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MicroRNA Delivery by Cationic Lipoplexes for Lung Cancer Therapy

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Abstract

Lung cancer is the leading cause of cancer deaths in western countries and carries a poor overall five year survival rate. Several studies demonstrate that microRNAs (miRNAs or miRs) are actively involved in tumor development by serving as tumor suppressors, oncogenes or both. In lung cancer, miRNAs may serve as both diagnostic and prognostic biomarkers as well as regulate *in vitro* and *in vivo* tumor progression. However, miRNA-based therapy is faced with several challenges including lack of tissue specificity, lack of optimal delivery systems, poor cellular uptake and risk of systemic toxicity. Here, we report a cationic lipid based miRNA delivery system to address some of these challenges. Among many lung cancer related miRNAs, miR-133b, a tumor suppressor, was selected as a therapeutic target because it directly targets the prosurvival gene MCL-1 thus regulating cell survival and sensitivity of lung cancer cells to chemotherapeutic agents. The efficacy of pre-miR-133b containing lipoplexes was evaluated in A549 non-small cell lung cancer (NSCLC) cells. Compared with siPORT NeoFX transfection agent, lipoplexes delivered pre-miR-133b in a more efficient manner with ∼2.3-fold increase in mature miR-133b expression and ∼1.8-fold difference in MCL-1 protein downregulation *in vitro*. In the *in vivo* biodistribution study, lipoplexes achieved ∼30% accumulation in lung tissue, which was ∼50-fold higher than siPORT NeoFX transfection agent. Mice treated with pre-miR-133b containing lipoplexes had mature miR-133b expression in lung ∼52-fold higher than untreated

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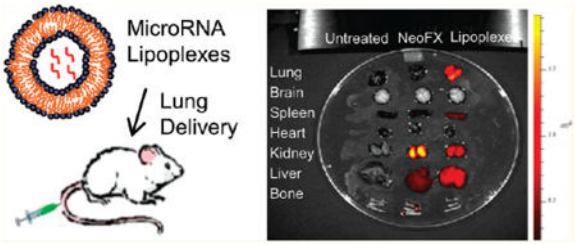
Supporting Information: Flow cytometry analysis and confocal microscopy analysis of cellular uptake of empty lipo-plexes. This material is available free of charge via the Internet at http://pubs.acs.org.

mice. Our results demonstrated that cationic lipoplexes are a promising carrier system for the development of miRNA-based therapeutics in lung cancer treatment.



Keywords

microRNA; lipoplexes; lung cancer; cationic lipids

1. Introduction

Lung cancer is the leading cause of cancer death in the United States, and non-small cell lung cancer (NSCLC) accounts for almost 80% of lung cancer deaths. Although significant therapeutic improvements have been made in recent years, the overall 5-year survival rate remains only about 15%[1–3].The poor survival rate is likely due to multiple factors including late presentation of disease, lack of biomarkers for early detection, and molecular heterogeneity. Thus, early detection, prognostication and targeted therapies may all contribute to improved outcomes in lung cancer.

MicroRNAs (miRNAs or miRs) are noncoding RNAs that are important in several biologic processes including proliferation, apoptosis, development and cellular differentiation, and may function as either oncogenes or tumor suppressors[2–9]. Many miRNAs, such as let-7[10–13], miR-21[2,3,7,14], miR-29,[2,7,15], miR- 126[16,17] andmiR-133b, [18] to name a few, are dysregulated in lung cancer and have a role in tumor development and progression. MiRNAs may also function as biomarkers for diagnosis and prognosis in lung cancer.[19,20] The rapid progress in miRNA studies has led to translational studies focused on miRNA targeted therapeutics in cancer.

Current strategies for miRNA-based delivery include the use of antisense oligonucleotides such as antagomirs, locked nucleic acid (LNA) anti-miR constructs, microRNA sponges, miR-masks, to block the oncogenic miRNAs, and the introduction of synthetic miRNA mimics to restore tumor suppressor miRNA expression.[7] Each of these approaches harbors its own limitations. The challenges for developing miRNA-based therapeutics include achieving efficient cellular uptake, tissue-specific delivery and minimizing systemic toxicity and off target effects.[7,21] “Naked” oligonucleotides are highly unstable due to rapid degradation in serum and renal clearance. The negative charge of oligonucleotides also interferes in their interaction with the cell membrane leading to poor cellular uptake. In order to overcome these obstacles, various delivery strategies have been developed, such as chemical modification of oligonucleotides and the use of nanoparticles as carrier systems[7,22].

Many investigators have demonstrated that nanoparticle carrier systems may be a promising approach to both *in vitro* and *in vivo* oligonucleotide delivery. Recently nanoparticles have been used for miRNA delivery and have shown great potential in the development of miRNA-based therapeutics in cancer treatment. Chen et al. developed liposome—polycation—hyaluronic acid (LPH) nanoparticles to effectively deliver siRNA and miR-34a to B16F10 lung metastasis in a syngeneic murine model[23]. Wiggins et al. and Trang et al. used a neutral lipid emulsion, MaxSuppressor *in vivo* RNALancerII (BIOO Scientific, Inc.), to deliver both miR-34a and let-7 to block tumor growth in lung cancer mouse models[24,25]. In the current study, we used cationic lipids to condense miRNAs to form lipoplexes in an attempt to enhance the cellular uptake and the pharmacological effectiveness both *in vitro* and *in vivo*. We selected miR-133b, a potential tumor suppressor in NSCLC, as a candidate for miRNA delivery. We previously demonstrated that MCL-1 is a direct target of miR-133b[18]. NSCLC cells overexpress MCL-1 protein, which reduces the sensitivity of lung cancer cells to apoptotic stimuli[26]. We have demonstrated in this work that cationic lipid formulations would be more effective for miR-133b delivery both *in vitro* and *in vivo* compared to standard transfection reagents, siPORT NeoFX transfection agent (NeoFX in short, Ambion, AM4511).

2. Experimental Section

Materials

1,2-Di-*O*-octadecenyl-3-trimethylammonium propane (chloride salt) (DOTMA) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol was purchased from Sigma-Aldrich (St. Louis, MO). D-Alpha-tocopheryl polyethylene glycol 1000 succinate (vitamin E TPGS) was purchased from Eastman (Kingsport, Tennessee). Pre-miR-133b (analogues that mimic precursor-miR-133b, mature miR sequence: 5′ UC-GUACCCG UGAGUAAUAAUGCG 3′, Ambion, PM10029), pre-miR-negative control #1 (pre-miR-NC in short, scrambled microRNA precursor with no target, Ambion, AM17110) and Cy3 dye labeled pre-miR-negative control #1 (Cy3-pre-miR-NC in short, Ambion, AM17120) were purchased from Ambion (Austin, Texas). Cy5 dye labeled oligodeoxynucleotides (Cy5-G3139, 5′-Cy5-TCT CCC AGC GTG CGC CAT-3′) was custom synthesized by Alpha DNA, Inc. (Montreal, Canada).

Experimental Animals

ICR mice of weight 18—20 g (age 4—6 weeks) were purchased from Harlan Laboratories, Inc. All work performed on animals was in accordance with and approved by the IACUC committee at The Ohio State University.

Preparation of Pre-miR-133b Containing Lipoplexes

Empty liposomes were prepared first by injecting the lipid mixture in ethanol (DOTMA:cholesterol:TPGS = 49.5:49.5:1 molar ratio) into 20 mM HEPES buffer (pH = 7.4) to achieve 10% ethanol and 90% aqueous in the final mixture[27]. Pre-miR-133b containing lipoplexes were prepared by adding pre-miR-133b to empty liposomes with the lipids to pre-miR-133b mass ratio of 12.5. The mixture was incubated at room temperature for 15 min and used immediately.[28]

Particle Size and Surface Charge Measurement

The size distributions of pre-miR-133b containing lipoplexes were measured by dynamic light scattering (Brookhaven Instruments Corporation, Holtsville, NY, BI 200SM). The wavelength of the laser was 632.8 nm, and the detection angle was 90°. The size distributions of three batches of lipoplexes prepared independently were measured at 20 °C, and the mean diameter by volume ± standard deviation was reported.

The surface charges of pre-miR-133b containing lipoplexes were measured using ZetaPALS zeta potential analyzer (Brookhaven Instruments Corporation, Holtsville, NY). Three batches of independently prepared lipoplexes were diluted in 20 mM HEPES buffer. Three measurements, each consisting of 5 runs, were performed at 20 °C. The Smoluchowski model was used to calculate the zeta potential, and the mean ± standard deviation was reported.

Cell Culture

A549 cells obtained from the American Type Culture Collection (ATCC) (Manassas, VA) were routinely cultured in a 25 cm^2 T flask containing 5 mL of RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA). The cells were seeded into T flasks at a concentration of 1×10^5 viable cells/mL and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Transfection Studies of Pre-miR-133b Containing Lipoplexes

A549 cells were seeded at 2×10^5 viable cells/well in 6 well plates containing 2 mL of culture medium supplemented with 10% FBS. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 overnight. Then the culture medium was replaced with the medium containing no FBS. Pre-miR-133b (100nM) containing lipoplexes were then added into the medium. The cells were incubated at 37 °C for 4 h, and then transferred into 2 mL fresh culture medium supplemented with 10%FBS. All transfection experiments were performed in triplicate.

Pre-miR-133b delivered by NeoFX transfection agent was the positive control. The transfection of pre-miR-133b via NeoFX was performed by following the manufacturer's manual. Briefly, 5 μL of NeoFX was mixed with 95 μL OptiMEM (Invitrogen, 11058021) and the mixture was incubated at room temperature for 10 min. Pre-miR-133b was diluted in OptiMEM and then added into the NeoFX transfection agent. The final mixture was incubated at room temperature for another 10 min and used for transfection by following the same transfection procedure as for transfection of lipoplexes. Untreated cells and cells transfected by scrambled microRNA precursor with no target, pre-miR-NC, which were delivered by both lipoplexes and NeoFX, were negative controls in our work.

Cytotoxicity of Pre-miR-133b Containing Lipoplexes

The cytotoxicity of pre-miR-133b containing lipoplexes was evaluated using alamarBlue assay (Invitrogen, A13262). 48 h post transfection, the cells were incubated with fresh culture medium containing 10% alamarBlue for 2 h at 37 °C in a humidified, 5% CO_2 atmosphere, protected from light. The fluorescence intensity was read at an emission

wavelength of 590 nm under the excitation wavelength of 570 nm using a microplate reader (GENios Pro, Tecan, USA).

Quantification of Mature miR-133b and MCL-1 mRNA Expression in A549 Cells by Quantitative Real Time PCR (qRT-PCR)

48 h post transfection, the cells were washed with cold 1× PBS twice and then treated with 1 mL of TRIzol reagent (Invitrogen, 15596-018). Total RNA was extracted by adding chloroform, further purified by isopropanol precipitation and washed by 70% ethanol. To measure mature miR-133b expression, the total RNA was first reverse transcribed into cDNA using the TaqMan MicroRNA reverse transcription kit (Applied Biosystems, 4366596). The qRT-PCR amplification of cDNA was then performed using TaqMan MicroRNA assay (Applied Biosystems, Assay ID 002247). The mature miR-133b expression was determined by the $\Delta\Delta C_T$ method and normalized to RNU44 (Applied Biosystems, Assay ID 001094), which was the endogenous control in the corresponding samples, and relative to the untreated control cells.

To measure MCL-1 expression at the mRNA level, the total RNA was transcribed into cDNA using the first-strand cDNA synthesis kit (Invitrogen, 18080051). The resulting cDNA was amplified by qRT-PCR (Applied Biosystems, assay ID Hs03043899_m1). Relative gene expression values were determined by the$\Delta\Delta C_T$ method. MCL-1 expression was normalized to GAPDH (Applied Biosystems, assay ID Hs02758991_g1), which was the endogenous reference in the corresponding samples, and relative to the untreated control cells.

Quantification of MCL-1 Protein Expression in A549 Cells by Western Blotting

48 h following transfection, the cells were washed with PBS twice and then incubated with the RIPA lysis buffer (Sigma-Aldrich, R0278) containing a protease inhibitor cocktail (Sigma-Aldrich, P8340) on ice for 15 min. The cell lysate was centrifuged for 15 min at 12000*g* at 4 °C. The protein concentration of the supernatant was measured by bicinchoninic acid (BCA) assay (Biorad, 500-0006), and 50 *μ*g of protein from each sample was loaded in a 4—15% Ready Gel Tris-HCl polyacrylamide gel (BioRed, Hercules, CA), and then transferred to a PVDF membrane. After blocking with 5% nonfat milk in Tris-buffered saline/Tween-20 for 1 h, the membranes were incubated with monoclonal rabbit anti-human MCL-1 (1:500, Cell Signaling Technology, 4572S) or monoclonal mouse anti-human β-tubulin (1:10000, Sigma, T8328) at 4 °C overnight, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000, Santa Cruz Biotechnology, SC2004) and horseradish peroxidase-conjugated goat anti-mouse IgG (1:1000, Santa Cruz Biotechnology, SC2005) for 1 h at room temperature. The membrane was then developed with Pierce SuperSignal West Pico or Dura Extended Duration Substrate (Pierce, Rockford, IL) and imaged with Kodak X-OMAT film (Kodak, Rochester, NY).

Cellular Uptake of Pre-miR Containing Lipoplexes in A549 Cells

The cellular uptake of the pre-miR containing lipoplexes was studied by flow cytometry (BD LSR II, San Jose, CA, USA) and laser scanning confocal microscopy (Olympus FV1000, Center Valley, PA, USA). A549 cells were transfected with Cy3-pre-miR-NC

containing lipoplexes. The cells were harvested 0, 24, and 48 h post transfection. To harvest the samples, the cells were first detached from culture plates using 0.25% trypsin, washed with PBS twice and fixed using 4% paraformaldehyde. In the flow cytometry experiments, the fluorescence signals of Cy3 were observed in the PE channel. 10,000 events were collected for each sample, and the average results of 3 replicates were reported. In confocal microscopy experiments, A549 cells were counterstained with DAPI and mounted on glass slides. The fluorescence signals of DAPI and Cy3 were observed in the DAPI (dichroic mirror 420—480 nm) and Cy3 (dichroic mirror 550—600 nm) channels respectively.

In Vivo Biodistribution Study of Lipoplexes

ICR mice were given iv injections of Cy5-labeled oligodeoxynucleotide (Cy5-G3139) containing lipoplexes. After 4 h, mice were euthanized and tissues were collected and fixed in 10% formalin for 12 h. The tissues were then soaked in 30 wt % sucrose solution for another 12 h. The fluorescence signals of Cy5 emitted by the whole tissues were measured using Xenogen IVIS-200 Optical In Vivo Imaging System (Caliper Life Sciences, Hopkinton, MA). All tissues were then cryopreserved in optimal cutting temperature (OCT) compound. The cross sections of tissues were counter-stained with Hoechst and mounted on glass slides for confocal microscopy analysis. The fluorescence signals of Hoechst and Cy5 were observed in the DAPI (dichroic mirror 420—480 nm) and Cy5 (dichroic mirror 655—755 nm) channels respectively.

In Vivo Delivery of Pre-miR-133b Containing Lipoplexes

Pre-miR-133b and pre-miR-NC containing lipoplexes were administered to ICR mice by tail vein injection at a concentration level of 1.5 mg/kg. 48 h after administration, mice were euthanized and lung tissues were collected and quickly frozen in liquid nitrogen. The frozen lung tissues were ground to powders and treated with TRIzol. The total RNA was extracted by adding chloroform, further purified by isopropanol precipitation and washed by 70% ethanol. The total RNA was then reverse transcribed into cDNA using the TaqMan MicroRNA reverse transcription kit. The qRT-PCR amplification of cDNA was then performed using TaqMan MicroRNA assay (Applied Biosystems, Assay ID 002247). The mature miR-133b expression was determined by the $\Delta\Delta C_T$ method and normalized to sno135 (Applied Biosystems, Assay ID 001230), which was the endogenous control in the corresponding samples, and relative to the untreated control tissue samples.

Statistical Analysis

Data are presented as the mean ± SD. The statistical significance was determined by using the analysis of variance (ANOVA). *P* values of <0.05 were considered significant.

3. Results

Characteristics of Pre-miR-133b Containing Lipoplexes

Cationic lipids, such as DOTMA used in our work, are widely used to condense nucleic acids to form lipoplexes. Typical size distributions of pre-miR-133b containing lipoplexes and pre-miR-133b NeoFX complexes are shown in Figure 1. The mean diameters by volume were 70.0 ± 5.8 nm for lipoplexes and 2427.7 ± 287.4 nm for NeoFX complexes. The zeta

potentials were 11.73 ± 1.47 mV for the lipoplexes and —19.91 ± 0.83 mV for NeoFX complexes.

Cytotoxicity of Pre-miR-133b Containing Lipoplexes

The cytotoxicity concern of cationic lipids usually limits their applications both *in vitro* and *in vivo*, and thus the cytotoxicity of pre-miR containing lipoplexes was first evaluated *in vitro* before we conducted any further experiments. A549 cells were transfected with pre-miR-133b containing lipoplexes, pre-miR-NC containing lipoplexes, pre-miR-133b NeoFX complexes, pre-miR-NC NeoFX complexes, empty NeoFX complexes and empty lipoplexes. At 48 h post transfection, cell viability was measured using alamarBlue assay. As shown in Figure 2, there was minimal loss of cell viability among cells transfected with either lipoplexes or NeoFX complexes compared with untreated cells, suggesting that pre-miR-133b containing lipoplexes did not show cytotoxicity in A549 cells.

Mature miR-133b Expression *in Vitro*

Mature miR-133b expression was measured 48 h post transfection. Figure 3 shows that A549 cells treated at the pre-miR-133b concentration level of 100 nM had ∼2.3-fold higher mature miR-133b expression when transfected with lipoplexes compared to NeoFX complexes. This suggests that lipoplexes may be a better carrier system. This is also demonstrated by flow cytometry and confocal microscopy analysis.

MCL-1 Expression

MCL-1 (myeloid cell leukemia 1) is an antiapoptotic member of the Bcl-2 family originally isolated from the ML-1 human myeloid leukemia cell line. NSCLC cells also overexpress MCL-1 protein, which reduces the sensitivity of lung cancer cells to apoptotic stimuli.[23] Our previous study showed that MCL-1 was a direct target of miR-133b.[18] Delivery of pre-miR-133b to NSCLC cell lines (H2009) resulted in reduced expression of MCL-1 expression and thus induced apoptosis when tumor cells were exposed to chemotherapeutic agents.[18]

MCL-1 mRNA and protein expression were measured using qRT-PCR (Figure 4) and Western blotting (Figure 5) respectively. No significant change of MCL-1 mRNA expression was observed among cells transfected with pre-miR-133b containing lipoplexes or NeoFX complexes, which was consistent with our previous results.[18] The expression of MCL-1 protein, however, was decreased 56.2% when cells were transfected with pre-miR-133b containing lipoplexes and 31.7% with pre-miR-133b NeoFX complexes. No significant change in MCL-1 mRNA or protein expression was observed when pre-miR-NC was delivered via lipoplexes or NeoFX complexes. These results suggested post-transcriptional regulation of MCL-1 by miR-133b.

Cellular Uptake of Pre-miR-133b Containing Lipoplexes

Flow cytometry and confocal microscopy were used to study the cellular uptake of Cy3-pre-miR containing lipoplexes and NeoFX complexes 0, 24, and 48 h post transfection (Figures 6 and 7; see Supporting Information for cellular uptake of empty lipoplexes). Generally, a typical used transfection period is 4 h. 0 h post transfection is the time point right after the

transfection process was stopped. This time point was selected to evaluate the cellular uptake of lipoplexes and NeoFX complexes, which helped explain why pre-miR delivered by lipoplexes had better efficacy than that delivered by NeoFX complexes. As shown in Figure 6, the fluorescence signal of cells transfected with Cy3-pre-miR containing lipoplexes was much higher than that of those transfected with Cy3-pre-miR NeoFX complexes and free Cy3-pre-miR. In addition, the lipoplexes treated cells showed narrower Cy3 fluorescence distribution than NeoFX complexes treated cells right after transfection. Confocal microscopy also showed stronger and more uniform cellular uptake of lipoplexes than NeoFX complexes (Figure 7). These results suggested that the lipoplexes were able to deliver pre-miR more efficiently and thus provided better efficacy than NeoFX complexes. In addition to cellular uptake, the intracellular fate of lipoplexes is also important. Therefore the fluorescence intensity of Cy3 was monitored 24 and 48 h post transfection. As shown in Figure 6, the fluorescence intensity of Cy3 progressively decreased at 24 and 48 h post transfection for both lipoplexes and NeoFX complexes, but the cells treated with lipoplexes still showed higher Cy3 fluorescence intensity than those treated with NeoFX complexes. The decrease of Cy3 fluorescence signal suggested that the delivered microRNA was either degraded or “diluted” by cell growth. Based on the results, when repeated treatment is required, 48 h might be a reasonable time interval between each delivery.

Biodistribution of Lipoplexes *in Vivo*

One of the major challenges for the use of lipoplexes *in vivo* is the tissue-specific delivery. Prior to *in vivo* evaluation of the efficacy of pre-miR containing lipoplexes, we used Cy5-G3139 containing lipoplexes to evaluate the biodistribution of lipoplexes in vivo. 200 μL of Cy5-G3139 containing lipoplexes was given to each mouse at 1.5 mg/kg through tail vain injection. Major organs including lung, liver, kidney, spleen, heart, brain and bone were harvested four hours later. The fluorescence signals of Cy5 were analyzed and compared with Cy5-G3139 NeoFX complexes and untreated mice. As shown in Figures 8a and 8b, it is challenging to deliver lipoplexes specifically to lung only. Compared with untreated mice, Cy5-G3139 NeoFX complexes mainly accumulated in kidney (∼64%) and liver (∼31%), and little was delivered to lung (∼0.6%). Lipoplexes achieved much higher lung accumulation (∼30%), which was about 50 times higher than NeoFX complexes. Confocal microscopy images of lung tissues (Figure 8c) further confirmed the results by showing that much more Cy5-G3139 delivered by lipoplexes accumulated in lung than by NeoFX complexes. In addition, hematoxylin and eosin staining of lung tissues (Figure 8d) indicated that no inflammation was induced by lipoplexes compared to untreated lung tissues, suggesting the lack of immediate lung toxicity with lipoplex delivery.

Mature miR-133b Expression *in Vivo*

Pre-miR-133b and pre-miR-NC containing lipoplexes were given to each mouse at 1.5 mg/kg through tail vain iv injection. 48 h later, mice were sacrificed and lung tissues were harvested. Mature miR-133b expression was measured using qRT-PCR. Mice treated by pre-miR-133b containing lipoplexes had mature miR-133b expression in lung ∼52-fold higher than untreated lung tissue (Figure 9).

4. Discussion

MicroRNAs are evolutionarily conserved small noncoding RNAs that regulate approximately up to one-third of genome. Studies increasingly show that miRNAs play important roles in tumor development, progression and metastasis by functioning as either tumor suppressors, oncogenes or both. In lung cancer, specific miRNA expression signatures may serve as biomarkers for lung cancer diagnosis and prognosis as well as potential targets. The development of miRNA-based therapeutics remains complicated as there are several challenges including tissue specific delivery, efficient cellular uptake and minimization of off-target effects.

In this work, miR-133b, a miRNA that directly targets MCL-1, was delivered by cationic lipoplexes and the delivery efficiency was evaluated both *in vitro* and in vivo. In the lipoplex formulation, DOTMA was chosen as the building block of our miRNA delivery system because of its high transfection activities *in vitro* and *in vivo* due to the close proximity of hydrocarbon chains to the cationic headgroup, two ether bonds and paired oleyl chains as a hydrophobic anchor.[29] Cholesterol was used in the formulation as a helper lipid. Cholesterol can improve transfection efficiency in vivo, protect oligonucleotides from degradation, decrease carrier permeability and increase circulation time.[30] In addition, TPGS was added to the formulation to increase the stability and circulation time of lipoplexes. When the molar ratio of DOTMA, cholesterol and TPGS is 49.5:49.5:1, the lipoplexes have size less than 100 nm (Figure 1a) and are protected by TPGS, thus they have prolonged blood circulation and relatively low rate of clearance by the mononuclear phagocyte system (MPS). This may help overcome in vivo barriers and increase the accumulation in lung.[31] On the contrary, NeoFX complexes have micrometer size and negative surface charge (Figure 1b). Therefore, they easily generate the phagocytic response and are cleared by the MPS in kidney, liver and spleen.[32] The pre-miR-133b containing lipoplexes carry positive surface charges. When the lipoplexes are injected intravenously, in the circulation, serum proteins (or opsonins) can bind to the surface of lipoplexes, which may lead to an increase in particle size. If aggregates are formed, they could be physically entrapped in the capillary bed of the lung, which may also contribute to the accumulation of lipoplexes in lung[32–34]. The positive surface charge also improves the interaction between lipoplexes and the negatively charged cell membrane, and thus provides more cellular uptake. In addition, the lipids used in this formulation might interact with certain receptors on the cell surface and thus facilitate the retention of lipoplexes in lung. Compared with other studies, where lipoplexes only resided in lung for the first hour post intravenous administration and then mostly accumulated in liver,[35,36] our formulation achieved ∼30% accumulation in lungs, which was ∼50-fold higher than for NeoFX complexes (Figure 8). In addition, mature miR-133b expression in the lung tissues of mice treated by pre-miR-133b containing lipoplexes was ∼52-fold higher than untreated mice 48 h after transfection (Figure 9). In summary, the size, surface charge and chemistry of nanoparticles influence their biodistribution *in vivo*.

The efficacy of lipoplexes was further examined *in vitro*. Pre-miR-133b containing lipoplexes did not show cytotoxic effects (Figure 2) and caused a greater decrease in target MCL-1 protein when compared to pre-miR-133b NeoFX complexes (Figure 5). This was

confirmed by the qRT-PCR analysis of mature miR-133b expression (Figure 3). Flow cytometry and confocal microscopy confirmed the higher delivery efficiency of lipoplexes by showing that higher and more uniform Cy3-pre-miR-NC cellular uptake was observed in cells treated by lipoplexes at 0, 24, and 48 h post transfection (Figures 6 and 7).

The lipoplex formulation for miRNA-based therapeutics shows great advantages both *in vitro* and *in vivo*. In the future, the capability of miRNA delivery to specific tissue/tumor sites may be further increased by introducing tumor-targeting moieties to the surface of lipoplexes. Antibodies, such as antiepithelial-cell adhesion-molecule (EpCAM, also known as TACSTD1), which is frequently overexpressed by carcinomas of lung, colorectal, breast, prostate, head and neck, and hepatic origin, may be incorporated in lipoplexes to provide tumor specific targeting function. Such approaches may contribute to the development of miRNA-based therapeutics.

5. Conclusions

A cationic lipid based delivery system was developed to condense miRNA to form pre-miR-133b containing lipoplexes in NSCLC treatment. *In vitro*, the pre-miR-133b containing lipoplexes provided much higher delivery efficiency and led to ∼2-fold higher mature miR-133b expression in A549 cells than NeoFX complexes. Post-transcriptional regulation of MCL-1 was evident with both transfection approaches. MCL-1 protein expression was successfully downregulated by 56.2% using lipoplex transfection compared to 31.7% downregulation by NeoFX complexes. *In vivo*, lipoplexes achieved ∼30% delivery to lung at four hours post intravenous injection, which was ∼50-fold higher than for NeoFX complexes. Mice systemically treated by pre-miR-133b containing lipoplexes had a 52-fold induction in mature miR-133b expression compared to untreated mice. Our results demonstrate that this lipoplex formulation may have potential for the development of miRNA-based therapeutics for lung disease. However, further *in vivo* miRNA-based delivery studies are required to evaluate long-term local and systemic toxicity and target gene effects prior to clinical application.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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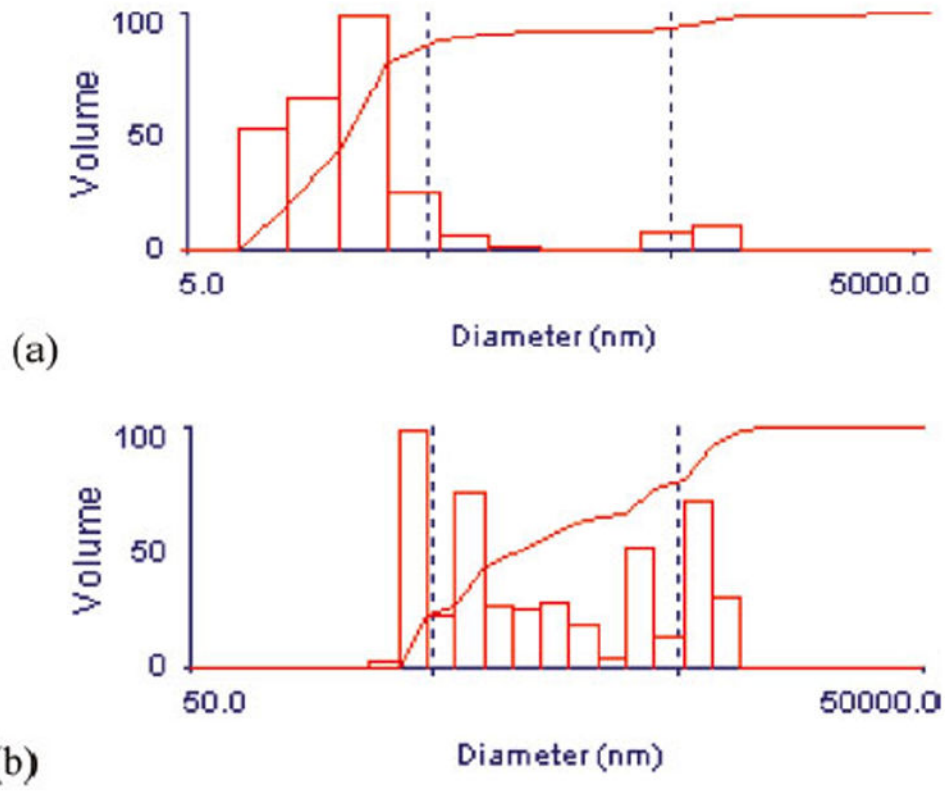

Figure 1.

Typical size distributions of (a) pre-miR-133b containing lipoplexes and (b) pre-miR-133b NeoFX complexes. The mean diameters by volume were 70.0 ± 5.8 nm for lipoplexes and 2427.7 ± 287.4 nm for NeoFX complexes (n = 3).

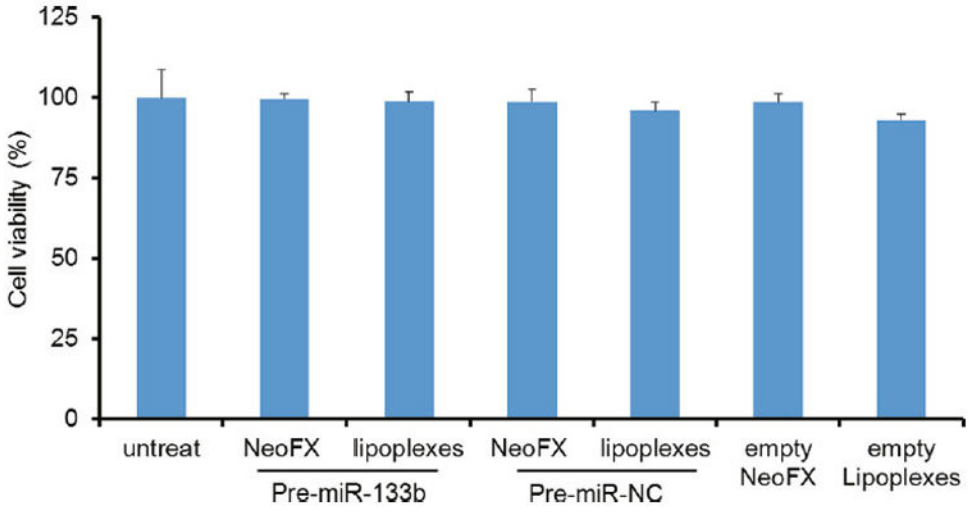

Figure 2.

Effects of pre-miR containing lipoplexes on A549 cell viability 48 h after cells were transfected with pre-miR-133b NeoFX complexes, pre-miR-133b containing lipoplexes, pre-miR-NC NeoFX complexes, pre-miR-NC containing lipoplexes, empty NeoFX complexes and empty lipoplexes at pre-miR concentration of 100 nM. No significant cell viability decrease at $p < 0.05$ level was observed when compared with untreated cells ($n = 3$).

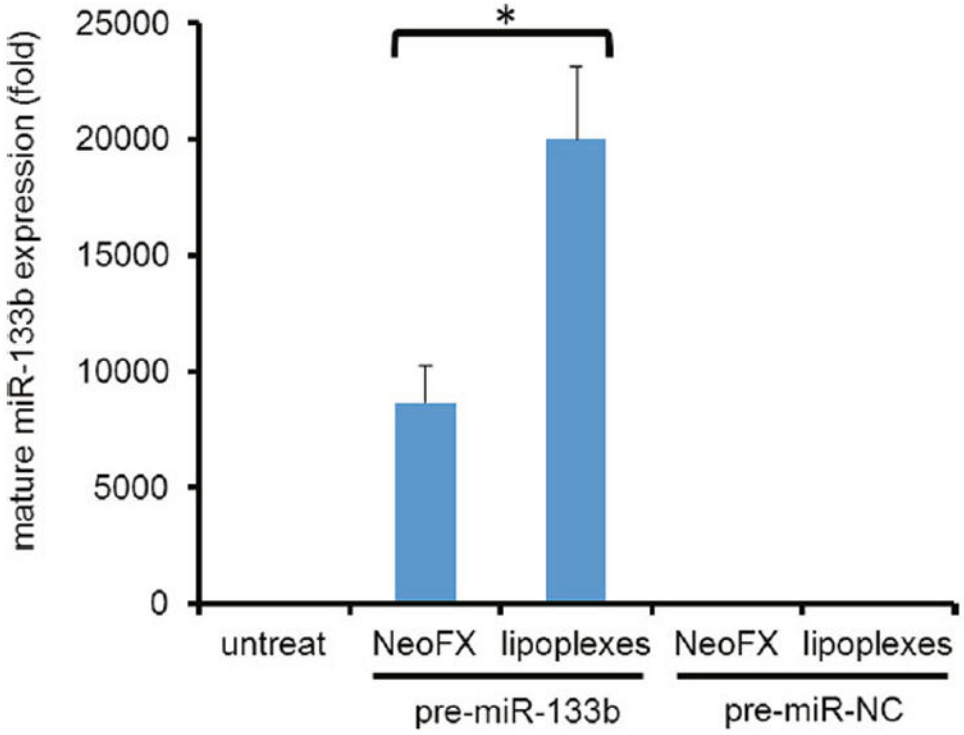

Figure 3.

Mature miR-133b expression 48 h after A549 cells were transfected with pre-miR-133b NeoFX complexes, pre-miR-133b containing lipoplexes, pre-miR-NC NeoFX complexes and pre-miR-NC containing lipoplexes at pre-miR concentration of 100 nM. Compared with untreated cells, cells treated by pre-miR-133b containing lipoplexes had mature miR-133b expression ∼2.3-fold higher than that of pre-miR-133b NeoFX complexes (*: $p < 0.05$) ($n = 3$).

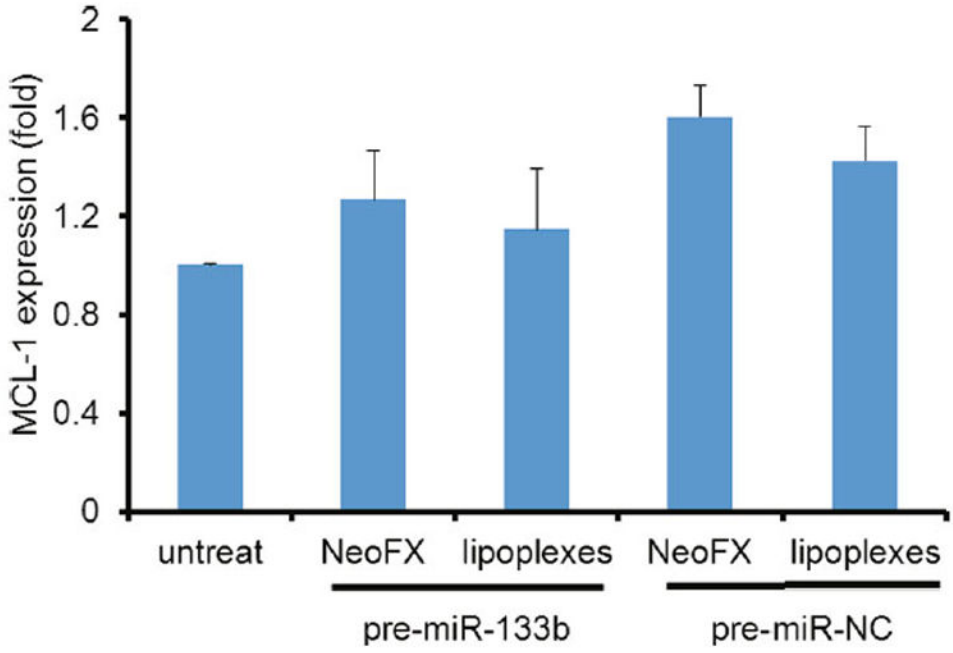

Figure 4.

MCL-1expression at mRNA level 48 h after A549 cells were transfected with pre-miR-133b NeoFX complexes, pre-miR-133b containing lipoplexes, pre-miR-NC NeoFX complexes and pre-miR-NC containing lipoplexes at pre-miR concentration of 100 nM. No significant difference at $p < 0.05$ level was observed between untreated cells and cells treated by lipoplexes and NeoFX complexes ($n = 3$).

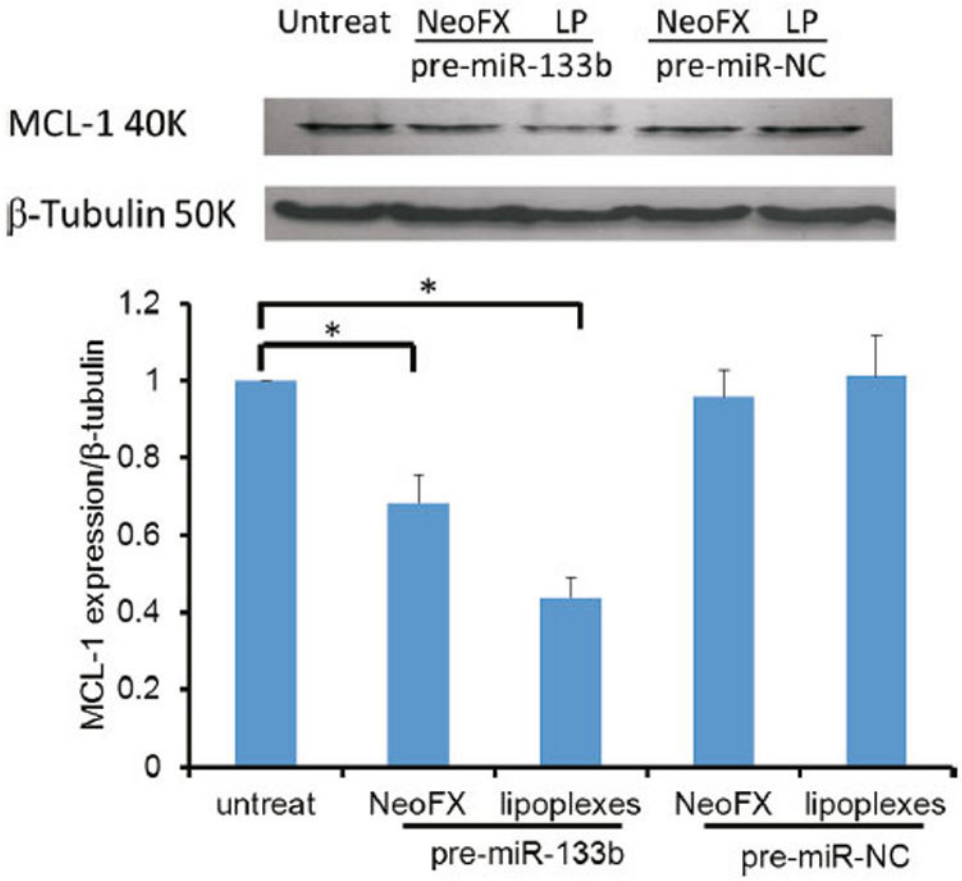

Figure 5.

MCL-1expression at protein level 48 h after A549 cells were transfected with pre-miR-133b NeoFX complexes, pre-miR-133b containing lipoplexes, pre-miR-NC NeoFX complexes and pre-miR-NC containing lipoplexes at pre-miR concentration of 100 nM. Compared with untreated cells, MCL-1 protein expression was downregulated 56.2% by pre-miR-133b containing lipoplexes and 31.7% by pre-miR-133b NeoFX complexes. No significant difference at $p < 0.05$ level was observed between untreated cells and cells treated by pre-miR-NC containing lipoplexes and pre-miR-NC NeoFX complexes (LP, lipo-plexes; *, $p < 0.05$; $n = 3$).

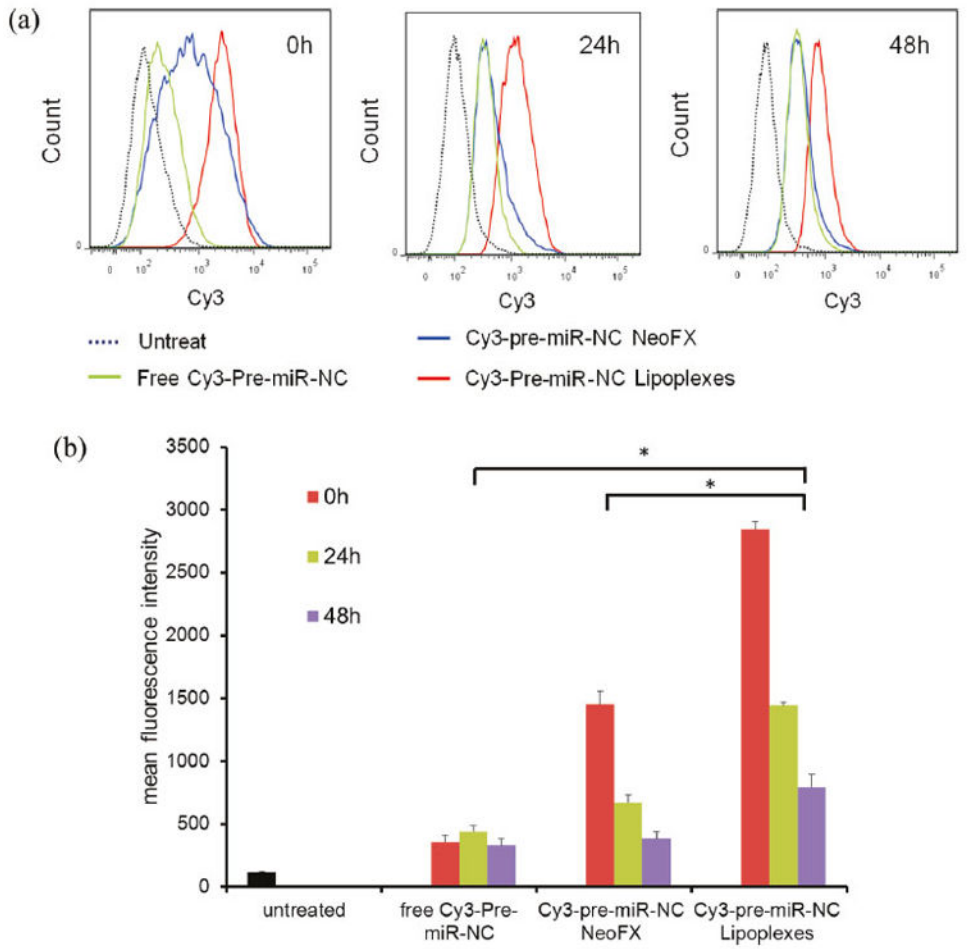

Figure 6.

Flow cytometry analysis of cellular uptake of Cy3-pre-miR-NC containing lipoplexes and NeoFX complexes 0, 24, and 48 h post transfection at Cy3-pre-miR-NC concentration of 100 nM. (a) A typical flow cytometry data set. (b) Mean fluorescence intensity of Cy3 averaged on 3 replicates (*: $p < 0.05$ at all three time points).

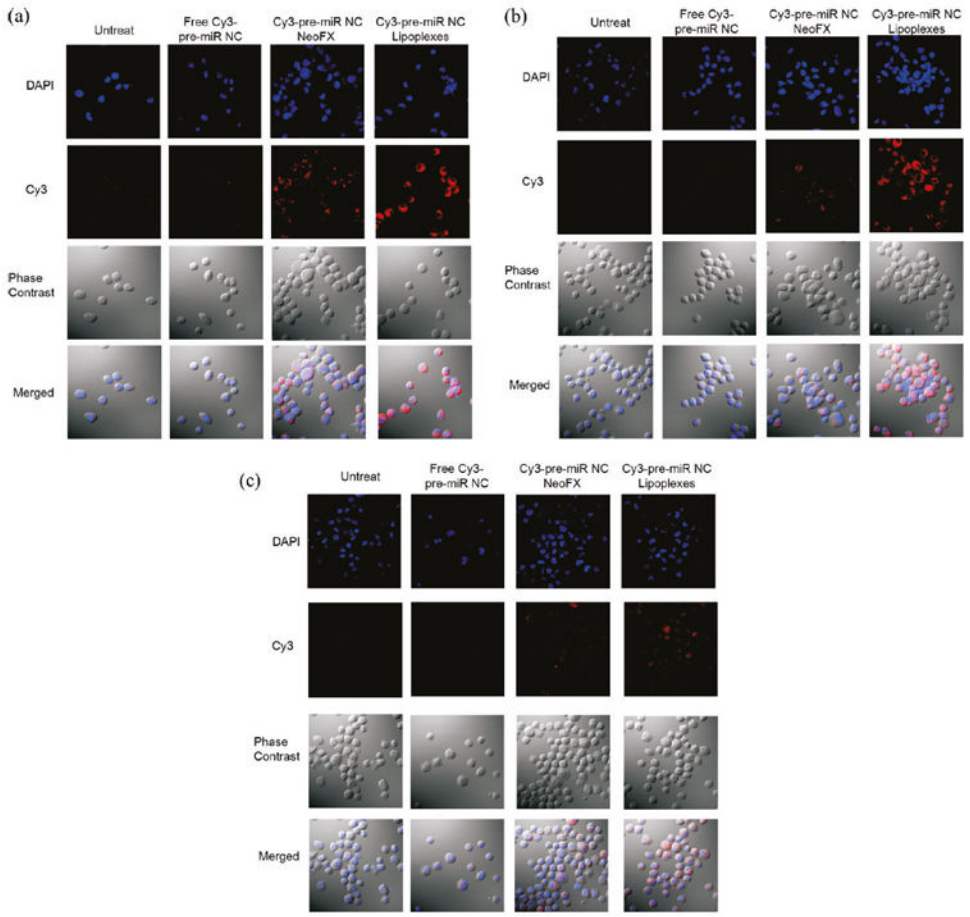

Figure 7.

Confocal microscopy analysis of cellular uptake of Cy3-pre-miR-NC containing lipoplexes and NeoFX complexes 0 h (a), 24 h (b) and 48 h (c) post transfection.

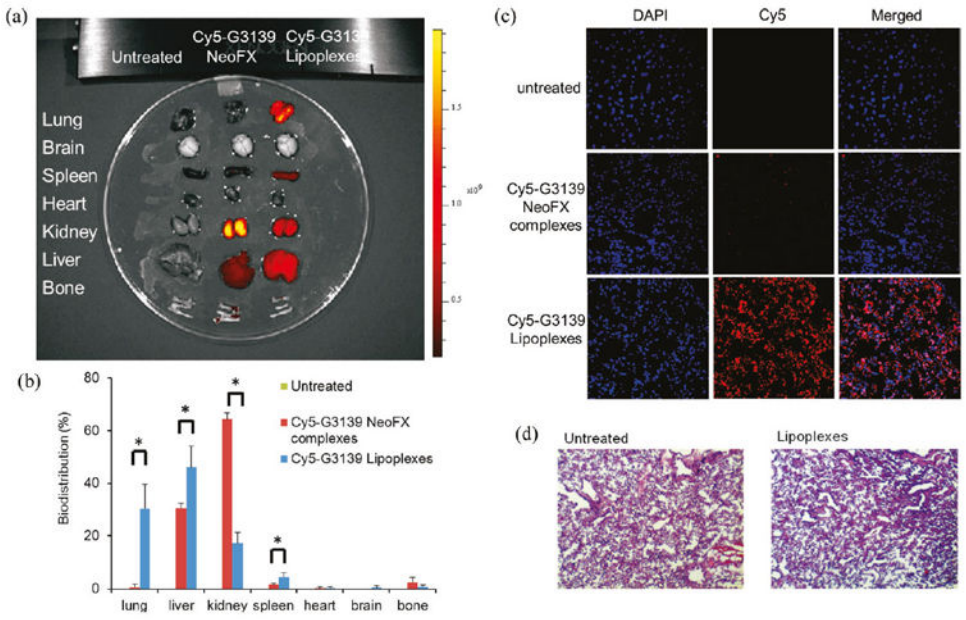

Figure 8.

Tissue distribution of Cy5-G3139 containing lipoplexes and Cy5-G3139 NeoFX complexes. 4 h after intravenous administration, tissues were harvested and then Cy5 fluorescence signals measured. (a) A typical Cy5 fluorescence images of whole tissues. (b) Biodistributions of lipoplexes and NeoFX complexes based on the fluorescence intensity of Cy5 signal. (c) Confocal images of lung tissues when mice were treated with NeoFX complexes and lipoplexes. (d) Hematoxylin and eosin staining of lung tissues of untreated mice and mice treated with lipoplexes. No inflammation was observed in either lung tissue samples ($n = 6$; *: $p < 0.05$).

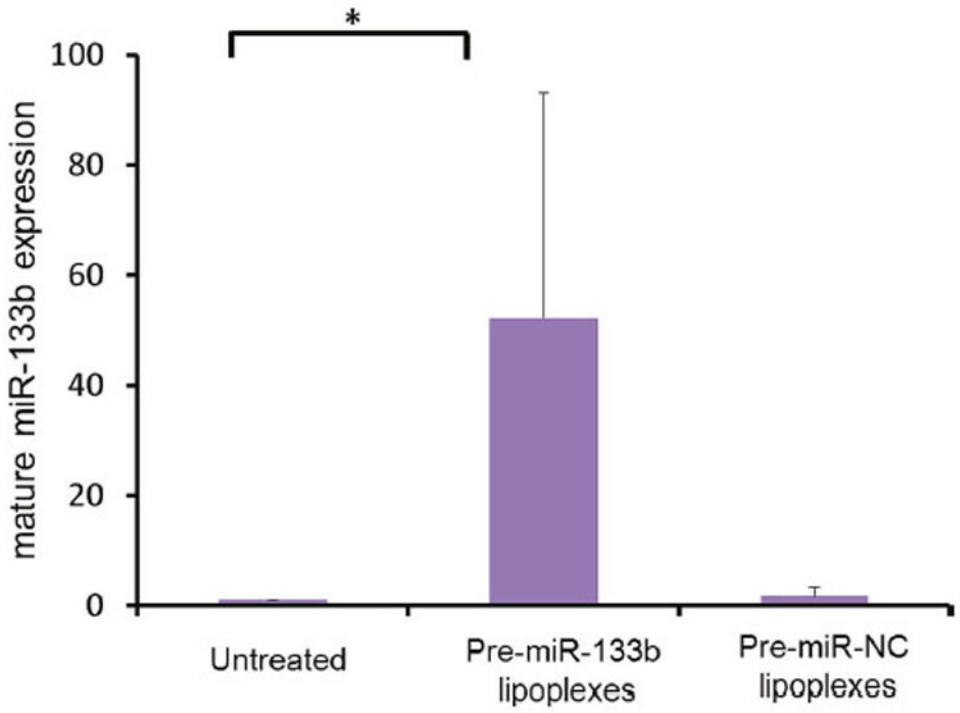

Figure 9.

Mature miR-133b expression in lung tissues 48 h after mice were iv injected with pre-miR-133b containing lipoplexes and pre-miR-NC containing lipoplexes at a pre-miR concentration of 1.5 mg/kg. Mice treated with pre-miR-133b containing lipoplexes had mature miR-133b expression in lung ∼52-fold higher than untreated lung tissues ($n = 3$; *: $p < 0.05$).