

Non-Small-Cell Lung Cancer Regression by siRNA Delivered Through Exosomes That Display EGFR RNA Aptamer

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Lung cancer is the second most common cancer in both men and women and is the leading cause of cancer death in the United States. The development of drug resistance to commonly used chemotherapeutics in non-small-cell lung cancer (NSCLC) poses significant health risks and there is a dire need to improve patient outcomes. In this study, we report the use of RNA nanotechnology to display ligand on exosome that was loaded with small interfering RNA (siRNA) for NSCLC regression in animal trials. Cholesterol was used to anchor the ligand targeting epidermal growth factor receptor on exosomes that were loaded with siRNA to silence the antiapoptotic factor survivin. The cytosolic delivery of siRNA overcame the problem of endosome trapping, leading to potent gene knockdown, chemotherapy sensitization, and tumor regression, thus achieving a favorable IC₅₀ of 20 nmol/kg siRNA encapsulated by exosome particles in the *in vivo* gene knockdown assessment.

Keywords: RNA nanotechnology, exosomes, non-small-cell lung cancer, siRNA, EGFR, aptamer

Introduction

LUNG CANCER IS the most common cancer worldwide, resulting in 2.09 million new patient cases per year, and causing 1.76 million deaths per year in 2018 according to World Health Organization (WHO) reports [1]. Non-small-cell lung cancer (NSCLC) accounts for more than 80% of all lung cancer cases [2]. First-line treatment for metastatic NSCLC includes chemotherapy, targeted therapy, and immunotherapy. Treatments targeting epidermal growth factor receptor (EGFR), including tyrosine kinase inhibitors (eg, erlotinib, gefitinib, osimertinib, etc.) [3–5] and monoclonal antibodies (e.g., bevacizumab, cetuximab, necitumumab, etc.) [6–8] have been widely used for various molecular forms of NSCLC. An RNA aptamer selectively targeting EGFR was discovered by Systematic Evolution of Ligands by Exponential enrichment (SELEX) and had been applied with RNA nanotechnology for targeting breast cancer [9–11]. Studies have shown that *BIRC5*, which codes for the survivin protein, is overexpressed in NSCLC and correlates with the development of resistance [12,13]. Moreover, downregulation of *BIRC5* can induce apoptosis, which has been proven to be a potent strategy for treatment in multiple cancer types [14–17].

RNA interference (RNAi), discovered by Andrew Fire and Craig Mello in 1998 and recognized by the Noble Prize

in 2006, was one of the most important breakthroughs in the history of gene therapy [18]. Instead of introducing the deficient gene, RNAi enables post-transcriptional gene silencing (PTGS) by suppressing mRNA translation or inducing mRNA cleavage. Small interfering RNA (siRNA) is double-strand artificially synthesized RNA ranging from 19 to 27 bp that can utilize the Dicer-RISC pathway to silence target mRNA [19]. This novel strategy created a new direction for gene therapy by allowing the specific design of siRNA to achieve precise manipulation of the target and minimize off-target effects.

However, challenges still exist and the development of RNAi-based gene therapy was more difficult than expected. The major challenge is drug delivery, the same problem facing the field of whole gene therapy [20,21]. First, naked RNA is vulnerable to RNases that widely exist in the circulatory system. Second, as a negatively charged macromolecule, the cellular uptake of RNA is low due in part to the lack of specific transporters that many small molecule drugs utilize for cellular entry. Third, due to these challenges, higher doses are needed, which increases the risk of side effects due to unwanted gene manipulation in healthy cells. Even though a small portion of RNA enters the cell, most of them will be trapped in the endosome that prevents the RNA from reaching the RNAi machinery, as well as its mRNA target located in the

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cytoplasm. Therefore, the development of a drug delivery vehicle that can protect RNA and improve target cell uptake, especially cytosolic distribution, is urgently needed. Despite the challenges aforementioned, three siRNA drugs (Patisiran, Givosiran, and Lumasiran) have been approved by the U.S. Food and Drug Administration (FDA) since 2018, leveraging the advantage of nucleic acid chemistry, GalNac conjugation, and nanoparticle-mediated delivery. However, the current success of systemic siRNA administration is still limited to liver applications, and the targeting strategy for other organ-associated disease is needed.

RNA nanotechnology was first proven by Peixuan Guo in 1998, a decade after his discovery of packaging RNA (pRNA) in bacteriophage phi29 DNA packaging motor [22,23]. The single-strand pRNA contains a 3-way-junction (3WJ) core that retains high thermostability in the physiological condition when truncated to three 16 ~ 20 nt single strands [24]. This unique feature provides feasibility to design self-assembling nanoparticles in a bottom-up strategy using single-strand RNA as a building block with precise control of size, shape, stoichiometry, and homogeneity [25–29]. Meanwhile, both the thermostability and the enzymatic stability can be significantly improved with the chemical modification of 2'-OH on ribose sugar [30]. Moreover, the cytotoxicity and immunogenicity can be controlled to a negligible level by rational design of sequence, size, shape, and chemical modifications [31–34]. Thereby, the development of RNA nanotechnology rebuilds the concept of RNA as a stable and biocompatible nanomaterial that can be used for drug delivery. The fully controllable feature of RNA nanotechnology enables the programmable design for tuning the pharmacokinetic (PK) and distribution profiles. In general, desired drug delivery should maximize the accumulation in the disease site and minimize healthy tissue accumulations. The nanometer scale allows RNA nanoparticles by themselves to nonspecifically accumulate in tumor tissue through enhanced permeability and retention effect due to the leaky texture in overgrowing tumor tissue [35,36]. The controllable size, along with the negatively charged and hydrophilic property, of RNA nanoparticle also eliminate the liver accumulation that reduces potential toxicity and immunogenicity [32,37,38]. Besides the passive targeting effect, RNA nanoparticles can also be actively internalized by cancer cells by the conjugation of ligands that can target to cancer cells, including RNA aptamers [10,39,40] and small molecule ligands [41–43]. However, challenges such as relatively low drug payload, endosome entrapment after receptor-mediated endocytosis, and rapid clearance are problems that remain to be addressed for RNA nanoparticle as a delivery vehicle for gene therapy. Nevertheless, RNA nanotechnology as an emerging solution holds great promise for RNAi therapeutics delivery.

Exosomes have been reported as a novel drug delivery vehicle with great potential [16,44–47]. Exosomes are derived from late endosome/multivesicular body with a diameter between 50 and 150 nm [48]. Exosomes have an endomembrane-like membrane property (structure, lipid, peptides, protein, etc.), which offers an innate ability to fuse with recipient plasma membrane or the membrane of the cellular organelles [47,49–52]. Exosomes were considered to be a relative “garbage disposal bucket” [53] until 2007, when three research groups discovered that genetic materi-

als, especially RNA, can be transferred and communicated in long distance among cells through exosomes [54–56]. In the following decade, exosomes have been demonstrated to serve as carriers for direct delivery of their RNA payloads into the cytosol, which enables the full functionality [47,57–59]. The advantages of exosomes include that they are the natural carriers of proteins and RNAs [55,60,61], and the high payloads they can carry while remaining a favorable size and are well tolerated *in vivo* [51]. Therapeutic payloads, such as siRNA, can remain fully functional after delivery to cells by exosomes [45,49,52,55,62]. However, the selectivity of naive exosomes is relatively low and vary from sources, which can cause random uptake in healthy cells. To generate cell-specific targeting exosomes, we used RNA nanotechnology for ligand displaying on naive exosomes to promote efficient cell targeting, cytosolic siRNA delivery, and cancer regression [16].

In this study, to leverage the targeting effect and therapeutic potential of EGFR RNA aptamer for NSCLC treatment, we designed an arrowtail RNA nanoparticle to display EGFR RNA aptamer on the surface of exosomes to deliver survivin siRNA (EGFR_{Apt}/Exo/siSurvivin) for NSCLC treatment as the formulation schematic shows in Fig. 1A.

Materials and Methods

Cell culture, chemicals, and antibodies

Human lung cancer cell line H596 and H1568 were obtained from American Type Culture Collection and maintained in Rosewell Park Memorial Institute (RPMI) 1640 medium (ATCC; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) (GE Healthcare, Chicago, IL) and 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA). Cells were cultured in 37°C with 5% CO₂. Cisplatin (Sigma, St. Louis, MO) was dissolved in dimethyl formamide (Sigma). Anti-CD63, TSG101, Survivin, EGFR, cleaved-poly (ADP-ribose) polymerase (PARP), cleaved-Caspase3, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primary antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-rabbit, and anti-mouse secondary antibodies were purchased from LI-COR Bioscience (Lincoln, NE).

Purification and characterization of exosomes

Exosomes were purified using a modified differential ultracentrifugation method as previously described [16,17,63]. HEK293T cells obtained from ATCC were cultured in FiberCell Hollow Fiber Bioreactor (C2011, 20 kDa MWCO) using Dulbecco's modified Eagle's medium (DMEM) with 10% exosome-free FBS, and the exosome-enriched medium was collected each week. Exosome-enriched medium was spun down at 300 rcf for 10 min to remove dead cells, followed by spinning at 10,000 rcf for 30 min at 4°C followed by 22 nm filtration to remove cell debris and microvesicles and store at -80°C until 500 mL accumulated.

Preprocessed medium was thawed slowly at 4°C overnight, then loaded into preconditioned Pall Minimate™ tangential flow filtration (TFF) system with 100 kDa MWCO capsule (OA100C12), precondition follows the standard operation by the manufacturer. Five hundred milliliters of exosome-enriched medium was processed at 6 mL/min by setting pump speed at 40 mL/min. When the volume reduced to ~5 mL, 200 mL sterile Dulbecco's phosphate buffered saline

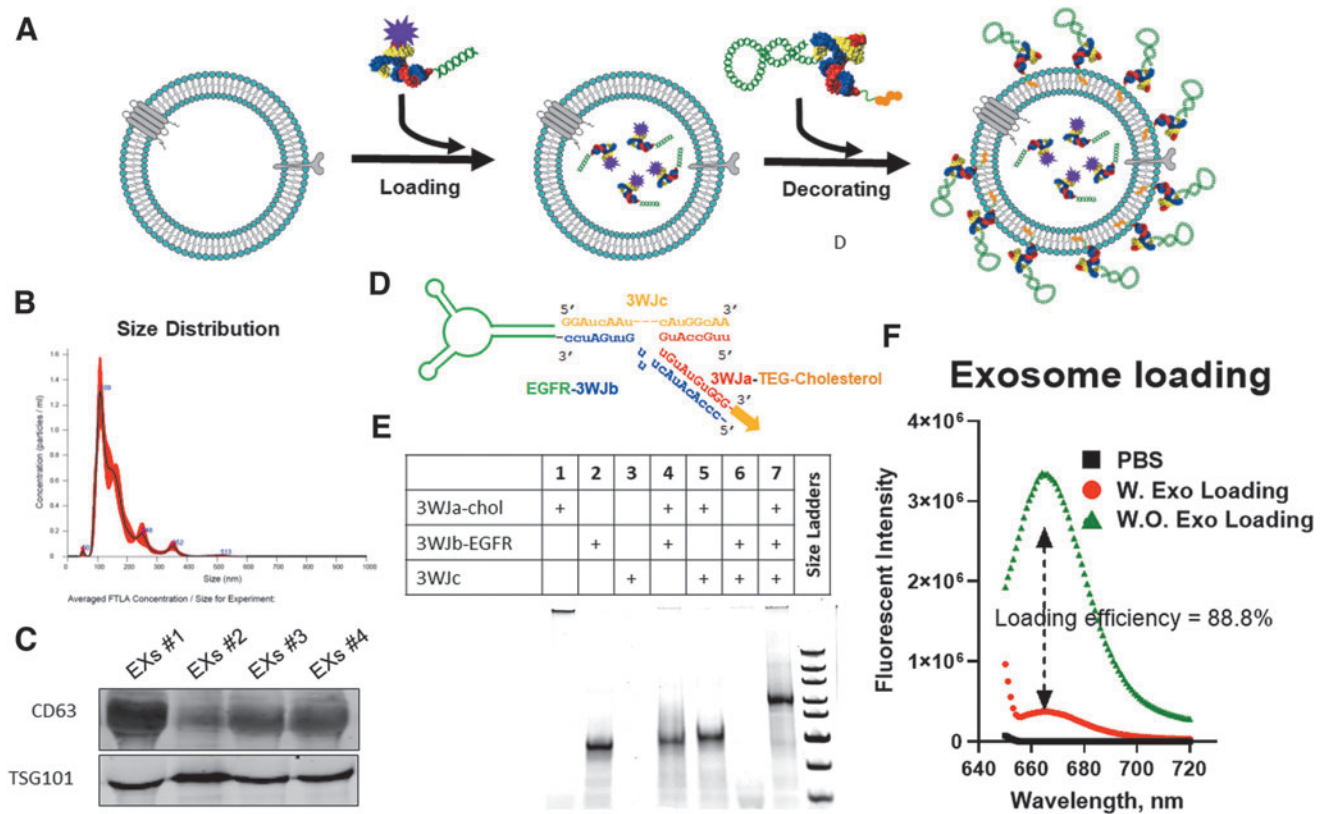


FIG. 1. Assembly of exosome vesicle loaded with siRNA and RNA nanotechnology decoration. (A) Formulation scheme of EGFR_{Apt} displaying exosomes loaded with siRNA. (B) Size distribution of exosomes. Purified exosomes were analyzed by NTA with peak size at 109 nm and average size of 177 ± 6 nm. (C) Surface marker characterization. Purified exosomes from four independent purification were assayed by immunoblotting to confirm exosome surface marker CD63 and TSG 101. (D) Design of EGFR_{Apt} arrowtail pRNA nanoparticles for ligand displaying. (E) Assembly of EGFR_{Apt} arrowtail pRNA nanoparticles assay by 10% Native PAGE in TBE buffer stained by EtBr. (F) Loading efficiency of siRNA into exosomes assayed by comparing fluorescent intensity in unloaded supernatant of siRNA loading with Exo and without Exo. EGFR, epidermal growth factor receptor; EtBr, ethidium bromide; NTA, nanoparticle tracking analysis; PAGE, polyacrylamide gel electrophoresis; pRNA, packaging RNA; siRNA, small interference RNA; TBE, tris/borate/EDTA buffer.

(DPBS) was added as washing step and continued to run until the volume reduced to ~5 mL again, then collected. Twice the 15 mL DPBS wash was performed and total 30 mL wash was collected and combined with the 5 mL sample from the last step.

The 35 mL post TFF medium was then further purified by 100,000 rcf ultracentrifugation using a SW28 rotor (Beckman Coulter) for 90 min at 4°C. Two-hundred microliters of 60% iodixanol (Sigma) was added to the bottom of each tube to serve as iso-osmotic cushion as we previously reported [16,17,63]. The supernatant was carefully removed from the top, and around 2 mL of the fraction close to the interface and cushion were collected. Nanoparticle tracking analysis (NTA) was performed using the Malvern NanoSight NS300 system following the standard protocol developed and reported before [16,17,63].

Assembly of exosomes loaded with siRNA and RNA nanotechnology decoration

Exosomes (0.05 pmole) and Alexa₆₄₇ or Alexa₇₅₀-conjugated siRNA (0.15 nmole) were mixed in 100 μL of 1 × PBS with 2.5 μL of ExoFect exosome transfection (System Bioscience) following the manufacturer's instruction. Unloaded siRNA from the supernatant was collected and Alexa₆₄₇ intensity on

supernatant was measured to evaluate loading efficiency compared with the control group. EGFR_{Apt}/3WJ arrowtail RNA nanoparticles were self-assembled in a one-pot manner by mixing individual RNA strands at equimolar concentrations (25 μM) in PBS buffer. Assembled RNA nanoparticles were confirmed by electrophoresis. Then RNA nanoparticles were incubated with exosomes at 37°C for 45 min, then left on ice for 1 h to prepare the RNA-decorated Exosome.

In vitro exosomes cellular binding assay

The ability of exosomes binding to human lung cancer cells was assessed *in vitro* by flow cytometry. In brief, 1 × 10⁵ H596 and H1568 cells were resuspended in 100 μL PBS buffer, and incubated with 50 nM Alexa₆₄₇-labeled exosomes loaded with siRNA at 37°C for 1 h. After washing three times with PBS, the cells were subjected to flow cytometry analysis using the BD LSRFortessa flow cytometer. Data were analyzed using the FlowJo 7.6.1 software.

Real-time quantitative polymerase chain reaction analysis

Survivin gene silencing by siRNA delivered by exosomes was detected by real-time quantitative polymerase chain

reaction (PCR) assay. Total cellular RNA was isolated using TRIzol (Invitrogen), and one microgram of total RNA was reverse transcribed using Superscript reverse transcriptase (Bio-Rad, Hercules, CA). PCR was performed on iCYCLER real-time PCR machine (Bio-Rad) using SYBR-Green chemistry (Bio-Rad). The gene expression levels were normalized to housekeeping gene *GAPDH*. The sequences of used primers are shown in Supplementary Table S1.

Immunoblotting

Cell lysates were prepared using RIPA buffer (Thermo Fisher, Waltham MA) supplemented with 1× protease inhibitors (Complete; Roche, Indianapolis, IN) and phosphatase inhibitors (PhosSTOP; Roche) followed by protein quantification with the Dc Protein Assay Kit (Bio-Rad). Equal amounts of protein were loaded and resolved by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were incubated in 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween-20 (TBST) blocking buffer for 1 h at room temperature. Primary antibodies with dilution of 1:200–1,000 were allowed to bind overnight at 4°C, or for 2 h at room temperature. After washing in TBST, the membranes were incubated with immunofluorescent secondary antibodies at a 1:5,000 dilution for 1 h at room temperature. Membranes were washed with TBST and allowed to air dry before imaging through LI-COR Odyssey® CLx Imaging System (Thermo Fisher). Original data refer to Supplementary Figure S3.

IncuCyte cell proliferation assay

Cells were treated with various exosomes accordingly for 48 h, and then seeded at 1,000–2,000 cells per well in 96-well plates. Cell confluence as a measure of cell growth over time was monitored every 4 h for up to 6 days using the IncuCyte ZOOM Live-Cell Imaging System (Essen Biosciences). Cell proliferation curve was plotted using GraphPad Prism.

Cell apoptosis analysis by flow cytometry

Cells were seeded into 6-well plates at a density of 200,000 cells per well in 2 mL medium for 16 h, then treated with exosomes for 48 h, followed by 5 μM cisplatin treatment for another 24 h. Cell apoptosis was assessed by Annexin V-FITC (Invitrogen) and Propidium Iodide (PI; Sigma-Aldrich) staining coupled with flow cytometry analysis using the BD LSRFortessa flow cytometer. Data were analyzed using the FlowJo 7.6.1 software.

Biodistribution and antitumor activity of exosomes in vivo

Animal studies were conducted in accordance with an approved protocol adhering to the Institutional Animal Care and Use Committee (IACUC) policies and procedures at The Ohio State University. Six- to eight-week-old male athymic nude mice (Taconic Farms, Inc., NY) were caged in groups of five or less, and fed with a diet of animal chow and water *ad libitum*. H596 cells were injected subcutaneously into the flanks of each mouse at 5×10^6 per injection. When the tumor sizes reached 150–200 mm³, the mice were randomly divided into four groups and tail vein injected with exosomes three times a week for 2 weeks. The tumor volume was monitored

three times a week, and tumor size was calculated using the formula: $V = \frac{L \times W^2}{2}$. For *in vivo* exosomes targeting and tumor imaging, mice were tail vein injected with 100 μL of 20 μM of RNA Alexa₇₅₀-labeled siSurvivin loaded with exosomes, and animal or tissue was imaged using the *in vivo* imaging system (IVIS) Lumina imaging system with Living Images 3.0 software (Caliper Life Sciences).

Data analysis

Data are presented as the mean ± standard deviation or standard error of the mean of at least three independent experiments. The difference among groups were calculated using Student's *t* test or one-way analysis of variance (ANOVA) analysis followed by Tukey's *post-hoc* test (GraphPad Prism).

Result

Isolation and characterization of exosomes derived from HEK293T cells

We first produced and isolated bulk exosomes from HEK293T cells using a hollow-fiber bioreactor, TFF, and cushion ultracentrifugation (Supplementary Fig. S1). With this workflow, the volume of exosomes can be concentrated from liter to microliter scale in a relatively straightforward process. Postexosome production, size distribution was analyzed by the NTA method, showing the size peak at 109 nm, and average size of exosomes as 177 ± 6 nm (Fig. 1B). We further characterized exosome marker expression by immunoblotting, which showed high expression of CD63 and TSG101 in various batches of the exosome solution (Fig. 1C).

Assembly, loading, and ligand displaying of exosomes by RNA nanotechnology

Following our rationale design reported previously to increase thermodynamic and enzymatic stability [16,17,24,63], we harbored survivin siRNA onto the pRNA 3WJ core with a 2'F modification on the sense strand. To determine siRNA loading efficiency into exosomes, the remaining siRNA was fused to an Alexa₆₄₇-labeled 3WJ core (Fig. 2) and then was loaded into exosomes using ExoFect method as we reported previously [16,17,63]. In short, 3WJ-siSurvivin-Alexa₆₄₇ mixed with equal volume of PBS and treated with ExoFect was used as background to evaluate loading efficacy. As shown in Fig. 1F, 88.8% of siRNA was loaded into exosomes. EGFR RNA aptamer (EGFR_{Apt}) was adapted to generate arrowtail RNA nanoparticles (Fig. 1D), which we had developed for ligand displaying on exosome surface [16,17,63]. As shown in Fig. 1E, EGFR_{Apt} arrowtail RNA nanoparticles were assembled with very high efficiency as indicated by gel shift assays showing stepwise assembly of the complex.

Specific binding and siRNA delivery to cells in vitro using EGFR_{Apt} displaying exosome

To prove the ligand-displaying strategy can improve NSCLC cell targeting, we next examined the cellular uptake of EGFR_{Apt}/Exo/siSurvivin on two EGFR-expressing lung cancer cell lines, H596, and H1568 (Fig. 3). As shown in Fig. 4A and Supplementary Fig. S2, flow cytometry revealed significantly higher binding efficiency (78.8% and 85.6%) for EGFR_{Apt}-decorated exosomes, compared with no EGFR_{Apt}

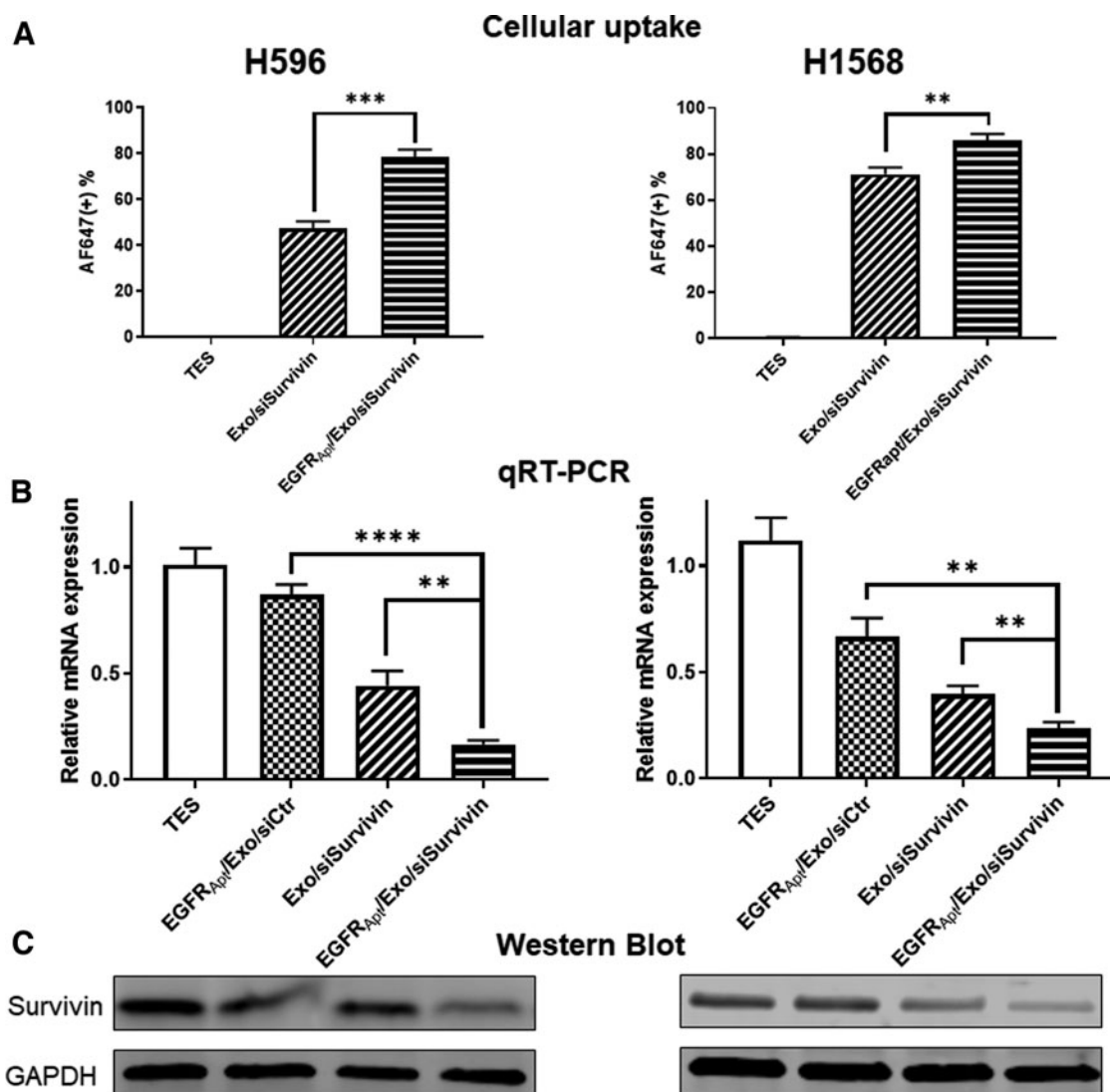


FIG. 4. EGFR_{Apt}/Exo/siSurvivin suppresses survivin expression in human lung cancer. (A) Cellular uptake of siSurvivin delivered by EGFR_{Apt}/Exo and Exo only compared by flow cytometry on both H596 and H1568 NSCLC cell lines, $**P < 0.01$, $***P < 0.001$ by Student's *t*-test, $n = 3$. Raw data and gate setting in Supplementary Fig. S4. (B) mRNA level of survivin gene quantified by qRT-PCR assay post 48 h treatment of EGFR_{Apt}/Exo/siSurvivin, EGFR_{Apt}/Exo/siCtr, Exo/siSurvivin, and TES buffer in 50 nM of siRNA concentration on both H596 and H1568 NSCLC cell lines. $**P < 0.01$, $***P < 0.0001$ by Student's *t*-test, $n = 3$. (C) Protein expression level of Survivin evaluated by western blot post 48 h treatment of EGFR_{Apt}/Exo/siSurvivin, EGFR_{Apt}/Exo/siCtr, Exo/siSurvivin and TES buffer in 50 nM of siRNA concentration on both H596 and H1568 NSCLC cell line. qRT-PCR, real-time quantitative reverse transcription polymerase chain reaction; TES, tris/EDTA/saline buffer.

evaluating the cleavage of PARP (c-PARP) and Caspase3 (c-Cas3), which are widely used as indicators of apoptosis [66,67]. Cotreatment of cisplatin and siSurvivin induced more cleavage of both indicator proteins with the same trends observed between EGFR-targeted and nontargeted formulation (Fig. 5C). The results above validated the siSurvivin delivery by EGFR_{Apt}/Exo nanoparticle and showed the potential application for combination therapy with cisplatin.

In vivo lung cancer cell targeting, and antitumor efficacy of EGFR_{Apt}/Exo/siSurvivin using tumor xenograft mouse model

To determine the *in vivo* siRNA delivery effects of EGFR_{Apt}/Exo/siSurvivin, we established nude mouse tu-

mor xenografts with H596 cells. Live animal imaging demonstrated that Alexa₇₅₀-conjugated EGFR_{Apt}/Exo/siSurvivin nanoparticles strongly accumulated in the xenograft tumor 24–48 h after tail vein injection of exosomes (Fig. 6A, Left). Further *ex vivo* biodistribution analysis confirmed that EGFR aptamer facilitated exosome accumulation in the xenograft tumors (Fig. 6A, Right). To optimize the best dose of exosomes for *in vivo* treatment, serial doses of EGFR_{Apt}/Exo/siSurvivin were intravenous (I.V.) administered every 2 days four times over 8 days, and tumors were isolated for extraction of total RNA and protein (Fig. 6B). As shown in Fig. 6C and D, survivin RNA and protein expression was suppressed in a concentration-dependent manner. Forty nanomole per kilogram dose has been shown to be the most effective dose

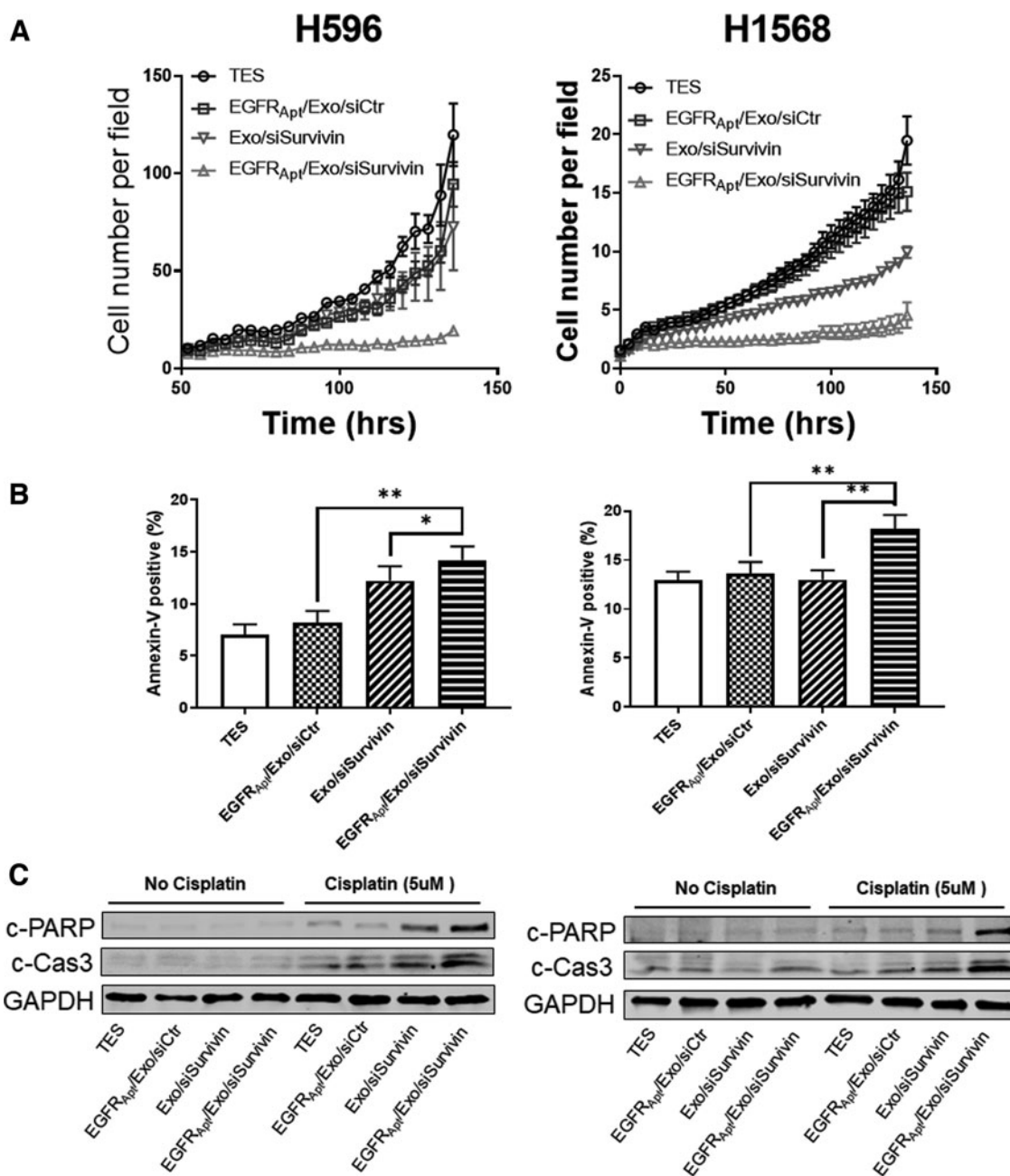


FIG. 5. Silencing of survivin suppressed tumor cell growth and sensitized cisplatin cytotoxicity in NSCLC Cells. **(A)** IncuCyte cell proliferation assay on both H596 and H1568 NSCLC cell lines posttreating with EGFR_{Apt}/Exo/siSurvivin, EGFR_{Apt}/Exo/siCtr, Exo/siSurvivin, and TES buffer in 50 nM of siRNA concentration. $n=3$, error bar \pm SEM. **(B)** Cell apoptosis evaluation by Annexin V-positive cells using flow cytometry on both H596 and H1568 NSCLC cell lines posttreating with EGFR_{Apt}/Exo/siSurvivin, EGFR_{Apt}/Exo/siCtr, Exo/siSurvivin, and TES buffer in 50 nM of siRNA concentration for 48 h followed by 24 h treatment with 5 μ M cisplatin, $n=3$, * $P<0.05$, ** $P<0.01$, error bar \pm SD. Original data and gating refer to Supplementary Fig. S4. **(C)** Cisplatin-induced apoptosis marker evaluation by western blot on both H596 and H1568 NSCLC cell lines posttreating with EGFR_{Apt}/Exo/siSurvivin, EGFR_{Apt}/Exo/siCtr, Exo/siSurvivin, and TES buffer in 50 nM of siRNA concentration for 48 h followed by 24 h treatment with or without 5 μ M cisplatin. SD, standard deviation; SEM, standard error of the mean.

for *in vivo* silencing of survivin expression. We chose this dose to compare the antitumor efficacy along with all different control groups. Finally, we found that treatment of tumor-bearing mice with EGFR_{Apt}/Exo/siSurvivin led to marked tumor growth regression, compared with the other treatment groups (Fig. 6E).

Discussion

Given the success in treating liver-associated disease by siRNA-based therapies, the exploration of nonliver therapeutic areas is of high interest. In this study, we report a novel strategy of delivering siRNA specific to NSCLC tumor,

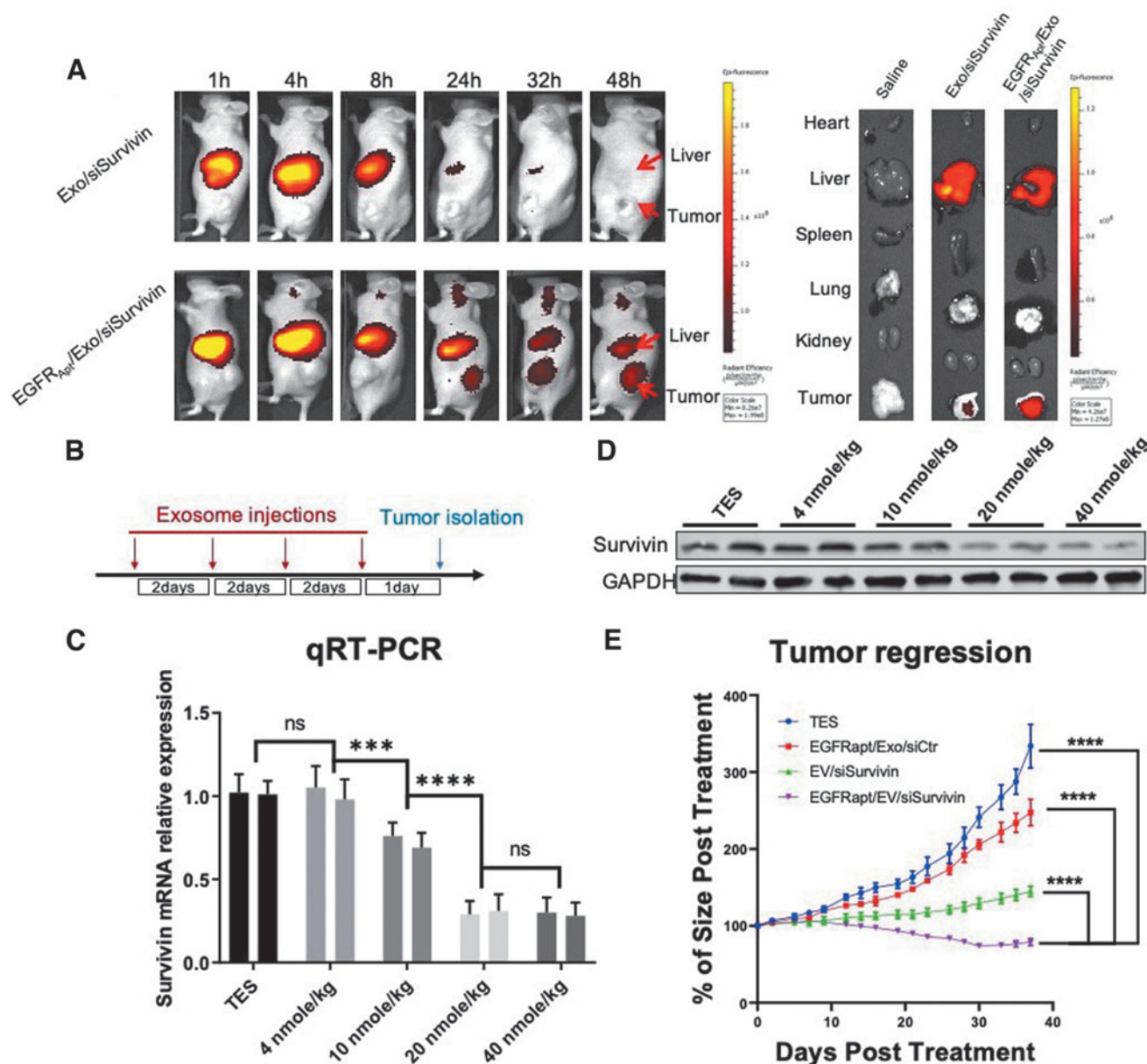


FIG. 6. *In vivo* evaluation of EGFR_{Apt}/Exo/siSurvivin on NSCLC tumor xenograft mice. (A) Biodistribution study by tracing Alexa₇₅₀-labeled siSurvivin delivered by exosomes. H596 NSCLC cell-developed tumor xenograft mice were I.V. administered with 100 μ L of 20 μ M of RNA Alexa₇₅₀-labeled siSurvivin loaded in exosomes or EGFR_{Apt}/Exo and imaging by IVIS post 1, 4, 8, 24, 32, and 48 h shown on the *left*. Organ was dissected at 48 h and imaged by IVIS shown on the *right*. (B) Scheme of exosome injection and tumor isolation time frame for dose optimization study. (C) mRNA level of survivin gene in tumor quantified by qRT-PCR assay post three repeated I.V. administration of EGFR_{Apt}/Exo/siSurvivin in 4, 10, 20, and 40 nmol/kg of siRNA concentration and TES buffer on H596 NSCLC cell-developed tumor xenograft mice, $n = 3$, error bar \pm SD. Protein level assayed by western blot shown in (D). (E) Tumor growth curve traced during six repeated I.V. administration of EGFR_{Apt}/Exo/siSurvivin, EGFR_{Apt}/Exo/siCtr, Exo/siSurvivin, and TES buffer in 40 nmol/kg of siRNA concentration on H596 NSCLC cell-developed tumor xenograft mice. *** $P < 0.001$, **** $P < 0.0001$, two-way ANOVA (mix model repeated measurement), $n = 5$, error bar \pm SEM. ANOVA, analysis of variance; I.V., intravenous; IVIS, in vivo imaging system; ns, no significance.

leveraging the advantage of exosomes and RNA nanotechnology, and demonstrating the antitumor effect on a xenograft mice model. With the engineerable arrow-tail RNA nanoparticle platform we developed [16,17,63], we expand the pipeline of ligand-displaying exosomes as vehicles for delivering anticancer siRNA on lung cancer treatment, meanwhile, demonstrating again the flexibility of this platform technology. Notably, with the combination of hollow-

fiber bioreactor, TFF system, and cushion ultracentrifugation, we increased the scale of exosome production over 80 times, demonstrating the efficiency of this technique (data not shown). This new exosome production workflow not only expands our bandwidth to pursue comprehensive studies in animals, but introducing these processes that are widely used in industrial manufacturing also support the potential for further scaling up for additional animal or human studies.

The display of EGFR_{Apt} ligands on exosomes shows obvious enhancement on cellular uptake, and gene knockdown in our *in vitro* experiments. It is also noticed that the HEK293T cell-derived exosomes had passive delivery capacity in both *in vitro* and *in vivo* settings, which is consistent with both of research previously published by this team, and exosome uptake published by peers [47,48,57,59,63,68–72]. Nevertheless, it is observed that more tumor accumulation and higher potency of EGFR_{Apt}/Exo/siSurvivin compared with nontargeting Exo/siSurvivin in the mouse model (Fig. 6A–E). The enhancement caused by EGFR targeting is favorable for consideration of dose reduction. Moreover, the *in vivo* gene knockdown assessment among different doses shown in Fig. 6C and D indicates that EGFR_{Apt}/Exo/siSurvivin at 20 nmol/kg already reaches the <50% knockdown efficacy, while no significant improvement was observed by increasing the dose to 40 nmol/kg. These results might explain the potency of nontargeting exosome groups and indicate the possibility for reducing dosing for future study.

We also reported the proof-of-concept study of combination therapy by cotreating NSCLC cells with EGFR_{Apt}/Exo/siSurvivin and first-line chemotherapy reagent cisplatin (Fig. 5). The results clearly showed the potential of this combination strategy for either reducing the doses of the toxic chemotherapy or enhancing the treatment of patients that have developed resistance to chemotherapy, both highly clinically relevant and translatable options. Furthermore, exosomes had been reported to improve the delivery of chemotherapy reagents like Taxol [73,74], which raises the possibility of coencapsulating the siRNA and cisplatin into our EGFR_{Apt}/Exo system. Taken together, these results demonstrate continued advances in combining RNA nanotechnology with the exosome delivery platform for enhanced targeting of cancer therapy.

Data and Materials Availability

All data generated or analyzed during this study can be found in the article or supplementary materials.

Author Disclosure Statement

P.G. is the consultant of Oxford Nanopore Technologies; the cofounder of Shenzhen P&Z Biomedical Co. Ltd., as well as the cofounder of ExonanoRNA, LLC and its subsidiary Weina Biomedical (Guangdong), Ltd.

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2

Supplementary Figure S3
Supplementary Figure S4
Supplementary Table S1

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