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Creatine Based Polymer for Codelivery of Bioengineered MicroRNA and Chemodrugs against Breast Cancer Lung Metastasis

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Abstract

Metastasis is the major cause for breast cancer related mortality. The combination of miRNA-based therapy and chemotherapy represents a promising approach against breast cancer lung metastasis. The goal of this study is to develop an improved therapy that co-delivers a novel bioengineered miRNA prodrug (tRNA-mir-34a) and doxorubicin (DOX) via a multifunctional nanomicellar carrier that is based on a conjugate of amphiphilic copolymer PEOG-VBC backbone with creatine, a naturally occurring cationic molecule. Co-delivery of DOX leads to more effective processing of tRNA-mir-34a into mature miR-34a and down-regulation of target genes. DOX +tRNA-mir-34a/POEG-PCre exhibits potent synergistic anti-tumor and anti-metastasis activity *in vitro* and *in vivo*. Interestingly, the enhanced immune response contributes to the overall antitumor efficacy. PEOG-PCre may represent a safe and effective delivery system for an optimal chemogene combination therapy.

Graphical Abstract

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Amphiphilic POEG-PCre polymer is able to self-assemble with DOX to form DOX-loaded micelles, which is then condensed by tRNA-mir-34a to form more compact and stable nanocomplexes. The multifunctional POEG-PCre micellar system is highly effective in co-delivery of DOX and tRNA-mir-34a to lungs and subsequently to tumor cells, which represents an optimal chemo-gene combination therapy against cancer lung metastasis.

Keywords

cancer metastasis; creatine; multifunctional nanocarriers; chemo-gene combination therapy; bioengineered miRNA prodrug

INTRODUCTION

Breast cancer is the most common type of cancer and second cause of cancer-related mortality in women worldwide.[1] The main cause of death from breast cancer is metastasis, with lung, liver and bones as the major secondary target sites.[2, 3] Among different subtypes of breast cancer, triple-negative breast cancer (TNBC) is notorious for its aggressive behavior and resistance to endocrine and anti-HER2 therapies.[4] Majority of patients with TNBC suffer from early relapse and metastatic spread.[5] Other than chemotherapy, treatments for metastatic TNBC are very limited.[6] Adjuvant treatment of TNBC with chemodrugs frequently failed due to the development of drug resistance.[7] Therefore, new and improved therapeutic strategies for metastatic TNBC are highly demanded.

MicroRNAs (miRNAs or miRs) are short endogenous non-coding RNAs, responsible for post-transcriptional regulation of many target genes that are involved in cancer cell

proliferation and tumor progression.[8] Due to the imperfect complementarity with target mRNAs, miRs are capable of regulating a broad set of genes simultaneously, which benefits the treatment of cancer as a heterogenic disease.[9] Therefore, there has been growing interest in developing miR-based therapies for TNBC. In particular, the expression levels of tumor suppressive miR-34a are often downregulated in TNBC cell lines and tissues. High expression of miR-34a in TNBC-patients confers better overall survival compared to those with low miR-34a expression, which suggests that miR-34a could be reintroduced into cancerous tissues to achieve replacement therapy.[10] Recent studies revealed that the introduction of exogenous miR-34a into cancer cells induced cell apoptosis and inhibited cell proliferation and migration through targeting Bcl-2, SIRT1, Rac1, Fra-1, Notch-1, and various cyclins.[11] In addition, miR-34a has been reported to sensitize breast cancer cells to first-line chemotherapies, such as doxorubicin (DOX), paclitaxel and 5-FU.[12] Moreover, a liposome-formulated miR-34a, namely “MRX34”, has entered Phase I clinical trials for the treatment of unresectable primary liver cancer.[13]

One major limitation for miR-based therapies is that the source of miRs has been limited to synthetic RNAs with artificial modifications, which raises concerns over the stability, cost, specificity and safety of these RNA-based therapeutics.[14] To improve the miR-based therapy, we have recently developed a novel miR-34a prodrug, pre-miR-34a fused to a transfer RNA (tRNA), namely tRNA-mir-34a.[15] These bioengineered miRs are produced and folded in *Escherichia coli* in large scale with high yield, which can effectively maintain the function and safety properties of natural RNAs and therefore represent a new class of more affordable and biocompatible miR-based agents for research and therapy. Indeed, this tRNA-carried pre-miR-34a was selectively processed into mature miR-34a in human carcinoma cells, resulting in reduced expression of the target genes and consequently the inhibition of cancer cell proliferation *in vitro* and *in vivo*. [15] However, the use of tRNA-mir-34a alone only exhibited modest effect against human lung cancer or hepatocarcinoma. The combination of tRNA-mir-34a with DOX-based chemotherapy might work synergistically to increase the antitumor efficacy while minimizing the toxicity associated with each single treatment. In this study, we aimed to develop a nanocarrier for systemic co-delivery of tRNA-mir-34a and DOX to treat metastatic TNBC.

Currently, a variety of delivery systems have been developed for gene delivery including viral carriers, peptides, liposomes and cationic polymers. Despite the high efficiency in gene delivery, viral vector-based therapies are limited by their safety concerns such as immunogenicity, and the difficulty of large-scale production.[16, 17] In contrast, non-viral nanocarriers have attracted increasing attention in nucleic acid delivery due to their favorable safety profiles and the ease of production.[18] In addition to nanocarriers designed exclusively for genes, there are growing interests in developing multi-functional nanocarriers that can simultaneously deliver multiple types of therapeutic payloads to disease sites in a targeted and controlled manner for combination therapy.[19] It has been suggested that the use of naturally occurring molecules as building blocks of the nanocarriers is superior in achieving the properties of non-toxic, biodegradable, biocompatible and non-immunogenic. [20-23]

We herein developed a multi-functional delivery system based on an amphiphilic polymer (POEG-PCre) with a naturally occurring cationic molecule creatine attached to the pendent side chain, for the co-delivery of bioengineered tRNA-mir-34a and small molecule chemodrugs. The backbone of POEG-PCre (POEG-PVBC), which consists of hydrophobic alkyl main chains and pendent benzyl rings, forms the core of micelles to load hydrophobic anticancer drugs through hydrophobic-hydrophobic interaction and π - π stacking. Creatine was post-conjugated to the backbone to introduce the positive charges, which further formed complexes with the negatively charged nucleic acids. In addition, the cationic creatine groups in the polymer can also facilitate the accumulation of nanoparticles in the lung tissue and thereby promote the subsequent interaction with tumor cells. That is because the lung is the first capillary bed encountered by cationic NPs after intravenous (i.v.) injection and therefore acts as the most effective target organ. [24] Also, the lung tissue has been reported to be endowed with a much more effective polyamine active uptake system than any other major organs,[25] which may benefit the targeted delivery of creatine-based nanocarrier with amine groups to the lungs. The excess amount of positive charges on the nanocarrier was shielded by PEG to improve *in vivo* stability of the system. We hypothesized that the co-delivery of tRNA-mir-34a and DOX via our multi-functional nanocarrier represents a safe and effective approach for the treatment of metastatic TNBC. We first examined the biophysical properties of the nanocarrier co-loaded with DOX and tRNA-mir-34a. The efficiency of delivery and transfection was then examined both *in vitro* and *in vivo*. Finally, the antitumor effect of DOX+tRNA-mir-34a/polymer as well as the underlying mechanism was investigated.

RESULTS

Synthesis of POEG-PCre Polymers.

The POEG-PCre polymer was synthesized by RAFT co-polymerization of OEG500 monomer and VBC monomer, followed by conjugation with creatine (Figure S1, Supporting Information). The number average molecular weight and polydispersity Index (PDI) of POEG-PVBC was measured to be 17200 and 1.39 by GPC (Figure S2a, Supporting Information). The ^1H nuclear magnetic resonance (NMR) spectra of the POEG-PVBC and POEG-PCre were shown in Figure S2b and c. The units of OEG and VBC were calculated to be 25 and 118, respectively, according to the monomer conversion. The conjugated units of creatine were calculated to be 47 by comparing the characteristic signals at 4.12-4.8 (f), 5.07 (e), and 5.95-7.88 (c and d) ppm (Figure S2c, Supporting Information). The calculated molecular weight by ^1H -NMR was $M_n=34901$.

Characterization of Micellar Nanoparticles.

POEG-PCre micelles were prepared by dialysis. As shown in Figure 1a, POEG-PCre copolymer self-assembled into spherical nanoparticles with a size around 180 nm as indicated by dynamic light scattering (DLS) and transmission electron microscopy (TEM). The critical micellar concentration (CMC) of POEG-PCre copolymer, determined using Nile red as a fluorescence probe,[26] was as low as approximately 0.05 mg/mL (Figure 1b). The low CMC indicated the stability of POEG-PCre micelles upon dilution in the blood stream after i.v. injection. The DOX-loaded POEG-PCre micelles were similarly prepared. DOX

could be incorporated into POEG-PCre micelles at a carrier/drug ratio of 5:1 (w/w) or higher (data not shown). A carrier/drug ratio of 10:1 was chosen for the subsequent studies due to the relatively high drug loading efficiency (90.0%) and drug loading capacity (8.2%) as well as the excellent colloidal stability (stable for one month and two months at RT and 4°C, respectively). DOX-loaded POEG-PCre micelles were comparable to blank POEG-PCre micelles in size and morphology (Figure 1a).

The surface zeta potential of POEG-PCre micelles was approximately +40 mV (Figure 1e). We then tested whether the cationic micelles could form stable complexes with tRNA-mir-34a via gel retardation assay. POEG-PCre micelles were mixed with tRNA-mir-34a at various N/P ratios (the molar ratio of nitrogen residues (N) in the cationic polymer over phosphate (P) of nucleic acids) from 1:1 to 40:1. N/P ratios were calculated as following: there are 47 units of creatine in POEG-PCre polymer with $M_n=34901$, and each creatine unit contains one positively charged nitrogen. Therefore, we assumed that 742.6 g/mol corresponded to each repeating unit of POEG-PCre containing one nitrogen atom, and 330 g/mol corresponded to each repeating unit of tRNA-mir-34a containing one phosphorus atom. As shown in Figure 1c, complete complexation of tRNA-mir-34a by POEG-PCre polymer was achieved at an N/P ratio of 5:1 or greater. Accordingly, at N/P ratios of below 5, the net charges of nanocomplexes were negative with the particle sizes similar to that of blank micelles (Figure 1e). At an N/P ratio of 5, a significant increase in particle size was observed with particle charges close to neutral. Interestingly, further increases in N/P ratios led to continuous increases in zeta potentials and decreases in particle sizes, suggesting gradual condensation of nucleic acid by POEG-PCre cationic polymer. In order to form more compact POEG-PCre/tRNA-mir-34a complexes as well as to overcome the neutralization by serum proteins, an N/P ratio of 20 was chosen for all subsequent studies. At this N/P ratio, the average size of the nanocomplexes decreased to around 120 nm (Figure 1a). In addition, DOX loading had negligible effect on the size distribution, zeta-potential and nucleic acid binding ability of POEG-PCre micelles (Figure 1a, 1c, 1e).

The binding ability of POEG-PCre to plasmid or siRNA was also investigated. Compared to complexation with tRNA-mir-34a, POEG-PCre polymer could form stable complexes with plasmid at a lower N/P ratio of 2.5:1 (Figure S3a, Supporting Information). However, POEG-PCre polymer was not effective in complexing with siRNA at an N/P ratio as high as 80:1 (Figure S3b, Supporting Information), suggesting that our POEG-PCre carrier was unique in forming complexes with nucleic acids with appropriate sizes and secondary structures including tRNA-mirs. To further investigate the interaction between POEG-PCre nanocarrier and tRNA-mirs, a competitive binding gel-shift assay with dextran sulfate was performed. As shown in Figure 1d, at an N/P ratio of 5:1, substantial amounts of tRNA-mir-34a began to be released from POEG-PCre nanocarrier or PEI at an S/P ratio (molar ratio between the sulfur from dextran sulfate and the phosphate from tRNA-mir-34a) of 5 or greater. When the N/P ratios reached 10:1 or higher, tRNA-mir-34a could not be replaced by dextran sulfate even at an S/P ratio as high as 80. In contrast, an obvious release of tRNA-mir-34a was observed from PEI carrier at the corresponding N/P ratios. These data suggest that, in addition to electrostatic interaction as seen with PEI, other interactions, such as π - π stacking between the pendent benzyl rings of our polymer and the base π -systems of nucleic acids, might also contribute to a more stable nanocomplexing system.

The release kinetics of DOX from DOX-loaded POEG-PCre micelles was investigated by dialysis against DPBS of pH 7.4 at 37 °C. As shown in Figure 1f, more than 80% of free DOX was diffused out of dialysis bag (MWCO=3500) in the first 4 h. On the contrary, only 16 % of DOX was released from DOX/POEG-PCre micelles in first 4 h, and less than 40 % of DOX was released for an extended period of 96 h. POEG-PCre micelles co-loaded with DOX and tRNA-mir-34a exhibited a comparable but slightly slower release profile compared with micelles loaded with DOX alone.

We further examined the stability of our nanocomplexes in various solutions that mimic commonly used buffer, routine cell culture medium or serum (i.e. DPBS of pH 7.4, DMEM medium with 2% FBS or 30 mg/mL of BSA), respectively. As shown in Figure 1g, no aggregation of nanoparticles was observed in all tested solutions and all of the nanocomplexes showed minimal changes in sizes for up to 48 h.

Cellular Internalization of Nanocomplexes.

To monitor intracellular co-delivery of DOX and tRNA-mir-34a, tRNA-mir-34a was labeled with MFP488 fluorescent dye in order to visualize the cell uptake of tRNA-mir-34a along with red fluorescence of DOX. 4T1.2 cells were incubated for 4 h with micelle complexes carrying DOX and/or MFP488-labeled tRNA-mir-34a and then observed under a confocal microscope. Naked tRNA-mir-34a without any transfection agent was used as a negative control. As shown in Figure 2a, DOX/POEG-PCre showed more DOX fluorescence signal compared to free DOX at the same dose of DOX. Interestingly, the DOX+tRNA-mir-34a^{MFP488} co-loaded POEG-PCre micelles showed even more uptake of DOX than that of the DOX/POEG-PCre micelles, which might be due to a more condensed structure after complexation with tRNA-mir-34a, leading to higher endocytosis efficiency. Overlay of DOX (red) and tRNA-mir-34a^{MFP488} (green) generated the yellow signals in the merged channel, suggesting the co-localization of DOX and tRNA-mir-34a^{MFP488} inside tumor cells. In addition, fluorescence signals of both DOX and tRNA-mir-34a^{MFP488} were present in the perinuclear regions of the cytoplasm in nanocomplexes-treated groups, while the DOX fluorescence signals in free DOX-treated group were profoundly observed in the nucleus overlapping with Hoechst staining (blue). These data suggest different cellular uptake routes of free DOX and DOX-loaded micelles, through passive diffusion and endocytosis, respectively. In Figure 2b and c, flow cytometry analysis further demonstrated that the percentage of DOX-positive cells as well as the mean fluorescence intensity (MFI) of DOX followed the order of free DOX < DOX/POEG-PCre < DOX+tRNA-mir-34a^{MFP488}/POEG-PCre. More importantly, more than 90% 4T1.2 cells were DOX/MFP488 double positive cells, suggesting that POEG-PCre nanocarrier was efficient in co-delivering DOX and tRNA-mir-34a into the same tumor cells simultaneously.

Generation of Mature MiR-34a and Suppression of Target Gene Expression.

The processing of tRNA-mir-34a into mature tumor suppressor miR-34a was evaluated by quantitative RT-PCR. As shown in Figure 3a, there was over a 200-fold increase in the amount of mature miR-34a in 4T1.2 cells after treatment with tRNA-mir-34a/POEG-PCre nanocomplexes. Interestingly, significantly greater amounts of mature miR-34a (~1200-fold increase) were found in cells treated with the DOX+tRNA-mir-34a co-loaded POEG-PCre

micelles. This is unlikely due to the differences in the amounts of tRNA-mir-34a delivered intracellularly since comparable levels of MFP488 fluorescence were found for the two groups (Figure 2). Consistently, Figure 3b shows that the amounts of chimeric ncRNA scaffold inside 4T1.2 cells were similar after treatment with either POEG-PCre/tRNA-mirs or POEG-PCre/DOX+tRNA-mirs, suggesting comparable efficiency of delivery of the two formulations. We hypothesized that it might be due to a more efficient processing of tRNA-mir-34a in cells following co-delivery of DOX. Figure S4 shows that treatment with free DOX or DOX-loaded POEG-PCre micelles led to the induction of Dicer, an RNase III enzyme involved in pre-miRs cleavage, in a dose-dependent manner. As a result, the level of pre-miR-34a in DOX+tRNA-mir-34a/POEG-PCre-treated cells was significantly lower than that in cells treated with tRNA-mir-34a/POEG-PCre, suggesting a more complete processing of pre-miR-34a into mature miR-34a as a result of the co-delivered DOX (Figure 3c). Similar trends were observed in MDA-MB-231 cells (Figure 3a-c; Figure S4, Supporting Information).

The anti-apoptotic Bcl-2 has been previously identified as a downstream target of miR-34a. [27] The expression of Bcl-2 was then evaluated at both the transcript and protein levels after intracellular delivery of tRNA-mir-34a. tRNA-MSA (Sephadex aptamer tagged methionyl-tRNA), the sole tRNA scaffold, was used as a control. As shown in Figure 3d, the mRNA expression levels of Bcl-2 in cells treated with blank POEG-PCre micelles, tRNA-MSA/POEG-PCre micelles, free DOX, DOX/POEG-PCre micelles or DOX+tRNA-MSA/POEG-PCre micelles were similar to that of untreated control cells, suggesting that POEG-PCre carrier, tRNA-MSA and DOX had minimal regulatory effects on the expression of Bcl-2. In contrast, significant down-regulation of Bcl-2 expression was observed in the tumor cells treated with POEG-PCre/tRNA-mir-34a or the co-loaded formulation. Consistently, the downregulation of Bcl-2 was reaffirmed at the protein level by Western blotting (Figure 3e). Importantly, the down-regulation of Bcl-2 was more significant in cells treated with the co-loaded formulation than cells treated with POEG-PCre/tRNA-mir-34a. Similar results were found in MDA-MB-231 cells (Figure 3d, 3e).

MTT Assay, Apoptosis Assay and Cell Migration.

The *in vitro* cytotoxicity of DOX+tRNA-mir-34a co-loaded POEG-PCre micelles was evaluated by MTT assay. Tumor cells were treated with various formulations for 48 h, with the doses of DOX, tRNA-mirs, and POEG-PCre carrier ranging from 7.8 to 1,000 ng/mL, 0.78 nM to 100 nM, and 78 ng/mL to 10000 ng/mL, respectively. As shown in Figure 4a, no obvious cell growth inhibition was noticed in cells treated with POEG-PCre nanocarrier alone or tRNA-MSA loaded POEG-PCre micelles, even at the concentration of the polymer as high as 10 µg/mL. TRNA-mir-34a/POEG-PCre micelles showed a moderate level of cytotoxicity at high concentrations. Free DOX inhibited the proliferation of tumor cells in a dose-dependent manner and the cytotoxicity of DOX/POEG-PCre was comparable to that of free DOX. Incorporation of tRNA-MSA into DOX/POEG-PCre micelles showed minimal impact on the overall cytotoxicity of the co-loaded formulation. In contrast, incorporation of tRNA-mir-34a into DOX/POEG-PCre micelles led to a significant enhancement in the cytotoxicity of nanocomplexes on 4T1.2 cells, indicating the synergistic effect between co-

delivered tRNA-mir-34a and DOX via our POEG-PCre system. A similar trend was also observed in MDA-MB-231 breast cancer cells (Figure 4a).

We then assessed the enhanced cytotoxicity of DOX+tRNA-mir-34a-co-loaded nanocomplexes via apoptosis assay. Cell apoptosis was evaluated quantitatively by Annexin V/propidium iodide flow cytometry. As shown in Figure 4b, few apoptotic cells were detected in control cells or cells treated with POEG-PCre carrier alone or tRNA-MSA loaded nanocomplexes, which was in agreement with the results of MTT assay. An increase in cell apoptosis (~21%) was observed in cells treated with tRNA-mir-34a loaded nanoparticles, suggesting that increased expression of mature miR-34a after delivery of tRNA-mir-34a into tumor cells can trigger the apoptosis of breast cancer cells due to the tumor suppressor functions of miR-34a. Free DOX, DOX/POEG-PCre and DOX+tRNA-MSA/POEG-PCre were comparable in inducing cell apoptosis to a significantly higher level (~31%). Importantly, a significantly higher percentage of necrosis (~13%), in addition to much greater degree of late apoptosis (~20%) and comparable level of early apoptosis, was obtained in cells treated with DOX+tRNA-mir-34a co-loaded formulation, which confirms a potent synergy between tRNA-mir-34a and DOX in inducing apoptosis and necrosis.

Besides cancer cell viability, cell migration is a critical process for tumor progression and metastasis. MiR-34a was reported to be functional as an anti-metastatic miRNA by directly targeting NOTCH-1.[28] Therefore, we investigated the effects of tRNA-mir-34a and DOX co-loaded nanocomplexes on the cell migration using the wound closure assay. As shown in Figure 4c and Figure S5, the wounded area was substantially repopulated by highly aggressive 4T1.2 cancer cells after 24 h in groups with no treatment or treated with POEG-PCre nanocarrier alone or tRNA-MSA loaded nanocomplexes. Significant inhibition (~75%) of cell migration was observed in cells treated with tRNA-mir-34a/POEG-PCre. Three DOX-containing formulations (free DOX, DOX/POEG-PCre or DOX+tRNA-MSA/POEG-PCre) had comparable potency with a modest inhibition of ~40 %. In contrast, the DOX/tRNA-mir-34a-co-loaded group was most effective in inhibiting the migration of cancer cells; the denuded area was well retained with only 4.9 % of repopulated cells.

***In Vivo* Biodistribution.**

To gain insight into the biodistribution of nanocomplexes, the fluorescence signals of DOX and MFP488-labeled tRNA-mir-34a in tissue sections were examined by confocal microscopy. As shown in Figure 5, substantial amounts of fluorescent signals of both DOX and MFP488 were detected in lungs treated with DOX+tRNA-mir-34aMFP488 co-loaded micelles. The yellow color in the merged channel indicated the co-localization of DOX and tRNA-mir-34a in lung tissues. Compared to the lungs, substantially lower levels of fluorescent signals were observed in other major organs (Figure S6, Supporting Information), suggesting that POEG-PCre nanocarrier is particularly suitable for targeted co-delivery of DOX and tRNA-mir-34a to lungs.

Plasma Pharmacokinetics.

The PK profiles of both DOX and tRNA-mir-34a after treatment with DOX+tRNA-mir-34a/POEG-PCre were determined by HPLC and RT-PCR respectively. The concentrations of

DOX in the blood as a function of time were illustrated in Figure S7a and Table S1. Compared to DOX-HCl, the plasma concentrations of DOX for DOX+tRNA-mir-34a/POEG-PCre were significantly higher at early time points. Although a rapid decline in plasma concentrations of DOX was observed for both formulations, the DOX concentrations for DOX+tRNA-mir-34a/POEG-PCre still maintained at relatively high levels until 12 h. Similar PK profiles were shown for tRNA-mir-34a following i.v. administration of DOX +tRNA-mir-34a/POEG-PCre and free tRNA-mir-34a (Figure S7b and Table S2).

***In Vivo* Therapeutic Study.**

The *in vivo* anti-tumor efficacy of DOX+tRNA-mir-34a co-loaded nanocomplexes was evaluated in a breast cancer lung metastasis model, generated by tail-vein injection of 4T1.2 tumor cells into female Balb/c mice. Five days after tumor inoculation, various treatments were applied to each group of mice (n=5) via i.v. administration every 3 days for 3 times (Figure 6a). The tumor burdens in the lungs were analyzed 5 days after the last treatment. As shown in Figure 6b, 6c and 6h, tRNA-MSA loaded in POEG-PCre micelles showed negligible therapeutic effect compared to the control group. tRNA-mir-34a/POEG-PCre nanocomplexes exhibited a moderate inhibition of lung metastasis ($P<0.05$). Dramatic reduction in the number of tumor nodules in lungs was found in groups treated with free DOX, DOX/POEG-PCre or DOX+tRNA-MSA/POEG-PCre micelles. More importantly, POEG-PCre micelles co-loaded with DOX and tRNA-mir-34a was significantly superior to all other treatments in the inhibition of 4T1.2 tumor metastasis.

Figure 6e showed that the levels of mature miR-34a was significantly and selectively upregulated in tumors treated with tRNA-mir-34a loaded micelles. In consistency to *in vitro* data, codelivery of tRNA-mir-34a and DOX via POEG-PCre micelles led to a further increase in the amounts of mature miR-34a likely due to the upregulation of Dicer expression by DOX-containing formulations in vivo (Figure S8, Supporting Information). Figure 6f and 6g showed that the expression levels of Bcl-2 in tumor tissues were significantly suppressed at both mRNA and protein levels following treatment with tRNA-mir-34a, particularly for the co-loaded formulation. To further evaluate the anticancer efficacy, H&E staining and immunohistochemical analysis of Ki67 were performed in tumor-bearing lungs. As shown in Figure 6i, H&E staining of lung tissues showed clearly infiltration of tumor cells (with large nuclei) in all of the groups except the DOX/tRNA-mir-34a co-delivery group. In addition, DOX/tRNA-mir-34a co-delivery group showed the lowest number of Ki67-positive tumor cells (Figure 6j). Together, the above data clearly demonstrate a synergy between DOX and tRNA-mir-34a in the overall antitumor activity in the lung metastasis model.

Safety Profile.

Compared to control or monotherapy, co-delivery of DOX and tRNA-mir-34a did not cause loss of body weight (Figure 6d) or any other signs of stress such as hunched posture and labored movement. In addition, blood chemistry tests were performed to evaluate the effect of our formulations on liver and kidney functions. As shown in Figure 7a-c, none of the measured blood biomarkers including alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine were significantly altered by our therapies, indicating

the absence of hepatic and renal toxicity. Moreover, the H & E staining of major organs was carried out to further evaluate the potential toxicity of different treatments. As shown in Figure 7d, there were signs of myocytolysis and myofibrillolysis with fibrils dearrangement in the heart sections in free DOX group. In addition, hepatocellular vacuolation was found in this group, suggesting possible cardiac and liver toxicities despite no significant alterations in ALT and AST levels. In contrast, no significant pathological changes were observed in the major organs in the groups treated with single-loaded or co-loaded nanocomplexes. These results suggested that POEG-PCre based formulations were well tolerated at the doses tested.

Immune Cell Infiltration Profile in Tumor-Bearing Lungs.

Figure 8a, 8b and 8g showed that DOX treatment led to a significant increase in the numbers of CD4 and CD8 cells in the tumorbearing lungs. DOX treatment also led to an increase in the numbers of functional CD8 (IFN- γ +CD8) cells (Figure 8c, 8h). However, the above changes were much more significant following the tRNA-mir-34a/DOX combination treatment (Figure 8a-c, 8g, 8h). Treatment with tRNA-mir-34a alone or tRNA-mir-34a/DOX combination reduced the numbers of PD1+ CD8 cells (Figure 8d, 8i) and T-regulatory cells (Tregs) (Figure 8e, 8j). Figure 8f and 8k show that the numbers of MDSC were significantly decreased following the tRNA-mir-34a/DOX combination treatment. There was a trend of decreases in the numbers of MDSC following the treatment with DOX alone.

DISCUSSION

Metastatic relapse, development of drug resistance in cancer cells and adverse side effects of chemo-therapeutic agents are the major obstacles for effective chemotherapy against TNBC. [29] Currently, chemotherapy is still the mainstay of adjuvant treatment of most TNBC patients. [30] DOX, an anthracycline DNA-damaging agent, serves as the first-line regimen for TNBC. However, repeated dosing of DOX in TNBC patients often leads to drug resistance. MiR-34a represents a type of therapeutics for TNBC that works through inducing cancer cell apoptosis, and inhibiting cancer cell proliferation and metastasis via targeting multiple pathways. This inspired us to develop a novel combination therapy that is based on codelivery of DOX and miR-34a via a multifunctional nanocarrier.

In this study, we have developed a novel co-delivery system for DOX and miR-34a, which is based on a natural and endogenous cationic molecule—creatine. A primary function of creatine is to facilitate recycling of ATP primarily in muscle and brain tissues; therefore it is widely used by athletes as an ergogenic aid to enhance anaerobic exercise performance.[31] Typically, creatine is produced endogenously or obtained through the diet at a rate of 1 g per day in young adult.[32] Excess amounts of creatine could be metabolized to creatinine and excreted through kidney.[33] Our carrier is a polymeric system in which creatine was post-conjugated to the polymer backbone via ester bond. The biodegradability of the polymer and the nontoxic nature of creatine may account for the excellent safety profile of our new nanocarrier as shown in Figure 6d and Figure 7. We further improved our DOX/miR-34a combination therapy via employing a bioengineered miR-34a prodrug that will be processed into mature miR-34a upon intracellular delivery. Currently miRNA replacement therapy is

limited to the use of synthetic RNAs that are chemically modified to improve their stability.[34] Such modifications may alter the folding, biologic activities, and more importantly the safety profiles, such as the proinflammatory cytokine response.[35] In contrast, bioengineered miR-34a prodrug is folded in living cells, which may better maintain the functions and safety properties of natural miR-34a.[15]

Besides the favorable safety, our creatine-based nanocarrier is also noted for its ability in codelivery of both small molecule drugs and nucleic acid-based therapeutics. Generally, most chemotherapeutic drugs are poorly water-soluble, while miRNAs are polyanionic molecules with high water-solubility, instability and high molecular weight.[36] Most carriers reported so far are designed for delivery of either small molecule drugs or nucleic acid therapeutics alone.[37, 38] Some carriers were developed for codelivery of the two different types of therapeutics but they often involved complicated preparation process.[39] POEG-PCre represents a simple nanocarrier for effective co-loading of small molecule drugs and nucleic acids-based therapeutics. POEG-PCre readily self-assembles to form a hydrophobic core that allows effective loading of hydrophobic drugs such as DOX. At the same time, the multiple positive charges from creatine at the interface of the POEG-PCre micelles facilitate the interaction with the negatively charged nucleic acids. Interestingly, the multivalent charge-charge interactions between the cationic polymer and nucleic acids may also serve as a simple approach to cross-link and stabilize the micelles.[40, 41] As a result, the size of tRNA-mir-34a complexed micelles (~120 nm) was much smaller than that of blank micelles or DOX-loaded micelles (~180 nm) (Figure 1a). In addition, DOX/tRNA-mir-34a-coloaded nanoparticles exhibited a slightly slower release kinetics compared to that of micelles loaded with DOX only (Figure 1f).

Complexation of cationic polymers with large-sized nucleic acids such as plasmid DNA is known to lead to formation of compact particles, which involves structure arrangement and condensation of DNA.[42] On the other hand, cationic polymers tend to form loose complexes with short oligonucleotides due to their small lengths.[43] Accordingly, our POEG-PCre polymer readily formed stable complexes with plasmid DNA at N/P ratios above 2.5 but failed to do so with short siRNA at N/P ratio as high as 80 (Figure S3, Supporting Information). Interestingly, tRNA-mir-34a appears to have an appropriate size (~200 nt) to form stable complexes with POEG-PCre polymer. It should be noted that POEG-PCre/tRNA-mir-34a complexes were more stable than PEI/tRNA-mir-34a complexes as evidenced by the fact that tRNA-mir-34a in POEG-PCre/tRNA-mir-34a complexes was more resistant to the replacement by the negatively charged dextran sulphate compared to PEI/tRNA-mir-34a (Figure 1d). This is likely due to additional mechanisms of interactions between POEG-PCre and nucleic acids in addition to charge interactions.[44] The backbone of POEG-PCre consists of hydrophobic alkyl main chains and pendent benzyl rings that can interact with the base π -systems of nucleic acids through hydrophobic interaction and π - π stacking.[45] The enhanced interaction of our polymer with nucleic acids may help to improve the stability of polymer/nucleic acid complexes in the blood circulation.

POEG-PCre was highly effective in mediating codelivery of tRNA-mir-34a and DOX to cultured tumor cells *in vitro* and pulmonary circulation *in vivo*. Creatine is a derivative of the guanidinium cation. Positively charged guanidinium group is able to form electrostatic

association and bidentate hydrogen bond with anionic cell surface phosphates, carboxylates and/or sulfates to initiate the event of cellular entry.[46] This feature has been widely utilized to design several new guanidinium-rich transporter scaffolds to improve the performance of cell penetration for small molecules, peptides and genes.[47-50] The efficient intracellular delivery of tRNA-mir-34a and DOX (Figure 2) shall likely benefit from this multivalent effect. Following systemic administration, the creatine-based polymer may take advantage of the lung-enriched polyamine transporters to guide the targeted accumulation of nanocomplexes to lung tissues.[25] Indeed, a more predominant distribution of the nanocomplexes in the lungs was observed (Figure 5; Figure S6, Supporting Information), which should be beneficial to the treatment of lung metastasis.

Codelivery of DOX and tRNA-mir-34a via POEG-PCre-based nanoparticles led to an enhanced combinational effect both *in vitro* and *in vivo*. It is interesting to note that codelivery of DOX facilitates the processing of tRNA-mir-34a, resulting in increased levels of mature miR-34a ($P<0.001$) (Figure 3a). The underlying mechanism is not clearly understood; however, our data suggest that DOX induced the expression of RNase III enzyme Dicer (Figure S4, Supporting Information). It has been reported that Dicer is required for activating the DNA damage response when double-strand DNA breaks and non-coding RNA is synthesized at the site of DNA damage.[51] Therefore, DOX may enhance the expression of Dicer through the DNA damage response. More studies are needed to better understand the underlying mechanism for the DOX-mediated enhancement of tRNA-mir-34a processing.

The improved antitumor activity of DOX/tRNA-mir-34a-based therapy is likely attributed to various factors. Overexpression of miR-34a is effective in inhibiting the proliferation of cancer cells and inducing apoptosis. In addition, miR-34a sensitizes cancer cells to chemotherapeutic agents such as DOX. Our data further suggest a role of immune response in the overall antitumor activity of DOX/tRNA-mir-34a combination therapy. DOX was reported to trigger immunogenic cell death by promoting tumor infiltration of IL-17-secreting $\gamma\delta$ T cells and enhancing the proliferation and activation of IFN γ -secreting CD8⁺ T cells in tumor draining lymph nodes.[52] Similar results were shown in our studies with a lung metastatic tumor model. However, treatment with DOX+tRNA-mir-34a co-loaded nanocomplexes resulted in even greater numbers of tumor-infiltrating CD4⁺, CD8⁺ and CD8⁺ IFN- γ ⁺ CD8⁺ T cells (Figure 8), which may be attributed to the effect of miR-34a on the down-regulation of PD-L1 expression (Figure S9, Supporting Information). In addition, the co-treatment led to significantly reduced numbers of MDSCs that are highly immunosuppressive and play an important role in suppressing the antitumor immunity. Therefore, our combination therapy helps to create an active tumor immune microenvironment that likely contributes to the overall antitumor activity.

CONCLUSION

In this study, we have developed a novel nanocarrier (POEG-PCre) for co-delivery DOX and bioengineered miR-34a prodrug, based on the built-in blocks of naturally occurring cationic molecule—creatine. Stable and nanosized micelles co-loaded with DOX and tRNA-mir-34a could be readily prepared and were highly effective in targeted delivery of payloads to lungs.

Significantly improved antitumor activity was demonstrated with codelivery of DOX and miR-34a prodrug in comparison with other control groups in a 4T1.2 lung metastasis model. POEG-PCre may represent a simple and effective delivery system for an optimal chemogene combination therapy.

METHODS

Reagents.

DOX·HCl was purchased from LC Laboratories (MA, U.S.A). Creatine, 4-Cyano-4 [(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid, vinylbenzyl chloride (VBC) monomer, and oligo(ethylene glycol) methacrylate (average Mn = 500, OEG500), 2-Azobis(isobutyronitrile) (AIBN), trypsin-EDTA solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma-Aldrich (MO, U.S.A). AIBN was purified by recrystallization in anhydrous ethanol. Opti-MEM medium, fetal bovine serum (FBS), Dulbecco's phosphate buffered saline (DPBS), penicillin-streptomycin solution and TRIzol lysis reagent were purchased from Invitrogen (NY, U.S.A.). QuantiTect Reverse Transcription Kit was purchased from Qiagen (MD, U.S.A). All solvents used in this study were HPLC grade.

Animals.

Female BALB/c mice (4–6 weeks) were purchased from Charles River (CA, U.S.A). All animals were housed under pathogen-free conditions according to AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) guidelines. All animal-related experiments were performed in full compliance with institutional guidelines and approved by the Animal Use and Care Administrative Advisory Committee at the University of Pittsburgh.

Cell Culture.

Mouse triple negative breast cancer cells 4T1.2 and human triple negative breast cancer cells MDA-MB-231 were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere with 5% CO₂. All cell lines used in this work were obtained from ATCC (VA. U.S.A).

Production of MiR-34a Prodrug.

The expression and purification of tRNA-mir-34a and control tRNA-MSA was carried out using a novel RNA bioengineering technology as described recently.[15] Briefly, recombinant RNAs were produced in HST08 *E. coli* and verified by denaturing urea (8 M) polyacrylamide (8%) gel electrophoresis (PAGE) analysis of total bacterial RNA isolated by phenol extraction. Target RNAs were isolated by an anion exchange fast protein liquid chromatography (FPLC) method[53] on a NGC QUEST 10PLUS FPLC system (Bio-Rad, CA, U.S.A). RNA concentrations were determined with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, MA, U.S.A), and RNA purity was further determined by a high performance liquid chromatography (HPLC) assay. [15, 54] RNAs over 98% pure (by HPLC) were used in this study.

Synthesis of POEG-PVBC Polymer.

OEG500 (550 mg, 1.1 mmol), VBC monomer (600 μ L, 4.27 mmol), 4-Cyano-4-(phenylcarbonothioylthio) pentanoic acid (8 mg, 0.0286mmol), AIBN (3 mg, 0.0186 mmol), and 1 mL of dried tetrahydrofuran were added into a Schlenk tube, and deoxygenated by free-pump-thawing for three times. Then the mixture was filled with N₂ and immersed into an oil bath thermostated at 82 °C to start the polymerization. After 16 h, the reaction was quenched, and the mixture was precipitated in hexane for 3 times. The product POEG-PVBC was obtained after vacuum drying. Conversion of OEG500 monomer was 66.0% and conversion of VBC monomer was 80.0%. Molecular weight and distribution of POEG-PVBC were measured using gel permeation chromatography (GPC, Waters). Tetrahydrofuran (THF) was used as the eluent with a flowing rate of 1.0 mL/min at 35 °C. A series of commercial polystyrene standards with narrow molecular weight distribution were applied to calibrate the GPC elution traces.

Synthesis of POEG-PCre Polymer.

The POEG-PVBC polymer (270 mg) and creatine (1 g) were mixed in 15 mL anhydrous DMF with K₂CO₃ (1 g). After stirring at 80 °C for 36 h, the reaction mixture was cooled down and transferred into a dialysis bag (MWCO=3500 Da). After dialysis against a dilute hydrochloric acid solution for 1 day and deionized water for 3 days, the solution in the dialysis bag was centrifuged at 4,500 rpm for 12 min and the supernatant was lyophilized to give the POEG-PCre polymer. ¹H NMR spectra were examined on a 600.0 MHz Bruker spectrometer using DMSO-d₆ as the solvent.

Preparation of DOX+tRNA-mir-34a Co-formulated Micelles.

DOX solution was first prepared by dissolving DOX·HCl in DMSO containing triethylamine (5 equiv) overnight to remove HCl. DOX-loaded POEG-PCre micelles were prepared by the dialysis method. Briefly, 2 mg of polymer was dissolved in 200 μ L DMSO and mixed with 20 μ L of DOX solution (10 mg/mL). The mixture was then dialyzed against distilled deionized (DD) water using a dialysis bag (MWCO=3500) overnight to remove the unloaded free DOX. Drug-free micelles were similarly prepared without adding DOX solution. For tRNA-mir-34a complexation, polymeric micelles were diluted to different concentrations in DD water and mixed with the equivalent volume of tRNA-mir-34a (in 10% of glucose) to obtain the desired N/P ratios. This nanocomplexes was allowed to incubate at RT for 20 min prior to further characterization. *in vitro* studies were performed with freshly prepared nanocomplexes. For *in vivo* studies, micelles and tRNA-mir-34a were first mixed in DD water for 20 min, and then lyophilized with glucose as a cryoprotectant.

Physical Characterization of DOX+tRNA-mir-34a Co-formulated Micelles.

The particle size and zeta potential of different POEG-PCre micellar formulations were assessed by dynamic light scattering (DLS) via a Zetasizer (Nano-ZS 90, Malvern Instruments, Malvern, UK). The morphology of POEG-PCre blank micelles and gene-drug-co-loaded micelles was observed by transmission electron microscopy (TEM). The micelles were mounted on a copper grid with nitrocellulose supporting film. The samples were

negatively stained with phosphotungstic acid and dried at room temperature before examination.

To quantify the incorporated DOX, the fluorescence intensity ($\lambda_{ex}/\lambda_{em}=480\text{ nm}/590\text{ nm}$) of DOX-loaded micelles and various known amounts of DOX dissolved in DMSO was measured with a SpectraMax M3 microplate reader (Molecular Devices, CA, U.S.A). The colloidal stability of POEG-PCre micellar formulations with various carrier/drug mass ratios at room temperature and 4°C was evaluated by following the changes in sizes of the particles or visible precipitates every hour in the first 12 h and daily after 12 h following sample preparation. The CMC of POEG-PCre micelles was determined by using Nile red as a fluorescence probe as described previously.[55]

Gel Retardation Assay and Dextran Sulfate Replacement Assay.

tRNA-mir-34a/polymer complexes were prepared at different N/P ratios, ranging from 1 to 40 (tRNA-mir-34a concentration was fixed at 0.02 mg/ml). The resulting nanocomplexes were then electrophoresed on a 2% agarose gel. The gels were prepared with 2% agarose in Tris-acetate-EDTA (TAE) buffer containing 0.5 µg/mL ethidium bromide (Biotium, CA, U.S.A). For electrophoretic mobility shift analysis, samples were incubated at room temperature for 20 min; BlueJuice™ Gel Loading Buffer (10X) (Invitrogen, NY, U.S.A) was then added to the samples. Gel electrophoresis was carried out at 120 V for 20 min and the gel was subsequently visualized using a UV illuminator. Free tRNA-mir-34a was served as a control. The gel retardation of plasmid/polymer complexes and siRNA/polymer complexes at different N/P ratios were performed similarly.

For replacement assay, tRNA-mir-34a/polymer complexes of various N/P ratios were first prepared. Dextran sulfate was then added to the samples with various S/P ratios (molar ratio between the sulfur from dextran sulfate and the phosphate from nucleic acid). The samples were then electrophoresed similarly as described above.

Stability of DOX+tRNA-mir-34a Co-formulated Micelles.

Nanocomplexes were suspended in Dulbecco's phosphate-buffered saline (DPBS) of pH=7.4, BSA (30 mg/mL in DPBS) and RPMI 1640 with 10% FBS, respectively. The stability of nanocomplexes at various time periods (0, 1, 2, 4, 6, 8, 24 and 48 hours) was followed by examining their sizes via DLS.

Release of DOX from POEG-PCre Micelles *In Vitro*.

The kinetics of DOX release from DOX/POEG-PCre or DOX+ tRNA-mir-34a/POEG-PCre was performed according to a previous report.[26]

Cellular Uptake of DOX+tRNA-miR-34a/Polymer Complexes.

tRNA-mir-34a was labeled with fluorescent dye MFP488 using Label IT® miRNA Labeling Kits (Mirus Bio, WI, U.S.A). Cellular uptake of nanocomplexes carrying tRNA-mir-34a^{MFP488} (green fluorescence) and DOX (red fluorescence) was evaluated by using confocal laser scanning microscopy (CLSM, FluoView 1000, Olympus, Japan). 4T1.2 cells were seeded in 6-well plates at a density of 2×10^4 cells/well followed by 24 h of incubation

in DMEM containing 10% FBS and 1% streptomycin/penicillin. The cells were then treated with tRNA-mir-34a^{MFP488}/POEG-PCre, DOX+ tRNA-mir-34a^{MFP488}/POEG-PCre, or DOX/POEG-PCre in Opti-MEM medium at a dose of 100 nM and 10 μ M for tRNA-mir-34a^{MFP488} and DOX respectively. Free DOX.HCl and naked tRNA-mir-34a^{MFP488} were used as controls. Four h later, the cells were washed three times with cold DPBS to remove the remaining nanocomplexes. Cell nuclei were then stained with Hoechst 33342 (1 μ g/mL) at room temperature for 15 min. Cells were then washed with cold DPBS three times before observation under a confocal scanning microscope.

For further quantification of the DOX and tRNA-mir-34a^{MFP488} uptake, 4T1.2 cells were seeded in a 12-well plate at a high density (1×10^5 cells/well) and incubated overnight at 37 °C and 5% CO₂. The cells then received different treatments as mentioned above for 4 h. Next, the medium was removed, and cells were washed three times with cold PBS. The cells were harvested following trypsin treatment and washed with PBS three times to remove traces of cell culture media. Then, the cells were fixed with 10% paraformaldehyde for 10 min on ice and washed with PBS twice. Finally, the cell pellet was suspended in 200 μ L PBS and transferred to the flow cytometry tubes for the flow cytometry analysis with CyAn ADP Analyzer (Beckman Coulter, CA, U.S.A). A total of 15,000 events were collected for each sample.

Real-Time PCR.

Real-Time PCR (RT-PCR) was applied to evaluate the expression level of mature miR-34a and its target genes Bcl-2 and PD-L1. Total RNA was extracted from cultured cells or frozen tumor tissues using Trizol reagent (Invitrogen, NY, U.S.A) following the protocols of the manufacturer.

For miRNA expression analysis, 1 μ g of total RNA was applied for reverse transcription to synthesize cDNA of miRNA using miScript PCR starter kit (Qiagen, MD, U.S.A). Quantitative real-time PCR was performed using miScript SYBR Green PCR Kit (Qiagen, MD, U.S.A) containing miScript Universal Primer (reverse primer) and QuantiTect SYBR Green PCR Master Mix on a 7900HT Fast Real-time PCR System. Relative miRNA levels were analyzed using delta-delta-Ct calculations and normalized to U6. Each sample was analyzed in triplicate. The primers were as follows: miR-34a: 5'-TGGCAGTGTCTTAGCTGGTTGT-3'; U6: 5'-CGCAAGGATGACACGCAAATTC-3'; chimeric ncRNAs forward 5'-GGCTACGTAGCTCAGTTGGT-3', chimeric ncRNAs reverse 5'-TGGTGGCTACGACGGGATTC-3'; pre-miR-34a forward 5'-GGCCAGCTGTGAGTGTTCCTTTGG-3', pre-miR-34a reverse 5'-GGGCCCCACAACGTGCAG-3'.

For mRNA expression analysis, cDNA was generated from the 1 μ g of total RNA using QuantiTect Reverse Transcription Kit (Qiagen, MD, U.S.A) according to the manufacturer's instructions. Quantitative real-time PCR was performed using SYBR Green Mix on a 7900HT Fast Real-time PCR System. Relative target mRNA levels were analyzed using delta-delta-Ct calculations and normalized to GAPDH. The primers were as follows: mouse Bcl-2 forward 5'-GTCGCTACCGTCGTGACTTC-3', mouse Bcl-2 reverse 5'-CAGACATGCACCTACCCAGC-3'; human Bcl-2 forward 5'-

GGTGGGGTCATGTGTGTGG-3', human Bcl-2 reverse 5'-CGGTTTCAGGTACTCAGTCATCC-3'; mouse Dicer forward 5'-GGTCCTTTCTTTGGACTGCCA-3', mouse Dicer reverse 5'-GCGATGAACGCTTCCCTGA-3'; human Dicer forward 5'-CGAGCCTCCATTGTTGGTCCAC-3', human Dicer reverse 5'-CCAGTTCGCCAATTTGTGCAG-3'; mouse PD-L1 forward 5'-GCATTATATTCACAGCCTGC-3', mouse PD-L1 reverse 5'-CCCTTCAAAAGCTGGTCCTT-3'.

Western Blot Assay.

Cultured cells or frozen tumors were harvested and lysed with RIPA lysis buffer (Thermo Fisher Scientific, MA, U.S.A) by gently shaking on ice for 30 min. After centrifugation at 12,500 g for 10 min, the supernatants were collected and the concentrations of proteins were measured using Pierce BCA Protein Assay Kit (ThermoFisher Scientific, MA, U.S.A). The protein samples were denatured by boiling for 5 min and loaded onto 10% SDS-PAGE gel for electrophoresis. The proteins were then incubated in blocking buffer (5% non-fat dry milk in TBST) at RT for 1 h after being transferred onto PVDF membranes (Bio-Rad, CA, U.S.A). The membranes were then incubated with anti-Bcl-2 or anti-PD-L1 antibody (Cell Signaling Technology, MA, U.S.A) in antibody dilution buffer (5% BSA in TBST) with gentle agitation overnight at 4°C. After washing with TBST for three times, the membranes were subsequently incubated with the secondary HRP-linked goat anti-rabbit IgG antibody (Cell Signaling Technology, MA, U.S.A) for 1 h at RT. After another three washes with TBST, the membranes were incubated with Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, MA, U.S.A) for 1 min, wrapped in plastic and exposed to X-ray film. Protein expression was normalized against GAPDH or β -Actin expression.

In Vitro Cytotoxicity.

4T1.2 (1×10^3 cells/well) or MDA-MB-231 (5×10^3 cells/well) cells were seeded in 96-well plates followed by incubation in DMEM with 10% FBS and 1% streptomycin/penicillin for 24 h. Cells were then treated with various formulations including free DOX.HCl, DOX-loaded POEG-PCre micelles, DOX+ tRNA-MSA/POEG-PCre micelles, DOX+ tRNA-mir-34a/POEG-PCre micelles, blank POEG-PCre micelles, tRNA-MSA/POEG-PCre micelles and tRNA-mir-34a/POEG-PCre micelles. The former four DOX-containing groups were compared at various equivalent concentrations of DOX. The concentrations of POEG-PCre carrier in the last three DOX-free micelles were same as those of the carrier in DOX-loaded groups. After 48 h, MTT assay was performed as described before[26].

Cell Apoptosis.

4T1.2 cells were seeded in 6-well plates at a density of 2×10^4 cells/well followed by 24 h of incubation in DMEM containing 10% FBS and 1% streptomycin/penicillin. Cells were then treated with blank POEG-PCre micelles, tRNA-MSA/POEG-PCre micelles, tRNA-mir-34a/POEG-PCre micelles, free DOX.HCl, DOX/POEG-PCre micelles, DOX+ tRNA-MSA/POEG-PCre micelles or DOX+ tRNA-mir-34a/POEG-PCre micelles. All DOX-loaded groups contained the equivalent DOX concentration of 100 nM while the dose of tRNA-mir was 100 nM of in all tRNA-miR complexed groups. After 48 h, cells were harvested,

processed by using AnnexinV/propidium iodide (PI) apoptosis detection kit (BD), and subjected to flow cytometric analysis (FACSCalibur, BD, U.S.A).

***In Vivo* Biodistribution.**

Female Balb/c mice bearing 4T1.2 tumor lung metastasis were used to investigate the biodistribution of nanocomplexes. tRNA-mir-34a was labeled with MFP488 similarly as described above. Mice were injected with free DOX.HCl, DOX-loaded POEG-PCre micelles, tRNA-mir-34a^{MFP488} loaded POEG-PCre micelles or DOX+ tRNA-mir-34a^{MFP488}-co-loaded POEG-PCre micelles through tail vein. Major organs including lung, liver, heart, spleen and kidney were harvest from mice at 1h post injection. Tissues were then embedded in OCT and frozen in liquid nitrogen. The specimens were cryosectioned into 5 μ m sections. The signals of DOX and tRNA-mir-34a^{MFP488} in different tissues were examined under a confocal microscope.

Plasma Pharmacokinetics.

Free DOX.HCl, free tRNA-mir-34a or DOX+ tRNA-mir-34a-co-loaded POEG-PCre micelles were injected intravenously into groups of 5 tumor-free female CD1 mice at a DOX dose of 5 mg/kg and a tRNA-mir-34a dose of 1.5 mg/kg, respectively. Blood samples were collected in tubes containing EDTA at designated time points (3min, 10 min, 30 min, 1 h, 2 h, 6 h and 12 h) post injection. The samples were centrifuged at 12,500 rpm for 10 min at 4 °C and 50 μ L of the supernatant was collected. Twenty μ L of above supernatant was used for DOX measurement. Two hundred μ L acetonitrile was added to 20 μ L sample and the mixture was vortexed for 1 min. The samples were centrifuged at 12,500 rpm for 10 min at 4 °C and 150 μ L of the clear supernatant was collected and injected into HPLC for DOX analysis.

For tRNA-mir-34a measurement, total RNA was isolated from 30 μ L plasma at different time points using miRNeasy Serum/Plasma kit (Qiagen, MD, U.S.A). Then RNA was applied for reverse transcription to synthesize cDNA using miScript PCR starter kit (Qiagen, MD, U.S.A) as described above. Pure tRNA-mir-34a was also reverse transcribed to cDNA. Serial diluted pure tRNA-mir-34a cDNAs as well as cDNA from samples were proceeded with RT-PCR using miScript SYBR Green PCR Kit (Qiagen, MD, U.S.A) as mentioned above. Then Ct values from each sample were extracted. A standard curve was generated by plotting the log copy number of tRNA-mir-34a against the mean Ct value. The copy number of tRNA-mir-34a in each sample was determined by applying the Ct value to the standard curve. Noncompartmental pharmacokinetic analysis was executed by WinNonlin.

***In Vivo* Anti-tumor Efficacy and Safety Profile.**

Female Balb/c mice at age of 4-6 weeks were injected with 4T1.2 cells (2×10^5 in 100 μ L DPBS) through the tail vein. Five days after tumor cell injection, mice were randomly divided into 7 groups (n=5). Mice were treated intravenously with DPBS, tRNA-MSA-complexed POEG-PCre micelles, tRNA-mir-34a-complexed POEG-PCre micelles, free DOX.HCl, DOX-loaded POEG-PCre micelles, DOX+tRNA-MSA-co-loaded POEG-PCre micelles or DOX+tRNA-mir-34a-co-loaded POEG-PCre micelles, at a DOX dose of 5 mg/kg and a tRNA/mirs dose of 30 μ g per mouse, respectively. The treatments were

conducted every three days for a total of three times. Sixteen days after the first injection, all mice were sacrificed, and lung tissues were harvested, weighted and photographed. Pulmonary metastases were enumerated by intra-tracheal injection of India ink solution. India ink-injected lungs were washed in Feket's solution (300 ml 70% EtOH, 30 ml 37% formaldehyde and 5 ml glacial acetic acid) and white tumor nodules against a dark blue lung background were counted. In addition, blood sample was collected, and serum was isolated after centrifuging at 12,500 rpm for 10 min. ALT, AST and creatinine levels in serum were measured.

Histopathological Analysis.

Major organs including lung, liver, heart, spleen and kidney were harvested and fixed in 10% formalin at the end of above treatments. The fixed samples were then embedded in paraffin and the tissue sections were performed with immunohistochemical analysis including H&E and Ki67 staining. H&E staining were performed for histological evaluation of the tumor nodules in lungs, and Ki67 staining was carried out to evaluate the cell proliferation rate of the tumor cells. The toxicity of formulations was also assessed by H&E staining of several major organs.

Immune Cell Infiltration Profile in Tumor-Bearing Lungs.

The immune cell population in tumor-bearing lungs with various treatments was conducted in an independent *in vivo* study. Mice received similar treatments as described in the therapeutic study. Twenty-four hours after the last injection, lungs and spleens were collected in serum free RPMI medium. Lungs were cut mechanically with scissors and digested with Liberase TL and DNase I. Tissues were further grinded and filtered through a 70 mm cell strainer with red blood cells lysed by ACK lysis buffer. TILs and MDSC cells were further purified and stained with fluorescence-labeled antibody for flow cytometry analysis using a FACS flow cytometer according to a previous report.[56]

Statistics.

In vitro or *in vivo* data are presented as mean $\pm$ standard deviation (SD) or mean $\pm$ standard error of mean (SEM), respectively. Two-tailed Student's T test or analysis of variance (ANOVA) was used to compare two groups or multiple groups, respectively. Significance was determined with Tukey simultaneous post hoc test. In all statistical analyses, $P < 0.05$ is considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- [1]. Kamangar F, Dores GM, Anderson WF, Patterns of Cancer Incidence, Mortality, and Prevalence Across Five Continents: Defining Priorities to Reduce Cancer Disparities in Different Geographic Regions of the World, *J. Clin. Oncol* 24(14) (2006) 2137–2150. [PubMed: 16682732]
- [2]. Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, Viale A, Olshen AB, Gerald WL, Massagué J, Genes that mediate breast cancer metastasis to lung, *Nature* 436 (2005) 518. [PubMed: 16049480]
- [3]. Weigelt B, Peterse JL, van't Veer LJ, Breast cancer metastasis: markers and models, *Nat. Rev. Cancer* 5 (2005) 591. [PubMed: 16056258]
- [4]. Foulkes WD, Smith IE, Reis-Filho JS, Triple-Negative Breast Cancer, *N. Engl. J. Med* 363(20) (2010) 1938–1948. [PubMed: 21067385]
- [5]. Carey L, Winer E, Viale G, Cameron D, Gianni L, Triple-negative breast cancer: disease entity or title of convenience?, *Nature Reviews Clinical Oncology* 7 (2010) 683.
- [6]. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, Lickley LA, Rawlinson E, Sun P, Narod SA, Triple-Negative Breast Cancer: Clinical Features and Patterns of Recurrence, *Clin. Cancer Res* 13(15) (2007) 4429–4434. [PubMed: 17671126]
- [7]. Zeichner SB, Terawaki H, Gogineni K, A Review of Systemic Treatment in Metastatic Triple-Negative Breast Cancer, *Breast cancer : basic and clinical research* 10 (2016) 25–36. [PubMed: 27042088]
- [8]. Bartel DP, MicroRNAs: Genomics, Biogenesis, Mechanism, and Function, *Cell* 116(2) (2004) 281–297. [PubMed: 14744438]
- [9]. Huntzinger E, Izaurralde E, Gene silencing by microRNAs: contributions of translational repression and mRNA decay, *Nat. Rev. Genet* 12 (2011) 99. [PubMed: 21245828]
- [10]. Bader AG, Brown D, Winkler M, The Promise of MicroRNA Replacement Therapy, *Cancer Res.* 70(18) (2010) 7027–7030. [PubMed: 20807816]
- [11]. Hermeking H, The miR-34 family in cancer and apoptosis, *Cell Death Differ.* 17 (2009) 193. [PubMed: 19461653]
- [12]. Adams BD, Parsons C, Slack FJ, The tumor-suppressive and potential therapeutic functions of miR-34a in epithelial carcinomas, *Expert Opin. Ther. Targets* 20(6) (2016) 737–753. [PubMed: 26652031]
- [13]. Bouchie A, First microRNA mimic enters clinic, *Nat. Biotechnol* 31 (2013) 577. [PubMed: 23839128]
- [14]. Chen Q-X, Wang W-P, Zeng S, Urayama S, Yu A-M, A general approach to high-yield biosynthesis of chimeric RNAs bearing various types of functional small RNAs for broad applications, *Nucleic Acids Res.* 43(7) (2015) 3857–3869. [PubMed: 25800741]
- [15]. Wang WP, Ho PY, Chen QX, Addepalli B, Limbach PA, Li MM, Wu WJ, Jilek JL, Qiu JX, Zhang HJ, Li T, Wun T, White RD, Lam KS, Yu AM, Bioengineering Novel Chimeric microRNA-34a for Prodrug Cancer Therapy: High-Yield Expression and Purification, and Structural and Functional Characterization, *J. Pharmacol. Exp. Ther* 354(2) (2015) 131–41. [PubMed: 26022002]
- [16]. Nayerossadat N, Maedeh T, Ali PA, Viral and nonviral delivery systems for gene delivery, *Adv. Biomed. Res* 1 (2012) 27–27. [PubMed: 23210086]
- [17]. Pack DW, Hoffman AS, Pun S, Stayton PS, Design and development of polymers for gene delivery, *Nat. Rev. Drug Discov* 4 (2005) 581. [PubMed: 16052241]
- [18]. Mintzer MA, Simanek EE, Nonviral vectors for gene delivery, *Chem. Rev* 109(2) (2009) 259–302. [PubMed: 19053809]
- [19]. Khan M, Ong ZY, Wiradharma N, Attia ABE, Yang Y-Y, Advanced Materials for Co-Delivery of Drugs and Genes in Cancer Therapy, *Adv. Healthcare Mater* 1(4) (2012) 373–392.
- [20]. Kratz F, Albumin as a drug carrier: Design of prodrugs, drug conjugates and nanoparticles, *J. Control. Release* 132(3) (2008) 171–183. [PubMed: 18582981]

- [21]. Sun J, Sheng R, Luo T, Wang Z, Li H, Cao A.J.J.o.M.C.B., Synthesis of diblock/statistical cationic glycopolymers with pendant galactose and lysine moieties: Gene delivery application and intracellular behaviors, *J. Mater. Chem. B* 4(27) (2016) 4696–4706.
- [22]. Wagner E, Zenke M, Cotten M, Beug H, Birnstiel ML, Transferrin-polycation conjugates as carriers for DNA uptake into cells, *Proc. Natl. Acad. Sci* 87(9) (1990) 3410–3414. [PubMed: 2333290]
- [23]. Zhao Y, Wang W, Guo S, Wang Y, Miao L, Xiong Y, Huang L, PolyMetformin combines carrier and anticancer activities for in vivo siRNA delivery, *Nat. Commun* 7 (2016) 11822. [PubMed: 27264609]
- [24]. Huang L, Li S, Liposomal gene delivery: A complex package, *Nat. Biotechnol* 15 (1997) 620. [PubMed: 9219259]
- [25]. Hoet PHM, Nemery B, Polyamines in the lung: polyamine uptake and polyamine-linked pathological or toxicological conditions, *Am. J. Physiol. Lung Cell Mol. Physiol* 278(3) (2000) L417–L433. [PubMed: 10710513]
- [26]. Xu J, Zhao W, Sun J, Huang Y, Wang P, Venkataramanan R, Yang D, Ma X, Rana A, Li S, Novel glucosylceramide synthase inhibitor based prodrug copolymer micelles for delivery of anticancer agents, *J. Control. Release* 288 (2018) 212–226. [PubMed: 30223045]
- [27]. Li L, Yuan L, Luo J, Gao J, Guo J, Xie X, MiR-34a inhibits proliferation and migration of breast cancer through down-regulation of Bcl-2 and SIRT1, *Clin. Exp. Med* 13(2) (2013) 109–117. [PubMed: 22623155]
- [28]. Bu P, Chen K-Y, Chen Joyce H., Wang L, Walters J, Shin Yong J., Goerger Julian P., Sun J, Witherspoon M, Rakhilin N, Li J, Yang H, Milsom J, Lee S, Zipfel W, Jin Moonsoo M., Gümü Zeynep H., Lipkin Steven M., Shen X, A microRNA miR-34a-Regulated Bimodal Switch Targets Notch in Colon Cancer Stem Cells, *Cell Stem Cell* 12(5) (2013) 602–615. [PubMed: 23642368]
- [29]. Bianchini G, Balko JM, Mayer IA, Sanders ME, Gianni L, Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease, *Nature Reviews Clinical Oncology* 13 (2016) 674.
- [30]. Joensuu H, Gligorov J, Adjuvant treatments for triple-negative breast cancers, *Ann. Oncol* 23(suppl_6) (2012) vi40–vi45. [PubMed: 23012301]
- [31]. Graham AS, Hatton RC, Creatine: A Review of Efficacy and Safety, *J. Am. Pharm. Assoc* 39(6) (1999) 803–810.
- [32]. Cooper R, Naclerio F, Allgrove J, Jimenez A, Creatine supplementation with specific view to exercise/sports performance: an update, *J. Int. Soc. Sports Nutr* 9(1) (2012) 33–33. [PubMed: 22817979]
- [33]. Wyss M, Kaddurah-Daouk R, Creatine and Creatinine Metabolism, *Physiol. Rev* 80(3) (2000) 1107–1213. [PubMed: 10893433]
- [34]. Van Rooij E, Kauppinen S, Development of microRNA therapeutics is coming of age, *EMBO Mol. Med* 6(7) (2014) 851–864. [PubMed: 24935956]
- [35]. Bramsen J, Kjems J, Development of Therapeutic-Grade Small Interfering RNAs by Chemical Engineering, *Front. Genet* 3(154) (2012).
- [36]. Kang L, Gao Z, Huang W, Jin M, Wang Q, Nanocarrier-mediated co-delivery of chemotherapeutic drugs and gene agents for cancer treatment, *Acta Pharm. Sin. B* 5(3) (2015) 169–175. [PubMed: 26579443]
- [37]. Trang P, Wiggins JF, Daige CL, Cho C, Omotola M, Brown D, Weidhaas JB, Bader AG, Slack FJ, Systemic Delivery of Tumor Suppressor microRNA Mimics Using a Neutral Lipid Emulsion Inhibits Lung Tumors in Mice, *Mol. Ther* 19(6) (2011) 1116–1122. [PubMed: 21427705]
- [38]. Suzuki R, Namai E, Oda Y, Nishiie N, Otake S, Koshima R, Hirata K, Taira Y, Utoguchi N, Negishi Y, Nakagawa S, Maruyama K, Cancer gene therapy by IL-12 gene delivery using liposomal bubbles and tumoral ultrasound exposure, *J. Control. Release* 142(2) (2010) 245–250. [PubMed: 19883708]
- [39]. Tsouris V, Joo MK, Kim SH, Kwon IC, Won Y-Y, Nano carriers that enable codelivery of chemotherapy and RNAi agents for treatment of drug-resistant cancers, *Biotechnol. Adv* 32(5) (2014) 1037–1050. [PubMed: 24924617]

- [40]. Rackstraw BJ, Martin AL, Stolnik S, Roberts CJ, Garnett MC, Davies MC, Tendler SJB, Microscopic Investigations into PEG–Cationic Polymer-Induced DNA Condensation, *Langmuir* 17(11) (2001) 3185–3193.
- [41]. Bishop CJ, Kozielski KL, Green JJ, Exploring the role of polymer structure on intracellular nucleic acid delivery via polymeric nanoparticles, *J. Control. Release* 219 (2015) 488–499. [PubMed: 26433125]
- [42]. Amiji MM, Polymeric gene delivery : principles and applications, CRC2005.
- [43]. Draz MS, Fang BA, Zhang P, Hu Z, Gu S, Weng KC, Gray JW, Chen FF, Nanoparticle-mediated systemic delivery of siRNA for treatment of cancers and viral infections, *Theranostics* 4(9) (2014) 872–892. [PubMed: 25057313]
- [44]. Mu Q, Jiang G, Chen L, Zhou H, Fourches D, Tropsha A, Yan B, Chemical Basis of Interactions Between Engineered Nanoparticles and Biological Systems, *Chem. Rev* 114(15) (2014) 7740–7781. [PubMed: 24927254]
- [45]. Matta CF, Castillo N, Boyd RJ, Extended Weak Bonding Interactions in DNA: π -Stacking (Base–Base), Base–Backbone, and Backbone–Backbone Interactions, *J. Phys. Chem. B* 110(1) (2006) 563–578. [PubMed: 16471569]
- [46]. Wender PA, Galliher WC, Goun EA, Jones LR, Pillow TH, The design of guanidinium-rich transporters and their internalization mechanisms, *Adv. Drug Del. Rev* 60(4-5) (2008) 452–72.
- [47]. Stanzl EG, Trantow BM, Vargas JR, Wender PA, Fifteen years of cell-penetrating, guanidinium-rich molecular transporters: basic science, research tools, and clinical applications, *Acc. Chem. Res* 46(12) (2013) 2944–54. [PubMed: 23697862]
- [48]. He Y, Nie Y, Cheng G, Xie L, Shen Y, Gu Z, Viral mimicking ternary polyplexes: a reduction-controlled hierarchical unpacking vector for gene delivery, *Adv. Mater* 26(10) (2014) 1534–40. [PubMed: 24757715]
- [49]. Zhang X, Xu X, Li Y, Hu C, Zhang Z, Gu Z, Virion-Like Membrane-Breaking Nanoparticles with Tumor-Activated Cell-and-Tissue Dual-Penetration Conquer Impermeable Cancer, *Adv. Mater* 30(27) (2018) e1707240. [PubMed: 29774608]
- [50]. Zhou J, Mohamed Wali AR, Ma S, He Y, Yue D, Tang JZ, Gu Z, Tailoring the Supramolecular Structure of Guanidinylated Pullulan toward Enhanced Genetic Photodynamic Therapy, *Biomacromolecules* 19(6) (2018) 2214–2226. [PubMed: 29689167]
- [51]. Patne K, Rakesh R, Arya V, Chanana UB, Sethy R, Swer PB, Muthuswami R, BRG1 and SMARCA1 transcriptionally co-regulate DROSHA, DGCR8 and DICER in response to doxorubicin-induced DNA damage, *Biochim. Biophys. Acta* 1860(9) (2017) 936–951.
- [52]. Ma Y, Aymeric L, Locher C, Mattarollo SR, Delahaye NF, Pereira P, Boucontet L, Apetoh L, Ghiringhelli F, Casares N, Lasarte JJ, Matsuzaki G, Ikuta K, Ryffel B, Benlagha K, Tesnière A, Ibrahim N, Déchanet-Merville J, Chaput N, Smyth MJ, Kroemer G, Zitvogel L, Contribution of IL-17-producing $\gamma\delta$ T cells to the efficacy of anticancer chemotherapy, *J. Exp. Med* 208(3) (2011) 491–503. [PubMed: 21383056]
- [53]. Ho PY, Duan Z, Batra N, Jilek JL, Tu M-J, Qiu J-X, Hu Z, Wun T, Lara PN, DeVere White RW, Chen H-W, Yu A-M, Bioengineered ncRNAs selectively change cellular miRNome profiles for cancer therapy, *J. Pharmacol. Exp. Ther* (2018).
- [54]. Chen QX, Wang WP, Zeng S, Urayama S, Yu AM, A general approach to high-yield biosynthesis of chimeric RNAs bearing various types of functional small RNAs for broad applications, *Nucleic acids research* 43(7) (2015) 3857–69. [PubMed: 25800741]
- [55]. Xu J, Sun J, Wang P, Ma X, Li S, Pendant HDAC inhibitor SAHA derivatised polymer as a novel prodrug micellar carrier for anticancer drugs, *Journal of Drug Targeting* 26(5-6) (2018) 448–457. [PubMed: 29251528]
- [56]. Chen Y, Xia R, Huang Y, Zhao W, Li J, Zhang X, Wang P, Venkataramanan R, Fan J, Xie W, Ma X, Lu B, Li S, An immunostimulatory dual-functional nanocarrier that improves cancer immunochemotherapy, *Nat. Commun* 7 (2016) 13443. [PubMed: 27819653]

Figure 1.

In vitro biophysical characterizations of micelles. a) Size distribution and morphology of blank micelles, tRNA-mir-34a complexed micelles, DOX-loaded micelles and micelles co-loaded with tRNA-mir-34a and DOX (carrier: drug = 10: 1, w/w; carrier: tRNA-mir-34a = 20: 1, N/P) were examined by DLS and TEM, respectively. Scale bar, 100 nm. b) CMC of POEG-PCre polymers determined from the plot of Nile red fluorescence intensity. c) Gel retardation assay of tRNA-mir-34a/polymer and tRNA-mir-34a+DOX/polymer complexes at different N/P ratios. Samples were incubated for 20 min at room temperature before being loaded onto a 2% (w/v) agarose gel (120 V, 20 min). d) Dextran sulfate competitive binding

gel electrophoresis assay for tRNA-mir-34a/vector complexes (N/P=5, N/P=10 and N/P=20) at various S/P ratios. The experiments were repeated three times. e) Particle sizes and zeta potentials of tRNA-mir-34a/polymer and tRNA-mir-34a+DOX/polymer complexes formed at different N/P ratios. f) In vitro drug release profiles of DOX from DOX/POEG-PCre and DOX+tRNA-mir-34a/POEG-PCre in comparison with free DOX diffusion in DPBS at 37°C. g) The stability of blank micelles, tRNA-mir-34a complexed micelles, DOX-loaded micelles and micelles co-loaded with tRNA-mir-34a and DOX was examined by incubating nanocomplexes with DPBS (pH=7.4), DMEM with 2% FBS or BSA (30mg/mL) at room temperature. Changes in sizes of the complexes were followed by DLS. Data are means $\pm$ SD (n=3).

Figure 2. Intracellular uptake of DOX+tRNA-mir-34a^{MFP488}/POEG-PCre in 4T1.2 breast cancer cells at 4h post incubation was monitored by confocal microscope and flow cytometry. a) From left to right: MFP488-labeled tRNA-mir-34a (green); DOX (red); nuclear staining with Hoechst 33342 (blue); an overlay of MFP488, DOX and Hoechst. Scale bar: 50 μ m. b) DOX-positive and MFP488-positive cells were quantitatively determined by flow cytometry. c) Mean fluorescence intensity of DOX and MFP488 was analyzed by flow cytometry. Data are means $\pm$ SD (n=3). The experiments were repeated three times.

Figure 3. Restoration of mature miR-34a down-regulates Bcl-2 expression in 4T1.2 and MDA-MB-231 cancer cells after treatment with different formulations. Cells were harvested at 48 h after treatment. a-c) Mature miR-34a (a), chimeric ncRNA (b) and pre-miR-34a (c) expression levels were detected by RT-PCR. d) The mRNA expression of Bcl-2 was quantitatively analyzed by RT-PCR. e) The protein expression of Bcl-2 was evaluated by Western blot. Data are means $\pm$ SD (n=3). P values were generated by one-way ANOVA using the Tukey test for multiple comparisons. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 4. *In vitro* antitumor efficacy. a) Cell cytotoxicity of 4T1.2 and MDA-MB-231 cancer cells treated with different formulations after 48 h. Data are means $\pm$ SD (n=3). b) Cell apoptosis of 4T1.2 cells detected by flow cytometry using an AnnexinV-FITC/PI apoptosis detection kit after 48 h. c) Inhibition migration of 4T1.2 cells examined by wound healing assay after 24 h. The experiments were repeated three times.

Figure 5.

Biodistribution of nanocomplexes in tumor-bearing lungs. Balb/c mice bearing 4T1.2 lung metastatic tumors were i.v. injected with DOX+tRNA-mir-34a^{MFP488}/POEG-PCre nanocomplexes. Fluorescent signals in tumor-bearing lungs were examined after 1 h. The experiments were repeated three times.

Figure 6.

Synergistic antitumor activity of tRNA-mir-34a and DOX co-delivered by POEG-PCre micelles in a mouse model of breast cancer (4T1.2) lung metastasis. a) Experimental scheme for the establishment of lung metastasis and treatment. b-c) Quantification of tumor nodules (b) and lung weights (c). d) Changes in mouse body weights during the treatment period. e-f) Quantification of mature miR-34a (e) and Bcl-2 mRNA (f) in tumor-bearing lungs by RT-PCR. g) Bcl-2 protein level was evaluated by Western blot. h) Representative images of lungs collected from mice receiving various treatments. i-j) H&E (i) and Ki67 (j) staining of

lung tissues. Data are means $\pm$ SEM (n=5). P values were generated by one-way ANOVA using the Tukey test for multiple comparisons. *P < 0.05, **P < 0.01, ***P < 0.001.

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Figure 7.

Safety profile of tRNA-mir-34a and DOX codelivered by POEG-PCre micelles in a mouse model. a-c) ALT (a), AST (b) and creatinine (c) level detected at the end of therapy. (d) H & E staining of major organs (heart, liver, spleen and kidney) harvested at the end of therapy. Data are means $\pm$ SEM (n=5).

Figure 8. Flow analysis of infiltration of immune cells in tumor-bearing lungs following various treatments. Balb/c mice were injected with 2×10^5 4T1.2 cells via tail vein. Five days later, mice were treated with tRNA-MSA/POEG-PCre, tRNA-mir-34a/POEG-PCre, free DOX, DOX/POEG-PCre, DOX+tRNA-MSA /POEG-PCre and DOX+tRNA-mir-34a/POEG-PCre respectively for three times. T lymphocyte subsets isolated from lung tissues with 4T1.2 metastasis was analyzed by flow cytometry. a-f) The percentage of CD4⁺ (a), CD8⁺ (b), IFN- γ positive intratumoural CD8 T cells (c), PD-1 positive intratumoural CD8 T cells (d), FoxP3⁺ Treg cells (e) and MDSC cells (f) in tumor-bearing lungs were detected by flow

cytometry. g-k) Flow cytometry gating and representative figures of CD4⁺ and CD8⁺ (g), IFN- γ positive intratumoural CD8 T cells (h), PD-1 positive intratumoural CD8 T cells (i), FoxP3⁺ Treg cells (j) and CD11b⁺/Gr-1⁺ MDSC cells (k). The results are shown as the means $\pm$ SEM with 3 mice in each group. The experiments were repeated two times. P values were generated by one-way ANOVA using the Tukey test for multiple comparisons. *P < 0.05, **P < 0.01, ***P < 0.001.