treated with modified vesicles with the GFP_{MAB} and containing mmu-miR-298 (abbreviated mEVs(GFP_{MAB}, mmu-miR-298) and mLNPs(GFP_{MAB}, mmu-miR-298-5p)). The highest miRNA uptake was observed in the mouse spleen after treatment with the functionalized vesicles. The IP injection puts the functionalized vesicle into the lymphatic system and the spleen (a lymphoid organ) [116]. The liver had about a third of the miRNA uptake than the spleen. The kidney and the lungs had only 20% of the miRNA uptake of the liver and 5% of the miRNA uptake of the spleen. Less than 1% of miRNA uptake was

observed in the brain. The miRNA uptake in mice follows a very similar pattern as biotin-labeled liposomal uptake in the organs of rats, with the exception of liposomal uptake in the lungs [117].

In vivo targeted miRNA delivery to the kidney by modified vesicles with the NPHS2 antibody

NPHS2 (a.k.a. Podocin) is a protein associated with the kidney [118]. Young female Nu/Nu mice were treated with functionalized vesicles engineered with $NPHS2_{PAB}$ and loaded with mmu-miR-298. Fig. 8A shows the relative miRNA uptake delivered by mEVs($NPHS2_{PAB}$, mmu-miR-298) to mouse organs, and Fig. 8B shows the relative distribution of miRNA uptake of mice treated with mEVs($NPHS2_{PAB}$, mmu-miR-298) compared to mice treated with mEVs(GFP_{PAB}, mmu-miR-298). In Fig. 8A, the relative miRNA uptake is evenly split between the kidney and the spleen or about 125-fold higher than the U6 snRNA expression level. The mouse lung and the mouse liver had 50% and 20% of the relative miRNA uptake, respectively than the kidney and spleen. The distribution of miRNA uptake shown in Fig. 8B shows miRNA uptake to the spleen decreased 50%, while the miRNA uptake increased 10-20-fold for mice treated mEVs($NPHS2_{PAB}$, mmu-miR-298) than mice treated with mEVs(GFP_{PAB}, mmu-miR-298). Off-target delivery of microRNA by this functionalized vesicle may be due to cross-reactivity of the polyclonal antibody ($NPHS2_{PAB}$) [119,120]. Figs. 8C shows the relative miRNA uptake of organs of mice treated with modified LNPs (abbreviated mLNPs($NPHS2_{PAB}$, mmu-miR-298) or mLNPs($NPHS2_{PAB}$, mmu-miR-298)) and it is about half the miRNA uptake as mice treated with mEVs. Still, the distribution of miRNA uptake to the organs was very similar (Fig. 8A). The normalized distribution of mice treated with

mLNPs(NPHS2$_{PAB}$, mmu-miR-298) and mLNPs(GFP$_{MAB}$, mmu-miR-298) in Fig. 8D was virtually identical to the distribution in Fig. 8B, implying that both types of functionalized vesicles engineered with NPHS2$_{PAB}$ had similar targeting mechanisms in mice.

In vivo targeted miRNA delivery to the liver by functionalized vesicles with the ASGR1 antibody

Male and female Nu/Nu mice were treated with mEVs(ASGR1$_{PAB}$, mmu-miR-298) and mLNPs(ASGR1$_{PAB}$, mmu-miR-298), respectively. Figs. 9A and C show relative miRNA uptake by various organs in adult male Nu/Nu mice were treated with mEVs(ASGR1$_{PAB}$, mmu-miR-298), and young female Nu/Nu mice were treated with mLNPs(ASGR1$_{PAB}$, mmu-miR-298) normalized to levels of mmu-miR-298 in mice only treated with SFM. Almost all mmu-miR-298 (94%) was in the liver for both adult male and young female Nu/Nu mice, which shows a lot of specificity for these functionalized vesicles. In the liver lobes of adult male Nu/Nu mice, the highest concentrations of mmu-miR-298 were found in the median lobe, which is the largest, and the caudate lobe, which directly interacts with inferior vena cava in mice (Fig. S1). The relative amount of mmu-miR-298 in adult male Nu/Nu mice was about the tenth of the young female Nu/Nu mice. These differences may be an artifact of normalizing with U6 snRNA, which likely differs significantly between adult male Nu/Nu mice and young female Nu/Nu mice. Figs. 9B and 9D show the distributions of miRNA uptake between mice treated with functionalized vesicles engineered with the ASGR1$_{PAB}$ and the GFP$_{MAB}$. Regardless of the age or gender, the panels show that virtually all miRNA uptake by mice treated with functionalized vesicles with ASGR1$_{PAB}$ was to the liver compared to 20% miRNA uptake by mice treated with control functionalized vesicles with GFP$_{MAB}$. The high specificity for mEVs and

mLNPs that target the ASGR1 receptor on hepatocytes may be due to the endocytotic nature of the receptor [121] and the access of the mesenteric circulation to the intraperitoneal cavity [122].

Immunogenic effects of miRNA delivery by modified vesicles with the relatively non-specific ACE2 antibody

To explore the immunogenicity of mEVs and mLNPs *in-vivo*, a targeting vesicle was engineered with a monoclonal antibody against the membrane-associated angiotensin-converting enzyme 2 (ACE2) ($ACE2_{MAB}$) with young female C57BL6/J mice with a functioning immune system. This enzyme is ubiquitously expressed in many organs throughout the body and plays a crucial role in controlling blood pressure [123–127]. In rodents, ACE2 mRNA levels were highest in the ileum in both mice and rats, followed by the kidney, almost equal levels in the lungs, bladder, stomach, colon, adipose tissue, atrium, brainstem, forebrain, and ventricle [128]. The lowest ACE2 mRNA was in the spleen of the rodents [128]. A different but consistent pattern in protein levels was observed in rats with the highest in the atrium (124.5%) and ventricle (131.7%), moderate expression in the kidney (100%), lesser in the lung (19.7%), testis (28.7%), thymus (44.4%), and least amount in the spleen [128]. Therefore, targeting mEVs and mLNPs with $ACE2_{MAB}$ will be relatively non-specific but more evenly distributed among the organs than mEVs and mLNPs with GFP_{PAB}, which primarily targeted the spleen (Fig. 7).

Fig. 10 shows the uptake of mmu-miR-298 and their effects on the cytokine levels after treatment of mice with miRNA-loaded mEVs and mLNPs engineered with $ACE2_{MAB}$. In Fig. 10A, from high to low fold miRNA uptake by mEV treated mice versus mice treated with SFM only, the miRNA uptake in mice by mEVs was ~280 fold in the liver, ~250 fold

in the kidneys, ~200 fold in the spleen, ~200 fold in the lungs, ~90 fold in the small intestine, ~70 fold in the heart and ~25 fold in the brain.

In Fig. 10B, the relative miRNA uptake was analyzed with mice treated with mLNPs($ACE2_{MAB}$, mmu-miR-298) versus SFM only treated mice (controls). The distribution of miRNA delivery to organs by mLNPs differed significantly from mEVs. The uptake of miRNA by treatment of mice with mLNPs($ACE2_{MAB}$, mmu-miR-298) was highest for the small intestine (~830 fold) followed by the kidney (~530 fold), spleen (~400 fold), liver (~400 fold), heart (~388 fold), and lungs (~100 fold). Virtually no miRNA uptake was observed in the brains of mice. Except for the spleen, these relative miRNA uptake levels are consistent with the protein expression levels of the ACE2 protein in rodents and humans [126–128]. Since there is almost no ACE2 enzyme in the spleen [128], the relatively high miRNA uptake after treatment with functionalized vesicles is likely due to IP administration rather than specific targeting by $ACE2_{MAB}$.

The immunogenicity of the functionalized vesicles was assessed by LegendPlex™ 8-panel Th1/Th2 Bio-plex assay, which tests for eight major anti- and pro-inflammatory cytokines from two types of CD4+ T helper lymphocytes [129]. Cytokine profiling has been used previously to determine the immunogenicity of EVs and LNPS [130,131]. Fig. 10C shows the cytokine levels in mice treated with mEVs($ACE2_{MAB}$, mmu-miR-298) and mLNPs($ACE2_{MAB}$, mmu-miR-298) in comparison to mice treated with SFM. No statistically significant change in cytokine levels was observed in mice treated with either mEVs (gray circles) or mLNPs (white circles) versus the SFM only treated mice (black circles). During the 72 hour treatment period (Figs. 7-10), no physical manifestations of immune response were observed, such as redness, itching, or sudden hair loss. The complete lack of

immune response to the functionalized vesicles in these pilot experiments is promising, but the adaptive immunity in mice may take significantly longer [132]. Therefore, experiments are planned to examine the immunogenicity of functionalized vesicle mouse treatment over several weeks.

Discussion

In this work, we present the "Functionalized Lipid Insertion Method" to bioengineer targeting functionalized vesicles with a surface coated with PEG-linked Abs that we refer to as mEVs or mLNPs. We explain how our approach differs significantly from the "Detergent-Dialysis Method" used since the 1980s [17–24]. These functionalized vesicles are versatile and can target surface receptors. Both mEVs and mLNPs could efficiently deliver miRNA to the liver cancer HepG2 cell line. The *in-vitro* miRNA uptake efficiency in HepG2 cells was 8-fold and 15-fold higher by mEVs and mLNPs, respectively, than by the transfection reagent. Functional effects of miRNA on the HepG2 cell line were demonstrated by the treatment of the cells with mEVs loaded with growth-affecting miRNA. Despite potential issues of using U6 snRNA for miRNA quantitation and other factors, functionalized vesicles engineered with antibodies appeared to deliver their intended miRNA to their intended mouse organs. The most specific miRNA delivery to mouse organs seemed to be from functionalized vesicles engineered with the $ASGR1_{PAB}$ (Fig. 9). The least specific miRNA delivery to mouse organs was from functionalized vesicles with the $ACE2_{MAB}$ (Fig. 10). At least over the 72 hour study period, the functionalized vesicles also did not appear to be non-immunogenic (Fig. 10).

Even unmodified extracellular vesicles can have oligonucleotide uptake efficiencies and target cells like mEVs [133,134]. Exosomes derived from the human

hepatoma Huh7 cell line targeted the human embryonic kidney line over human PBMCs or a human lymphoblast cell line [133]. The miRNA uptake efficiencies for the human embryonic kidney cell line treated with exosomes were 5-200-fold higher than the other cell lines [133]. A study with exosomes derived from MDA-MB-231 cells and H-29 colon cancer cells suggested that efficient exosomal targeting relied on complementary interactions [134]. Unmodified exosomes are the simplest to produce but predicting the exosomal target requires analyzing the oligonucleotide uptake in various tissues and cell lines to determine specificity.

Several methods have been developed to modify the surfaces of natural vesicles to control targeting to cells and improve uptake of oligonucleotides [15,53,134]. Targeting and miRNA uptake of CD8+ T-cell-derived exosomes to effector T-cells was enhanced by having a constitutive exosomal protein bound with antibodies [15]. Unfortunately, this simple approach does not have broader applicability to exosomal targeting beyond T-cells. Another strategy to exploit constitutive exosomal proteins is to fuse them with targeting proteins by genetic engineering [135]. Exosomes were genetically engineered with a lysosomal protein (Lamp2b) and the rabies viral glycoprotein (RVG) [72]. They were able to enhance siRNA uptake efficiency in cells and overcome the blood-brain barrier (BBB) in mice [53]. Exosomes have been genetically engineered using a similar technique with fusion proteins of the lysosomal protein (Lamp2) with the green fluorescent protein (GFP) for fluorescent tracking and with the HER2 affibody for targeting [136]. Exosomes have also been genetically engineered with a construct made from the vesicular stomatitis virus glycoprotein with improved loading, delivery, and tracking [137]. The development of modified exosomes by genetic engineering is limited by the

complexity of engineering the fusion protein construct and getting the construct to express in budding exosomes [135].

A highly PEGylated exosome with a relatively low surface density of Abs than Abi-exosomes was developed using a two-stage process to deliver anticancer drugs cells [16]. The modified exosomes provided only a ~30% increase in anticancer drug uptake by a human pancreatic cancer cell line [16].

Similarly, modified and unmodified LNPs have been used to deliver miRNA previously. Cationic liposomes have been used as transfection agents to deliver miRNA such as miR-7 to ovarian cancer cells, miR-143 and -145 in human colorectal tumors, and miR-122 in liver cells achieving up to 80% efficiency [91,138,139]. Cationic liposomes typically consist of a cationic lipid, neutral lipid and/or cholesterol, and a PEG-lipid. Cationic liposomes are known to form ion pairs with anionic phospholipids of the endosomal membrane leading to the release of cargo efficiently, which makes them one of the promising drug delivery vehicles. Cationic liposomes can form complexes with oligonucleotides such as miRNA and are known as lipoplexes. For instance, a cationic-based lipoplex delivery system has been used to deliver miR-29b and miR-133b to non-small cell lung cancer cells. The lipoplexes achieved over 2-fold delivery efficiency in comparison to the transfection reagent *in vitro*. In addition to low delivery efficiency, when injected intravenously, only 30% accumulation was achieved in the target organ, lungs, with the highest amount found in the liver, followed by kidney and spleen [140,141]. The process of preparing lipoplexes, unfortunately, renders them unoptimizable down the process and increases the risk of interaction of cationic lipids with other tissues making them non-specific, limiting their potential as targeted drug delivery vehicles.

In addition to cationic liposomes, liposomes can also be conjugated with target-specific vectors to design cell-specific vehicles. Targeted liposomes have been prepared by conjugating Aptamer (AS1411), a target-specific single-stranded oligonucleotide, using thioether linkage between DSPE-PEG2000 maleimide on the liposomal surface. These Aptamer functionalized liposomes were used to deliver miR-29b to A2780 cells, ovarian cancer cells [142]. Another study prepared a liposome-polycation-hyaluronic acid (LPH) modified with GC4 single-chain variable fragment, a tumor-targeting monoclonal antibody to deliver siRNA and miRNA to B16F10, metastatic lung cancer cells [143]. The GC4 targeted liposomes achieved 40% delivery of siRNA in the tumor, and the highest accumulation was achieved in the liver (>50%) when dosed intravenously to the tumor-bearing mice [143]. An antibody against the CD59 receptor, a receptor overexpressed in cervical cancers, was conjugated to Cisplatin (CDDP)/miR-1284-loaded liposomes. CD59 conjugated vehicles showed a significant increase in miRNA-induced cytotoxicity in cervical cancer cells and prolonged blood circulation of cisplatin [144]. All these studies have used the post-conjugation method to modify liposomes, where they attach derivatized PEGylated lipids to the liposomes while preparing liposomes followed by conjugation of antibodies. Although successful, this method is more complicated as you functionalize lipid derivatives even before preparing liposomes. However, the post-insertion method, also known as the detergent dialysis method, attachment of antibodies to derivatized lipids first, and then inserting antibody derivatized lipids in PEGylated liposomes, gives more room to play with designing or modifying liposomes as desired. The Detergent-dialysis method has been shown to be as effective as if not more than the post conjugation method [145]. This method has been used to attach target-specific

antibodies such as CD44 and CD133 to deliver cargoes like small molecules like doxorubicin or antibodies like anti-IL6R antibodies [146,147]. However, as mentioned earlier, this method requires extensive dialysis over a long time, increasing the risk of degradation (in the case of natural vesicles), aggregation, and attachment of functionalizing ligands both internally and externally.

Considering the versatility of the "Functionalized Lipid Insertion Method," the approach seems to be the simplest and most efficient way to modify therapeutic delivery vehicles. Fluorescence tracking, PEGylation, and Ab targeting are integrated into a single dialysis step followed by electroporation for oligonucleotide loading. Many of the pieces for industrial-scale production of mEVs are already available. Industrial methods for dialyzing and electroporation have already been around for decades [148,149]. Synthetic methods are available to produce large amounts of miRNA [150]. Innovative large-scale production of monoclonal Abs is being developed from plants [151]. Currently, getting EVs is rate-limiting, but methods are on the horizon for getting from abundant sources such as milk [138,139]. Liposomes have been around for more than a few decades, with different methods available to upscale their preparation, including microfluidics [152,153].

Several challenges exist for implementing mEVs and mLNPs therapeutically. For long-term viability, EVs and, by extension, mEVs are most often stored at -80°C. However, month-long room temperature storage has been shown after lyophilization and in the presence of trehalose [41,154]. In contrast, lyophilized LNPs can last up to a year at 4°C or room temperature in an oxygen-free environment [155–158]. MicroRNAs also have their challenges and must often be stored at -80°C [159], but methods are available to

increase temperature stability through chemical modification [160]. MiRNA also has the potential for slowly leaking from functionalized vesicles [161].

Going beyond these pilot studies

In these pilot studies, oligonucleotide delivery through our functionalized engineering approach in HepG2 cells and its function was demonstrated. Oligonucleotides were also delivered to mice from different strains, different genders, and different ages with these bioengineered functionalized vesicles (Table S1). Over the narrow 72 hour time frame of these experiments, the functionalized vesicles were non-immunogenic. These pilot studies gave us a tantalizing look at the potential of our novel approach to bioengineer functionalized vesicles to deliver macromolecules both *in vitro* and *in vivo*. However, more in-depth studies will be required for use as a potential therapeutic and for studying the biological functions of oligonucleotides such as miRNA.

For the *in vitro* experiments, treating a range of cell lines with functionalized vesicles will firmly establish the *in vitro* miRNA uptake efficiency of this technology. One possible cell line to examine in future studies is the extrahepatic Jurkat T-cell line, which is known to express the ASGR1 receptor [162]. An immortalized human kidney podocyte cell line that expresses NPHS2 (podocin) protein would be a great way to investigate the uptake efficiency of functionalized vesicles engineered with the NPHS2 Abs [163]. In addition, functionalized vesicles engineered with a GFP Ab can serve as an additional control for the *in vitro* experiments. The liver cancer HepG2 cell line used in these pilot experiments is well known to express the ASGR1 receptor, so Western blots were not essential for qualitatively demonstrating efficacy [e.g., 162,164]. Because different cell lines will be used in future studies, Western blots with appropriate Abs should be used to

establish protein expression in the cell lines. Because miRNA affects multiple mRNAs [87], a wound-healing assay of HepG2 was used to determine the functional effects of the hsa-miR-26 (Fig. 5). To examine the functional effects of specific miRNA on proteins, a Western blot should be used to quantify the protein of interest in addition to the relative protein mRNA level.

In Fig. 2B, we discuss the theoretical possibility of targeting secreted receptor protein ligands by our functionalized vesicles. To test the technology, cell lines that produce a lot of a receptor protein ligand can be compared to cell lines that produce little or none of it. For example, the miRNA uptake efficiency of the MB-MDA-435 cell line that secretes the receptor protein-ligand autotaxin can be compared to the miRNA uptake efficiency of the MB-MDA-231 cell line after treating them with functionalized vesicles engineered with the autotaxin antibody [165–167].

In vivo experiments using our functionalized vesicles will require careful selection of non-coding RNAs (ncRNAs) for normalization of miRNA uptake and mice. The mice should also be strain, gender, and age-matched. Mouse studies should include both genders, and the numbers of mice used in the study should be statistically high enough.

Choosing appropriate non-coding RNA controls (ncRNAs) is essential for accurate miRNA uptake quantitation. Therefore, several ncRNAs from each mouse tissue should be examined for expression stability and variability. Several different approaches have been developed for this purpose [168–170]. A tool called BestKeeper estimates the stability by the standard deviation (SD) of mean cycle quantitation (C_q) values between candidate ncRNAs [168]. The stability of ncRNAs can also be determined using the ΔC_q method, where the SD is calculated by the ΔC_q of ncRNA gene pairs [169]. The GeNorm

algorithm is another approach to gauge ncRNA stability with a calculated gene stability M value [170]. Finally, the calculation of variation between groups of candidate control genes can be accomplished with the NormFinder algorithm [171]. For the best results, these four methods can be used together to determine the optimum reference genes for miRNA quantitation, as recently shown for CNS tissues of mice [108]. Even after optimum reference ncRNA genes have been identified, control RNA gene expression should be monitored in the presence of the miRNA interest to ensure that the control genes are not significantly affected by the miRNA (i.e., ΔC_q = ~0).

Once appropriate control RNAs are selected for monitoring the miRNA in each mouse tissue, the effects of miRNA delivery by our functionalized vesicles can be demonstrated experiments analogous to hsa-miR-26a delivery to HepG2 cells in Fig. 5. First, HepG2 cells can be implanted in immune system deficient mice (i.e., Nu/Nu) mice as described [172]. Then the mice can undergo treatment by functionalized vesicles loaded with hsa-miR-26 to determine their effect on HepG2 tumor size.

Authors contributions

Authorship was determined using guidelines recommended by *The International Committee of Medical Journal Editors* [173–176], which requires significant and novel contributions to scholarship and significant contributions to the organization, writing, and intellectual ideas within this manuscript. The original development of the "Functionalized Lipid Insertion Method" was based on membrane protein reconstitution in liposomes previously done in Dr. Arthur G. Roberts laboratory [52,53]. Ms. Pragati Jain made significant improvements to the functionalized vesicle experimental protocol that was part of Dr. Sudeepti Kuppa's Ph.D. thesis. Ms. Pragati Jain and Dr. Arthur G. Roberts played the largest roles in designing the experiments within the manuscript, as evidenced by their many individual meetings. They both played major roles in the writing and the organization of the manuscript. Ms. Pragati Jain selected the specific antibodies and most of the other materials that were used within the project. Dr. Arthur G. Roberts selected the fluorescent and reactive lipids used in this project and designed the original protocol for functionalized vesicles, as well as contributed to modified versions by Dr. Sudeepti Kuppa and Ms. Pragati Jain. Ms. Pragati Jain did all the data collection and almost all of the data analysis.

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Figure Legends.

Figure 1. Bioengineering functionalized vesicles by the "Detergent-Dialysis Method" and the "Functionalized-Lipid Method." A) Shows the "Detergent-Dialysis Method" that has been used since the early 1980s to produce functionalized vesicles from LNPs. In Step 1, functionalized lipids containing a targeting molecule such as an Ab are mixed with lipids and high concentrations of detergent, leading to complete solubilization of all the components. In Step 2, the high concentrations of detergent are dialyzed over time to remove the detergent. Once the detergent reaches a specific concentration, the mLNPs will spontaneously form, but the orientations of the functionalized lipids become randomized (red arrows) within the lipid bilayer. Even after this long dialysis period, column chromatography is often used to remove detergent-solubilized components in Step 3. B) Shows the same process of producing functionalized vesicles from natural vesicles like EVs. Because EVs are intact vesicles, they are lysed into their individual components in Step 1 prior to detergent solubilization and dialysis in Steps 2 and 3. In Step 3, because proteins are likely to be embedded in the natural vesicle lipid bilayer, their orientations will be randomly oriented (green arrows) like the functionalized lipids (red arrows). Column chromatography can be used in Step 4 to remove detergent-solubilized contaminants. C) Shows the process of producing mLNPs using the "Functionalized Lipid Insertion Method" that we developed. In Step 1, detergent-solubilized lipids with reactive functional groups are dialyzed for two hours to remove excess detergent. These solubilized lipids are mixed with targeting molecules such as antibodies (Abs) for 1 hour to form functionalized lipids in Step 2. In Step 3, the functionalized lipids are then incubated (or mixed gently) with preformed LNPs for another

hour to ensure the integrity of the preformed vesicle. In Step 4, because the detergent concentration is relatively small compared with the detergent concentrations used in the "Detergent-Dialysis Method," a lot shorter time is required for detergent removal. Removal of detergent from the functionalized lipids causes them to directionally insert into the preformed liposomes, becoming mLNPs. D) Shows natural vesicles like EVs being produced by the "Functionalized Lipid Insertion Method." The process of producing mEVs is virtually identical to producing mLNPs. In Steps 1 and 2, a detergent-solubilized functionalized lipid is produced from dialysis and incubation with targeting molecules such as Abs. Then in Step 3, they are incubated (or gently mixed) with the EV to maintain vesicle integrity. Detergent removal through dialysis inserts the functionalized lipid directionally into the EV forming an mEV.

Figure 2. Potential mechanisms for cellular endocytosis of functionalized vesicles. A) A functionalized vesicle binds to a cell surface endocytotic receptor in Step 1 and then undergoes endocytosis in Step 2. The receptor-bound functionalized vesicle forms an endosome in Step 3 and eventually disintegrates in Step 4 to release its contents like the miRNA (R). B) A soluble receptor-ligand migrates to the plasma membrane surface from the Golgi apparatus, where it binds to a functionalized vesicle in Step 1. In order for the soluble protein-bound functionalized vesicle to be internalized by a cell, the complex binds to a cell surface receptor forming a ternary complex in step 2. If the surface receptor is endocytotic, the functionalized vesicle-bound receptor ligand is endocytosed to form an endosome in Step 3. Alternatively, if the functionalized vesicle is connected to the receptor-ligand through a long linker like PEG2000, the functionalized vesicle might

contact the plasma surface leading to endocytosis. The resulting endosome disintegrates and releases its contents, such as R in Step 4. Abbreviations: R, miRNA.

Figure 3. Size distribution of functionalized vesicles determined by dynamic light scattering (DLS). A) Detergent DDM micelles (solid line) and DDM-solubilized FAs (dashed line). The detergent solubilized FAs are an equal mixture of NBD-DSPE and DSPE-PEG2000-maleimide. The "Detergent-Dialysis Method" was used B) to produce the size distributions of LNPs (solid line) and mLNPs (dashed line) and C) to produce the size distributions of EVs (solid line) and mEVs (dashed line). The solid lines in panels D) and E) show the size distributions produced for LNPs using the extrusion approach [44] and EVs through isolation and purification protocols described in the *Materials and Methods*. The dashed lines in panels D) and E) show the size distributions produced for mLNPs and mEVs using the "Functionalized Lipid Insertion Method" described in the text.

Figure 4. Relative miRNA uptake efficiency into HepG2 cells with different treatments. A) The relative mmu-miR-298-5p (miRNA) uptake into HepG2 cells under the various treatments: Untreated HepG2 cells (Cells), HepG2 cells treated with DharmaFect 4 transfection reagent and miRNA (Cells + DharmaFect(miRNA)), HepG2 cells treated with EVs and miRNA (Cells + EVs(miRNA)), HepG2 cells treated with mEVs with an $ASGR1_{PAB}$ and containing miRNA (Cells + mEVs($ASGR1_{PAB}$, miRNA), and HepG2 cells treated with mLNPs with an $ASGR1_{PAB}$ and containing miRNA (Cells + mLNPs($ASGR1_{PAB}$, miRNA). The relative miRNA uptake was normalized against the miRNA uptake by treatment with DharmaFect(miRNA). The values in the panel represent the mean $\pm$ SD of at least three independent experiments. B) Temporal changes in miRNA uptake efficiency for mLNPs($ASGR1_{PAB}$, miRNA) (solid squares) and

mEVs(ASGR1$_{PAB}$, miRNA) (solid circles) over 72 hours with respect to U6 snRNA. The points and bars represent the average and SD, respectively. The p-values are * $p \leqslant 0.05$, ** $p \leqslant 0.01$, *** $p \leqslant 0.001$, **** $p<0.0001$. Abbreviations: SD, standard deviation.

Figure 5. Anti-proliferative effects miRNA hsa-miR-26a-5p with HepG2 cells under various treatments. A) Microscopic images of a HepG2 layer during the migration assay with the following treatments after 0 (top) and 72 hours (bottom): Untreated HepG2 cells (Cells), HepG2 cells treated with empty EVs (Cells + EVs()), HepG2 cells treated with EVs containing miRNA (EVs(miRNA)), HepG2 cells treated with empty mEVs engineered with an ASGR1$_{PAB}$ (Cells + mEVs(ASGR1$_{PAB}$)), HepG2 cells treated with mEVs with the ASGR1$_{PAB}$ and miRNA (Cells + mEVs(ASGR1$_{PAB}$, miRNA)), and HepG2 cells treated mLNPs with the ASGR1$_{PAB}$ and miRNA (Cells + mLNPs(ASGR1$_{PAB}$, miRNA)). The black square bracket in the lower right-hand image represents a distance of 0.5 μm. B) Shows quantitative measurements of HepG2 cell migration using the data and same abbreviations from panel A. The values in the bar graphs represent the mean ± SD of at least three independent experiments. C) Temporal changes in HepG2 cell migration in untreated cells (open circles) and cells treated with EVs(miRNA) (open squares), mEVs(ASGR1$_{PAB}$, miRNA) (closed circles), and mLNPs(ASGR1$_{PAB}$, miRNA) (closed squares) over 72 hours. To differentiate the functionalized vesicles, the time course for HepG2 cells treated with mEVs(ASGR1$_{PAB}$, miRNA) and mLNPs(ASGR1$_{PAB}$, miRNA) is shown as thick black lines. The error bars represent the mean of three independent experiments ± SD. The dose miRNA in each of the experiments was 0.35 μg. The p-values are * $p \leqslant 0.05$, ** $p \leqslant 0.01$, *** $p \leqslant 0.001$, **** $p<0.0001$. Abbreviations: SD, standard deviation.

Figure 6. Process of administering functionalized vesicles into mice. A) On the first day, functionalized vesicles, mEVs or mLNPs, with miRNA (R) and targeting ligands such as Abs are intraperitoneally (IP) injected into mice. B) Three days later, the mice are euthanized, and the organs are harvested. C) RNA is harvested and purified from the mouse organs. D) The amount of purified RNA from each of the organs is analyzed against U6 snRNA by qRT-PCR. E) The relative level of miRNA uptake by the mouse organs from treatment with the functionalized vesicles is determined using the $\Delta\Delta C_t$ method. Abbreviations: R, miRNA.

Figure 7. Uptake of miRNA in organs of mice treated with functionalized vesicles containing the non-targeting polyclonal green fluorescent GFP (GFP_{PAB}) antibodies. The figure shows the relative-fold uptake of miRNA in the organs of young female Nu/Nu mice (n=4) treated with (A) mEVs(GFP_{PAB}, mmu-miR-298) (gray) and (B) mLNPs(GFP_{PAB}, mmu-miR-298) (white) versus mice that were treated with SFM only (black and barely visible in the figure). The data were normalized against the U6 snRNA and expressed as mean ± SEM. The p-values are * $p \leqslant 0.05$, ** $p \leqslant 0.01$, *** $p \leqslant 0.001$, **** $p<0.0001$. The dosage for each of the mice was ~100 μg miRNA with an injection volume of 250-300 μl. Abbreviations: SEM, standard error of the mean.

Figure 8. Relative mmu-miR-298 distribution by organs with the treatment of mEVs and mLNPs bioengineered with the NPHS2 antibody. The following figure shows the fold-difference of mmu-miR-298 uptake and distribution of 5-7 week old female Nu/Nu mice being treated with (A,B) mEVs when bioengineered with NPHS2 antibody (gray, n=3) (A) versus SFM only treated mice, and (B) distribution in comparison to 5-7 week old C57BL6/J female mice treated with mEV(GFP, miRNA) (black, n=4) and (C,D) mLNPs

when bioengineered with NPHS2 antibody (gray)(n=6) versus (C) SFM only young female Nu/Nu mice, and (D) in comparison to when bioengineered with GFP antibody (black)(n=4) in various organs of 5-7 week old female C57BL6/J mice. All the data were normalized to the constitutive level of U6 snRNA to show uptake as well as to the total amount of miRNA delivered to show distribution and represent the mean± SEM. The dosage for each of the mice was ~100 μg with an injection volume of 250-300 μl.

Figure 9. Targeting of mmu-miR-298 with the ASGR1 antibody in mice. The following figure shows the fold-difference of mmu-miR-298 uptake (A,C) and the distribution (B,D) after being treated with (A,B) mEVs when bioengineered with ASGR1 antibody (gray)(n=3) in male Nu/Nu mice aged between 15-17 weeks old versus (A) SFM only treated mice (black), and (B) versus mice treated with mEVs bioengineered with GFP antibody (black)(n=4) in female C57BL6/J mice aged 5-7 weeks old and (C,D) mLNPs when bioengineered with ASGR1 antibody (gray)(n=6) versus (C) SFM only treated female Nu/Nu mice aged 5-7 weeks old, and (D) versus mice treated with mLNPs bioengineered with GFP antibody (black) (n=4) in female C57BL6/J mice aged between 5-7 weeks old. All the data were normalized to the constitutive level of U6 snRNA to show uptake as well as to the total amount of miRNA delivered to show distribution and represent the mean± SEM. The dosage for each of the mice was ~100 μg miRNA with an injection volume of 250-300 μl.

Abbreviations: SEM, standard error of the mean.

Figure 10. ACE2 targeting and immune reactivity of mEV(ACE2) and mLNP(ACE2) in-vivo. The relative ratio of mmu-miR-298 uptake in various organs of mice after being treated with (A) mEVs($ACE2_{PAB}$, miRNA) (gray) and (B) mLNP($ACE2_{PAB}$, miRNA) (white)

versus SFM only treated female C57BL6/J mice aged 5-7 weeks (black) (n=6). The data were normalized to U6 and expressed as mean± SEM of at least three independent experiments. C) Levels of 8-major cytokine factors as obtained after conducting cytokine assay on the blood samples withdrawn from mice treated with mEVs($ACE2_{PAB}$, miRNA) (gray circles), mLNP($ACE2_{PAB}$, miRNA) (white circles), and SFM only (black circles) (n=6) 72 hours after the treatment. The dosage for the mice was ~100 μg and the injection volume was 250-300 μl. The p-values are * $p \leqslant 0.05$, ** $p \leqslant 0.01$, *** $p \leqslant 0.001$, **** $p<0.0001$.

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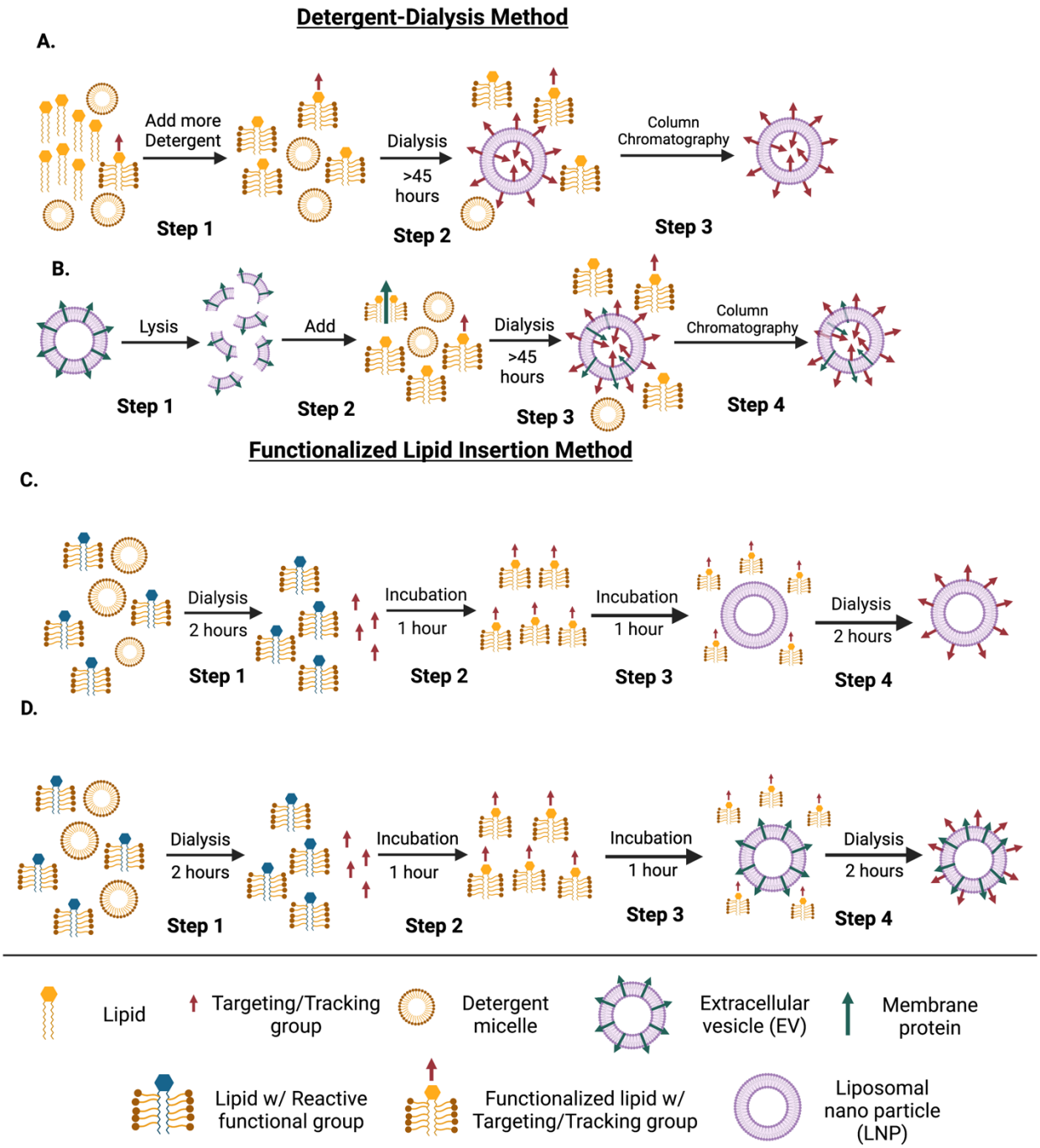

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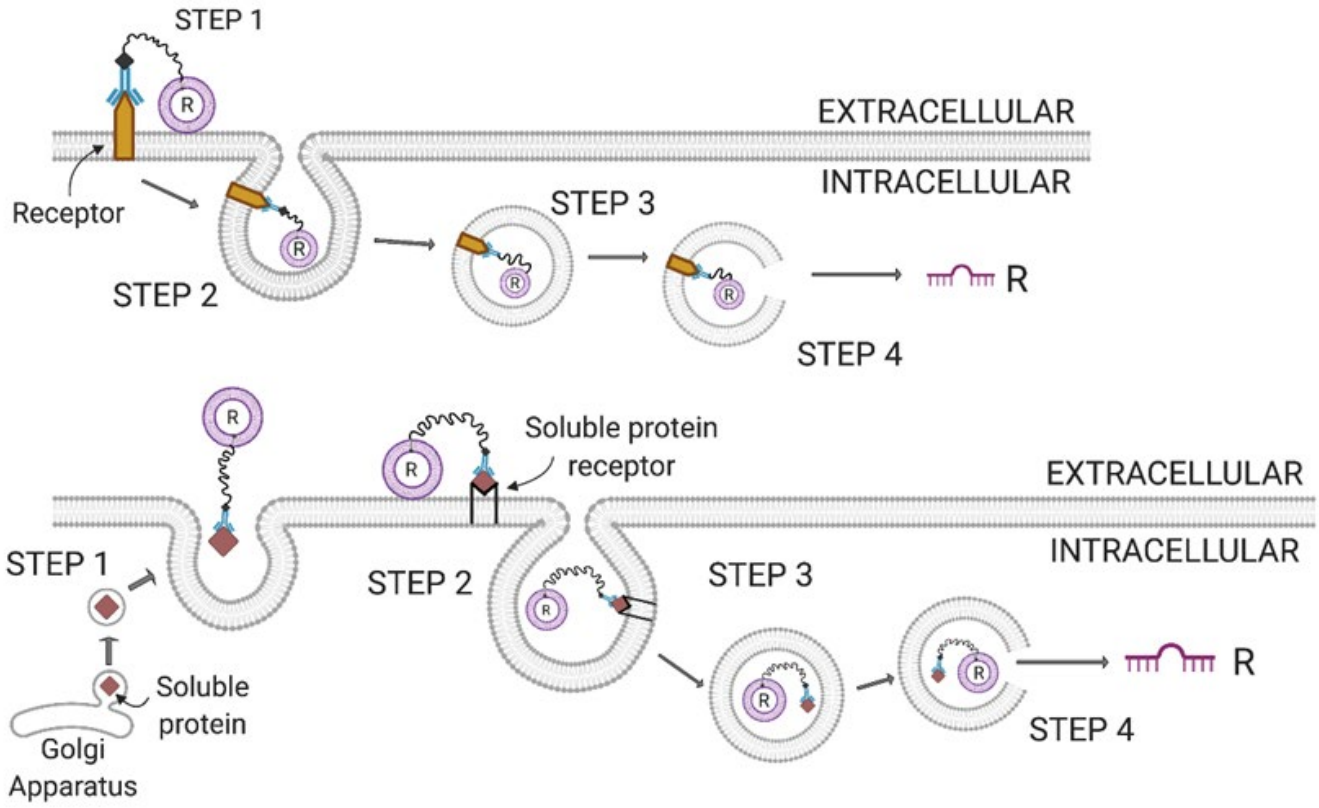
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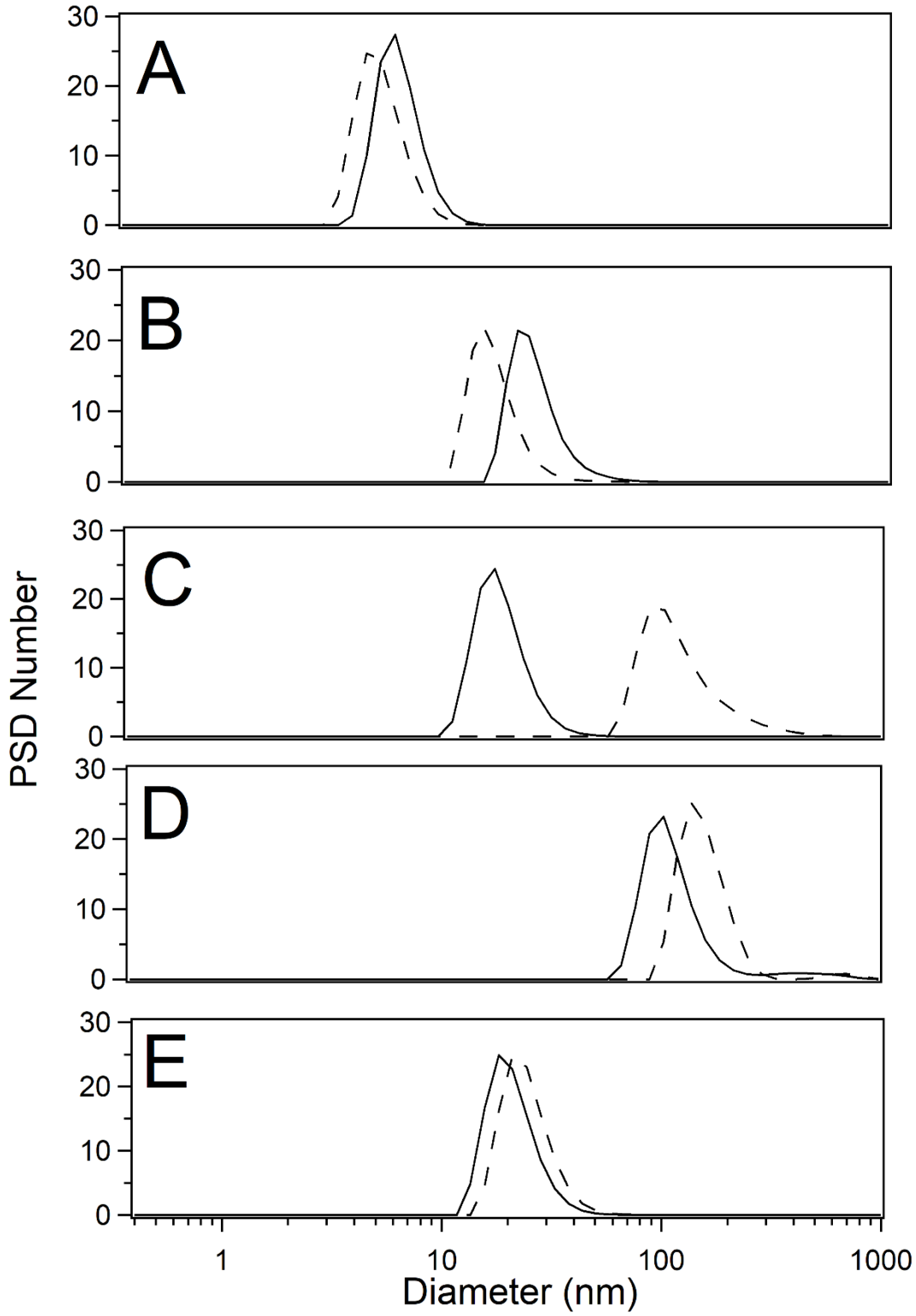

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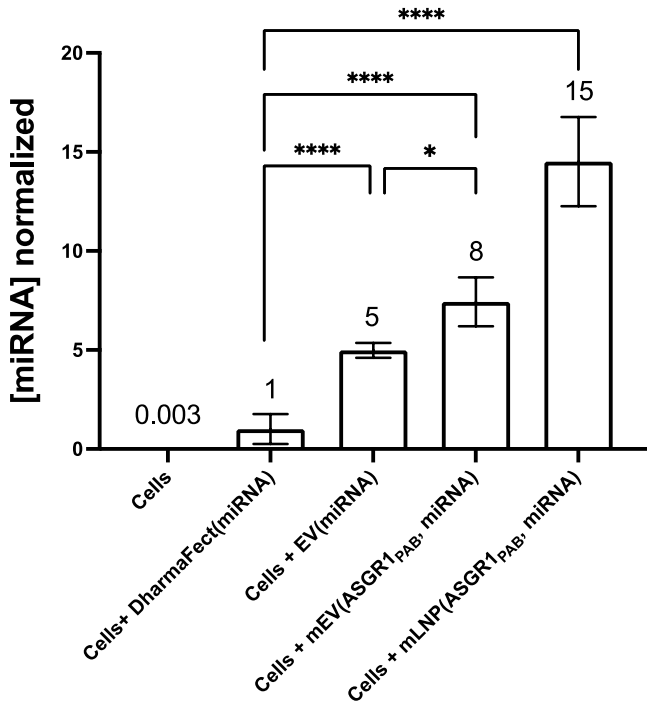
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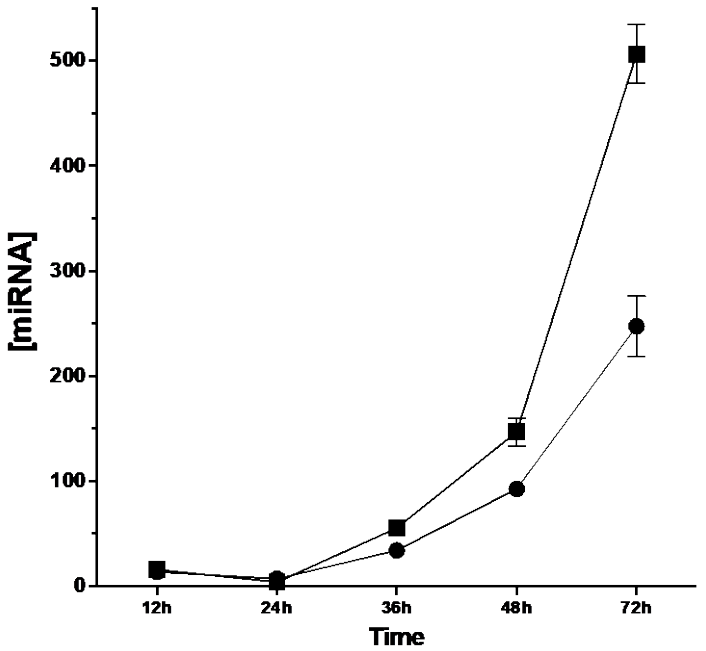
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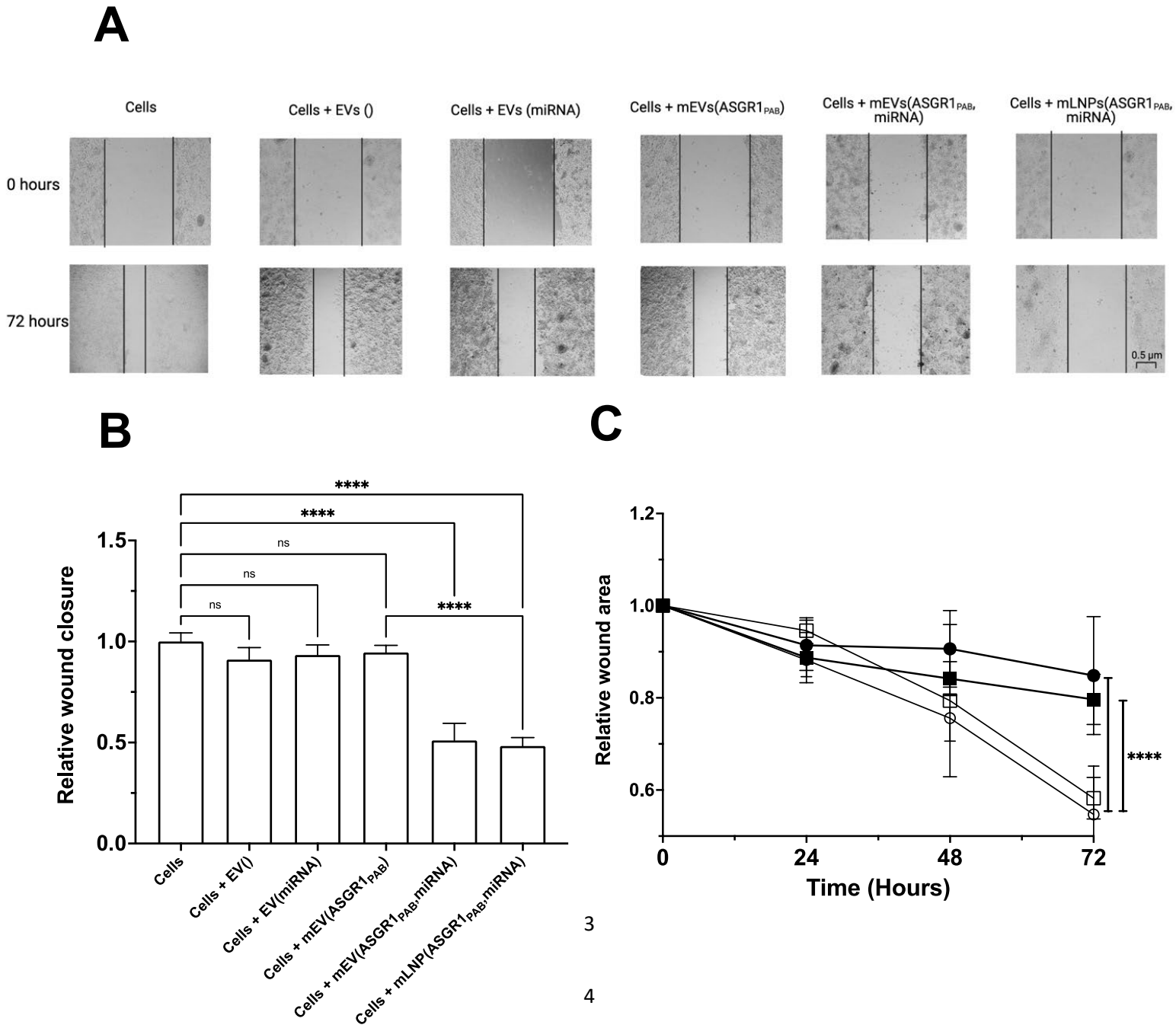

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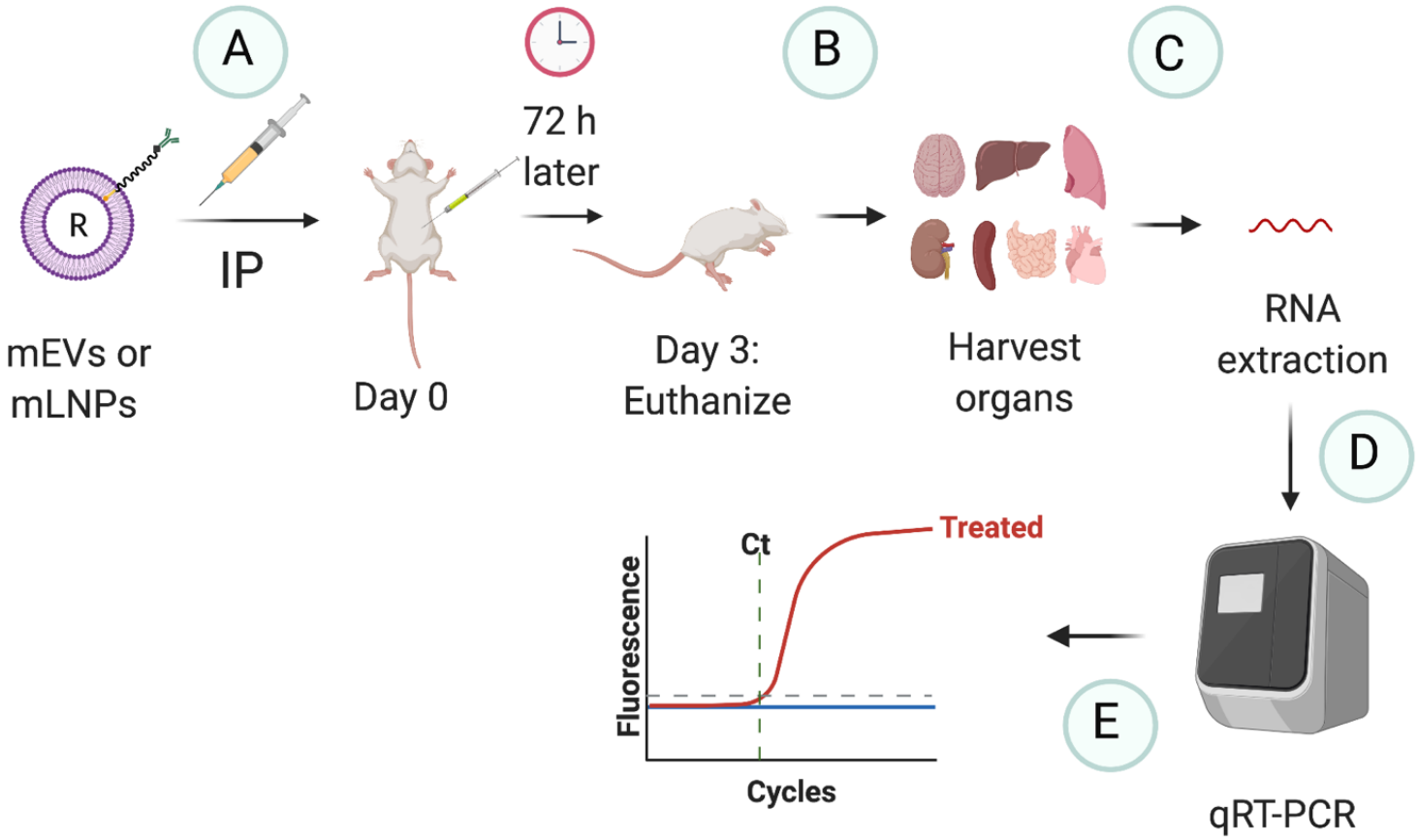
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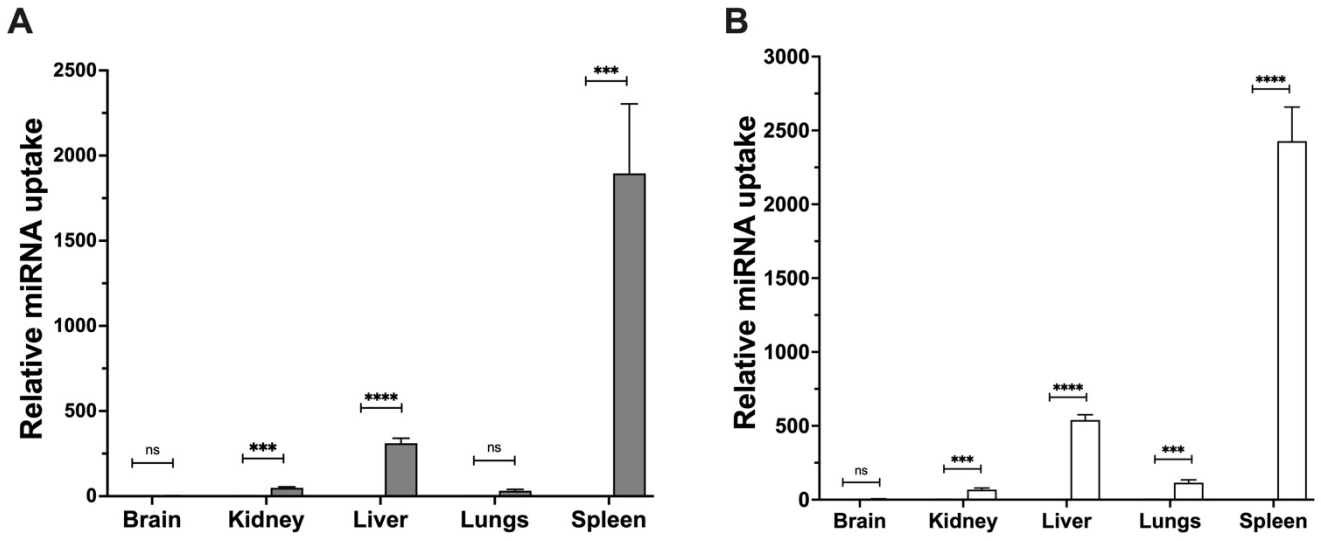

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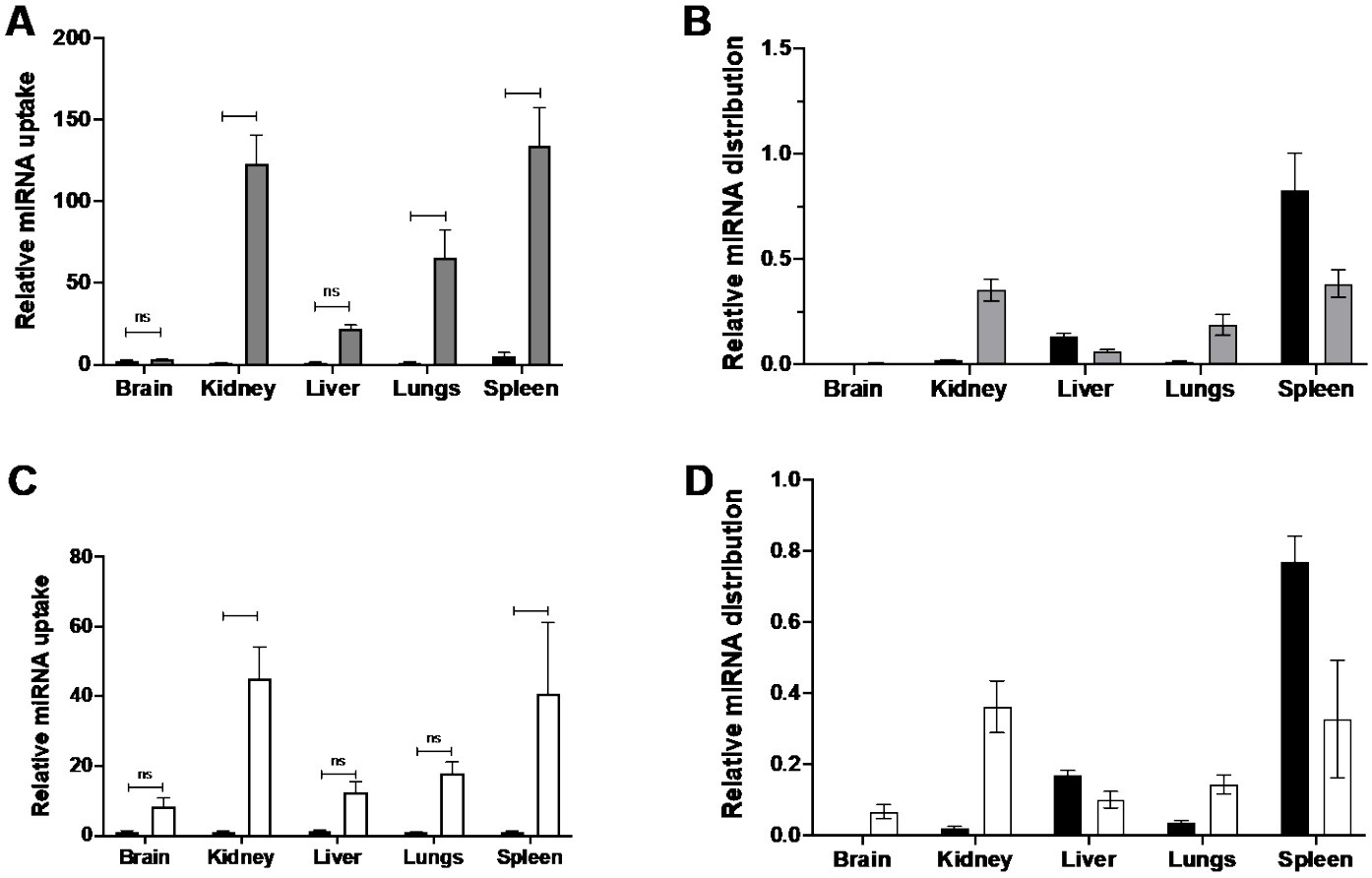

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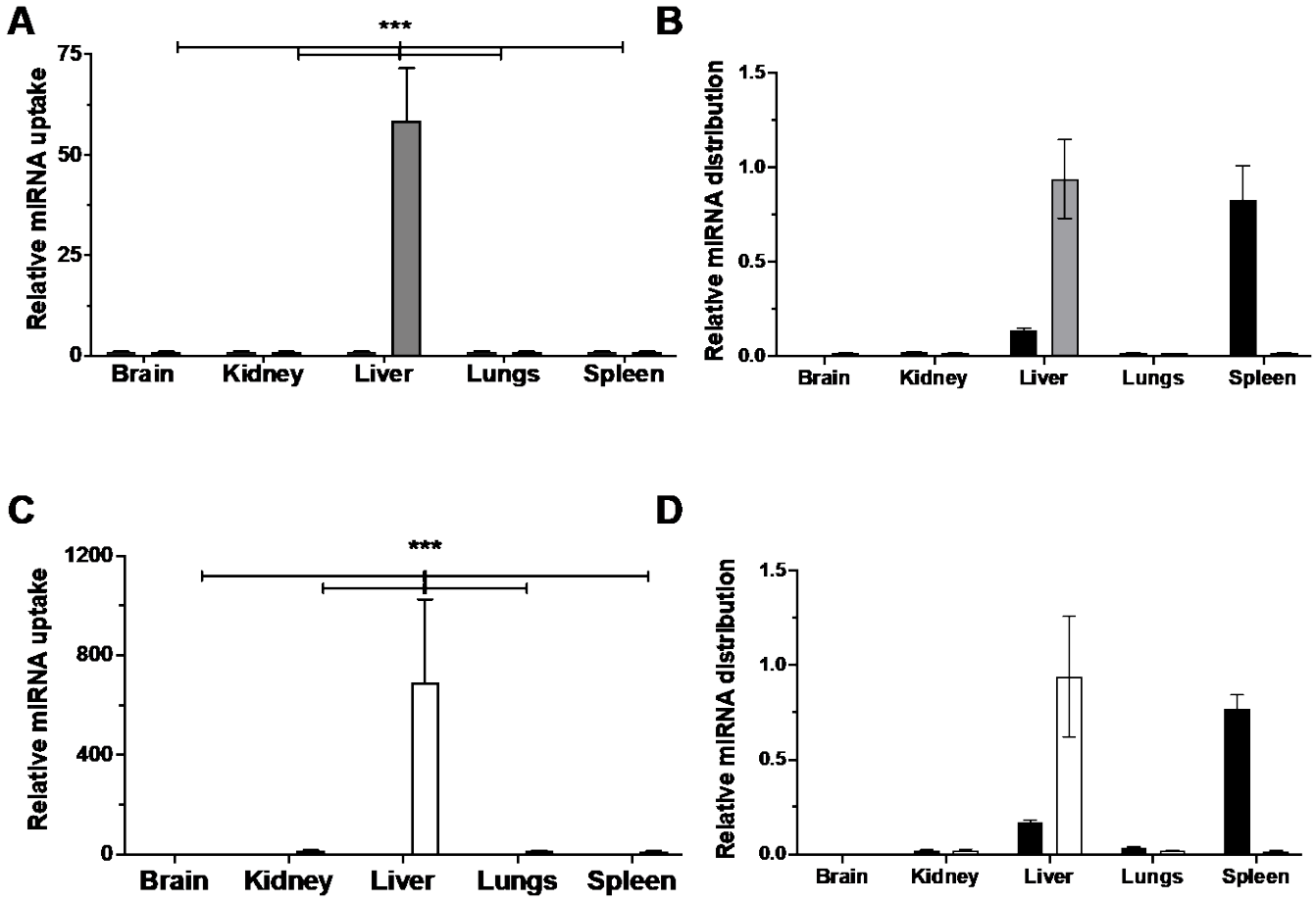
9

1 **Figure 9.**



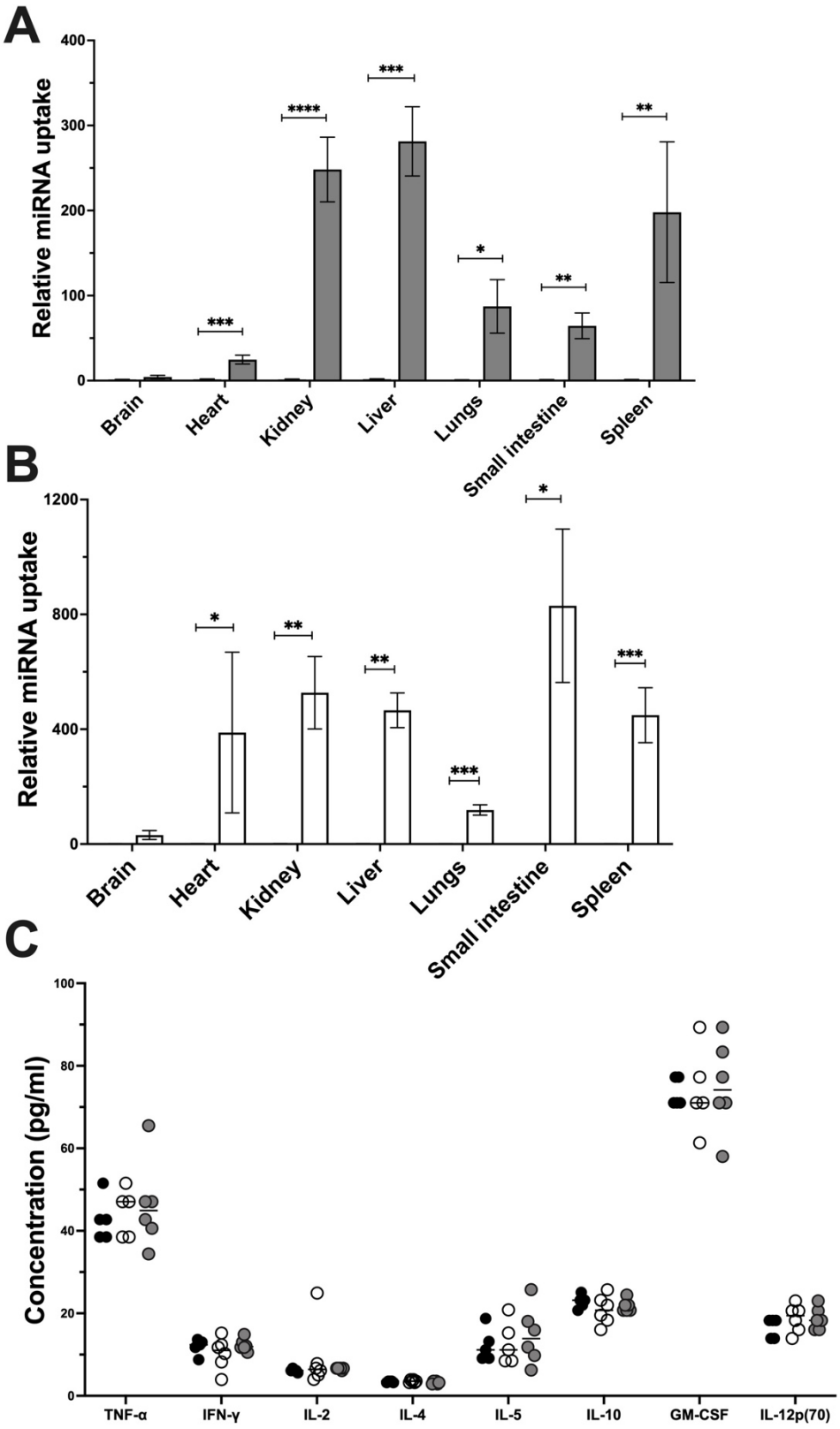
3

4

5

6

1 **Figure 10.**


A
Relative miRNA uptake
400
300
200
100
0
***
****
***
*
**
**
Brain
Heart
Kidney
Liver
Lungs
Small intestine
Spleen
B
Relative miRNA uptake
1200
800
400
0
*
**
**
***
*
***
Brain
Heart
Kidney
Liver
Lungs
Small intestine
Spleen
C
Concentration (pg/ml)
100
80
60
40
20
0
TNF-α
IFN-γ
IL-2
IL-4
IL-5
IL-10
GM-CSF
IL-12p(70)

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Supplementary Information for

A novel method for producing functionalized vesicles that efficiently deliver oligonucleotides *in vitro* in cancer cells and *in vivo in mice*[3,4,5]

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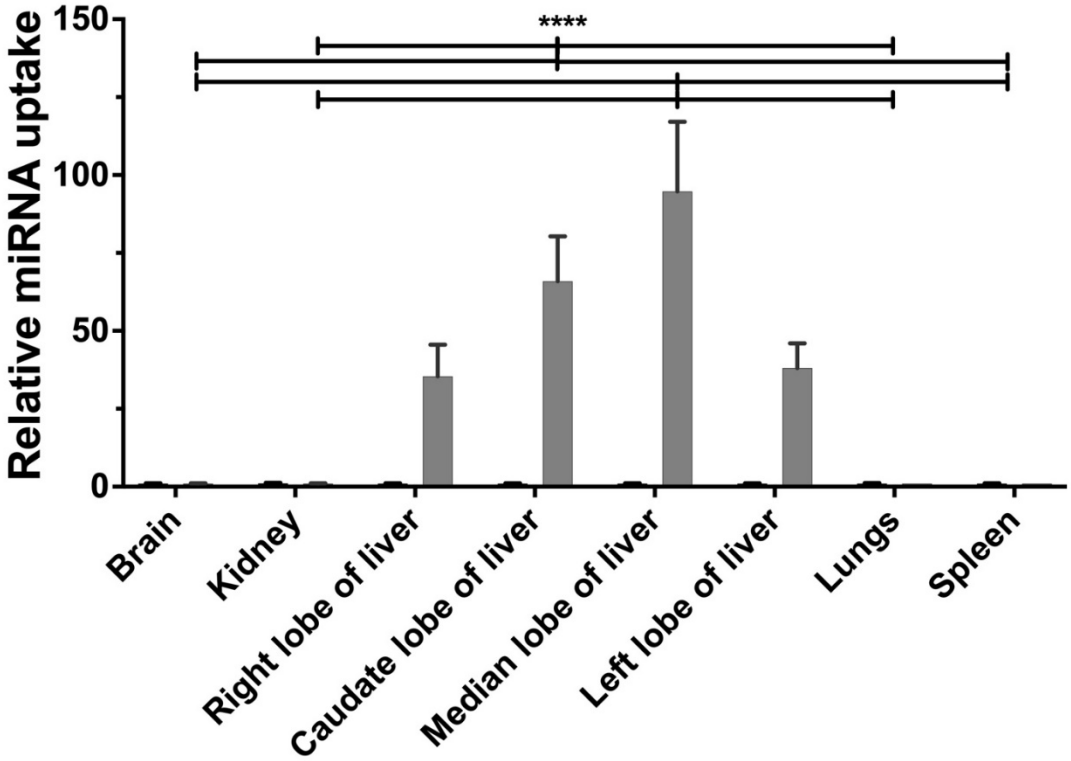
Figure	Strain	Common Name	Gender	Age	#	Treatment
7A	C57BL6/J	C57 black 6 mouse	Female	5-7 weeks	4	mEV(GFP_{MAB}, mmu-miR-298-5p)
7B	C57BL6/J	C57 black 6 mouse	Female	5-7 weeks	4	mLNP(GFP_{MAB}, mmu-miR-298-5p)
7A,B	C57BL6/J	C57 black 6 mouse	Female	5-7 weeks	4	SFM only
8A	Nu/Nu	Immunodeficient nude mouse	Female	5-7 weeks	3	mEV($NPHS2_{PAB}$, mmu-miR-298-5p)
8B	Nu/Nu	Immunodeficient nude mouse	Female	5-7 weeks	6	mLNP($NPHS2_{PAB}$, mmu-miR-298-5p)
8A,B	Nu/Nu	Immunodeficient nude mouse	Female	5-7 weeks	9	SFM only
9A, S1	Nu/Nu	Immunodeficient nude mouse	Male	15-17 weeks	3	mEV($ASGR1_{PAB}$, mmu-miR-298-5p)
9B	Nu/Nu	Immunodeficient nude mouse	Female	5-7 weeks	6	mLNP($ASGR1_{PAB}$, mmu-miR-298-5p)
9A, S1	Nu/Nu	Immunodeficient nude mouse	Male	15-17 weeks	3	SFM only
9B	Nu/Nu	Immunodeficient nude mouse	Female	5-7 weeks	6	SFM only
10A	C57BL6/J	C57 black 6 mouse	Female	5-7 weeks	6	mEV($ACE2_{MAB}$, mmu-miR-298-5p)
10B	C57BL6/J	C57 black 6 mouse	Female	5-7 weeks	6	mLNP($ACE2_{MAB}$, mmu-miR-298-5p)
10A,B	C57BL6/J	C57 black 6 mouse	Female	5-7 weeks	6	SFM only

Table S1. Mice used in these pilot studies showing the strain, gender, age and functionalized vesicle treatment given to the mice. Mice 5-7 weeks old are considered young, while mice aged 15-17 weeks are considered adults.

Abbreviations: $ASGR1_{MAB}$, ASGR1 receptor monoclonal antibody; GFP_{MAB}, green fluorescent protein (GFP) monoclonal antibody; $NPHS2_{PAB}$, NPHS2 or podocin polycolonal antibody; mEV, modified extracellular vesicle; mLNP, modified liposomal nanoparticle; SFM, Serum Free Media;

1



13 **Figure S1: The relative miRNA uptake by different mouse liver lobes after mEV**
14 **treatment of vesicles engineered with the ASGR1 receptor polyclonal antibody and**
15 **loaded with mmu-miR-298.** The following figure shows the relative miRNA uptake after
16 being treated with mEVs bioengineered with the ASGR1 receptor polyclonal antibody and
17 loaded with mmu-miR-298 (mEV(ASGR1$_{PAB}$, mmu-miR-298)) (gray) (n=3) in adult male
18 Nu/Nu mice versus SFM only treated adult male mice (black). All the data were
19 normalized to the constitutive level of U6 snRNA and represented the mean ± SEM of
20 three independent experiments. The mEV dosage given for each of the mice was ~100
21 μg miRNA with an injection volume of 250-300 μl.

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