IN-VITRO AND IN-VIVO GENETIC REGULATION OF P-GLYCOPROTEIN

by

PRAGATI JAIN

(Under the Direction of Arthur G. Roberts)

ABSTRACT

The ATP binding-cassette (ABC) transporters are a protein superfamily that efflux endogenous molecules and drugs through ATP hydrolysis. This superfamily has five subfamilies, but the most studied and ubiquitous ABC transporter isoform is ABCB1 (P-glycoprotein or P-gp). This isoform plays a critical role in drug disposition, serves as a gatekeeper across many critical biological barriers, and plays a major role in human disease. The protein expression within the superfamily is highly regulated on genetic, translational, and post-transcriptional levels. In particular, short oligonucleotides called microRNAs are known to have significant effects on ABC transporter gene expression. A lot of interest exists in therapeutic applications for microRNAs since they can potentially reduce disease-causing effects from these transporters. Specific inhibitors have been developed for P-gp and other ABC transporters to combat disease, but they have been plagued by unacceptable toxicity in the clinic. To overcome this therapeutic roadblock, we have developed a novel method to bioengineer miRNA delivering vesicles to specific tissues called the “Functionalized Lipid Insertion Method.” The method produces miRNA delivering vehicles from extracellular vesicles (mEVs) and liposomal nanoparticles (mLNPs), which can be loaded with microRNA by electroporation. In vitro experiments with hepatoma HepG2 cells show that microRNA uptake and functional effects are significantly enhanced with these modified

vesicles over other approaches. Functionalized vesicles were designed for in vivo experiments with different antibodies to determine the organ miRNA delivery specificity in mice. Functionalized vesicles made with non-targeting antibodies delivered microRNA to the spleen due to the intraperitoneal route of administration. Functionalized vesicles targeting the endocytotic liver receptor delivered microRNA specifically to the mouse liver, while functionalized vesicles targeting a non-endocytotic kidney receptor were less specific. To determine the immunogenic effects of these vesicles, functionalized vesicles were designed with antibodies that are known to target multiple organs. Mice treated with these functionalized vesicles exhibited no physical manifestations of an immune response or showed significant changes in their cytokine levels. Ultimately, our technology has a lot of potential to treat devastating diseases such as cancer as well as the ability to scale up production for the pharmaceutical industry.

INDEX WORDS: P-gp, ABC transporters, Extracellular vesicles, Liposomes, MicroRNA delivery, Genetic regulation

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DEDICATION

I would like to dedicate this thesis to my parents, Adarsh Kumar Jain, and Rajani Jain. If it was not for their sacrifices and hard work, I wouldn't have made it till here. For as long as I can remember, my parents have nurtured my curiosity, and encouraged me in every avenue of my life. From solving math problems before going to bed to running behind a cotton candy vendor to make sure I get one, my dad has always carried my dreams in his eyes and cleared a path for me to reach there. Papa, thank you, for fighting the prejudices set by our society, and for being the wind beneath my wings. Although my dad supported me financially and is my backbone, my mom is the crackle in my laughter and the sparkle in my eyes. She is the one responsible for me wanting to be a scientist. I remember how she would wake up at 4 am in the morning to make sure she finishes all her chores by the time I wake up so that she can teach me. She often says that she stopped teaching me after I entered seventh grade, however, I know her teachings never left me and it was all those early mornings she put in, that made me a stronger woman. Mumma, thank you, for believing in me, for showing me that I can be anything, do anything, and achieve everything. I know I have a long road ahead of me still, but without the blessings and love of my parents I wouldn't have reached this important platform of my life. I hereby dedicate my life's work to them.

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CHAPTER 1

GENETIC REGULATION OF ABC TRANSPORTERS

1. Pragati Jain[3] and Arthur G. Roberts[3]
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Abstract

ATP binding cassette (ABC) transporters are superfamily of proteins that move drugs and other molecules unidirectionally across membranes through ATP hydrolysis. More than forty ABC transporters isoforms have been identified in humans and are involved in various cellular processes such as maintenance of lipid homeostasis, antigen processing, cell division, and immunity. They exhibit many roles including drug disposition and several diseases. The genetic regulation of these transporters holds paramount significance, as the world is moving more and more towards gene therapy. Understanding genetic regulation becomes even more cardinal as we are beginning to understand the role that oligonucleotides play in modulating protein expression post-transcriptionally and how this can be exploited therapeutically. This review will thus summarize essential factors involved in the transcriptional and post-transcriptional regulation of ABC transporters. The labyrinth of signaling pathways and transcription factors, as well as the miRNAs that have been implicated in regulating these transporters directly or indirectly, will be discussed in detail.

Keywords: Transcriptional, ABC transporters, MicroRNAs, Post-transcriptional, Genetic

1.1 Introduction

ATP Binding Cassette (ABC) transporters represent a superfamily of integral membrane proteins that play a critical role in molecular transport [1,2]. They function as energy-dependent uniporters by effluxing molecules unidirectionally across the lipid bilayer through the ATP hydrolysis [1,2]. The superfamily is classified into seven subfamilies, ABCA-ABCG, based on their gene structure, amino acid sequence, domain organization, and phylogenetic analysis [3–5]. In humans, the superfamily plays a significant role in drug disposition by being highly expressed within the apical and basolateral sides of cells within the intestines, livers, and kidneys [1]. They also play an important role as gatekeepers of drugs such as the blood-brain barrier, blood-testes barrier, and the blood-placental barrier [1]. They also play a significant role in human diseases such as cancer [6] and cystic fibrosis [7].

The expression levels of transporters are highly regulated [8,9]. ABC transporter regulation affects all stages of ABC transporter maturation from genetics to post-transcriptional modification and ultimately localization within the cells [10]. The expression of ABC transporters is regulated by transcription factors, signaling pathways, ligands, and polynucleotides [8,9]. This review provides an overview of the different ABC transporter subfamilies followed by the major signaling pathways, nuclear transcription factors, and microRNAs involved in their regulation.

1.2 ABC Transporter Subfamilies

All functional ABC transporters consist of two transmembrane domains (TMD) comprised of six helices and two nucleotide-binding domains (NBDs [2,3,11,12]. Most isoforms within the ABCA-ABCC subfamilies have two TMDs and two NBDs with the TMD-NBD-TMD-NBD topology [2,3,11,12]. The ABCA subfamily has additional extracellular (EC) domains, and some

isoforms of the ABCC subfamily have an extra TMD known as TMD0 [2,3,11,12]. Some isoforms of the ABCB subfamily, ABCB2, ABCB3, and ABCB10, and the members of the ABCD subfamily function as homodimers and heterodimers, with each monomer having TMD-NBD topology [2,3,11,12]. The ABCG transporter subfamily forms homodimers like the ABCD subfamily, but the monomer topology is reversed with NBD-TMD instead of TMD-NBD topology [2,3,11,12]. Finally, members of the ABCE and the ABCF subfamilies contain two connected NBDs but lack the ability to transport due to the lack of TMD units [13–15]. Since proteins of the ABCE and ABCF subfamilies are not transporters, they are not discussed in the review.

1.2.1 ABCA

The human ABCA transporter subfamily encodes 12 genes: ABCA1-10 and ABCA12-13 [16]. The break in the gene naming is due to a non-functional gene that was incorrectly named ABCA11 [16]. A distinct feature of the ABCA subfamily of transporters is that they contain two extracellular (EC) domains [17,18]. The EC domains have been implicated in substrate binding and contribute to lipid trafficking by the transporter [19–21]. The ABCA subfamily of transporters has broad substrate specificity and is essential in maintaining lipid homeostasis [22–24].

Several isoforms within the subfamily have been associated with several human diseases [25]. Mutations in the ABCA1 transporter isoform and decreased expression of the transporter are associated with atherosclerosis, high-density lipoprotein deficiency, and Tangier disease that causes cholesterol ester depositions [26–28]. Defects with the ABCA3 transporter isoform are associated with neonatal surfactant deficiency and pediatric interstitial lung diseases [25,29]. The ABCA4 transporter isoform is associated with the retinal Stargardt disease and the age-related eye disease called macular degeneration [30]. Mutations in the ABCA12 transporter isoform gene lead

to a rare and lethal skin disease called Harlequin ichthyosis [31]. Several ABCA transporters are associated with Alzheimer's disease, including the ABCA1, ABCA2, and ABCA7 transporter isoforms through regulating amyloid-ß availability in the brain [24,32,33]. In addition, the ABCA2 and ABCA3 transporter isoforms are associated with anticancer drug resistance in children suffering from leukemia [34].

1.2.2 ABCB subfamily

The human ABCB subfamily (MDR transporters) has 11 genes, ABCB1 to ABCB11 [35]. Four isoforms within the transporter subfamily function as monomers and are known as full transporters [35]. The remaining seven transporters can only function by forming homodimers and heterodimers with each other [35]. The individual transporter monomers are called half transporters [35]. A characteristic structural feature of this subfamily is TMDs, where helices 4 and 5 cross each other [35]. The most studied human ABCB transporter subfamily member is ABCB1, which is also known as P-glycoprotein (P-gp) and the multiple drug resistance 1 (MDR1) transporter in humans [1,35]. The transporter plays a major role in drug disposition and strongly affects drug absorption, distribution, and excretion [36–40]. The transporter isoform is highly expressed at several critical biological barriers such as the blood-brain barrier and the blood-retinal barrier [36–40]. ABCB1 recognizes a wide range of drug substrates, including many cytotoxic anticancer drugs, and thus, its overexpression is significantly correlated with the development of multidrug resistance in different types of cancers [41–45]. Expression of ABCB1 is associated with higher relapse rates, decreased survival rates in cancer therapy[44–46]. The ABCB4 transporter isoform expresses on the apical membranes of hepatocytes [47]. The transporter facilitates phosphatidylcholine transportation from hepatocytes into the bile canaliculus [47]. As a

result of this function, ABCB4 transporter isoform mutations cause intrahepatic cholestasis type 3 [47]. Another ABCB11 transporter isoform is also expressed on the apical membranes of hepatocytes [48]. The transporter is a bile salt export pump, and ABCB11 transporter isoform mutants cause intrahepatic cholestasis type 2 [48]. The ABCB2 and ABCB3 transporter isoforms are known as transporter-associated with antigen processing 1 (TAP1) and transporter-associated with antigen processing (TAP2), respectively [49]. These transporter isoforms are half transporters with an extra N-terminal TMD consisting of 4 helices [49]. They transport antigenic peptides from the cytosol to the lumen of the endoplasmic reticulum (ER), thus, playing an important role in the adaptive immune system [49]. The ABCB9 transporter isoform expresses in the lysosomes and mediates peptide efflux to the cytosol [50]. The ABCB6, ABCB7, ABCB8, and ABCB10 transporter isoforms are half transporters expressed in mitochondria [51–54]. They are involved in iron metabolism and transport of Fe/S cluster protein precursors [51–54]. Specifically, the ABCB6 transporter isoform maintains porphyrin concentrations with the assistance of ABCG2 transporter isoform [51,55,56]. They both maintain the hemoprotein homeostasis in mitochondria [51,55,56]. Mutations in the ABCB6 transporter isoform correlate to the severity of hereditary porphyria or familial pseudohyperkalemia characterized by fever, tiredness and weight loss [55,57].

1.2.3 ABCC subfamily

The ABCC transporter subfamily has 13 genes, which is the most of any subfamily member [58]. Except for the pseudogene ABCC13, ABCC8, and ABCC9, the remaining transporter isoforms in this subfamily are known as multidrug resistance-associated proteins (MRPs) [58]. Additionally, the ABCC7 transporter is also known as the cystic fibrosis transmembrane conductance regulator, because of its association with cystic fibrosis, functions as a chloride ion

channel conductor across epithelial membranes [59,60]. Specific mutations in the ABCC7 gene lead to cystic fibrosis, characterized by coughing from mucus buildup and frequent lung infections [61].

All MRPs, except for MRP4 and MRP5, transporters possess an additional c-terminal 5-helix domain called TMD0 [58]. The ABCC8 and the ABCC9 transporters are known as the sulfonylurea receptors 1 (SUR1) and 2 (SUR2), respectively [58,62]. SUR1 and SUR2 isoforms get their name from their substrates, namely, sulfonylurea antidiabetic drugs that they transport [58]. They also possess a c-terminal domain TMD0 domain like many MRPs [58,63,64]. These receptors are targeted by various compounds that bind and either stimulate or inhibit K(ATP) channel activity [62,65]. Mutations in ABCC8 and ABCC9 are associated with Brugada (BrS) and Early repolarization (ERS) syndromes that have been characterized as J wave syndromes associated with sudden cardiac death [66].

Several isoforms in the ABCC subfamily are promiscuous transporters, contributing to multiple drug-resistance like the ABCB subfamily [67]. ABCC1 (MPR1) and ABCC3 (MPR3) transporter isoforms express significant amounts on the basolateral membrane of most epithelial cells [68]. ABCC1 (MRP1) transporter isoforms function as a protective barrier to drug penetration into the cerebrospinal fluid and are overexpressed in many cancers exhibiting chemoresistance to many drugs, especially natural products [69–72]. ABCC2 (MPR1) and ABCC4 (MRP2) transporter isoforms play an essential role in drug disposition through drug secretion [67,73–75]. As a result, they are highly expressed in the liver, the intestine, and the kidney [67,73–75]. Defects in the ABCC2 gene and expresses the MRP2 transporter isoform protein cause Dubin-Johnson

syndrome related to bilirubin metabolism in the liver characterized by jaundice during adolescence or early adulthood [76].

1.2.4 ABCD subfamily

The ABCD transporter subfamily has four genes, ABCD1-4 [77]. The ABCD transporters are half transporters expressed in the peroxisomes, endoplasmic reticulum, and lysosomes [77,78]. ABCD1-3 are peroxisomal proteins, and one of the functions of these transporters is to mediate the efflux of substrates for β-oxidation by specific acyl CoA synthetases and further metabolism [79,80]. ABCD1 and ABCD2 function as homodimers and are known to transport long and very long chain fatty acids (VLCFA) or their Co-A derivatives across the peroxisomal membrane [81]. ABCD3 has been postulated to play a role in the oxidation of dicarboxylic acids, an intermediate in the synthesis of bile acids, which is a crucial step in bile acid biosynthesis [82]. Mutations in the ABCD1 and ABCD3 genes lead to X-linked adrenoleukodystrophy, which causes a fatty acid buildup and makes individuals with the disease, prone to seizures and hyperactivity [83]. In addition, defects in ABCD3 can cause hepatosplenomegaly, a liver disease that can cause jaundice and liver failure [84]. ABCD4, on the other hand, is mainly expressed in the endoplasmic reticulum (ER) and not peroxisomes because it lacks the NH2-terminal hydrophilic region required for peroxisomal targeting [85]. ABCD4 is translocated to lysosomes through an interaction with the lysosomal membrane protein LMBD1, where it is involved in the intracellular processing of cobalamin [86]. Mutation in lysosomal ABCD4 causes a newly identified inborn error of Vitamin B12 (cobalamin) metabolism by failing to release Vitamin B12 from lysosomes that can lead to dementia or even paralysis [87].

1.2.5 ABCG subfamily

The ABCG transporter subfamily is comprised of five genes: ABCG1-5 [3,88]. Many members of the ABCG family are known to play a role in lipid transport across membranes [89]. The ABCG1 and ABCG4 isoforms efflux excess cholesterol to high-density lipoprotein, which plays a vital role in transporting cholesterol from macrophage to liver [88,90]. ABCG1 is abundantly expressed in the liver, lung, and spleen and is associated with the endoplasmic reticulum and the golgi membrane of macrophages [90–92]. ABCG4 is homologous to ABCG1; however, the expression of ABCG4 is more restricted to nervous tissues such as the brain [91,93]. Patients with non-small lung cancer with high expression of the ABCG4 transporter isoform had a poor prognosis suggesting a role for this isoform in multiple drug resistance [94]. Mutations in ABCG5 and ABCG8 can cause sitosterolemia, a disease characterized by xanthomatosis and premature atherosclerosis [95,96]. ABCG5 and ABCG8 are expressed at high levels in the canalicular membrane of hepatocytes and in the apical membranes of enterocytes in the small intestines and colon to mediate hepatobiliary cholesterol transport and limit the absorption of sterols from the lumen [95,97,98]. ABCG1, ABCG4, ABCG5, and ABCG8 translocate steroids and lipids, while ABCG2, also known as the breast cancer resistance protein (BCRP), because it originated from a breast cancer cell line, is known to confer multiple drug resistance in cells [96,98,99]. Human ABCG2 is expressed in the canalicular membrane of hepatocytes, the apical membrane of small and large intestines and human proximal tubule cells, the luminal membrane of the capillary endothelium of brain, and in the brush border membrane of placental syncytiotrophoblasts [100]. ABCG2 has been identified as a urate transporter and a urate excretion promoter in the kidney [89]. ABCG2 expression leads to the development of resistance on a range

of cancer cells to toxic ions and various anticancer agents [101–105]. ABCG2 has also been shown to play a protective role in Alzheimer's neuroinflammatory response [106].

1.3 Regulation of ABC transporters

It is essential to understand the regulatory aspects of these transporters especially from a therapeutic point of view. One aspect that affects the expression of any protein is the transcription and the factors regulating transcription. A major factor in ABC transporter expression is regulation of its transcription and post-transcription. Transcriptional regulation consists of complex networks modulating the transcription of mRNA from DNA, and post-transcriptional regulation includes the factors affecting mRNA expression [85,86]. The fate of a gene is thus determined by the complexity and accessibility of a wide range of response elements as well as the transcription factors interacting with these elements [109]. Protein expression can thus be affected by a myriad of signaling pathways, transcription factors, and oligonucleotides such as miRNA.

1.3.1 Transcription factors and Signaling pathways

A network of signaling pathways and transcription factors intersect together to regulate ABC transporters at the transcription level. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) has been found at the centre of the network. A lot of factors such as cytokines or downstream substrates of Mitogen-activated protein kinase (MAPK) signalling cascades modulate the transcription of different ABC transporters via affecting NF-κB phosphorylation directly or indirectly. Some NF-κB independent pathways directly regulating the transporters have also been identified. (Figure 1.1)

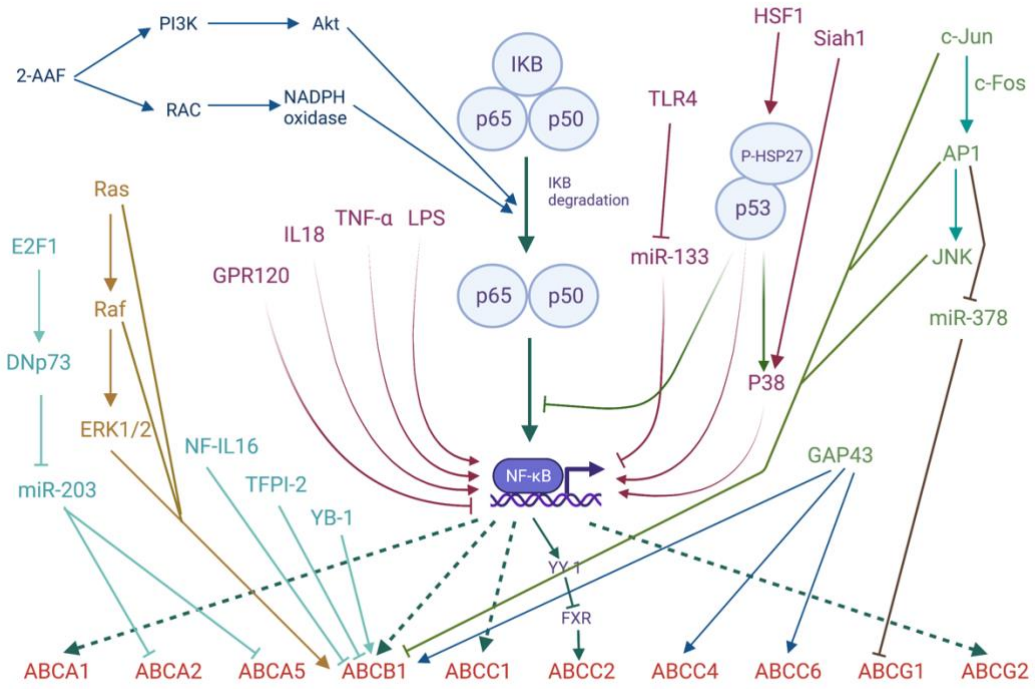

Figure 1.1: Transcription factors and signaling pathways involved in regulation of ABC transporters.

1.3.1.1 Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)

NF-κB is a protein complex found in almost all cell types and plays an essential role in mediating cellular survival [110]. It is present in an inactive state in the cytoplasm bound to IκB. The common pathway that can lead to the activation of NF-κB is where IκB is phosphorylated and degraded by the IKKb (Inhibitor of κB Kinase) complex enabling the NF-κB dimers to enter the nucleus and activate specific target gene expression [111–113]. Several stimuli and factors are known to target gene expression by activating NF-κB. For instance, Lipopolysaccharides downregulate the mRNA expression of ABCA1 and Scavenger receptor-1 (SR-B1) in macrophages like RAW 264.7 cell line possibly by activating NF-κB thus inducing cholesterol

accumulation in macrophages contributing to atherogenesis [114]. Furthermore, NF-κB was shown to downregulate ABCA1 and ABCG1 by directly binding to the promoter of Sterol regulatory element binding protein-2 (SREBP-2), a transcription factor regulating cholesterol and fatty acid synthesis, and miR-33a in mice [114–116]. Another study found that GPR120, a receptor for long-chain fatty acids, upregulates the expression of ABCB1, ABCC1, and ABCG2 by inhibiting NF-κB activation leading to the development of Epirubicin resistance in breast cancer cells [117]. Toll-like receptor-2 (TLR2), a cytokine, negatively regulated ABCA1 expression via NF-kB activation and miR-33 in THP-1 macrophage-derived foam cells treated with *Chlamydia pneumoniae* [118].

In contrast, another cytokine, TNF-α acts via NF-κB to induce ABCA1 expression in macrophages that helps phagocytes to deal with the oxidant stress and reactive oxygen species generation, protecting against atherosclerosis [119]. TNF-α also upregulated ABCC1 expression and functional activity in glial cells in a concentration-dependent manner. The release of TNF-α in glial cells was triggered by NF-κB after exposure to HIV-1 gp-120 protein [120]. IL-18 represses ABCC2 at both the mRNA and protein levels in a dose-dependent manner in human hepatoma HepG2 cells and in the bile duct ligated Sprague Dawley rat livers. IL-18 activates NF-kB, which then increases the expression of YinYang 1 (YY-1), a transcription factor. YY-1 then inhibits the nuclear receptor, Farnesoid X receptor, which has been shown to induce ABCC2 expression, thus, leading to decreased ABCC2 expression [121]. In another study, transcriptional inhibition of the oxidative stress-responsive heat shock factor 1(HSF1) and expression of heat shock proteins (Hsp) such as Hsp27 induced MDR1 gene and P-glycoprotein by mutating p53, a tumor suppressor protein, resulting in a multidrug-resistant phenotype. An NF-kB mediated regulation in ABCB1

expression was also observed by both Hsp27 and mutant p53 [122]. Interestigly, P53 also targets the Mdr1 promoter gene in NIH3T3 cells, an immortalized mouse embryonic fibroblasts cell line directly [123]. 2-acetylaminofluorene (2-AAF) is known to activate ABCB1 in rat and human HepG2 cells or Hek293 cells. The activation of ABCB1 involved an NF-kB binding at -6029 site located distal to the ABCB1 promoter in human cells [124]. The study showed that 2-AAF activates phosphoinositide 3-kinase (PI3K) which then further activates two downstream targets: a serine/threonine kinase, Akt and a small G-protein, Rac. Rac is a mitogenic protein that activates NADPH oxidase which catalyzes the production of reactive oxygen species (ROS). These downstream targets (ROS and Akt) phosphorylate IKKb leading to the activation of NF-kB [124].

Additionally, NF-κB induces Daunomycin resistance by transactivating the ABCB1 gene by binding to an intronic site on the gene in HCT15 colon cancer cells [125]. NF-κB similarly induces Sulfasalazine resistance in human T cells by upregulating ABCG2 [126]. NF-kB inhibition by dehydroxymethylepoxyquinomicin (DHMEQ) significantly increased anti-tumor activity of 5-fluorouracil, cisplatin, and doxorubicin and decreased ABCB1, ABCC1, and ABCG2 levels in Cholangiocarcinoma cell lines [127,128]. Arsenic trioxide, an anticancer against acute promyelocytic leukemia, increased the ABCG2 concentration in human lung adenocarcinoma A549 cells by activating NF-kB [129]. Pretreating the rats with pyrrolidine dithiocarbamate salt (PDTC), a selective inhibitor of NF-κB abolished the increased ABCB1 expression in the hippocampus and amygdala complex area 24 hours after the onset of kainic acid-induced seizures [130]. Increased expression of acid ceramidase has also been associated with activated NF-kB induced increase in ABCB1 expression in acute myeloid leukemia cell lines [103].

In parallel to these studies, natural inhibitors of NF-kB such as licocalchone A, anacardic acid, celastrol, xanthumol, magnolol, and honokiol increase the cellular accumulation of daunorubicin and rhodamine-123 in a concentration-dependent manner by decreasing ABCB1 activity in MDR1 transfected cervical cancer (KB-1-3) cells. In addition, these compounds exhibited chemosensitizing effects by reversing ABCB1 mediated multidrug resistance with the highest effects shown by anacardic acid[132]. Along with chemical and natural inhibitors, knocking down NF-kB by siRNA also decreased ABC transporters levels such as ABCB1 and ABCC1 [108]. siRNA mediated inhibition of NF-kB in doxorubicin-resistant breast cancer (MCF-7) cells consequently decreased the IC50 of cells against doxorubicin from 2525 to 879 nM, thus increasing the drug-associated cytotoxic effects in resistant cells [133].

These studies when brought together show that the role of NF-kB in mediating the levels of ABC transporters is very well defined. NF-kB has been able to target ABC transporters directly as well as indirectly by activating other downstream targets. However, the role of NF-kB in upregulation or downregulation can vary with different cell types and depend on the pathways leading to its activation.

1.3.1.2 MAPK cascades

Mitogen-activated protein (MAP) kinase signaling pathways are evolutionarily conserved kinase modules that control fundamental cellular processes such as growth, proliferation, differentiation, migration, and apoptosis [134–136]. They are generally classified into three well-characterized signaling pathways: the c-Jun N-terminal kinase (JNK), the p38 mitogen-activated protein kinase (p38-MAPK), and the extracellular signal-related kinase (ERK) [135,137]. Multiple stimuli can activate members of the MAPK family, and once activated, they can function as

effector protein kinases to phosphorylate several substrates such as p53, NF-κB, Signal transducer and activator of transcription-1 (STAT1), and Heat shock factor-1 (HSF-1), leading to several changes in cells [138,139]. These upstream and downstream substrates are known to then affect the transcription of ABC transporters (Figure 1.1).

JNK signaling is one of the most cardinal cascades for that matter(Figure 1.1). This cascade starts with the combination of c-Jun and c-Fos to form Activated protein-1(AP-1) [140]. AP-1 is a transcription factor that controls many cellular processes, including differentiation, proliferation, and apoptosis[140]. Activated c-Jun and AP-1 enhances the JNK signaling pathway playing vital roles in cellular processes by activating or repressing various transcription factors such as c-Jun, p53, HSF1, and STAT3 [141]. These transcription factors and signaling cascades, thus, form a feedback loop regulating one another's expression and functions, have been shown to play an important role in regulating target genes expressions, including ABC transporters. For example, the ovarian cancer cell lines, when treated with c-jun antisense oligodeoxynucleotide, have exhibited increased sensitivity to cisplatin and an elevation in the steady-state mRNA level of c-fos was observed in mouse sarcoma and human KB cells with multidrug resistance phenotype [142,143]. In another instance, Salvicine, a topoisomerase II inhibitor, downregulated ABCB1 expression in multidrug-resistant human leukemia cell lines by increasing c-Jun expression in both resistant and sensitive cells. An increase in DNA binding activity of AP-1 was also observed [144]. Overlapping AP-1 and CAAT box binding elements were revealed on the MDR1 promoter sequence in Adriamycin-resistant human breast cancer MCF-7 cells. AP-1 binds to the -123 to -108 promoter region of Mdr1 mRNA to enhance MDR1 expression in the cells [145]. Increased c-Jun/AP-1 levels were observed in etoposide-resistant human leukemia cell line, suggesting that

the post-transcriptional changes in c-Jun mRNA might play a role in acquiring etoposide resistance in leukemia cell lines [146]. Adenovirus-mediated enhancement of c-Jun NH2-terminal kinase (JNK) decreased the ABCB1 protein and mRNA level in a dose and time-dependent manner. The adenoviral JNK also increased the AP-1 binding activity of the mdr1 gene in the multidrug-resistant cells [147].

A study by Bark and Choi observed downregulation of ABCB1 by PSC833, a cyclosporin analog, via activating JNK/c-Jun/AP-1 and suppressing NF-kB in colon cancer and its resistant sublines [148]. Another transcription factor that regulates the ABCB1 gene via JNK signaling is Seven in absentia homologue 1 (Siah1). Siah1 is an E3 ubiquitin ligase that regulates the ubiquitination and proteasome-dependent degradation of several proteins [149]. It decreases MDR-1 expression transcriptionally by promoting c-Jun transcription factor binding to the AP1 site in the MDR1 promoter. Siah1 triggers c-Jun NH2-terminal kinase (JNK) activation to increase phosphorylation of c-Jun, and the JNK/c-Jun signaling axis is critical for Siah1 to decrease MDR1 expression [150].

In addition to JNK signaling, studies have also implicated p38-MAPK in regulating ABC transporters (Figure 1). p38 MAPKs are generally activated in response to stress and mitogenic stimuli and leads to activation of a divergent downstream signaling pathways consequently regulating multiple cellular processes [151]. One of those pathways is the regulatory with Akt. As mentioned earlier, PI3K activates Akt which is also activated by other factors including p38/MAPK [152]. Active p38 promotes phosphorylation of the scaffold protein HSP27, which further recruits Akt and phosphorylates it. Active Akt then phosphorylates HSP27 alongwith numerous substrates such as STAT3 involved in cell survival. These substrates regulate each other

and maintain cellular processes [153,154]. For example, one study found that Imatinib prevents intrinsic resistance in cancer cells by inhibiting the activation of Abl family of non-receptor tyrosine kinases (c-Abl/Arg). This leads to decreased doxorubicin resistance, decreased activation of STAT3/HSP97/p38/Akt cell-survival pathway, repressing NF-kB targets, and by downregulating ABCB1. Although this study did not find a direct link, increased NF-kB activation might be involved in the decreased ABCB1 expression [155]. Another study found increased phosphorylation of p38-MAPK in Vincristine-resistant gastric cancer cells. However, no phosphorylation of ERKs and JNKs was observed. Inhibition of p38-MAPK reduced AP-1 and ABCB1 activity and increased chemosensitivity in the resistant cells indicating the role of p38-MAPK signaling in affecting ABCB1 expression [156]. In contrast, SB203580, a specific inhibitor of p38-MAPK, reversed the ABCB1-mediated multidrug resistance in the multidrug-resistant mouse leukemia cell lines [157].

Activation of another transcription factor, nuclear factor erythroid 2–related factor 2 (Nrf2) at the rat and mouse blood-brain barriers increased the protein expression of ABCB1, ABCG2, and ABCC2 both in-vitro and in-vivo after exposure to Sulphorafane (SFN), a ligand of Nrf2. The study demonstrated that Nrf2 increases ABCB1 by upregulating p53 leading to activation of p38, which induces nuclear translocation and activation of NF-kB in the brain capillaries [158,159]. Amongst ERK signaling substrates, activation of the c-Raf kinase and Ras oncogene is involved in the regulation of ABCB1. Raf responsiveness to mdr1 promoter in NIH3T3 cells, an immortalized embryonic fibroblast cell line, has been localized to sequences between -69 and -41, relative to the initiation site [160].

Together, these investigations have suggested that different downstream substrates of MAPK signaling pathways mediate the expression of ABC transporters. Different mechanisms have been explored in analyzing the regulatory pathways of the transporters. All three MAPKs pathways can modulate the transporters independently or by working together and affect transcription factors regulating components of all the pathways depending on the stimuli and the conditions of activations.

1.3.1.3 Other Transcription factors

Besides transcription factors related to MAPK cascades, studies have identified other factors that can affect the expression of ABC transporters too, such as Y-box binding protein-1 (YB-1), E2F transcription factor 1 (E2F1), Tissue factor pathway inhibitor-2 (TFPI-2), NF-IL6, Myc factors, Growth associated protein 43 (GAP43), and GATA transcription factor [125,129–136]. Y-box binding protein-1 (YB-1) is a member of a family of DNA binding proteins. Its nuclear localization in MCF-7 breast cancer cells has been correlated with MDR-1 gene expression [161] and acts as a major transcription factor for the activation of the MDR1 promoter [162]. YB-1 is also involved in the development of resistance to Paclitaxel in breast cancer. In a study on 27 breast cancer tissues, nine showed translocation of YB-1 from cytoplasm to nucleus correlated with increased expression of P-gp [169]. E2F1 is a transcription factor involved in cell cycle progression upregulated in many high-grade tumors with chemoresistance [170]. E2F1 has been shown to target ABCA5 directly as well as through a miRNA, miR-205. Knockdown of E2F1 in resistant melanoma cells led to upregulation of miR-205 and increased chemosensitivity [163]. Tissue factor pathway inhibitor-2 (TFPI-2), an extracellular matrix-associated Kunitz-type serine proteinase inhibitor, is known to induce apoptosis and suppress tumor metastasis in several types

of cancer cells [171–173]. TFPI-2 inhibits mRNA expression of ABCB1 and enhances 5-FU induced apoptosis in 5-Fluorouracil resistant human hepatocellular cancer cells (BEL-7402/5-FU) and [164]. NF-IL6, a member of the C/EBP family of transcription factors, transactivates the MDR1 gene promoter by binding between -157 to -125 sequences of the promoter [165]. GAP43 has been found to induce the expression of ABCB1, ABCC4, and ABCC6 in colon cancer cells [167]. These studies thus outline the role of different transcription factors in regulating ABC transporters along with the ones involved in MAPK cascades (Figure 1.1).

1.3.2 Nuclear receptors

Nuclear recptors are a family of transcription factors activated by lipid soluble ligands able to cross the plasma membrane [174]. Once activated nuclear receptors recognize and bind to DNA sequences to modulate the expression of their target genes that control a variety of biological functions [174]. Nuclear receptors have been shown to modulate the expression of different ABC transporters (Table 1). One of the most recognized nuclear receptors to regulate the expression of ABC transporters and affect chemosensitivity is the Pregnane X receptor (PXR), a xenobiotic sensing receptor. Ligands targeting human PXR have been shown to upregulate the expression of ABCB1/P-glycoprotein in the blood-brain barrier both *in-vitro* and *in-vivo* [175]. Rifampicin and hyperforin induced PXR activation upregulated mRNA and protein level of ABCB1/P-gp and ABCG2/BCRP and enhanced activity of both the transporters in porcine brain capillaries [176]. Besides the blood-brain barrier, a positive correlation between PXR expression and mRNA amount of ABCB1, ABCC2/MRP2, and ABCG2 in peripheral blood molecular cells (PBMCs) and small intestines has been observed. Similarly, an association has been discovered between PXR upregulation and increased ABCG2 mRNA levels in breast cancer MCF-7 cells when treated with

cytokines such as IL-1β and TNF-α [177]. A DR4 motif present in the upstream enhancer of the MDR1 promoter has been identified as a binding site for PXR in human colon carcinoma cells when treated with Rifampin [178]. MRP2 expression was strongly enhanced in tamoxifen-resistant MCF-7 cells after PXR was found to be activated by PI3-kinase[179]. Constitutive Androstane receptor (CAR), another xenobiotic sensing receptor, has been found to be a positive regulator of ABCB1, ABCC2, and ABCG2 in rat and mouse brain capillaries [180].

Table 1: List of ABC transporters regulated by different nuclear receptors

Receptor	ABC transporter regulated	Reference
PXR	ABCB1	[175][176][181][178]
	ABCG2	[176][181][177][180]
	ABCC2/MRP2	[181][182][179]
	ABCC3/MRP3	[183]
CAR	ABCC2	[182,183]
	ABCC4	[183]
AhR	ABCC4	[183][184]
	ABCB1	[185]
	ABCC2	[185]
	ABCG2	[185]
FXR	ABCC2	[182]
	ABCA1	[186]

LXR	ABCA1	[187][188]

In addition to PXR and CAR, other nuclear receptors such as the Aryl hydrocarbon receptor (Ahr) have been shown to regulate MRP4 expression by directly binding to it [184]. Liver X receptor (LXR), a key regulator in maintaining lipid homeostasis, has demonstrated regulation of hepatic ABCA1 gene expression through a dual promoter system with Sterol regulatory element-binding protein-2 [188]. LXR agonists also increased the ABCA1 mRNA in conditionally immortalized brain capillary endothelial cell line (TR-BBB13) but did not affect the expression of ABCG2 [187]. Farnesoid X receptor (FXR), the nuclear receptor activated by bile acids, has been shown to regulate hepatic ABCA1 and high-density lipoprotein through a microRNA, miR-144 [186]. Nuclear receptors have been shown to act individually as well as induce coordinated regulations on the expression of ABC transporters.

A plethora of nuclear receptors was found to be involved in the export of bile salts and organic anions from the hepatocyte into the bile through transporters such as MRP2 and BSEP. Activation of PXR, CAR, and FXR induces the expression of MRP2 responsible for transporting the metabolites from the hepatocytes into the bile canaliculi. PXR also increases the expression of MDR1 that mediates the transportation of amphipathic compounds across the canalicular membrane [182]. Another study has similarly demonstrated coordinated regulation of various hepatic phase I and II drug-metabolizing genes and transporters by different transcription factors: Aryl hydrocarbon receptor (AhR), CAR, PXR, Peroxisome proliferator-activated receptor α, and nuclear factor erythroid 2-related factor 2 (Nrf2) in the livers of male and female mice. Amongst

the ABC transporters induced, AhR induces mRNA expression of MRP4, CAR induces MRP2-4, PXR induces MRP3, PPAR-α induces MRP4 and Nrf2 induces MRP3-4 [183]. Together these findings show the importance of nuclear receptors in regulating the transcription of ABC transporters (Table 1) and their potential in serving as critical targets in gene therapy for chemotherapy as well as lipid regulation.

1.3.3 MiRNAs

MicroRNAs are short non-coding strands of RNA around 25-45 bp long involved in the post-transcriptional regulation of many genes by either inactivating the mRNA by binding to the 3' Untranslated region of the mRNA or degrading the mRNA [189,190]. MicroRNAs play important roles in maintaining different functions ranging from cellular survival, apoptosis, maintenance, and many others [191,192]. MicroRNAs have been known to regulate the expression of different ABC transporters by either directly binding to the protein-encoding mRNAs or indirectly binding to other transcription factors affecting the expression of ABC transporters (Figure 1.2-1.4).

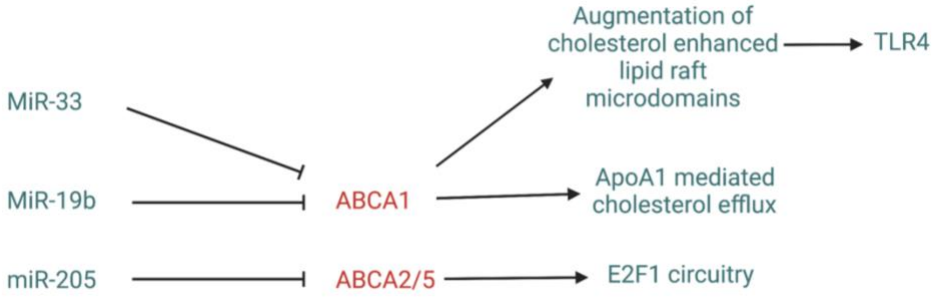

Figure 1.2: MiRNAs regulating ABCA family

For example, miR-19b binds to 3110-3116 sites within 3'UTR of ABCA1 and has been shown to directly regulate the expression of endogenous ABCA1 in foam cells derived from human THP-1 macrophages and mouse peritoneal macrophages. This miRNA thus suppresses cholesterol efflux in the macrophages leading to increased cholesterol accumulation in the macrophages and promoting atherosclerotic development [193]. MiR-33 binds to 3' UTR of ABCA1 and ABCG2, mediating augmentation of cholesterol-enhanced lipid shaft microdomains, subsequently enhancing proinflammatory signaling by inducing expression of toll-like receptor-4 (TLR-4) and other TLRs [194]. MicroRNA responsive elements matching the seed sequences of another miRNA, miR-205, have been identified in ABCA 2/5. This prediction was confirmed by a luciferase reporter assay as well as gene expression analysis in metastatic melanoma cells resistant to chemotherapy [163].

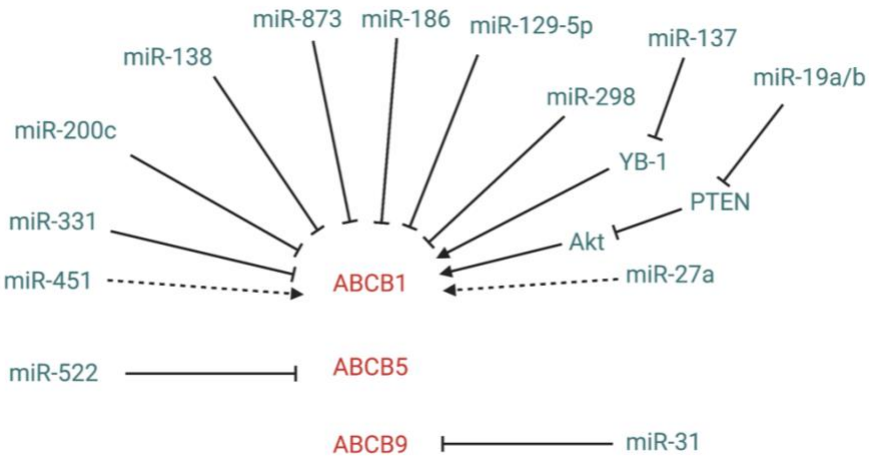

Figure 1.3: MiRNAs regulating ABCB family

Additionally, several miRNAs have been shown to modulate the expression of the ABCB family (Figure 1.3). For instance, Hsa-miR-137 indirectly inhibits the expression of P-gp/ABCB1

by downregulating Y-box binding protein-1, a member of a family of DNA binding proteins [195]. Localization of YB-1 has been correlated with multidrug resistance and acts as a major transcription factor for the activation of the MDR1 promoter [162]. Restoration of YB-1 by miR-137 sensitizes Adriamycin-resistant breast cancer lines to Adriamycin treatment [195]. MiR-137 is an essential regulator of MDR1 as it also regulates MDR1 expression through CAR, as mentioned earlier [196]. Reduced expression of hsa-miR-298 in doxorubicin-resistant cancer cell lines has been associated with increased expression of P-gp. Hsa-miR-298 binds directly with the 3'UTR region of ABCB1 in a dose-dependent manner. Overexpression of miR-298 downregulates ABCB1 expression increasing Doxorubicin's nuclear accumulation and drug sensitivity in chemoresistant metastatic breast cancer [197].

Upregulation of another miRNA, hsa-miR-200c, enhanced the chemosensitivity of breast cancer cells to Epirubicin and was associated with reduced expression of ABCB1 mRNA and P-gp [198]. Hsa-miR-138, a tumor suppressor miRNA, is downregulated in Vincristine-induced multidrug resistance leukemia cell line, HL-60/VCR. The cells showed higher sensitivity to P-gp related drugs such as vincristine and Adriamycin when transfected with miR-138 mimics with decreased expression of MDR1 mRNA and P-gp protein. Luciferase assays confirmed that the microRNA binds directly to the ABCB1 mRNA, thus inhibiting its expression [199]. Sun et al. found that the overexpression of Hsa-miR-186 increased the sensitivity of ovarian cancer cells to Paclitaxel and Cisplatin. Computational analysis predicted 3'UTR regions of both ABCB1 and ABCC1 contains potential miRNA binding site for miR-186. However, luciferase reporter assays demonstrated that the miRNA directly binds to ABCB1 3'UTR but not of ABCC1, suggesting miR-186 induced increase in the chemosensitivity of ovarian cancer cell lines is mediated by

targeting ABCB1 and not ABCC1 [200]. MiR-873 is another microRNA that has been shown to bind with the 3' UTR of ABCB1 by both the web-based MicroRNA programs and luciferase activity assay in HEK-293T cells. MiR-873 downregulated expression of MDR1 at both mRNA and protein levels in four ovarian carcinoma cell lines. In parallel, treatment with anti-miR-873 upregulated MDR1 expression in all four cell lines. Increased efficacy of Cisplatin in a mouse xenograft model of human carcinoma was also observed. Similar results were obtained in multidrug-resistant ovarian cancer cells [201].

A putative binding site for hsa-miR-451 at 4742 to 4763 nucleotides has been identified in the Mdr1 gene. MCF-7 doxorubicin-resistant cells, when transfected with miR-451, exhibited increased sensitivity to doxorubicin [202]. MiR-451 also exhibits a similar profile in cancer stem cells (CSC). It decreased self-renewal, tumorigenicity, and increased chemosensitivity of CSCs for Irinotecan by downregulating ABCB1[203]. In contrast, treatment with miR-451 inhibitor decreased the expression of MDR1 mRNA in multiple myeloma SP cells [204] and ovarian cancer cells [205]. Interestingly, a similar contrast in regulation was observed in hsa-miR-27a. Hsa-miR-27a directly downregulated the expression of P-gp in K562, a leukemia cell line, and HL-6, human promyelocytic cell line [206], but indirectly upregulated the expression of P-gp in ovarian cancer cells and cervical carcinoma cells [205]. These contrasting results in different cell lines show that miR-451 and miR-27a might exhibit different effects on ABCB1 depending on cellular subpopulations and the associated malignancy.

Other members of the ABCB subfamily, for example, ABCB5, contains a miRNA binding site on its 3'UTR promoter for miR-522 [207]. MiR-31 directly targets 3'UTR of ABCB9 as shown by luciferase activity assay. Interestingly, MiR31 was significantly upregulated in the cisplatin-

resistance cell line compared with its level in the sensitive cell line and has been shown to induce cisplatin resistance in the cisplatin-sensitive non-small cell lung cancer cell line. It repressed cisplatin-induced apoptosis by targeting ABCB9, which regulates cisplatin resistance [208]. In contrast, miR-24, another miRNA that has been shown to bind to the 3'UTR region of ABCB9 directly, was downregulated in paclitaxel-resistant breast cancer patients and in MCF-7/PR human breast carcinoma cells. In vivo experiments have also demonstrated that overexpression of miR-24 could increase the sensitivity of drug-resistant MCF-7 cells to paclitaxel [209].

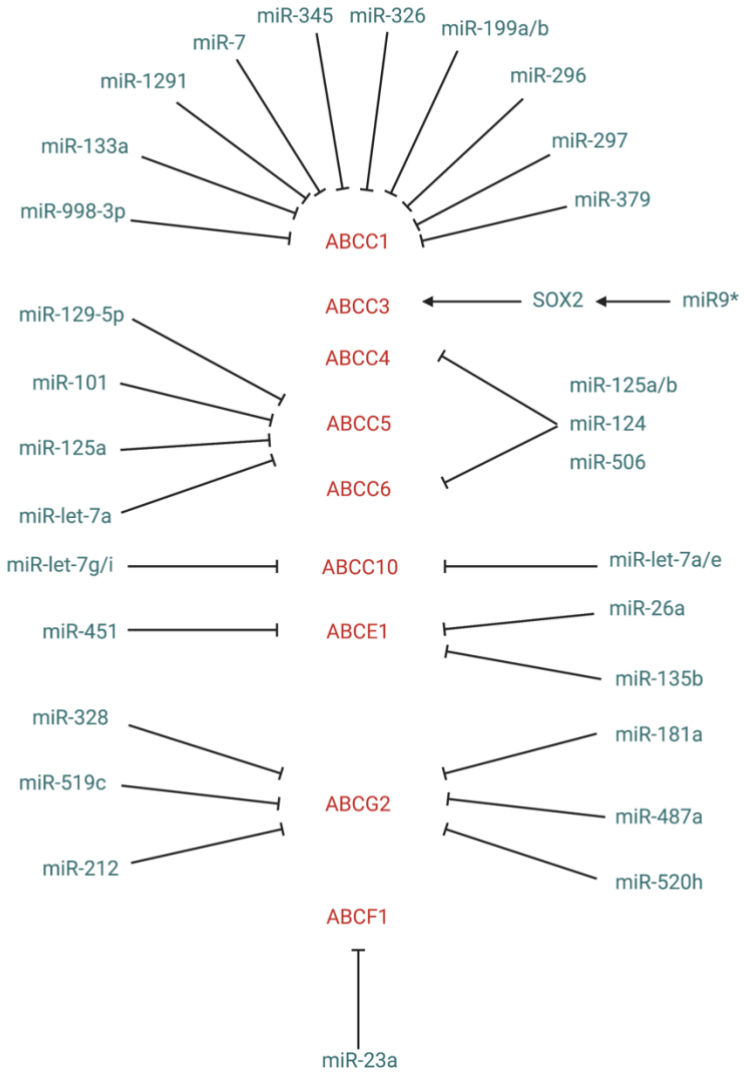

Figure 1.4: MiRNAs regulating ABCC-G family

Along with the ABCB family, the ABCC family has been known to transport a wide range of therapeutic agents and may play an important role in developing multidrug resistance (Figure 1.4). Several miRNAs have been identified regulating ABCC transporters. For example, miR-326 was downregulated in various breast cancer cell lines and was inversely correlated with ABCC1/MRP1. Etoposide (VP-16) resistant MCF-7 cells have been found to overexpress

ABCC1/MRP-1 but not ABCB1 or ABCG2 When transfected with miR-326 mimics, MRP-1 was found to be downregulated in MCF-7/VP cells and increased sensitivity to etoposide, and doxorubicin was demonstrated [210]. Another study identified over 100 miRNAs dysregulated in MCF-7 cell lines resistant to cisplatin. They identified MRP1 3'UTR as the target of two miRNAs, miR-345 and miR-7 [211]. Bioinformatic analysis and luciferase reporter assays have shown that miR-1291 binds to the 3'UTR of ABCC1 and regulates its expression in pancreatic cancer (Panic-1) cells transfected by miR-1291. A miRNA degradation study further showed that miR-1291 might be affecting ABCC1 expression by translation inhibition or targeting of transcription factors and not mRNA degradation [212]. Another study examined miR-7 expression in 44 small cell lung cancer (SCLC) samples and their correlation with the clinicopathological feature. They showed that miR-7 was downregulated in the drug-resistant group in comparison to the sensitive group, and miRNA expression was closely correlated with chemotherapy responsiveness. MRP1 expression was found to be negatively correlated with miR-7 levels in SCLC tissues. Further studies showed ABCC1 as a direct target of miR-7 through bioinformatic analysis and luciferase reporter assays. Knockdown of miR-7 elevated MRP-1 expression in human small cell lung cancer cells [213].

In parallel to previous studies, few other miRNAs have been shown to target ABCC1, such as miR-133a, miR-326, miR-199a/b and miR-296 [214,215]. Along with ABCC2, ABCC3 and ABCC6 are indirectly targeted by miR-9* by binding to 3' UTR of SOX-2, conferring stemness potential and chemoresistance to glioma stem cells [216]. ABCC4 has been identified to be targeted by miR-124a and miR-506 [217]. An additional mRNA and miRNA expression study in hepatocellular carcinoma and adjacent healthy liver tissues identified ABCC4 as a target of miR-

125a/b, ABCC5 as a target of miR-101, miR-125a and let-7a, ABCC10 as a target of let-7a/e, and ABCE1 as a target of miR-26a, -135b, and miR-451. These results were also confirmed by the luciferase reporter assays [214]. Another miRNA known to directly target 3' UTR of ABCC10 is miR-let-7g/I in esophagus cancer cells [218] and ABCC5 is miR-129-5p in Vincristine resistant gastric cancer cells [219].

Another ABC transporter with high relevance when it comes to chemoresistance is ABCG2/BCRP. 3' UTR of ABCG2 is targeted by hsa-miR-328 in mitoxantrone resistant breast cancer cell lines [220] and side population (SP) cells in colorectal cancer cells [221]. MiR-328 influenced drug disposition in the breast cancer cell line [220][222] and reversed drug resistance, and inhibited cell invasion of SP cells [221]. Hsa-miR-328 was also found to be inversely correlated with the ABCG2 expression when chronic myeloid leukemia (CML) K-652 cells were long-term treated with 0.3 umol/l Imatinib and decreased gradually at high concentrations. However, direct binding of the miRNA with ABCG2 mRNA was not found [223].

Another study by To et al. found a microRNA, hsa-miR-519c, truly targeting 3'UTR of ABCG2 mRNA, thus, regulating ABCG2 expression by translational repression in the parental S1 colon cancer cell lines, but similar results were not achieved in the resistant cell line [224]. Interestingly, another study conducted in mitoxantrone-resistant MCF-7 cells showed miR-519c targeting ABCG2 in both the sensitive and the resistant cell line. This study suggested the involvement of mRNA degradation as a mechanism of ABCG2 downregulation [222]. These discrepancies in the binding ability of miR-519c to ABCG2 mRNA might be attributed to the shortening of ABCG2 3'UTR in several ABCG2-overexpressing colon cancer cell lines [225] which leads to suppression of miR-519c binding site in drug-resistant colon cancer cells. To et al.

have thus found an involvement of miR-519c in the initial step of selection when the resistant cell line acquires multidrug resistance, leading to its decreased expression causing overexpression of ABCG2 [225].

To et al. also reported another putative miRNA, hsa-miR-520h, reported to target ABCG2 in hematopoietic stem cells during their differentiation into progenitor cells. Hsa-miR-520h binds to the 3'UTR of ABCG2 in both the resistant (S1MI80 cells) and the sensitive (S1) cells [224]. However, the expression of miR-520h is reduced in the resistant cells in comparison to the sensitive cells [224]. A miRNA that has repeatedly been found regulating ABCG2 in different cell lines is hsa-miR-520h. Hsa-miR-520h binds to ABCG2 in both parental and resistant colon cancer cell lines, decreasing ABCG2 expression and increasing chemosensitivity [224]. Similar results were found when the pancreatic cancer cell line, Pacn-1, was treated with miR-520h. MiR-520h decreased cell migration and invasion but did not affect cell proliferation ad cell cycle distribution, or apoptosis suggesting the miRNA might be influencing ABCG2 expression by affecting side population cells [226]. Two other miRNAs, Hsa-miR-487a and Hsa-miR-181a, have exhibited ABCG2 as their targets in mitoxantrone resistant breast cancer cells (MCF-7/MX) [227,228]. Both the miRNAs resensitized the resistant cells to BCRP-substrates [227,228]. ABCF1, unlike other ABC transporters, lacks the transmembrane domains and has been shown to be involved in ribosome assembly and some xenobiotic resistance [229,230]. Mir-23a has been shown to directly target ABCF1. The miRNA has been found to be inversely correlated with the expression of ABCF1 in Colorectal cancer tissues (CRC). Repressing ABCF1 expression by either miR-23a overexpression or siABCF1 led to the recovery of 5-FU sensitivity in microsatellite instability (MSI) CRC cells [231].

ABC transporters play a vital role in the disposition of endo and xenobiotics within the body. Regulation of these transporters, especially genetic regulation, thus, holds a high significance, especially when it comes to battling multidrug resistance in chemotherapy. All the studies mentioned here highlight the important role microRNAs serve in regulating these transporters and subsequently drug resistance (Figure 1.2-1.4)(Table 2). This presents miRNAs as interesting targets and biomarkers, especially when it comes to enhancing the chemosensitivity of malignant cells. Variations in regulations of the same transporter in different cell types require further studies to shed light on the interactions of miRNAs with the transporters. Another thing that stands before achieving the true potential of miRNAs as therapeutic modulators of ABC transporters is the lack of a proper delivery vector to avoid degradation as well as off-target effects and lack of complete understanding of the field yet.

Table 2: MiRNAs interfering with the expression of ABC transporters					
MicroRNA	ABC transporter affected	Effect on gene	Mechanism	Tissue	Action
Hsa-miR-19b	ABCA1	↓	Directly binds to 3'UTR	Human THP-1 and peritoneal macrophages [193]	Promotes Macrophage cholesterol accumulation and aortic atherosclerosis.
Hsa-miR-33	ABCA1 ABCG1	↓	Directly binds to 3'UTR	Murine macrophages [194]	Augments macrophage lipid rafts and enhances proinflammatory responses to lipopolysaccharides.
Hsa-miR-205	ABCA2/5	↓	Directly binds to 3'UTR	Metastatic melanoma cells [163]	Increases drug sensitivity to anti-neoplastic agents.
Hsa-miR-451	ABCB1	↓	Directly binds to 3'UTR	Doxorubicin resistant MCF-7 cells [202]	Increased the sensitivity of cells to doxorubicin and Irinotecan.

				Colonospheres with the properties of cancer stem cells [203]	
	ABCB1	↑	Via PI3K/Akt/MTOR signaling pathway	Multiple myeloma Side population (SP) cells [204]	Regulates stemness of cells.
	ABCB1	↑		Multidrug resistant cancer cell lines of ovarian cancer cells and cervical carcinoma cells [205]	Increased drug resistance in the parental cell lines.

	ABCE1	↓	Directly binds to 3'UTR	Hepatocellular carcinoma [214]	-
Hsa-miR-27a	ABCB1	↓	Directly binds to 3'UTR	Leukemia cell line, K652, and a human promyelocytic cell line, HL60 [206]	Increased the sensitivity of cells to doxorubicin.
	ABCB1	↑		Multidrug resistant cancer cell lines of ovarian cancer cells and cervical carcinoma cells [205]	Increased drug resistance in the parental cell lines.
Hsa-miR-331-5p	ABCB1	↓	Directly binds to 3'UTR	Leukemia cell line, K652, and a human promyelocytic	Increased the sensitivity of cells to doxorubicin.

				cell line, HL60 [206]	
Hsa-miR-137	ABCB1	↓	Via Y-box binding protein-1	Adriamycin resistant breast cancer cell lines [162,195]	Restoration of miR-137 or inhibition of YB-1 sensitizes cell lines to P-gp related drugs.
Hsa-miR-298	ABCB1	↓	Directly binds to 3'UTR	Doxorubicin resistant breast cancer cells [197]	Increased Doxorubicin's nuclear accumulation and increased drug sensitivity.
Hsa-miR-200c	ABCB1	↓	Directly binds to 3'UTR	Human breast cancer cells [198]	Increased sensitivity of resistant cells to Epirubicin.
Hsa-miR-138	ABCB1	↓	Directly binds to 3'UTR	Multidrug resistance leukemia cell line HL-60/VCR [199]	Increased sensitivity of the resistant cells to both P-gp related drugs such as vincristine and adrimaycin and non-

					related drugs 5-flu and cisplatin.
Hsa-miR-186	ABCB1	↓	Directly binds to 3'UTR	Ovarian cancer cell lines [200]	Increased the sensitivity of ovarian cancer cells to paclitaxelb and cisplatin.
Hsa-miR-873	ABCB1	↓	Directly binds to 3'UTR	Four ovarian carcinoma cell lines [201]	Increased the efficacy of cisplatin in a mouse xenograft model of human ovarian carcinoma.
Hsa-miR-19a/b	ABCB1	↑	Via PI3K/Akt pathway	Gastric cancer cells [232]	Decreased the sensititvity of gastric cancer cells to anticancer drugs

MicroRNA	ABC transporter affected	Effect on gene	Mechanism	Tissue	Action
Hsa-miR-522	ABCB5	↓	Directly binds to 3'UTR	HT29 colon cancer cells [207]	Reverses doxorubicin induced resistance.
Hsa-miR-31	ABCB5	↓	Directly binds to 3'UTR	Non-small cell lung cancer cells [208]	Inhibits cisplatin-induced apoptosis.
Hsa-miR-24	ABCB5	↓	Directly binds to 3'UTR	Paclitaxel resistant breast cancer cells and human breast carcinoma cells [209]	Increased the sensitivity of drug-resistant MCF-7 cells to paclitaxel.
Hsa-miR-326	ABCC1	↓	Directly binds to 3'UTR	Etoposide resistant MCF-7 cells [210] Hepatocellular carcinoma cells (HepG2) [215]	Sensitized cells to Adriamycin, Etoposide and doxorubicin.

Hsa-miR-345	ABCC1	↓	Directly binds to 3'UTR	Cisplatin resistant MCF-7 cells [211]	-
Hsa-miR-7	ABCC1	↓	Directly binds to 3'UTR	Cisplatin resistant MCF-7 cells [211]	-
	ABCC1	↓	Directly binds to 3'UTR	Human small cell lung cancer cells [213]	Increased chemosensitivity of the resistant cell lines.
Hsa-miR-1291	ABCC1	↓	Directly binds to 3'UTR	Pancreatic cancer cells (Panc-1) [212]	Modulates drug disposition.
Hsa-miR-133a	ABCC1	↓	Directly binds to 3'UTR	HepG2 cells [215]	Sensitized cells to Adriamycin.
Hsa-miR-199a/b	ABCC1	↓	Directly binds to 3'UTR	Hepatocellular carcinoma [214]	-
Hsa-miR-296	ABCC1	↓	Directly binds to 3'UTR	Hepatocellular carcinoma [214]	-

Hsa-miR-297	ABCC2	↓	Directly binds to 3'UTR	Oxaliplatin resistant human colorectal cancer cells (HCT116/L-OHP) [233]	Increased the sensitivity of human CRC to anticancer drugs.
Hsa-miR-379	ABCC2	↓	Directly binds to 3'UTR	HepG2 cells [234]	Mediates ABCC2 downregulation in Rifampicin treated HepG2 cells.
Hsa-miR-998-3p	ABCC2	↓	Binds to the coding sequence (CDS) of ABCC2	Three representative lepidopteran pests, including *Helicoverpa armigera*, *Spodoptera exigua* and *Plutella xylostella*.[235]	Increases tolerance to cry protein toxins.

Hsa-miR-9*	ABCC2	↓	Via SOX2	Glioma stem cells [216]	Confers stemness potential and chemoresistance.
	ABCC6	↓	Via SOX2	Glioma stem cells [216]	Confers stemness potential and chemoresistance.
Hsa-miR-125a/b	ABCC4	↓	Directly binds to 3'UTR	Hepatocellular carcinoma [214]	-
Hsa-miR-124a	ABCC4	↓	Directly binds to 3'UTR	Hek293T cells [217]	-
Hsa-miR-506	ABCC4	↓	Directly binds to 3'UTR	Hek293T cells [217]	-
Hsa-miR-101	ABCC5	↓	Directly binds to 3'UTR	Hepatocellular carcinoma [214]	-

Hsa-miR-125a	ABCC5	↓	Directly binds to 3'UTR	Hepatocellular carcinoma [214]	-
Hsa-miR-let-7a	ABCC5	↓	Directly binds to 3'UTR	Hepatocellular carcinoma [214]	-
Hsa-miR-let-7a/e	ABCC10	↓	Directly binds to 3'UTR	Hepatocellular carcinoma [214]	-
Hsa-miR-26a	ABCE1	↓	Directly binds to 3'UTR	Hepatocellular carcinoma [214]	-
Hsa-miR-135b	ABCE1	↓	Directly binds to 3'UTR	Hepatocellular carcinoma [214]	-
HSa-miR-let 7g/i	ABCC10	↓	Directly binds to 3'UTR	Esophagus cancer cells [218]	Inhibits cell proliferation.
Hsa-miR-141-3p	ABCD2	↓	Via Ascl4	Human chondrocytes [236]	Involved in cartilage homeostasis.

Hsa-miR-328	ABCG2	↓	Directly binds to 3'UTR	Mitoxantrone resistant breast cancer cell lines [220] SP cells in colorectal cancer cells [221] Mitoxantrone resistant MCF-7 cells [222] Chronic myeloid leukemia (CML) K-652 cells [223]	Reverses drug resistance and inhibits cell invasion.
Hsa-miR-519c	ABCG2	↓	Directly binds to 3'UTR	Colon cancer cell lines [224] Drug resistant MCF-7 cell lines [222]	Increases chemosensitivity.

Hsa-miR-520h	ABCG2	↓	Directly binds to 3'UTR	Colon cancer cell lines [224] Drug resistant MCF-7 cell lines [222] Pancreatic cancer cells [226]	Increases chemosensitivity. Inhibits migration, invasion, and side populations.
Hsa-miR-212	ABCG2	↓	Directly binds to 3'UTR	Chronic myeloid leukemia K-652 cells [223]	Enhanced cell viability, reduced apoptosis and cytotoxicity under Imatinib treatment.[237]
Hsa-miR-487a	ABCG2	↓	Directly binds to 3'UTR	Mitoxantrone resistant MCF-7 cells [227]	Increases intracellular accumulation of mitoxantrone.
Hsa-miR-181a	ABCG2	↓	Directly binds to 3'UTR	Mitoxantrone resistant MCF-7 cells [228]	Increases intracellular accumulation of mitoxantrone.
Hsa-miR-129-5p	ABCG1	↓	Directly binds to 3'UTR	Vincristine resistant gastric	-

				cancer cells [219]	
	ABCC5	↓	Directly binds to 3'UTR	Vincristine resistant gastric cancer cells [219]	-
	ABCB1	↓	Directly binds to 3'UTR	Vincristine resistant gastric cancer cells [219]	-
Hsa-miR-23a	ABCF1	↓	Directly binds to 3'UTR	Colorectal cancer tissues [231]	Recovery of 5-fluoro uracil in microsatellite instability CRC cells.

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CHAPTER 2

A NOVEL METHOD FOR PRODUCING FUNCTIONALIZED VESICLES THAT EFFICIENTLY DELIVER OLIGONUCLEOTIDES *IN VITRO* IN CANCER CELLS AND *IN VIVO* IN MICE

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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 with liver cancer HepG2 cells and in vivo studies in mice were demonstrated to show feasibility of the "Functionalized Lipid Insertion Method." The dynamic light scattering (DLS) technique suggests that the original vesicular structure was retained. 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 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

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

2.1 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]. Naturally-occurring 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]. Complete solubilization of the vesicular components by this approach 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 4oC and 40oC, which may destabilize constitutive 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 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 several cardiovascular disorders, cancer, and allergic responses [39]. They can also elicit anticancer drug resistance in cancerous tumors [40]. 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]. These polymers suffer from similar problems as other oligonucleotide drugs [34]. To compensate, 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]. Currently, several miRNA therapeutics are going through different stages of drug development 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 produced with any targeting ligand or protein, such as 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.

2.2 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 (ASGR1PAB) (ab49355) and recombinant ACE2 monoclonal antibody (ACE2MAB) (ab108252) were purchased from Abcam (Cambridge, MA). NPHS2 (podocin) polyclonal antibody (NPHS2PAB) (MBS3013144) was acquired from MyBioSource (San Diego, CA). GFP monoclonal antibody (GF28R) (GFPMAB) (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% CO2 at 37ºC in Thermo Fisher Scientific Napco Series 8000 WJ CO2 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 4oC 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 4oC. 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 Na2HPO4, 1.8 mM KH2PO4) 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 37oC and 5% CO2 in Thermo Fisher Scientific Napco Series 8000 WJ CO2 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 allowed to incubate 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, were combined in one tube, and were 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 had 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 KD cut-off filter (Thermo Fisher Scientific, Waltham, MA) against 2 L isotonic PBS for 2 h at 4oC 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 4oC. Dialysis slowly removes the DDM detergent that is surrounding 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 a membrane protein transporter into a liposomal bilayer [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, and the functionalized vesicle pellet was resuspended in 100 μl of isotonic PBS, which will be called the functionalized vesicle solution. The purpose of centrifuging the functionalized vesicles and removing the supernatant is 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 experiments.

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. 2.1.

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 detergent-solubilized solutions in 200 μl microcentrifuge tubes were sonicated in a Kendal ultrasonic cleaner HB23 (Kendal) at 25oC 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. 2.3), 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 4oC. 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. The miRNA in the in-vitro experiments with the HepG2 cells varied between 1 and 4 μg, while the miRNA varied between 80 and 110 μg for the in-vivo mouse 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 4oC 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% CO2 in a Thermo Fisher Scientific Napco Series 8000 WJ CO2 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 (CT) method with the U6 snRNA expression as a

reference to calculate ΔΔCT 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 ΔΔCT values were normalized against expression of the housekeeping U6 snRNA 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% CO2 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 ASGR1PAB (abbreviated mEVs(ASGR1PAB)) 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.

Six Nu/Nu male nude mice, 54 Nu/Nu female nude mice for targeting studies, and 30 female C57/BL6 mice for immune reactivity studies were ordered from Jackson labs (Maine). All mice were intraperitoneally (IP) injected with a 25-gauge needle and 200 μl or 400 μl of solutions containing SFM or functionalized vesicles in SFM. Unless otherwise specified, each of the functionalized vesicles had 114 μg of mmu-miR-298 microRNA. All the mice were anesthetized using isoflurane (Millipore Sigma, Burlington, MA).

As a negative control, mEVs and mLNPs were bioengineered with the GFPMAB since they are not known to interact with any mouse proteins (Fig. 2.7). The functionalized vesicles are abbreviated mEV(GFPMAB) and mLNP(GFPMAB) to denote their Ab modifications. In the experiment, 12 female C57BL/6J mice aged 5-7 weeks were divided into three groups (n=3). One group of mice were IP-administered 200 µl of SFM. The next two groups were IP-administered 200 µl mEVs(GF28RMAB) and mLNPs(GF28RMAB) electroporated with miRNA (i.e., mmu-miR-298). These functionalized vesicles are abbreviated mEV(GF28RMAB, mmu-miR-298) and mLNP(GF28RMAB, mmu-miR-298) to denote their Ab modifications and their miRNA cargo.

The targeting ability against the endocytotic liver ASGR1 receptor was tested with mLNPs and mEVs bioengineered with the ASGR1PAB (Fig. 2.9). For experiments involving mEVs(ASGR1PAB), six Nu/Nu male mice aged 15-17 weeks were randomly divided into two groups (n=3). For the experiments involving mLNPs(ASGR1PAB), 18 Nu/Nu female mice aged 5-8 weeks were divided into three groups (n=6). The mice then received IP injections with a total volume of 400 µl per mouse. The injections contained SFM only for the untreated mice and mEVs(ASGR1PAB, mmu-miR-298) or mLNPs(ASGR1PAB, mmu-miR-298) for treated mice.

Functionalized vesicles, mLNPs, and mEVs were bioengineered with the NPHS2 polyclonal antibody abbreviated mLNP(NPHS2PAB) and mEV(NPHS2PAB), which targets the kidney-related NPHS2 integral membrane protein (Fig. 2.8). Twelve Nu/Nu female mice, aged 5-8 weeks, were divided into two groups (n=6). These mice were IP administered 400 µl containing SFM only or mLNPs(NPHS2PAB, mmu-miR-298). Parallelly, Six Nu/Nu female mice, aged 5-8

weeks, were divided into two groups (n=3). These mice were IP administered 400 µl containing SFM only or mEVs(NPHS2PAB, mmu-miR-298).

Functionalized vesicles, mEVs, and mLNPs were bioengineered with the Angiotensin-converting enzyme 2 monoclonal antibodies (ACE2MAB) against the membrane-associated ACE2 enzyme, which is part of the renin-angiotensin system that controls blood pressure (Fig. 2.10) [59]. This antibody was chosen to test the immunoreactivity of functionalized vesicles made by our method because ACE2 is expressed in many tissues ensuring direct interaction with them by these vesicles [60]. Eighteen female C57/BL6 mice, aged 5-8 weeks, were divided into three groups (n=6). These groups received 400 µl IP injections of SFM only, mEVs(ACE2MAB, mmu-miR-298), mLNPs(ACE2MAB, mmu-miR-298).

In-vivo Quantification of miRNA

After 72 hours, the mice were euthanized using carbon dioxide followed by a necropsy as described [61]. 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-ArtTM 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) [62]. 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 (μg/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 [63]. About a milliliter of blood was withdrawn immediately after euthanizing the animals in BD Microtainer® tubes containing serum separator (SSTTM) (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).

2.3 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. 2.1. In the "Detergent-Dialysis Method" (Figs. 2.1A-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. 2.1B 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. 2.1C and 2.1D. 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 [64]. The specific mechanism of vesicular uptake has not been entirely resolved but appears to be cell-dependent [64].

Fig. 2.2 shows the 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. 2.2 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) [65]. The Golgi apparatus produces secretory vesicles containing the receptor ligands that migrate to the plasma membrane [65]. At the plasma membrane, they are secreted into the extracellular (EC) space, where they are bound by a functionalized vesicle (Step 1) [65]. 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., 66]. 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) [64]. 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 [67]. The technique is well suited to estimate particle size distributions [e.g., 50,68]. 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 initial components of the detergent-solubilized mixture will strongly influence the vesicular size distribution.

Fig. 2.3 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. 2.3A 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. 2.3B and 2.3C show vesicles produced using the "Detergent-Dialysis Method." In Fig. 2.3B, 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. 2.3C 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 another example of how the initial mixtures can influence vesicle size.

Fig. 2.3D and Fig. 2.3E show the unmodified vesicles and modified vesicles produced using the "Functionalized-Lipid Insertion Method" described in this manuscript. Fig. 2.3D 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 [69,70]. The unmodified EVs shown in Fig. 2.3E (solid line) were 21.2 ± 5.9 nm, which is similar to the EV sizes produced with the "Detergent-Dialysis Method" (Fig. 2.3C, solid line). In Fig. 2.3E (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., 71]. 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 have the same functionalized lipid density on 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 [72–74]. The endocytotic asialoglycoprotein receptor 1 (ASGR1) is highly expressed on the surface of HepG2 cells [75]. The endocytotic process involves receptor-ligand binding and ASGR1 trimerization through receptor-mediated endocytosis [76]. This receptor has already been exploited to mediate small molecule uptake into HepG2 cells [77–79]. For example, galactosamine-linked albumin and nanoparticles loaded with pullulan and arabinogalactan delivered the anti-cancer drug doxorubicin to HepG2 cells [77,78].

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 [79]. Finally, ASOs have been delivered to HepG2 cells by linking them to the ASGR1 receptor substrate N-acetylgalactosamine [80]. As far as we know, ASGR1PAB 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 ASGR1PAB, 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 [81]. Despite being studied for decades, transporter measurements with P-gp and other transporters remain challenging [82]. The P-gp expression can vary between different cancer cell lines [83]. Even variants of the same mammalian cancer cell line can have significantly different properties [84]. Transporter transfection of mammalian cells can produce expression of other transporters in the cell [85]. 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 [83]. Additionally, culturing conditions can significantly affect P-gp expression levels [86].

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 [87,88] and to be compliant with NIH funding for this project. The miR-298 in [87,88] turned out to be human miRNA-298, which has completely different targeting properties and biological functions than mouse miRNA-298

[89]. As far as we are aware, 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, and miRNA could be used to test its feasibility since it would not have any physiological effects on the HepG2 cells.

MiRNA nomenclature can be confusing for a novice. The longer names for mouse and human miR-298 are mmu-miR-298-5p and hsa-miR-298-5p, respectively [89]. The first three letters are the species [89]. Mmu is for Mus (M.) musculus (house mouse) and hsa is for Homo (H.) sapiens [89]. The number typically reflects the order of miRNA deposition into the miRNA database called miRbase and is not related to the biological function [89]. The 5p refers to the 5' side of the precursor miR-298 stem-loop that the miRNA emanates [87]. The mouse miR-298 step loop can even have a second miRNA on the 3' side called mmu-miR-298-3p, which can be functionally distinct from the miRNA on the 5' side [90].

Fig. 2.4 are experiments to estimate the relative uptake of mmu-miR-298-5p in HepG2 cells. The experiments shown in Fig. 2.4A 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

ASGR1PAB and loaded with mmu-miR-298-5p (abbreviated mEVs(ASGR1PAB, 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 endocytotic mechanism of the ASGR1 receptor than the ASGR1PAB that targets different parts of the EC domains [76]. Also, internalization through the Apolipoprotein A1 targeting ligand only requires binding to the scavenger receptor, class B, type I (SR-BI) to initiate endocytosis [76,91,92]. Modified LNPs with ASGR1PAB were also made and loaded with 4 μg mmu-miR-298 (abbreviated mLNP(ASGR1PAB, miRNA)). Relative miRNA uptake was almost 2-fold higher in HepG2 cells treated with mLNPs (ASGR1PAB, miRNA) than cells treated with mEVs (ASGR1PAB, 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 compared to EVs.

The miRNA uptake's temporal efficiency depends on the miRNA endocytotic mechanism, the miRNA carrier vehicle, and the miRNA type [93,94]. 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 [93]. The uptake of miRNA in multiple myeloma treated with stable nucleic acid-lipid particles (SNALPs) loaded with hsa-miR-34a decreased after 48 hours [94]. In this study, the miRNA uptake in HepG2 cells after treatment with mLNP(ASGR1PAB, miRNA) and mEV(ASGR1PAB, mRNA) was examined over 72 hours (Fig. 2.4B). 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 approximately 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. The relatively high miRNA uptake after 72 hours in these experiments 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(ASGR1PAB), and mLNPs(ASGR1PAB) loaded with miRNA hsa-miR-26a-5p (abbreviated mEV(ASGR1PAB, miRNA) and mLNP(ASGR1PAB, 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 [95,96]. The approach was used to estimate the effect of mEVs and mLNPs treatment of HepG2 cells in Fig. 2.5. Fig. 2.5A shows the wound closures of HepG2 cells after various treatments at 0 and 72 hours. For untreated cells, the wound closed to about half its original width, 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(ASGR1PAB)) 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(ASGR1PAB, miRNA) and mLNPs(ASGR1PAB,

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

This wound closure over 72 hours was assessed quantitatively in Fig. 2.5B. 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(ASGR1PAB) 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(ASGR1PAB, hsa-miR-26a) or mLNPs(ASGR1PAB, hsa-miR-26a) inhibited cell proliferation by 50%.

Along with wound closure, the relative wound area was also analyzed temporally in Fig. 2.5C. 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. 2.5B 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(ASGR1PAB, miRNA) decreased the rate of closure to 5 ± 4.3% per day (closed circles) and with mLNPs(ASGR1PAB, 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. 2.5B 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. 2.6. 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 and more reliable than intravenous (IV) administration [97–101]. Also, vesicles administered by IP injection distribute broadly to the organs and the visceral adipose tissue because of access to the lymphatic system [97–101], which was preferred for testing our functionalized vesicles.

The treated mice are euthanized after three days using carbon dioxide followed by cervical dislocation, and their organs are harvested (Fig. 2.6B). The tissue was analyzed using RNA extraction protocols described in the Materials and Methods (Fig.2.6C). The relative miRNA concentration taken up in the organs is compared to the relative concentration of the U6 snRNA (Fig. 2.6D). The crossover threshold (Ct) is determined for the miRNA and the U6 snRNA. Finally, the relative-fold miRNA uptake in the mouse organs is determined by the ΔΔCt method (Fig. 2.6E).

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 [87,88,102–104]. As far as we are aware, no microRNAs are known to affect mouse P-gp expression or any related mouse transporters within its transporter superfamily. Our former collaborator made miRNA sound

specific, especially for P-gp transporter expression, but a single miRNA can affect several physiological functions [105]. 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 [102,106]. The miRNA is also known to regulate the expression of β-Amyloid Precursor Protein-converting Enzyme 1 [103] and regulate the expression of the insulin-like growth factor-1 β (IGF1Rβ) [104]. The miRNA also has a wide variety of theoretical targets that remain to be investigated in the TargetScanVert and miRDB databases [107,108]. 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 [53,109]. 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 should be considered qualitative

To determine the relative miRNA uptake, miRNA should be normalized against a stably expressing endogenous control gene or a combination of genes. Ideal reference genes should have low expression variability and comparable expression levels, since they can significantly impact miRNA quantitation [e.g. 110].

For these pilot studies the relative miRNA uptake was normalized against the non-coding U6 snRNA [111]. Normalizing against U6 snRNA demonstrates feasibility of our approach but is not ideal the following reasons. U6 snRNA expression can vary in mouse tissues (even within the

same organ), can vary significantly between genders, and can be affected by a mouse's age [110,112–115]. U6 snRNA expression also likely varies significantly between mouse strains and sub-strains [116,117]. 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 the treatment (data not shown).

A table of mice used in these pilot studies is provide in Table S1. Because of potential differences in the U6 snRNA expression in the mice, the relative miRNA uptake in the mouse organs in this work should be considered qualitative. To roughly compare the miRNA uptake between the control in Fig. 2.7 and the functionalized vesicles in Figs. 2.8 and 2.9, 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 GFPMAB as a control antibody. Fig. 2.7 shows the uptake of mmu-miR-298 treated with modified vesicles with the GFPMAB and containing mmu-miR-298 (abbreviated mEVs(GFPMAB, mmu-miR-298) and mLNPs(GFPMAB, mmu-miR-298-5p)). The highest miRNA uptake with the functionalized vesicles was observed in the mouse spleen, which is not surprising considering the IP administration of the functionalized vesicle. The IP injection puts the functionalized vesicle into the lymphatic system and the spleen (a lymphoid organ) [118]. 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 [119].

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 [120]. Young female Nu/Nu mice were treated with functionalized vesicles engineered with NPHS2PAB and loaded with mmu-miR-298. Fig. 2.8A shows the relative miRNA uptake delivered by mEVs(NPHS2PAB, mmu-miR-298) to mouse organs and Fig. 2.8B shows the relative distribution of miRNA uptake of mice treated with mEVs(NPHS2PAB, mmu-miR-298) compared to mice treated with mEVs(GFPPAB, mmu-miR-298). In Fig. 2.8A, 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. 2.8B shows miRNA uptake to the spleen decreased 50%, while the miRNA uptake increased 10-20-fold for mice treated mEVs(NPHS2PAB, mmu-miR-298) than mice treated with mEVs(GFPPAB, mmu-miR-298). Off-target delivery of microRNA by this functionalized vesicle may be due to cross-reactivity of the polyclonal antibody (NPHS2PAB) [121,122]. Figs. 2.8C shows the relative miRNA uptake of organs of mice treated with modified LNPs (abbreviated mLNPs(NPHS2PAB, mmu-miR-298) or mLNPs(NPHS2PAB, mmu-miR-298)) and it is about half the miRNA uptake as mice treated with mEVs, but the distribution of miRNA uptake to the organs was very similar (Fig. 2.8A). The normalized distribution of mice treated with mLNPs(NPHS2PAB, mmu-miR-298) and mLNPs(GFPMAB, mmu-miR-298) in Fig. 2.8D was virtually identical to the distribution in Fig.

2.8B implying that both types of functionalized vesicles engineered with NPHS2PAB had similar targeting mechanisms in mice.

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

After successfully delivering miRNAs by both mEVs and mLNPs bioengineered with ASGR1 antibody to hepatocellular carcinoma HepG2 cells (Fig. 2.4) and achieving 40% targeting of kidney (Fig. 2.8A-D), we wanted to assess the capability of these vehicles in targeting the liver in vivo utilizing the same antibody used in-vitro for liver targeting in female and male Nu/Nu mice. Mice were treated with mEVs(ASGR1PAB, mmu-miR-298) and mLNPs(ASGR1PAB, mmu-miR-298) like the in vitro experiments.

Figs. 2.9A and C shows relative miRNA uptake by various organs in adult male Nu/Nu mice were treated with mEVs(ASGR1PAB, mmu-miR-298) and young female Nu/Nu mice were treated with mLNPs(ASGR1PAB, 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 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. 3.1). 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. 2.9B and 2.9D show the distributions of miRNA uptake between mice treated with functionalized vesicles engineered with the ASGR1PAB and the

GFPMAB. Regardless of the age or gender, the panels show that all of miRNA uptake by mice treated with functionalized vesicles with ASGR1PAB was to the liver compared to 20% relative miRNA uptake by mice treated with control functionalized vesicles with GFPMAB. The high specificity for mEVs and mLNPs that target the ASGR1 receptor on hepatocytes may be due to the endocytotic nature of the receptor [123] and the access of the mesenteric circulation to the intraperitoneal cavity [124].

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) (ACE2MAB) with a 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 [60,125–128]. 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 [129]. The lowest ACE2 mRNA was in the spleen of the rodents [129]. 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 [129]. Therefore, targeting mEVs and mLNPs with ACE2MAB will be relatively non-specific but more evenly distributed among the organs than mEVs and mLNPs with GFPPAB, which primarily targeted the spleen (Fig. 2.7).

Fig. 2.10 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 ACE2MAB. In Fig. 2.10A, 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. 2.10B, the relative miRNA uptake was analyzed with mice treated with mLNPs(ACE2MAB, 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(ACE2MAB, 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 [60,128,129]. Since there is almost no ACE2 enzyme in the spleen [129], the relatively high miRNA uptake after treatment with functionalized vesicles is likely due to IP administration rather than specific targeting by ACE2MAB.

The immunogenicity of the functionalized vesicles was assessed by LegendPlex™ 8-panel Th1/Th2 Bio-plex assay, which tests for 8 major anti- and pro-inflammatory cytokines from two types of CD4+ T helper lymphocytes [130]. Cytokine profiling has been used previously to determine the immunogenicity of EVs and LNPS [131,132]. Fig. 2.10C shows the cytokine levels in mice treated with mEVs(ACE2MAB, mmu-miR-298) and mLNPs(ACE2MAB, 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. 2.7-2.10), 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 to fully manifest itself [133]. Therefore, experiments are planned to examine the immunogenicity of functionalized vesicle mouse treatment over a weeks time frame.

2.4 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 HepG2 cell line were demonstrated by 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 ASGR1PAB (Fig. 2.9), while the least specific miRNA delivery to mouse organs was from functionalized vesicles with

the ACE2MAB (Fig. 2.10). At least over the 72 studied period, the functionalized vesicles also did not appear to be immunogenic (Fig. 2.10).

Even unmodified extracellular vesicles can have oligonucleotide uptake efficiencies and target cells like mEVs [134,135]. Exosomes derived from the human hepatoma Huh7 cell line targeted the human embryonic kidney line over human PBMCs or a human lymphoblast cell line [134]. The miRNA uptake efficiencies for the human embryonic kidney cell line treated with exosomes were 5-200-fold higher than the other cell lines [134]. 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 [135]. 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 [15,53,135]. 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 [136]. 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 [137]. Exosomes have

also been genetically engineered with a construct made from the vesicular stomatitis virus glycoprotein with improved loading, delivery, and tracking [138]. 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 [136].

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 [93,139,140]. 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 [141,142]. 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 [143]. 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 [144]. 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 [144]. 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 [145]. 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 [146]. 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 [147,148]. 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 [149,150]. Synthetic methods are available to produce large amounts of miRNA [151]. Innovative large-scale production of monoclonal Abs is being developed from plants [152]. 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 [153,154].

Several challenges exist for implementing mEVs and mLNPs therapeutically. For long-term viability, EVs and, by extension, mEVs are most often stored at -80oC. However, month-long room temperature storage has been shown after lyophilization and in the presence of trehalose [41,155]. In contrast, lyophilized LNPs can last up to a year at 4oC or room temperature in an oxygen-free environment [156–159]. MicroRNAs also have their challenges and must often be

stored at -80oC [160], but methods are available to increase temperature stability through chemical modification [161]. MiRNA also has the potential for slowly leaking from functionalized vesicles [162].

2.5 Going beyond these pilot studies

In these pilot studies, oligonucleotide delivery through our functionalized engineering approach in HepG2 cells and affect its function are clearly demonstrated. Oligonucleotides are 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 [163]. A 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 [164]. 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., 163,165]. 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 affect multiple mRNAs [89], a wound healing assay of HepG2 was used to determine the functional affects of the hsa-miR-26 (Fig. 2.5). 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. 2.2B, we discuss the theoretical possibility of targeting secreted receptor protein ligands being effective for our functionalize 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 [166–168].

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) are 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 [169–171]. A tool called BestKeeper estimates the stability by the standard deviation (SD) of mean cycle quantitation (Cq) values between candidate ncRNAs [169].

Stability of ncRNAs can also be determined using the ΔCq method where the SD is calculated by the ΔCq of ncRNA gene pairs [170]. The GeNorm algorithm is another approach to gauge ncRNA stability with a calculated gene stability M value [171]. Calculation of variation between groups of candidate control genes can be accomplished with the NormFinder algorithm [172]. 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 [110]. 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., ΔCq = ~0).

Once appropriate control RNAs are selected for monitoring the miRNA in each tissue of the mouse, the effects of miRNA delivery by our functionalized vesicles can be demonstrated experiments analogous to hsa-miR-26a delivery to HepG2 cells in Fig. 2.5. HepG2 cells can be implanted in immune system deficient mice (i.e., Nu/Nu) mice as described [173]. Then the mice can undergo treatment by functionalized vesicles loaded with hsa-miR-26 to determine their effect on HepG2 tumor size.

2.6 Authors contributions

Authorship was determined using guidelines recommended by The International Committee of Medical Journal Editors [174–177], 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.

2.7 Acknowledgments

We would like to thank the National Cancer Institute Grant (1R01CA204846-01A1) for the resources necessary for this research. We would like to thank the generosity of several Pharmaceutical and Biomedical Sciences (PBS) Department faculty members at the University of Georgia (UGA) for sharing their instrumentation, their cell culture hoods, their animal use protocols (AUPs), and space within the animal facilities at the Davidson Life Sciences and the Coverdell buildings at the University of Georgia, Athens, GA 30602. We greatly appreciate the help of Dr. Charnel Byrnes, who trained Ms. Pragati Jain in mammalian cell culture, PBMC vesicle isolation, and assisted her in optimizing electroporation for loading vesicles with miRNA. We want to thank Drs. Mandi M. Murph and Yao Yao for assistance with the mice experiments. We would also like to thank Dr. Balazs Rada for assisting us in getting PBMCs from human volunteers. We want to thank Dr. Sergiy Minko for providing the DLS machine for vesicle size determination from his laboratory. We would also like to thank various PBS faculty members at the UGA for giving comments and suggestions for this project. Most importantly, we would like to thank Dr. Sudeepti Kuppa, who used the original protocol that I designed and got the method to work in vitro in the triple-negative breast cancer MDA-MB-231 cell line, which was a significant part of her Ph.D. thesis. Figs. 2.1, 2.2, and 2.6 were created with BioRender.com.

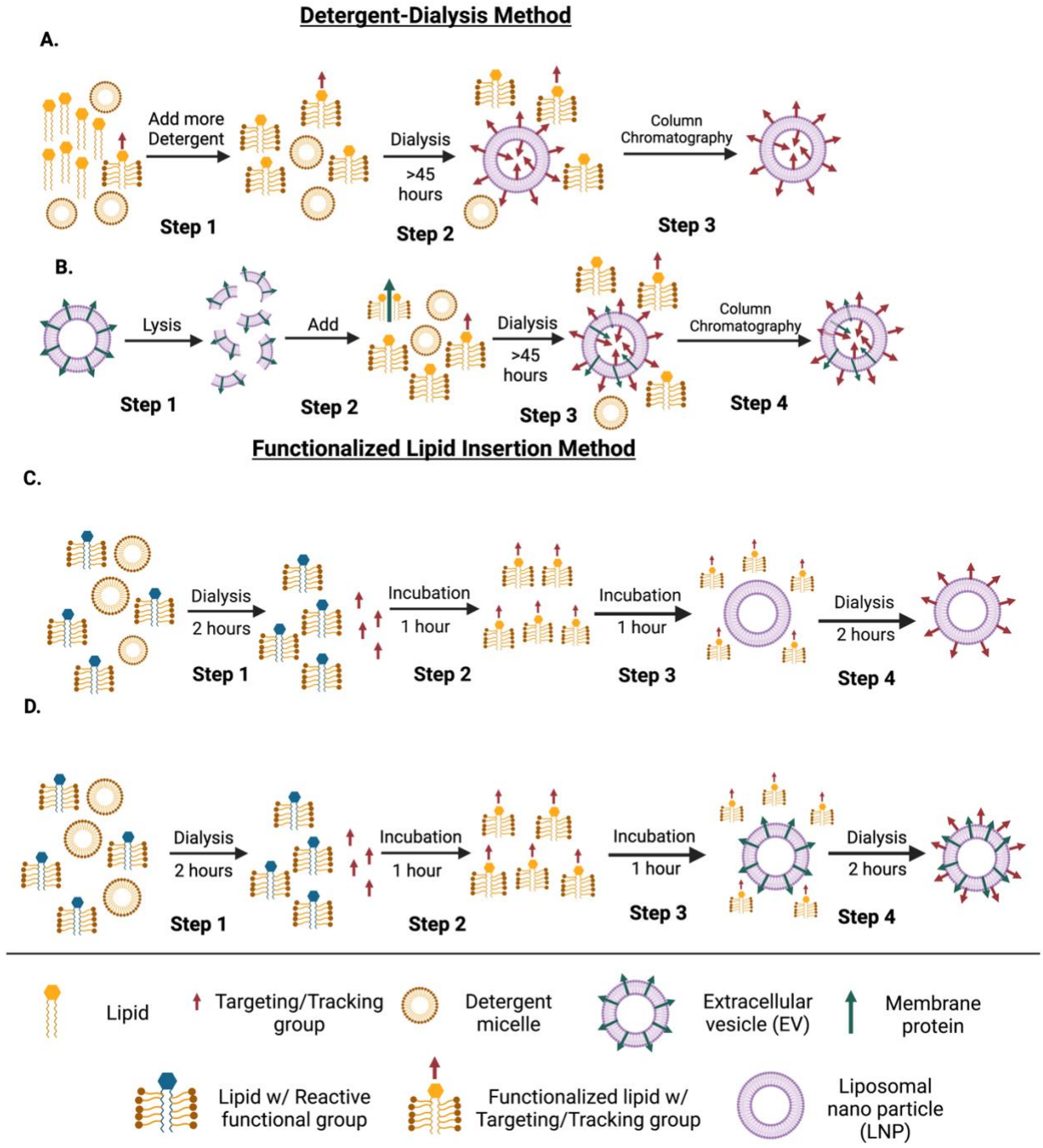


Figure 2.1. Bioengineering functionalized vesicles by the "Detergent-Dialysis Method" and the "Functionalized-Lipid Method." A) Shows the "Detergent-Dialysis Method" that has been

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.

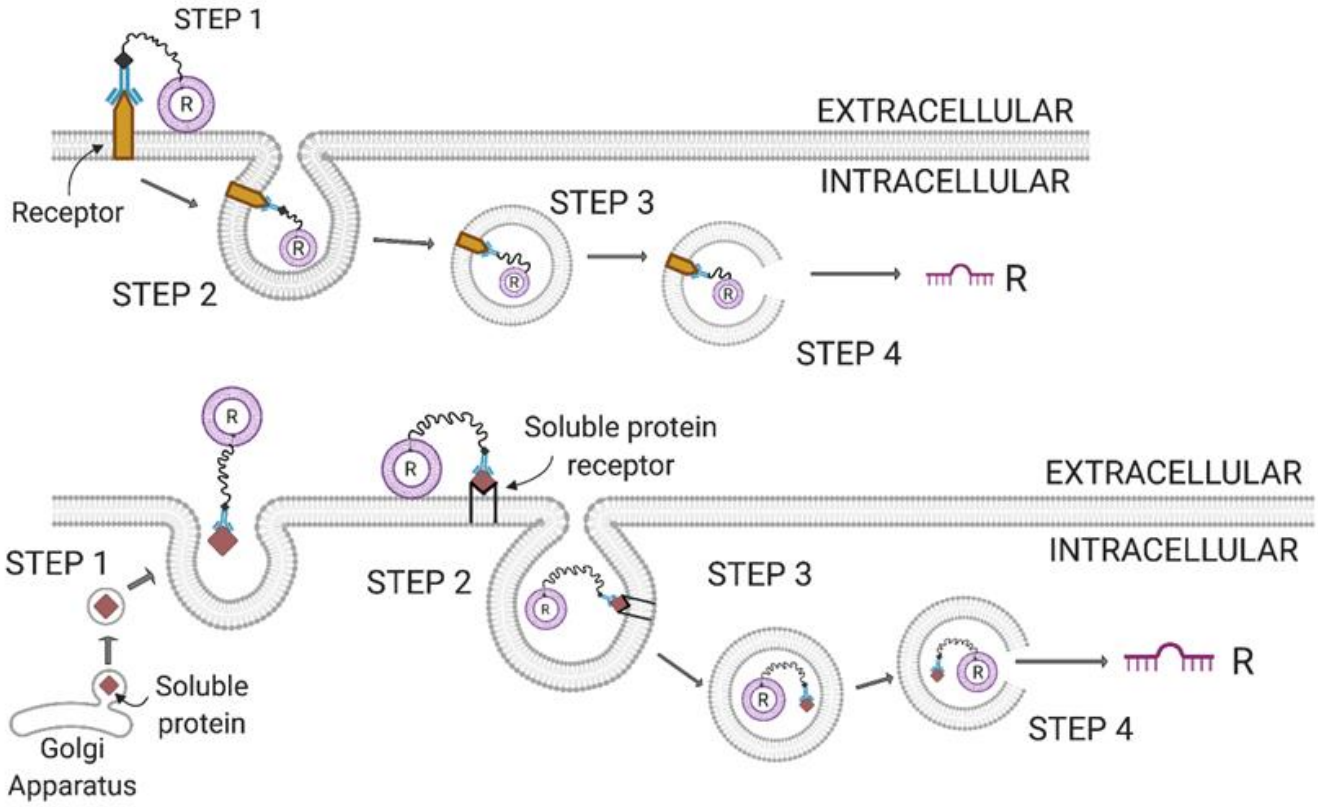


Figure 2.2. Potential mechanisms for cellular endocytosis of functionalized vesicles. A) A functionalized vesicle binds to a cell surface endocytotic receptor in Step 1 and then undergoes endocytosis in Step 2. The receptor-bound functionalized vesicle forms an endosome in Step 3 and

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.

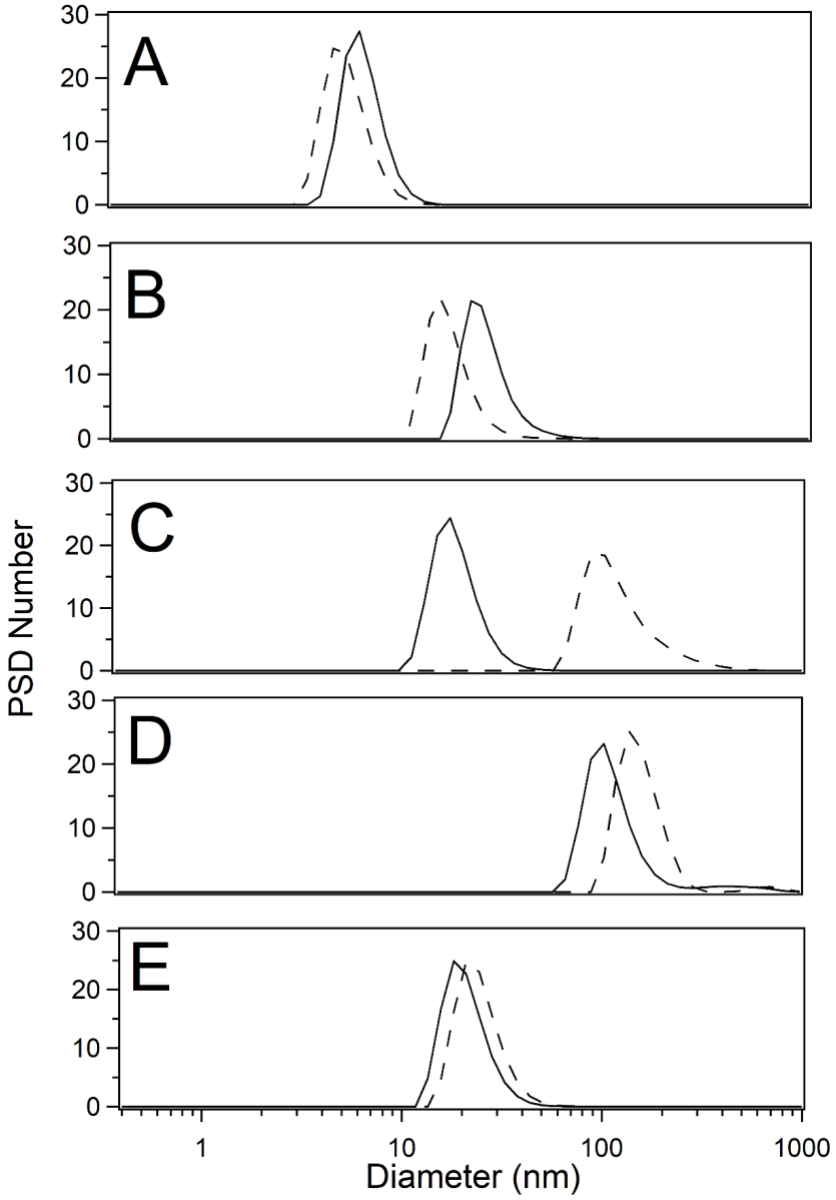
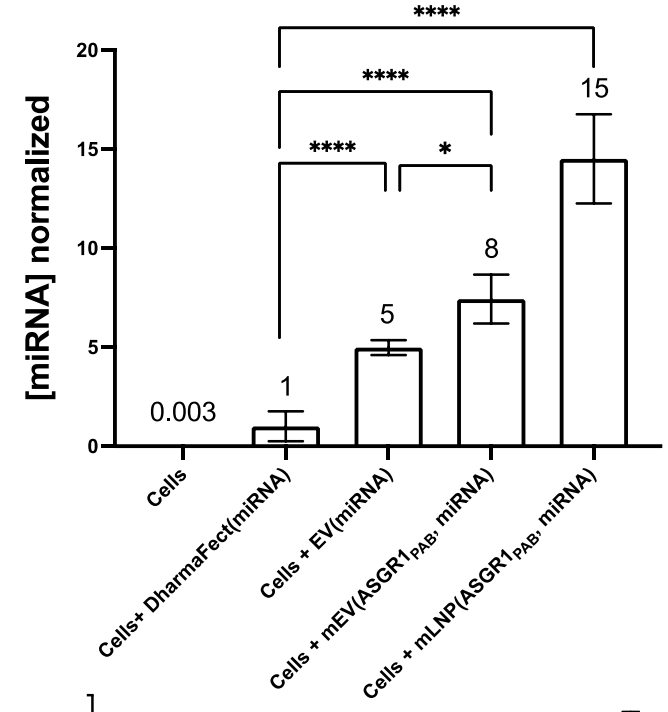


Figure 2.3. Size distribution of functionalized vesicles determined by dynamic light scattering (DLS). A) Detergent DDM micelles (solid line) and DDM-solubilized FAs (dashed line). The detergent solubilized FAs are an equal mixture of NBD-DSPE and DSPE-PEG2000-maleimide. The "Detergent-Dialysis Method" 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.

A



B

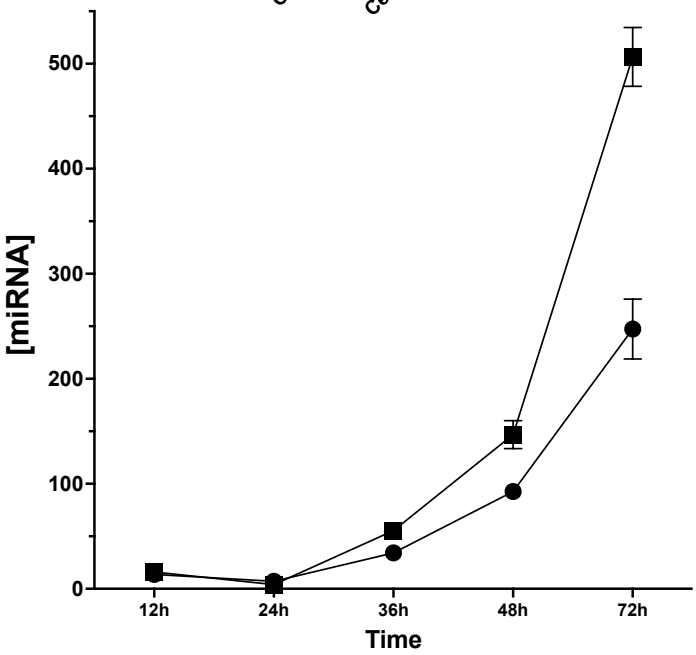


Figure 2.4. Relative miRNA uptake efficiency into HepG2 cells with different treatments. A) The relative mmu-miR-298-5p (miRNA) uptake into HepG2 cells under the various treatments: Untreated HepG2 cells (Cells), HepG2 cells treated with DharmaFect 4 transfection reagent and

miRNA (Cells + DharmaFect(miRNA)), HepG2 cells treated with EVs and miRNA (Cells + EVs(miRNA)), HepG2 cells treated with mEVs with an ASGR1PAB and containing miRNA (Cells + mEVs(ASGR1PAB, miRNA), and HepG2 cells treated with mLNPs with an ASGR1PAB and containing miRNA (Cells + mLNPs(ASGR1PAB, miRNA). The relative miRNA uptake was normalized against the miRNA uptake by treatment with DharmaFect(miRNA). The values in the panel represent the mean ± SD of at least three independent experiments. B) Temporal changes in miRNA uptake efficiency for mLNPs(ASGR1PAB, miRNA) (solid squares) and mEVs(ASGR1PAB, 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 * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p<0.0001$. Abbreviations: SD, standard deviation.

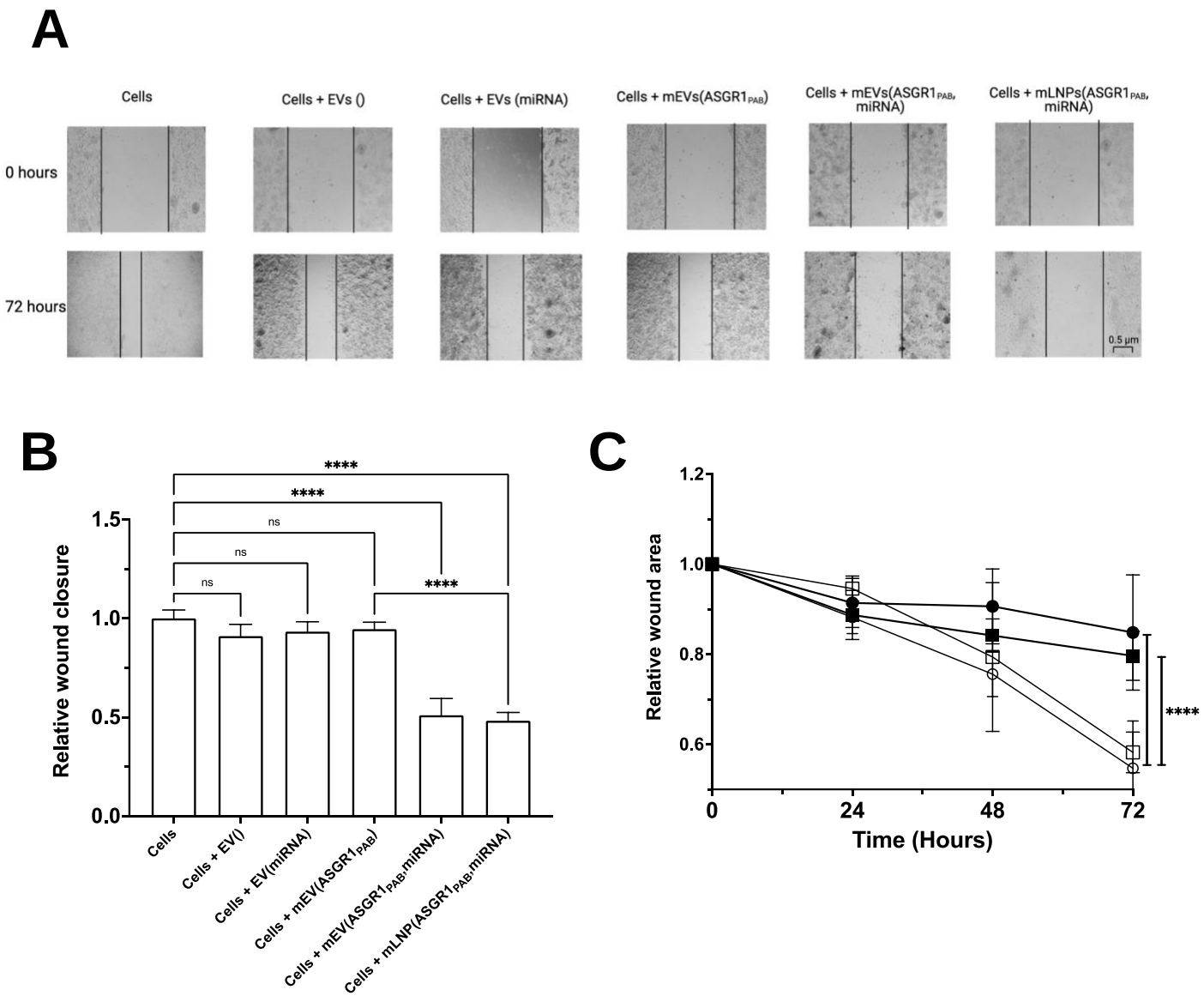


Figure 2.5. Anti-proliferative effects miRNA hsa-miR-26a-5p with HepG2 cells under various treatments. A) Microscopic images of a HepG2 layer during the migration assay with the following treatments after 0 (top) and 72 hours (bottom): Untreated HepG2 cells (Cells), HepG2 cells treated with empty EVs (Cells + EVs()), HepG2 cells treated with EVs containing miRNA

(EVs(miRNA)), HepG2 cells treated with empty mEVs engineered with an ASGR1PAB (Cells + mEVs(ASGR1PAB)), HepG2 cells treated with mEVs with the ASGR1PAB and miRNA (Cells + mEVs(ASGR1PAB, miRNA)), and HepG2 cells treated mLNPs with the ASGR1PAB and miRNA (Cells + mLNPs(ASGR1PAB, 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(ASGR1PAB, miRNA) (closed circles), and mLNPs(ASGR1PAB, miRNA) (closed squares) over 72 hours. To differentiate the functionalized vesicles, the time course for HepG2 cells treated with mEVs(ASGR1PAB, miRNA) and mLNPs(ASGR1PAB, 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 * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p<0.0001$. Abbreviations: SD, standard deviation.

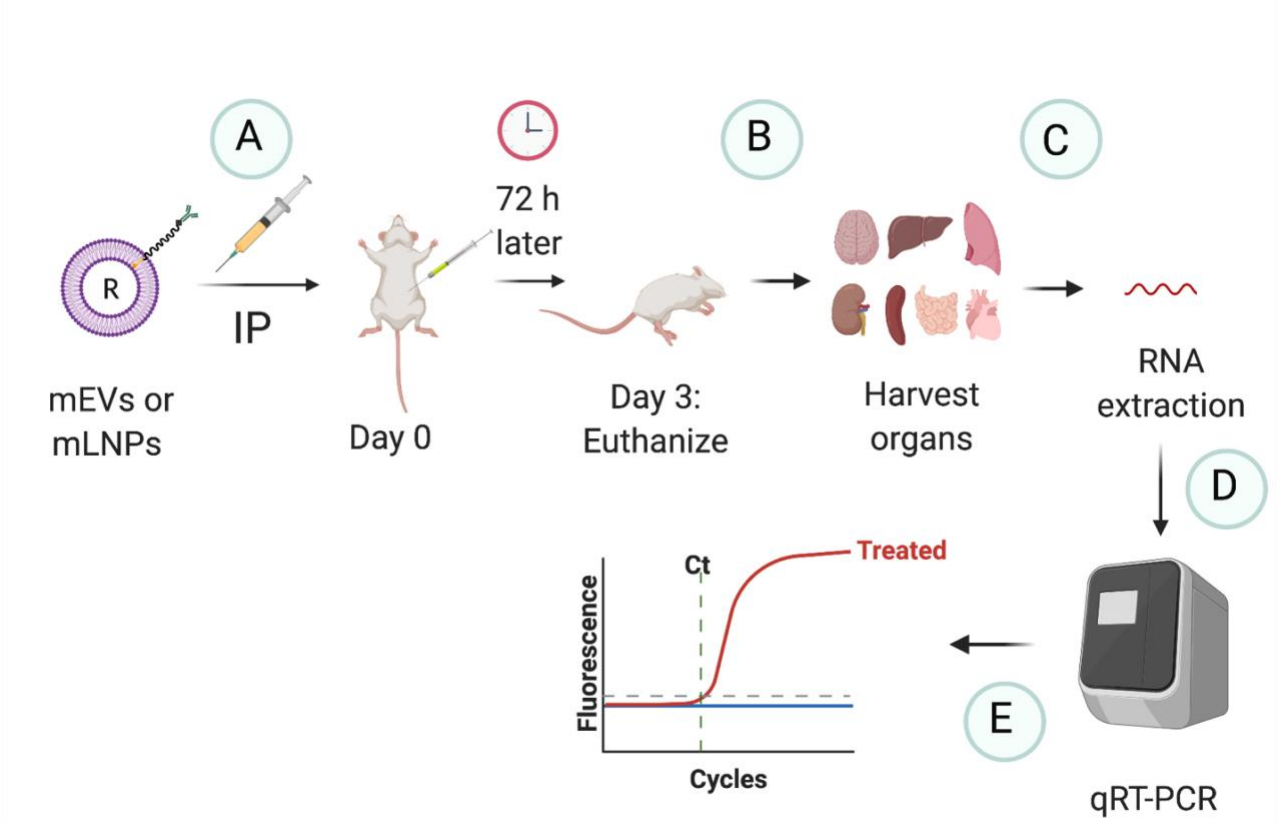


Figure 2.6. Process of administering functionalized vesicles into mice. A) On the first day, functionalized vesicles, mEVs or mLNPs, with miRNA (R) and targeting ligands such as Abs are intraperitoneally (IP) injected into mice. B) Three days later, the mice are euthanized, and the organs are harvested. 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 ΔΔCt method. Abbreviations: R, miRNA.

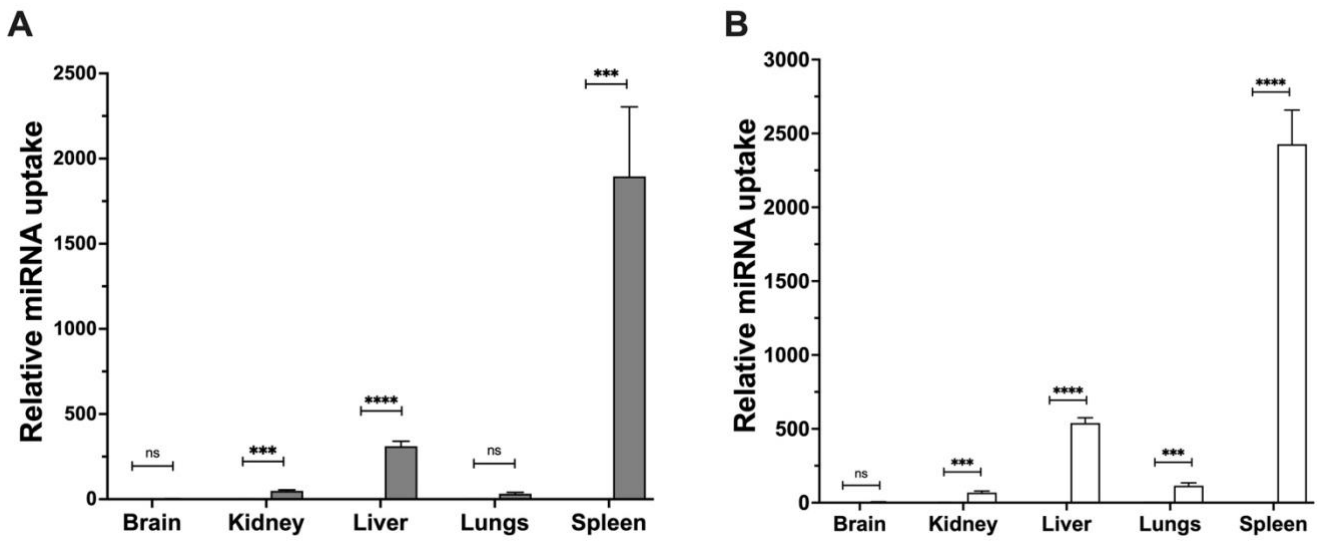


Figure 2.7. Uptake of miRNA in organs of mice treated with functionalized vesicles containing the non-targeting polyclonal green fluorescent GFP (GFPPAB) antibodies. The figure shows the relative-fold uptake of miRNA in the organs of young female Nu/Nu mice (n=4) treated with (A) mEVs(GFPPAB, mmu-miR-298) (gray) and (B) mLNPs(GFPPAB, 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 * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p<0.0001$. 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.

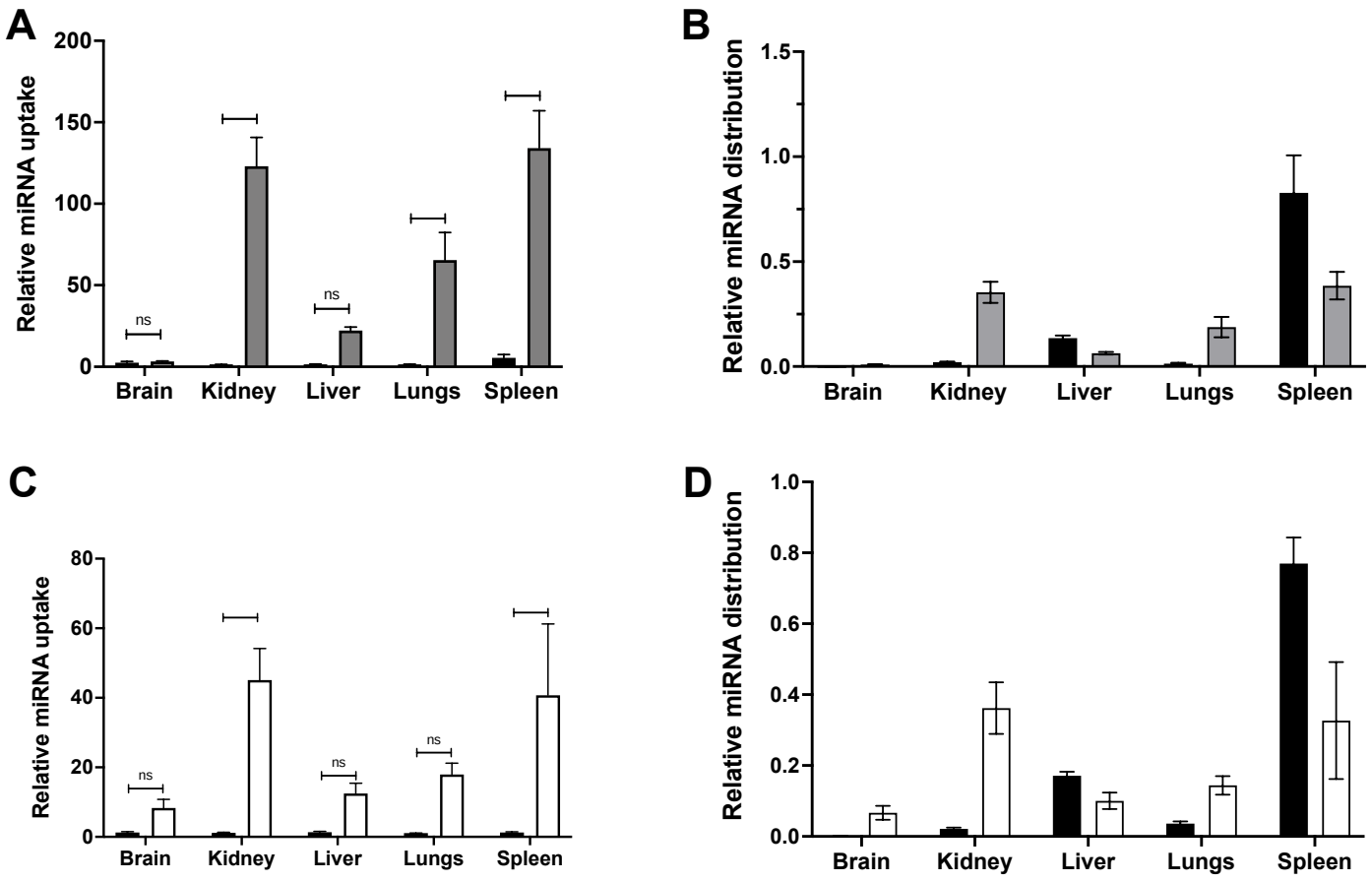


Figure 2.8. Relative mmu-miR-298 distribution by organs with the treatment of mEVs and mLNPs bioengineered with the NPHS2 antibody. The following figure shows the fold-difference of mmu-miR-298 uptake and distribution of 5-7 week old female Nu/Nu mice being treated with (A,B) mEVs when bioengineered 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.

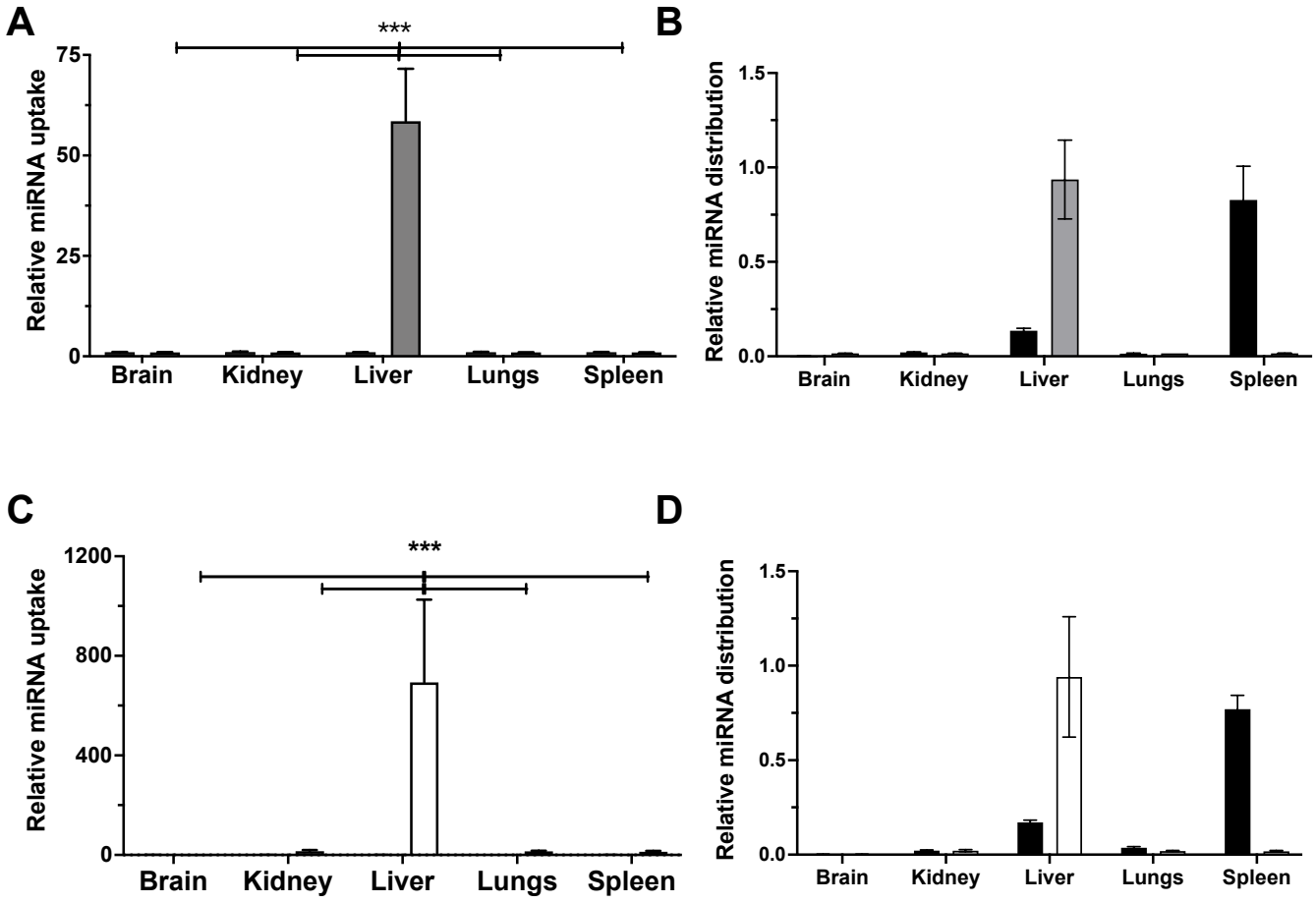


Figure 2.9. Targeting of mmu-miR-298 with the ASGR1 antibody in mice. The following figure shows the fold-difference of mmu-miR-298 uptake (A,C) and the distribution (B,D) after being treated with (A,B) mEVs when bioengineered with ASGR1 antibody (gray)(n=3) in male Nu/Nu mice aged between 15-17 weeks 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.

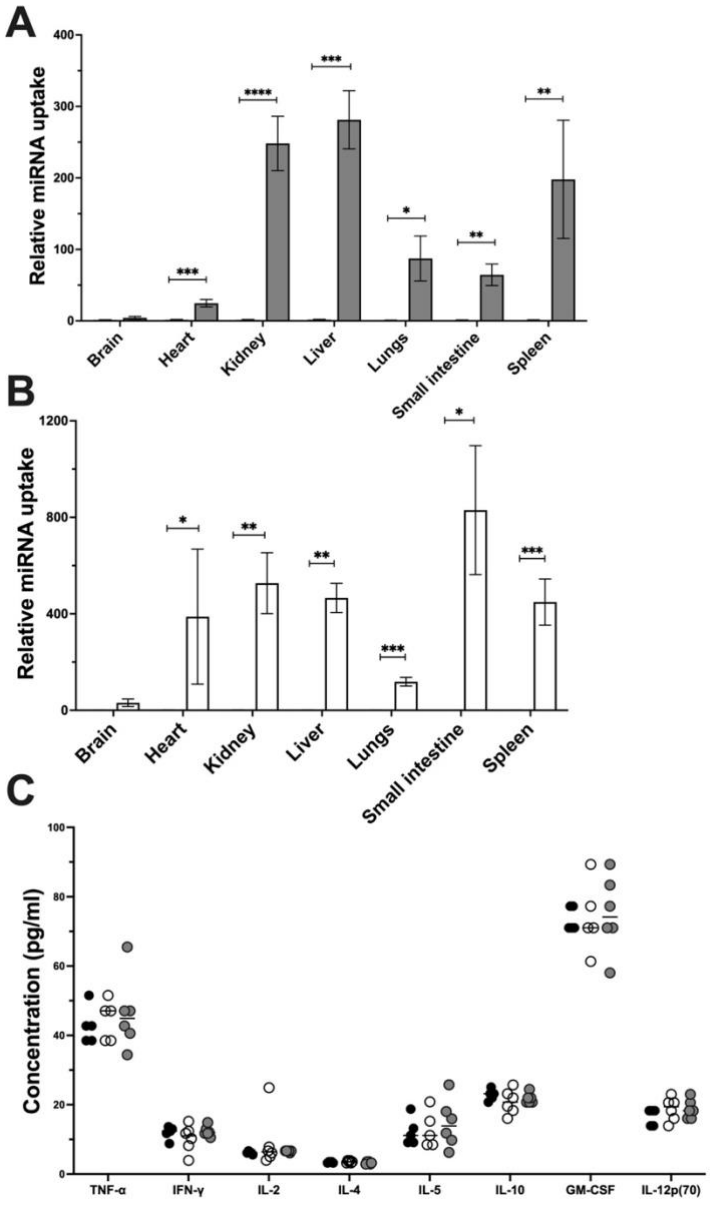


Figure 2.10. ACE2 targeting and immune reactivity of mEV(ACE2) and mLNP(ACE2) in-vivo. The relative ratio of mmu-miR-298 uptake in various organs of mice after being treated with (A) mEVs(ACE2PAB, miRNA) (gray) and (B) mLNP(ACE2PAB, miRNA) (white) versus SFM only treated female C57BL6/J mice aged 5-7 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(ACE2PAB, miRNA) (gray circles), mLNP(ACE2PAB, 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 * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p<0.0001$.

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CHAPTER 3

SUPPLEMENTARY INFORMATION FOR:

A NOVEL METHOD FOR PRODUCING FUNCTIONALIZED VESICELS THAT EFFICIENTLY DELIVER OLIGONUCLEOTIDES *IN VITRO* IN CANCER CELLS AND *IN VIVO* IN MICE

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3. The general idea of this work is protected by the provisional patent application number is 62/824,901 and Patent Cooperation Treaty (PCT) application number PCT/US2020/025333.

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Table 3. Mice used in these pilot studies showing the strain, gender, age and functionalized vesicle treatment given to the mice. Mice that are 5-7 weeks are considered young, while mice of 15-17 weeks are considered adult.

Abbreviations: ASGR1MAB, ASGR1 receptor monoclonal antibody; GFPMAB, green fluorescent protein (GFP) monoclonal antibody; NPHS2PAB, NPHS2 or podocin polycolonal antibody; mEV, modified extracellular vesicle; mLNP, modified liposomal nanoparticle; SFM, Serum Free Media;

Figure	Strain	Common Name	Gender	Age	#	Treatment
7A	C57BL6/J	C57 black 6 mouse	Female	5-7 weeks	4	mEV(GFPMAB, mmu-miR-298-5p)
7B	C57BL6/J	C57 black 6 mouse	Female	5-7 weeks	4	mLNP(GFPMAB, 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(NPHS2PAB, mmu-miR-298-5p)
8B	Nu/Nu	Immunodeficient nude mouse	Female	5-7 weeks	6	mLNP(NPHS2PAB, 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(ASGR1PAB, mmu-miR-298-5p)
9B	Nu/Nu	Immunodeficient nude mouse	Female	5-7 weeks	6	mLNP(ASGR1PAB, 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(ACE2MAB, mmu-miR-298-5p)
10B	C57BL6/J	C57 black 6 mouse	Female	5-7 weeks	6	mLNP(ACE2MAB, mmu-miR-298-5p)
10A,B	C57BL6/J	C57 black 6 mouse	Female	5-7 weeks	6	SFM only

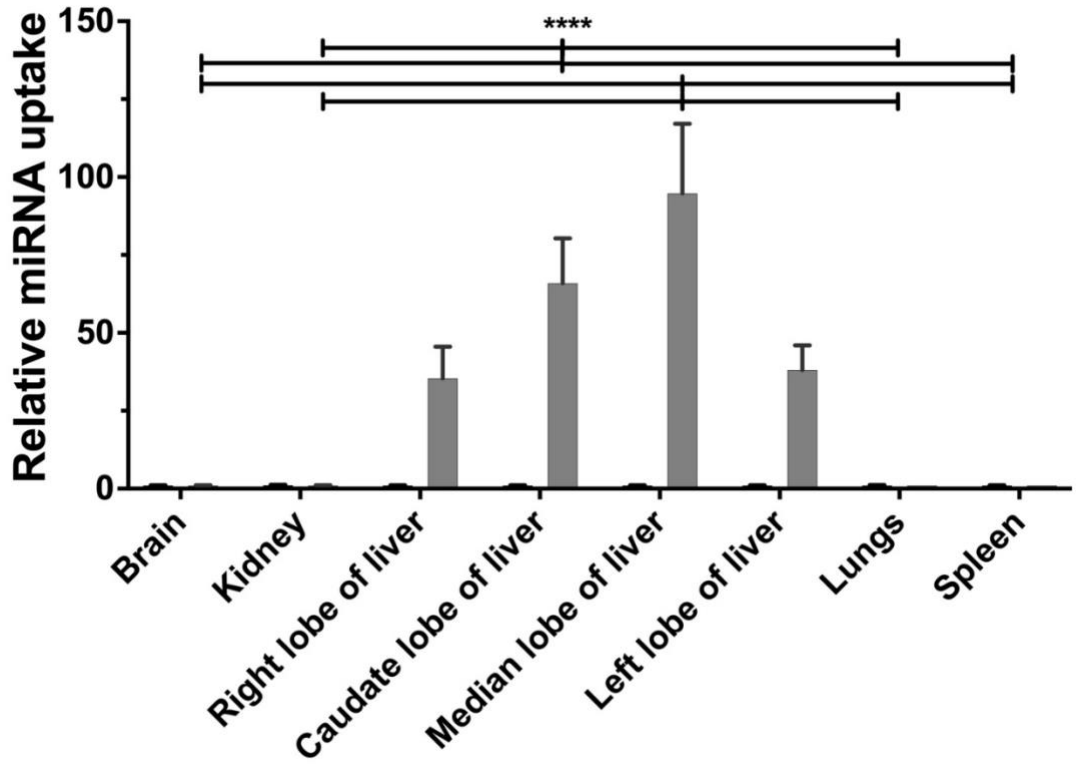

Figure 3.1: The relative miRNA uptake by different mouse liver lobes after mEV treatment of vesicles engineered with the ASGR1 receptor polycolonal antibody and loaded with mmu-miR-298. The following figure shows the relative miRNA uptake after being treated with mEVs bioengineered with the ASGR1 receptor polyclonal antibody and loaded with mmu-miR-298 (mEV(ASGR1PAB, mmu-miR-298)) (gray)(n=3) in adult male Nu/Nu mice versus SFM only treated adult male mice (black). All the data were normalized to the constitutive level of U6 snRNA and represent the mean± SEM of three independent experiments. The mEV dosage given for each of the mice was ~100 μg miRNA with an injection volume of 250-300 μl.