Cancer Letters 449 (2019) 186–195

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Milk exosomes - Natural nanoparticles for siRNA delivery

Farrukh Aqil[a,b], Radha Munagala[a,b,1], Jeyaprakash Jeyabalan[a,1], Ashish K. Agrawal[a,2], Al-Hassan Kyakulaga[d], Sarah A. Wilcher[c], Ramesh C. Gupta[a,d,*]

[a] James Graham Brown Cancer Center, University of Louisville, Louisville, KY, 40202, USA
[b] Department of Medicine, University of Louisville, Louisville, KY, 40202, USA
[c] Research Resources Center, University of Louisville, Louisville, KY, 40202, USA
[d] Department of Pharmacology & Toxicology, University of Louisville, Louisville, KY, 40202, USA

ARTICLE INFO

Keywords:
Milk exosomes
siRNA
Cancer
Gene silencing
Nanoparticles

ABSTRACT

Gene-silencing with targeted siRNAs has great potential as a therapeutic approach for various diseases including cancer. However, intracellular delivery of siRNA is challenging. We used bovine milk exosomes as a novel system for siRNA delivery. First, we demonstrated that exosomes can deliver endogenous RNA payloads into recipient cells. Next, we loaded siRNA against specific genes including *VEGF*, *EGFR*, *AKT*, *MAPK*, and *KRAS*. We utilized 5′$^{-32}$P-labeled si*KRAS* as a tracer and found exosome loading with siRNA could be variable. We demonstrated that the siRNA of loaded exosomes is stable and resist degradation. Our results indicated that siRNAs against target genes ranged from 2 to 10-fold knockdown in expression levels in various cancers. Since mutated *KRAS* has been implicated in the development of various cancers including lung cancer, we tested a mutant-allele specific siRNA against *KRAS*^{G12S}, in A549 cells. We observed a dose-dependent anti-proliferative activity against A549 cells treated with exosomes carrying si*KRAS*^{G12S}. We observed significant inhibition of A549 tumor xenografts in animals treated with folic acid-functionalized exosomes carrying si*KRAS*. In summary, milk-derived exosomes represent a viable natural nano-carrier for the delivery of siRNA for therapeutic application against cancer.

1. Introduction

Exosomes are naturally occurring, nano-sized (30–100 nm) extracellular vesicles composed of lipids, proteins and nucleic acids. Exosomes play an important role as mediators in cell-to-cell communication by trafficking these macromolecules [1]. Exosomes are released from most cell cultures, and from tissues as well as other biological fluids including blood, milk, urine, tears and cerebrospinal fluid. Owing to their unique size as well as structural and functional properties, exosomes have emerged as a novel delivery system for biological therapeutics including siRNAs. The ability of exosomes to transfer mRNA and miRNA between cells and subsequently to mediate changes in the expression of target genes in the recipient cells, together with their high abundance in most body fluids, highlights their potential as delivery vehicles for exogenous small interfering RNAs (RNAi or siRNA). Accumulating evidence indicates that an exosomal drug delivery system is feasible, safe, and may be efficacious compared to traditional (synthetic) nanoparticles used.

siRNAs are emerging as a new generation of therapeutics. With increasing knowledge of the molecular mechanisms of endogenous RNA interference, siRNAs, is evolving as innovative nucleic acid medicines for treatment of various diseases including cancer [2]. Despite their great therapeutic potential, successful implementation of siRNAs *in vivo* is hampered by the lack of effective delivery into cells. Chemical modifications to siRNA to enhance cellular delivery have been attempted but some have resulted in undesirable outcomes such as loss of biological activity [3,4], and toxicity [5].

Numerous nanoparticle delivery systems have been reported to target siRNA, including polymeric nanoparticles, lipids and liposomes, peptides and synthetic nanocarriers; liposome delivery system has advanced the most [6–8]. Despite great promise, these approaches must overcome challenges including immune response, non-specific targeting, and particle size control. Thus, there is a need to find efficient and biocompatible vectors that can overcome the hurdles of targeted siRNA delivery.

Exosomes have many of the desirable features of an “ideal” drug

* Corresponding author. Room 304E, University of Louisville, 580 S. Preston Street, Louisville, KY, 40202, USA.
E-mail address: rcgupta@louisville.edu (R.C. Gupta).
[1] Current affiliation – 3P Biotechnologies, Inc., 320 Delia Baxter II, 580 S. Preston Street, Louisville, KY 40202.
[2] Current affiliation – Department of Pharmaceutical Engineering and Technology, Indian Institute of Technology (BHU), Banaras, India.

https://doi.org/10.1016/j.canlet.2019.02.011
Received 1 November 2018; Received in revised form 22 January 2019; Accepted 10 February 2019

delivery system, such as a long circulating half-life, an intrinsic ability to target tissues, biocompatibility, and minimal or no inherent toxicity issues [9] and thus appear to be a superior choice, overcoming the limitations observed with the majority of liposomal or polymeric drug delivery systems [10,11]. As an alternative strategy, exosomes derived from cell culture have been explored for delivery of siRNA. Several studies have indicated cell-derived exosomes to be capable of efficient delivery of exogenous RNAi cargoes to targeted tissues compared to synthetic nanoparticles [12,13]. Due to their unique composition, exosomes are speculated to enter the cell and deliver the payload. Recent evidence suggests enhanced retention of fibroblast-like mesenchymal cell derived exosomes, compared to liposomes, in the circulation of mice due to CD47-mediated protection ('don't eat me' signal) [14] of exosomes against phagocytosis by monocytes and macrophage [12].

Until now, numerous cell types have been used to produce exosomes, these include model cell lines such as HeLa and HEK-293 [15,16] and different types of murine melanoma cell lines [17,18]. Immature dendritic cells have favorable properties with respect to immunogenicity due to their special surface-protein composition, making them relevant exosome donor cells [19]. However, the workload required to scale-up the production of these cells combined with low exosome yield make them a limited system for clinical translatability [20]. Mesenchymal stem cells (MSCs) represent better exosome donor cells [21,22] as they produce larger amounts of exosomes and thus are suitable for clinical translatability [23,24]. However, as the cancer-stimulating properties of MSC exosomes and microvesicles are disputed, care needs to be exercised for using these cells as exosome donors in cancer treatment [25].

In view of the current limitations of cell-derived exosomes and scalability issues for clinical applications, we recognized bovine milk as a viable source for exosomes that could serve as a potential drug delivery platform. Our recent pioneering work indicates that bovine milk exosomes provide a viable alternate with high impact because of cost effectiveness, biocompatibility, capacity of exosomes to provide stability to the drugs, tumor targeting characteristics and lack of toxicity. Due to their known stability in acidic environment [26,27], milk exosomes may provide the added benefit as a desirable oral drug delivery carrier, with wide therapeutic applications. We have demonstrated the uptake, biodistribution and effective delivery of various small molecules using milk-derived exosomes.

In this study, we investigated the potential of milk exosomes for the delivery of siRNA. We demonstrated that milk exosomes can be loaded with siRNAs by electroporation and chemical transfection (Exo-Fect) methods. Our findings show that siRNA delivered via milk exosomes is capable of silencing key oncogenes in multiple cancers *in vitro*. We also demonstrate anti-tumor activity of siRNA-loaded exosomes against lung tumor xenograft in nude mouse model when administered intravenously.

2. Materials and methods

2.1. Cell lines

All cells were cultured in their respective media described below in an atmosphere of 95% humidity and 5% CO_2 at 37 °C except for MDA-MB-231, which was grown in the absence of CO_2. Human lung cancer (A549 and H1299), breast cancer (MDA-MB-231 and MCF7), pancreatic (Panc1 and Mia PaCa2), and ovarian (A2780) cancer cell lines were obtained from American Type Cell Culture (ATCC, Manassas, VA) or provided by colleagues at the University of Louisville, James Graham Brown Cancer Center, Louisville, KY. All cancer cell lines were grown in the media supplemented with fetal bovine serum (10%) and antibiotics (1%, penicillin/streptomycin). The A549 cells were grown in F-12K media. The MCF7, Panc1 and MiaPaCa2 received Dulbecco's Modified Eagle's Medium (DMEM). Media for MCF7 and ovarian cancer cells were also supplemented with 0.2 unit/ml insulin. MDA-MB-231 cells were grown in L-15 media. The H1299 and A2780 received RPMI media.

2.2. Isolation of exosomes

Exosomes were isolated by differential centrifugation of raw milk pooled from pasture-raised cow during the mid-lactation period as described [28,29]. Briefly, milk was centrifuged at 13,000 × *g* for 30 min and then centrifuged at 100,000 × *g* for 60 min. The upper two thirds portion of the supernatant was collected and centrifuged at 135,000 × *g* for 90 min to obtain exosomal pellet. The exosome pellet was washed with PBS two times and suspended in PBS. Exosome suspension was stored in aliquots at ≤ 6 mg exosomal proteins/ml at − 80 °C until used.

2.3. Loading of exosomes with siRNA

Electroporation: Exosomes were loaded with siRNA by electroporation using the Gene Pulser Xcell (Bio-Rad, Hercules, CA) electroporation system. Exosomes were diluted to 0.5 mg/ml in electroporation buffer (Bio-Rad, Hercules, CA). siRNA against AKT or fluorescently-labeled control siRNA (Block-iT; Life Technologies, Grand Island, NY) were added at a final concentration of 100 nM. The mixtures were transferred into ice-cold 0.4-cm cuvettes and electroporated at 400 V and 125 μF capacitance for a pulse time of 10–15 ms. Samples were kept on ice for at least 10 min pre- and post-electroporation pulse. Post electroporation, samples were centrifuged at 7000 × *g* for 5 min to pellet aggregates. siRNA without exosomes was also electroporated to serve as control.

Exo-Fect (chemical transfection): Exosomes were loaded with siRNA using a chemical transfecting reagent, Exo-Fect (System Biosciences, Palo Alto, CA, USA). Briefly, exosomes (150–300 μg in 50 μL) were incubated with Exo-Fect and siRNA (50–300 pmol) at 37 °C for 10 min. The exosomes were then collected by incubating with ExoQuick-TC reagent (System Biosciences, Palo Alto, CA) on ice for 30 min followed by centrifugation at 13,000 × *g* for 5 min. Exosome pellets were washed with PBS and re-suspended in PBS and then used directly for transfection studies.

2.4. Quantifying siRNA loading in exosomal formulation

Labeling of siRNA: si*KRAS* was 5′−^{32}P labeled by T4-polynucleotide kinase and [γ-^{32}P] ATP (> 6,000 Ci/mmol). Unused ATP was removed using illustra ProbeQuant G-50 Micro Column (GE Healthcare), and the labeled si*KRAS* was purified by small RNA isolation kit. The purity of the labelled si*KRAS* was determined by polyacrylamide gel electrophoresis with detection by Packard InstantImager.

Loading determination: To determine siRNA loading capacity, the ^{32}P-labeled si*KRAS* was used as tracer. The reaction conditions were as described above for Exo-Fect except that varying amounts of si*KRAS* were added along with fixed amount of the labeled si*KRAS*. After the reaction termination, the exosomes were collected by precipitating with ExoQuick-TC reagent (System Biosciences, Palo Alto, CA) on ice for 1 h followed by centrifugation at 13,000 × *g* for 5 min and the pellet was re-suspended in PBS. The supernatant was collected separately. Radioactivity in the pellet and supernatant was determined by applying a known volume to a PEI-cellulose layer and the radioactivity was detected and quantitated by Packard InstantImager. Percent siRNA loading was calculated by the following formula.

siRNA loading = (CPM in pellet/CPM in supernatant) *100

2.5. siRNA stability

5′−^{32}P-labeled siRNA loaded exosomes were incubated with varying concentrations of RNase A (0.01, 0.003, and 0.001 μg/mL).

After incubation at 37 °C for 45 min, heparin (10 μL of 10 mg/mL) was added to the reaction mixture to release the siRNA. Heparin was used to dissociate siRNA from the Exosomes. Heparin has ability to interact with proteins by mimicking the polyanionic structure of nucleic acid [30]. siRNA alone was used as control. The siRNA degradation products were resolved by polyacrylamide gel electrophoresis and detected by Packard InstantImager.

2.6. In vitro transfection

All *in vitro* transfection experiments with cancer cells were conducted with 60%–80% confluent cultures. Block-iT Alexa Fluor Red Fluorescent Oligo (Thermo Fisher Scientific, Waltham, MA) was used as transfection control. For siRNA loaded by electroporation, human lung cancer H1299 cells were seeded in 24-well plates at a density of 0.5×10^4 cells/well and treated for 72 h with 300 μg exosomes carrying the Block-iT Alexa Fluor Red Fluorescent Oligo.

Exo-siRNA formulation was prepared using exosomes (300 μg in 50 μL) and test siRNA (EGFR, VEGF, AKT, MAPK, BCL2 or survivin) (50–300 pmol) in presence Exo-Fect and incubated at 37 °C for 10 min. Additional reactions with siRNA and Exo-Fect, in absence of exosomes and siRNA and exosomes, in absence of the transfection reagent were also included. The reaction mixture was then incubated with ExoQuick-TC reagent (System Biosciences, Palo Alto, CA) on ice for 30 min, followed by centrifugation at 13,000 × *g* for 5 min. Pellet was re-suspended in PBS and used for transfection studies. The lung cancer H1299 and A549 cells were seeded in 6-well plates (BD Biosciences, San Jose, CA) at a density of 0.4×10^6 cells/well and transfected with 25 μL of Exo-siRNA for 24–48 h. The treated cells were analyzed by fluorescent microscope and western blot to determine the siRNA incorporation and functionality, respectively.

2.7. Confocal microscopy

Loading of Control siRNA Block-iT was achieved by electroporation or Exo-Fect was checked by confocal microscopy. For Exo-Fect method, Exo-siRNA formulation was prepared using exosomes (300 μg in 50 μL) and Texas red-labelled control siRNA (System Biosciences, Palo Alto, CA, USA) as described above. Human lung cancer H1299 and A549 cells were seeded into BD Falcon 8-well chamber culture slides (BD Biosciences, San Jose, CA). Cells were transfected with Texas red-labelled control siRNA and incubated for 8–12 h. For confocal microscopy, samples were fixed with 4% methanol-free paraformaldehyde for 10 min, permeabilized in 0.1% Triton-X 100 in PBS for 3 min. After fixation and permeabilization, cells were washed three times with PBS and visualized under the confocal microscopy. DNA was visualized through DAPI staining (Molecular Probes, Life Technologies, Grand Island, NY).

2.8. Western-blot analysis

Cell lysates were prepared using the RIPA buffer (Cell signaling Technology, Danvers, MA) and protein concentration was determined by BCA reagent. Western-blot analysis was performed as described [31]. Blots were probed for EGFR, VEGF, AKT, MAPK, BCL2 and survivin (Cell signaling Technology, Danvers, MA) and KRAS (Thermo Fisher Scientific, Waltham, MA) antibodies and their respective secondary antibodies (Cell Signaling Technology). β-actin (Sigma, St. Louis, MO) was used as loading control.

2.9. Cell uptake of the exosome internal nucleic acid materials

To confirm that milk exosomes were able to deliver the exogenously loaded siRNA to the cells, we first determined delivery of the endogenous payload of the exosomes by labeling the internal nucleic acids by the RNA and DNA labeling reagent ExoGlow (SBI, Palo Alto, CA). Briefly, 300 μg milk exosomes were mixed with the Exo-Red- and Exo-Green-labeling reagent. After incubation for 10 min, exosomes were collected using ExoQuick-TC reagent. The Exo pellet was washed with PBS and resuspended in PBS. Lung cancer H1299 and A549 cells were treated with the exosomes in 8-chambered slides. After overnight incubation, the slide was prepared for confocal microscopy as described above. The florescent signals from labelled exosomal RNAs were monitored using confocal microscopy.

2.10. Cell viability assay

Cells were plated in 96-well microtiter plates at an initial density of 3×10^3 cells per well and treated with exosomes, siRNA alone or exosomes loaded with survivin siRNA (si*SUR*) or si*KRAS* and incubated for an additional 72 h. Exosome concentration was maintained constant in all the treatments. Inhibition of the cell proliferation was determined by MTT assay as described previously [27].

2.11. Tumor targeting of exosomes

Folic acid (FA) has been used to functionalize nanoparticles for enhanced drug delivery to the tumor site. Our recent data have shown that milk exosomes loaded with FA by hydrophobic interactions can enhance therapeutic activity of small drug molecule co-loaded onto the exosomes. In this study we tested if covalently attached FA will also target the tumor site. We covalently attached FA and near infrared fluorescent Alexa Fluor 750 (AF750) to exosomes by using standard stable amide chemistry. Briefly, exosome (10 mg/ml) were mixed with 100 μl of $NaHCO_3$ buffer (1M, pH 8.4) followed by mixing with 100 μl of freshly prepared FA NHS (N-hydroxysuccinimidyl) ester (2.5–3 mg activated FA was dissolved in 0.1 ml of 0.1 N NaOH). AF750 NHS-ester was also attached similar to FA accept that it was dissolved in PBS. The mixture was incubated for 1 h at room temperature. The unbound FA or AF750 were removed by ultrafiltration using 300,000 MWCO filters (Sartorius, Goettingen, Germany). Female athymic nude (nu/nu) mice (5–6 weeks old) were purchased from Charles River, maintained according to the Institutional Animal Care and Use Committee (IACUC) guidelines. Lung tumor xenografts were produced by subcutaneously injecting human lung A549 cells (2.5×10^6) in serum-free media mixed with Matrigel matrix (Becton Dickinson, Bedford, MA) in the left flank of the mice. Animals were provided purified AIN-93M diet and water *ad libitum*. Once tumors grew to > 200 mm^3, animals were administered a single *i.v.* dose of AF750 alone, Exo-AF750, or FA-functionalized Exo-AF750. The dose of AF750 in each treatment was maintained at 20 μg per mouse. First, the animals were live imaged after 4, 12 and 24 h using Advanced Molecular Imager AMI 1000 'Advanced Molecular Imager (Spectral Instruments Imaging, LLC 420 N. Bonita Ave. Tucson, AZ 85745 USA) and then euthanized. Tumor tissues were collected at the time of euthanasia, and imaged *ex vivo*. The relative intensities were measured and compared with untreated animals.

2.12. Antitumor activity against lung cancer xenograft

Lung cancer A549 cells having $KRAS^{G12S}$ mutations were used for the xenograft assay. Female athymic nude (nu/nu) mice (5–6-week old), from Charles Rivers were maintained in accordance with the Institutional Animal Care and Use Committee guidelines. Lung Cancer $KRAS^{G12S}$ A549 cells (2.5×10^6) in serum-free media were mixed with Matrigel matrix (Becton Dickinson, Bedford, MA) and subcutaneously injected into the left flank of each mouse. Animals were provided purified AIN-93M diet and water *ad libitum*. Once average tumor size reached about 80 mm^3, mice were divided into 2 groups (n = 8–10: one group was treated *i.v.* with exosomes loaded with si*KRAS* twice a week. Based on the si*KRAS* loading, the total dose was 7 μg/mouse. Tumor size and animal weights were monitored weekly. After 8 weeks of treatment, the animals were euthanized by CO_2 asphyxiation and select

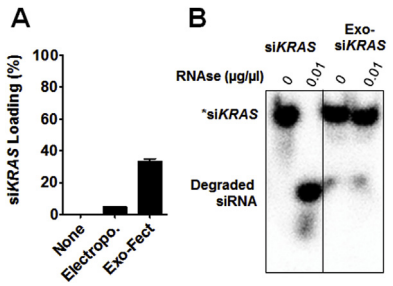

Fig. 1. Loading of siRNA in milk exosomes and its stability. 5′$^{-32}$P-labeled si*KRAS* prepared by T4 polynucleotide kinase-mediated phosphorylation of si*KRAS* using carrier-free [γ^{-32}P]ATP (> 6,000 Ci/mmol), followed by purification by RNA isolation kit. (A) Exosomes were incubated with siRNA in the presence of the chemical transfection reagent, Exo-Fect. The resulting exosomes loaded with the siRNA was harvested by precipitation with ExoQuick and applied on a PEI-cellulose thin layer. Radioactivity was measured by Packard InstantImager and the siRNA load was calculated. Data represent mean of 3 replicates ± SD. (B) Radiolabeled si*KRAS*, without and Exo-Fect-mediated exosome loading was treated with different concentrations of RNAse A and following the heparin treatment was resolved by polyacrylamide gel electrophoresis, and imaged with the Packard InstantImager.

tissues were collected.

2.13. Statistical analysis

All statistical analyses were performed with GraphPad Prism statistical software (version 4.03; La Jolla, CA). For cell proliferation assay, IC_{50} values were calculated. Differences between the means of the treatments were calculated for tumor volume and p-values were determined by Student's *t*-test. Differences were considered a priori to be statistically significant if the p value was < 0.05.

3. Results

3.1. siRNA loading in the exosomes and stability

To determine loading of exosomes with exogenous siRNA 5′$^{-32}$P-labeled si*KRAS* was used as a tracer. The loading was achieved by the chemical transfecting agent, Exo-Fect as well as by electroporation. Using 300 µg exosomes, Exo-Fect provided about 30% si*KRAS* loading; the loading was significantly lower (4–5%) by electroporation (Fig. 1A). The siRNA loading with Exo-Fect mediation increased when exosomes quantity was reduced; however, it was unchanged by electroporation.

To determine nuclease stability, the si*KRAS* with and without loading in exosomes were treated with increasing concentrations of RNase A. The hydrolysates were resolved on polyacrylamide gels and scanned using Packard InstantImager. Fig. 1B shows that the si*KRAS* was essentially undegraded under the experimental conditions when presented as loaded exosomes. However, the naked si*KRAS* was completely degraded.

3.2. Labeling endogenous nucleic acids of milk exosomes and cellular uptake

We first determined if the milk exosomes could deliver their endogenous payloads to cancer cells. The exosome endogenous payload of nucleic acid was labeled with ExoGreen (nucleic acid labeling dye) and precipitated with ExoQuick prior to transfecting lung cancer A549 and H1299 cells. Cells were visualized under confocal microscope after 24 h of incubation. As expected, there were no fluorescence signals from the cells treated with exosomes or ExoGreen alone. However, cells treated with the labelled exosomes showed strong green fluorescence, suggesting uptake of exosomes and delivery of its nucleic acid payloads to the recipient cells (Fig. 2A and B).

3.3. Uptake of siRNA-loaded milk exosomes

To examine the efficiency of Exo-Fect-mediated loading of exosomes, H1299 lung cancer cells were transfected with Texas-red labeled control siRNA-loaded exosomes, and cells were examined under fluorescent microscope. Data in Fig. 3 show strong signals when cells were treated with the Texas Red-labeled siRNA-loaded exosomes. Cells treated with the labeled siRNA and Exo-Fect, in absence of exosomes, did not show any signal suggesting the Exo-Fect mediation led to the siRNA loading in the exosomes. Similarly, treatment of cells with the labeled siRNA and exosomes, in absence of the transfection reagent, did not yield any signals, indicating inability of exosomes to entrap siRNA and presumable degradation of the free siRNA in the culture media.

Evidence of electroporation-mediated loading in the milk exosomes and its transfection efficiency was studied using the alexa fluor red fluorescent oligo control, Block-iT. We observed strong signals in the H1299 cell treated with the Block-iT-loaded exosomes (Fig. 4A). The transfection efficiency was confirmed by the silencing of *AKT* gene. Fig. 4B shows that si*AKT*-loaded exosomes significantly reduced the expression of AKT protein in H1299 cells. When cells were treated with si*AKT* and exosomes without the electroporation, AKT expression remained essentially unaltered (Fig. 4B).

3.4. Gene knock-down efficiency is siRNA concentration dependent

To study the effect of siRNA concentration on reducing expression of the target gene, we loaded 50–200 pmol of si*EGFR* into exosomes using the Exo-Fect and transfected the lung cancer H1299 cells for 24 h, and analyzed the protein lysates by western blot. The exosomes loaded with si*EGFR* resulted in a dose-dependent silencing of EGFR expression with the highest concentration (200 pmol) resulting in over 5-fold reduction of EGFR levels compared to vehicle treatment. Treatment of the cells with Exo-Fect and si*EGFR* or exosomes and si*EGFR* had no effect on the EGFR expression (Fig. 5A).

Next, we tested several siRNAs against genes such as *VEGF*, *AKT*, *MAPK*, *KRAS*, *SUR* and *BCL2* in multiple cancer cell types. Since the expression levels of these genes vary in different cancer types, we used several cancer cell lines to evaluate the gene silencing effects of these siRNAs.

The effect of si*VEGF*, si*AKT*, si*MAPK*, and si*KRAS* was studied in lung cancer cells; si*VEGF*, si*SUR* and si*BCL2* were investigated in breast cancer cells; si*VEGF* and si*MAPK* were investigated in pancreatic cancer cells; and si*VEGF* was tested in ovarian cancer cells. In all the cases, 100 pmol of siRNA was loaded into the exosomes. As shown in Fig. 5B the various Exo-siRNA formulations silenced the target genes from nearly 20% to as much as over 80%. si*AKT* and si*VEGF* knocked down the respective genes by 81% and 76%, respectively in lung cancer cells while si*SUR* was extremely effective in breast cancer cells (~80% silencing). si*VEGF* was also extremely effective in silencing the gene (75%) in ovarian cells.

3.5. Antiproliferative activity of Exo-siKRAS

We determined the antiproliferative activity of mutant-allele specific si$KRAS^{G12S}$ against A549 lung cancer. We also determined antiproliferative activity of this siRNA against the H1299 cells which lacks mutant-allele specific si$KRAS^{G12S}$. Exo-si*KRAS* showed a dose-dependent inhibition of the A549 cells (Fig. 6A). As expected, the H1299 cells remained unaffected with this treatment (Fig. 6B). It may be noted that some but statistically insignificant antiproliferative effect of the vehicle treatment (both the exosomes and Exo-Fect) was observed in both the A549 and H1299 cells (Fig. 6A and B).

3.6. Biodistribution of siRNA-loaded exosomes

To determine if FA functionalization of exosomes can target tumor,

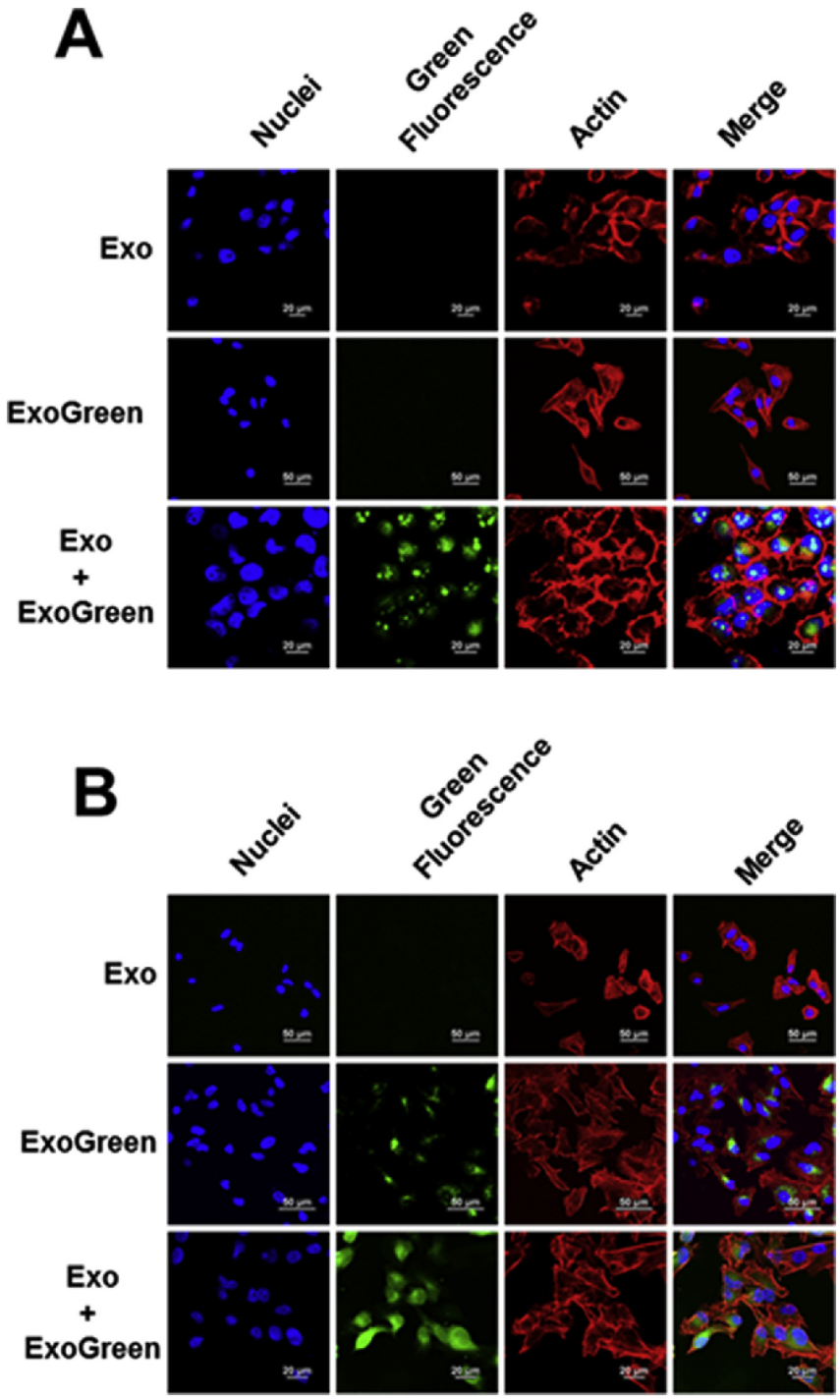

Fig. 2. Milk exosome tracking by labeling endogenous exosomal RNA. Exosomal RNA was labelled with RNA labelling dye, ExoGreen. Labelled exosomes were collected by precipitation with ExoQuick and applied on the lung cancer H1299 (A) and A549 cells (B). After 24 h incubation cells were visualized by confocal microscope. Nuclei of cells were stained with DAPI while actin was labelled with phalloidin.

we established biodistribution studies in lung subcutaneous tumor xenograft-bearing mice. Live animal imaging by AMI1000 showed significantly higher fluorescence intensity with the Exo formulations compared with the dye alone administered *i.v.*; the fluorescent signals were detectable a few minutes after the treatment and peaked between 4 and 8 h and then which considerably decreased after 24 h (Supplementary Fig. S1). E*x vivo* imaging of tumor tissues after 24 h of treatment showed higher fluorescence signal with the Exo formulation of AF750 (ExoAF750) compared to AF750 alone. The AF750 accumulation was significantly ($p = 0.007$) higher in exosomes functionalized with FA suggesting higher uptake of exosomes to the tumor tissue (Fig. 7).

3.7. Antitumor activity of siKRAS against lung tumor xenograft

We examined the antitumor activity of mutant-allele specific siRNA against $KRAS^{G12S}$ on A549 xenograft tumor growth in nude mice. Since FA functionalization provided higher accumulation of exosomes in tumor tissues, we loaded siRNA in the exosomes functionalized by FA attachment. As shown in Fig. 8, si$KRAS^{G12S}$ significantly inhibited the

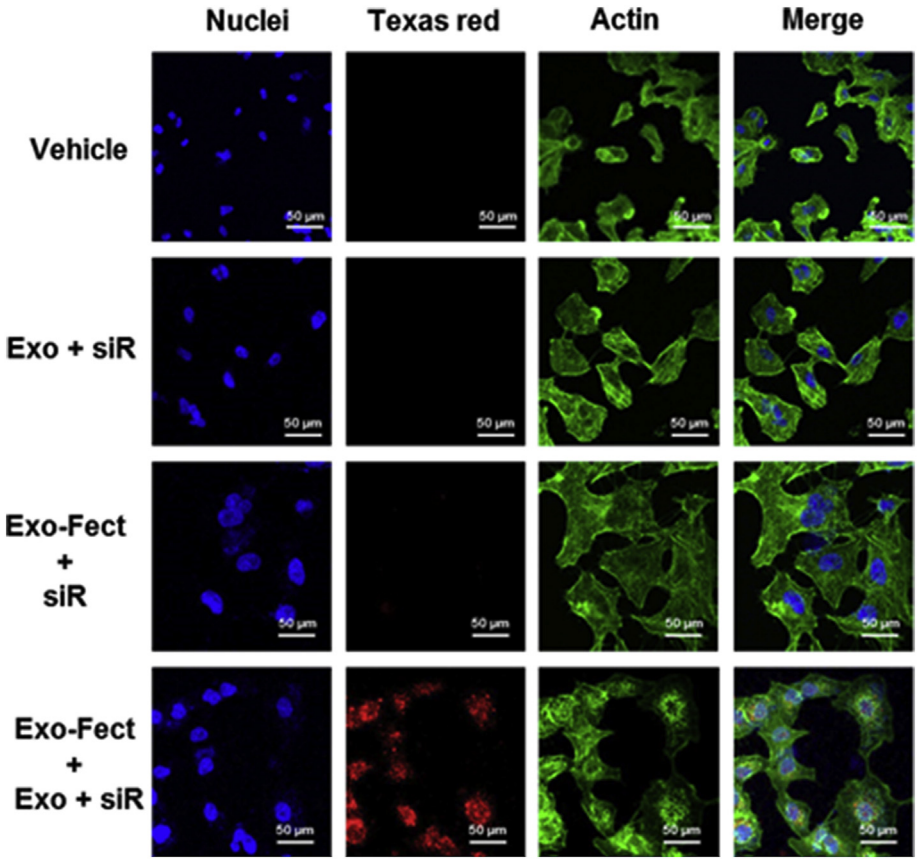

Fig. 3. Loading of Texas red-labelled siRNA into milk exosomes using Exo-Fect. Control siRNA (Texas-red fluorescent) was loaded into milk exosomes using Exo-Fect. The siRNA-loaded exosomes were collected by precipitation with ExoQuick and applied on the H1299 cells. Nuclei of cells were stained with DAPI while actin was labelled with phalloidin. After 24 h incubation cells were visualized by confocal microscope (scale 20 μm).

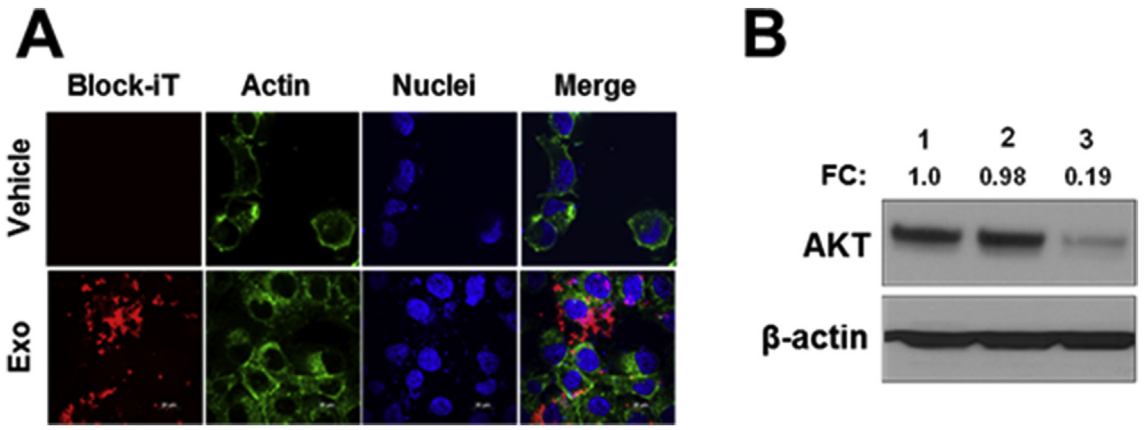

Fig. 4. siRNA loading of milk exosomes by electroporation and gene silencing. (**A**) siRNA (Block-iT red fluorescence) was loaded into milk exosomes by electroporation and applied on the H1299 cells. After 24 h incubation cells were visualized by confocal microscope (scale 20 μm). (**B**) siRNA against *AKT* was loaded into milk exosomes and H1299 cells were treated for 24 h. Cell lysates were analyzed by western blot. **1**, untreated control; **2**, si*AKT* + Exo no electroporation; and **3**, si*AKT* + Exo electroporation. FC = fold change.

tumor growth. The inhibitory effect of si*KRAS* was evident as early as two weeks, however, the anti-tumor effect was significant starting from the fifth week onward. It is important to note that only a modest dose of siRNA (7 μg/mouse) was administered *i.v.* two times a week. Since we have shown in our previous study a modest but insignificant effect of the exosomes alone, this group was not included in the study. The mean tumor volume at the end of the study in siRNA-treated mice was $327 \pm 181\ mm^3$ as compared to $709 \pm 409\ mm^3$ in the control group (a reduction of 54%; $p < 0.03$).

3.8. Toxicity study

Serum samples for the nude mice study was tested for any systemic toxicity due to si*KRAS*-loaded exosomes. No signs of gross toxicity were observed in Exo-si*KRAS*-treated mice based on gross visual pathology of internal organs and body weight gains between the treated and untreated groups. Systemic toxicological effects were determined by hematological and serum biochemical parameters of liver and kidney functions (Fig. 9). si*KRAS*-loaded exosomes did not show any significant effects on hematological parameters and liver and kidney functions and leukocytes, except neutrophils which were reduced. A significant decrease in the GGT level was also observed in the treatment

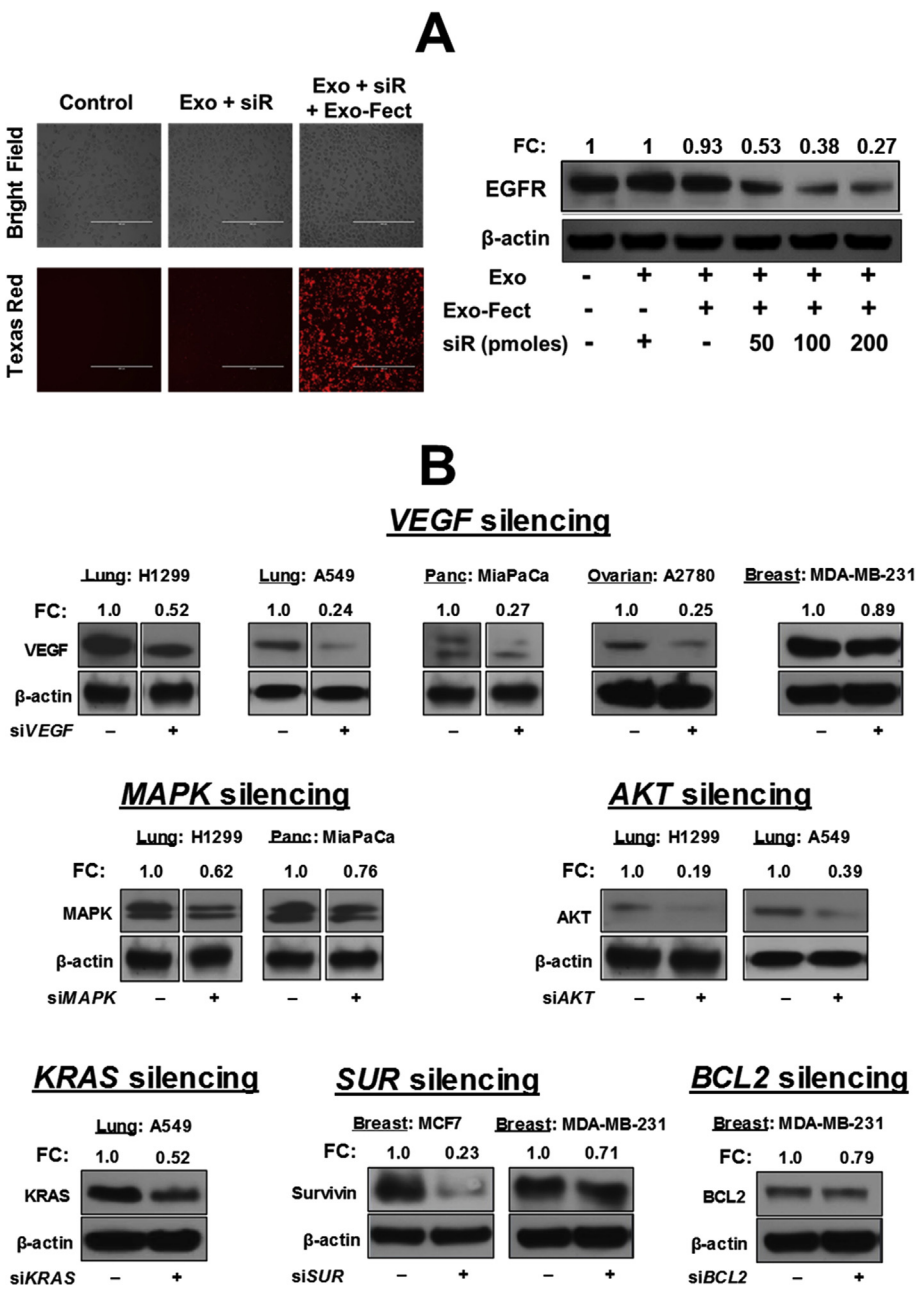

Fig. 5. siRNA loading of milk exosomes by Exo-Fect and gene silencing. (A) siRNA (Texas red fluorescent control) was loaded in milk exosomes by Exo-Fect and applied on the lung cancer H1299 cells. After 24 h incubation cells were visualized by confocal microscope (scale 20 μm). For functionality, different concentrations (0–200 pmol) of si*EGFR* were loaded in milk exosomes using Exo-Fect, the loaded exosomes were collected by ExoQuick and then applied on the H1299 cells for 24 h. Cell lysates were analyzed by western blot. Equal loading was confirmed by β-actin. **(B)** siRNAs against *VEGF*, *MAPK*, *AKT*, *KRAS*, *SUR* and *BCL2* were loaded into milk exosomes by Exo-Fect mediation, followed by precipitation with ExoQuick reagent. Lung cancer (H1299 and A549), ovarian cancer (A2780), pancreatic cancer (MiaPaCa) and breast cancer (MCF-7 and MDA-MB-231) cells were treated with indicated siRNA-loaded exosomes for 24 h. Cell lysates were analyzed by western blot; β-actin was used as loading control. FC = fold change.

group. These reductions in the neutrophils and GGT might be due to various factors including traces of the transfection reagent (Exo-Fect) and/or the precipitating agent (ExoQuick) as no such effects by treatment with milk exosomes have been observed in our previous studies [28]; though the neutrophil levels in the treatment group are well within the normal physiological range. Further toxicity studies with wild type mice are needed to fully understand the effects on these markers in relation to the use of Exo-Fect and ExoQuick reagents.

4. Discussion

The application of RNA interference technology, such as siRNA, to knockdown gene expression has been of great interest as a therapeutic strategy for cancer and other diseases. Intracellular delivery of siRNA is a challenging task, given that naked siRNAs are rapidly degraded in circulation, and their size and negative charge limit membrane passage and cellular uptake. Chemical modifications and carrier development for siRNA delivery has been attempted to address the siRNA delivery issue. Some siRNA sequence motifs elicit undesired immune responses, and targeting to specific tissues and cells is required to reduce adverse effects caused by off-target silencing [32]. Two major classes of delivery vehicles have been employed in delivery of siRNA - viral and non-viral such as polymeric nanoparticles and lipid and liposomes. These delivery systems have shown some successes, especially in inducing RNAi in the liver. At the same time, there have been some major issues such as viruses can be cleared in the bloodstream by pre-existing antibodies and polymer-based vectors accumulate in the liver, lung, spleen and kidney etc. Lipids are currently the preferred approach for siRNA delivery, and efficient delivery has been achieved particularly to the liver. Nonetheless, liver toxicity remains a serious concern. Encapsulation of siRNA in biological nanoparticles, e.g., exosomes, is being explored as a promising novel strategy to overcome most of these limitations. Exosomes have many of the desirable features of an “ideal” drug delivery system, such as a long circulating half-life, the intrinsic ability to target tissues, biocompatibility, and minimal or no inherent toxicity issues [9] and appear to be a superior choice, overcoming the limitations observed

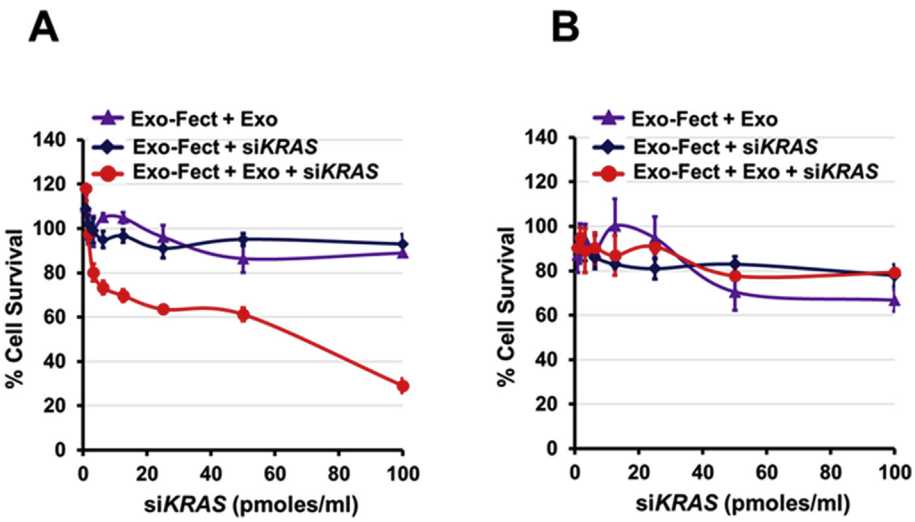

Fig. 6. Antiproliferative activity of si*KRAS* against lung cancer cells. siRNAs against *KRAS* (200 pmol) was loaded into milk exosomes using Exo-Fect reagent, and the loaded exosomes were collected by ExoQuick precipitation. Lung cancer A549 cells (*KRAS*^{G12S} mutant) and H1299 (*KRAS* wild type) were treated with different concentrations of si*KRAS*-loaded exosomes. Antiproliferative activity was determined by MTT assay after 72 h. Exosomes without siRNA were used as negative control.

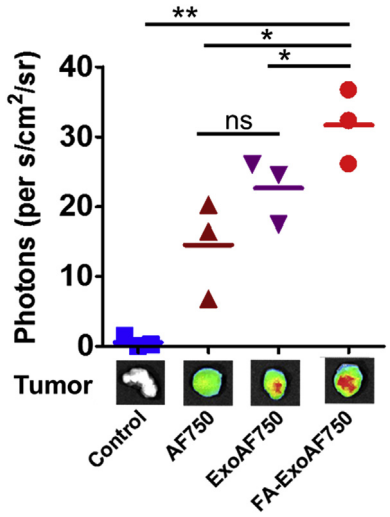

Fig. 7. Tumor targeting of exosomes. Lung subcutaneous tumor xenograft-bearing female nude mice (n = 3) were administered *i.v.* either AF750 dye alone, the dye covalently linked to the exosomes, or folic acid (FA)-functionalized exosomes, and animals were euthanized after 24 h and the tumor imaged *ex vivo*.

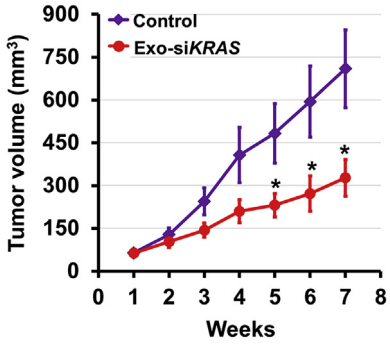

Fig. 8. Inhibition of lung A549 tumor xenograft by Exo-si*KRAS*. The A549 cells (2.5×10^6) were injected subcutaneously in athymic nude mice (n = 8–10). Exosomal formulation of si*KRAS* (7 μg/mouse) or vehicle was given *i.v.* two times a week and tumor volume measured once a week. Data represent mean ± SE; (*p < 0.05, *t*-test).

with the earlier approaches.

In exosomal delivery, several researchers have employed physical (electroporation [30]) and chemical (lipofectamine and Exo-Fect [27]) transfection strategies to allow efficient loading of siRNA in cell culture-derived exosomes. Here, we demonstrate successful loading of siRNA into milk exosomes by electroporation and chemical transfection (Exo-Fect) methods. However, chemical transfection achieved several fold higher loading of siRNA into milk exosomes compared to electroporation method.

We employed a rapid assay for determining siRNA loading and stability in exosomes using 5′$^{-32}$P-siRNA as tracer. Previous methods to determine siRNA loading involve isolation of siRNA and analysis by PCR, which are time consuming and due to low levels of RNA, have produced large errors. In a recent paper, extracellular vesicles isolated from embryonic kidney HEK293T cells were shown to deliver the siRNA and the loading was achieved similarly using Exo-Fect [33].

Since exosomes are naturally trafficking and play important roles in carrying payloads including RNA between cells, an interesting approach for the delivery of siRNA was tested by manipulating cells to secrete the exosomes containing sequence of interest. In one of such reports, hepatic cells transduced with lentiviral vectors were shown to secrete exosomes expressing siRNA and mediated an RNAi response in recipient cells [34]. Evidence of RNAi transmission *in vivo* was further observed in NOD-SCID mice engrafted with human hepatoma cells producing siRNA targeting CD81, causing suppression of CD81 expression in mouse hepatocytes. These studies collectively show the enormous potential these biological nanoparticles hold for therapeutic RNAi intervention, either used *per se* or when produced from cells in a cell therapy context (Reviewed in Ref. [35]). In spite of promise of these approaches, cell culture-derived exosomes face several challenges, including problems in the large-scale production, high costs and potential toxicity of particles.

We have shown here that bovine milk exosomes, which represent biocompatible and scalable nanoparticles [27,28,36], can deliver exogenous nucleic acids to recipient cells and could elicit target gene silencing in cell culture studies and antitumor activity against lung tumor xenograft, in addition to delivery of the endogenous payload of nucleic acids. In our previous studies with small drug molecules administered orally using milk-derived exosomes, we have shown the higher efficacy in term of tumor growth inhibition against lung and ovarian tumor xenografts, suggesting higher drug concentrations at the tumor site. Further, in our previous study, we reported a modest but insignificant effect of the exosomes alone [28] and lack of systemic and immunotoxicity of bovine milk exosomes in wild-type mice [37]. We [27] and others [38] have shown that exosomes could survive the harsh

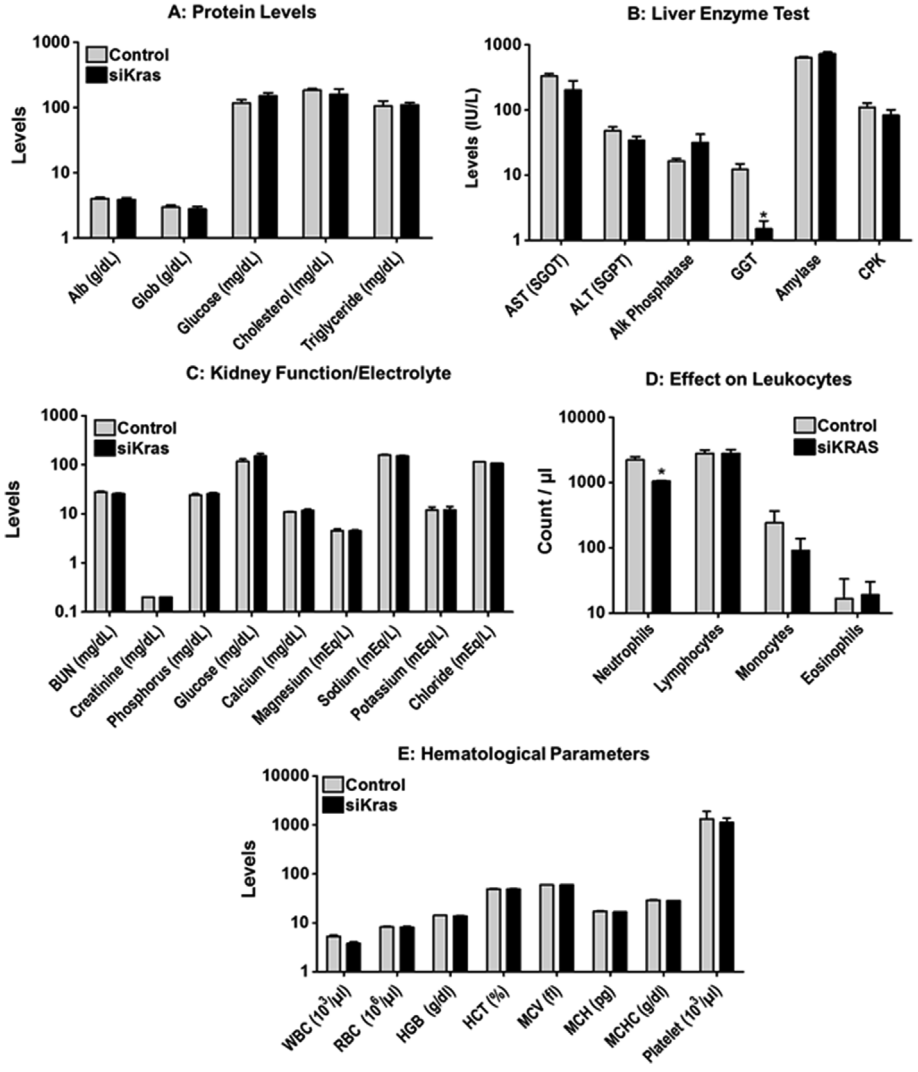

Fig. 9. Systemic toxicity due to Exo-si*KRAS*. Female athymic nude mice bearing subcutaneous lung tumor xenograft were treated with either vehicle or Exo-si*KRAS* (7 µg/dose) *i.v.* twice weekly for 7 weeks. At euthanasia blood was collected and analyzed using an automated AU640® Chemistry Analyzer by Antech diagnostics. Data represent average ± SD of 4–5 animals. Statistical analysis was performed by student t-test.

conditions and are stable at low pH of gastric conditions. Only a handful of previous studies have utilized exosomes for delivery of macromolecules. Alvarez-Erviti et al. [13] showed the delivery of siRNA using exosomes from immature murine dendritic cells by electroporation. They demonstrate *in vitro* and *in vivo* delivery into the circulation that deliver siRNA to neurons, microglia and oligodendrocytes in the mouse brain. They further demonstrate the BACE1 inhibition by siRNA delivery leading to a significant decrease in brain β-amyloid levels of wild-type mice. Recently, Kamerkar et al. [39] exhaustively studied exosomes derived from normal fibroblast-like mesenchymal cells to deliver si*KRAS*. They demonstrate that the treatment with siRNA-loaded exosomes suppressed cancer in multiple mouse models of pancreatic cancer and significantly increased overall survival. This study further indicated that CD47 marker on the exosomes enhances the retention of exosomes, compared to liposomes, in the circulation of mice, and is likely due to protection of exosomes from phagocytosis by monocytes and macrophages [39].

In view of our earlier encouraging findings with milk exosomes for delivery of various small drug molecules by eliciting higher tissue drug levels, uniform biodistribution, lack of immune response, enhanced anti-tumor efficacy and ability of achieving tumor targeting, we foresee a great potential of milk exosome utilization in gene delivery. Our continuing research in this area focus on optimization of siRNA loading, functionalization to achieve tumor targeting and perform anti-tumor efficacy studies following oral delivery.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgements

This research work was supported from Agnes Brown Duggan Endowment, Helmsley Trust funds, and James Graham Brown Cancer Center. RCG holds the Agnes Brown Duggan Chair of Oncological Research. Drs. Wendy Spencer and David Schultz are acknowledged for critical reading of the manuscript and useful edits.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.02.011.

References

[1] G. Raposo, W. Stoorvogel, Extracellular vesicles: exosomes, microvesicles, and

friends, J. Cell Biol. 200 (2013) 373–383.
[2] H. Dana, G.M. Chalbatani, H. Mahmoodzadeh, R. Karimloo, O. Rezaiean, A. Moradzadeh, N. Mehmandoost, F. Moazzen, A. Mazraeh, V. Marmari, M. Ebrahimi, M.M. Rashno, S.J. Abadi, E. Gharagouzlo, Molecular mechanisms and biological functions of siRNA, Int. J. Biomed. Sci. 13 (2017) 48–57.
[3] M.A. Behlke, Chemical modification of siRNAs for in vivo use, Oligonucleotides 18 (2008) 305–319.
[4] A.L. Jackson, J. Burchard, D. Leake, A. Reynolds, J. Schelter, J. Guo, J.M. Johnson, L. Lim, J. Karpilow, K. Nichols, W. Marshall, A. Khvorova, P.S. Linsley, Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing, RNA 12 (2006) 1197–1205.
[5] M. Amarzguioui, T. Holen, E. Babaie, H. Prydz, Tolerance for mutations and chemical modifications in a siRNA, Nucleic Acids Res. 31 (2003) 589–595.
[6] A. Schroeder, C.G. Levins, C. Cortez, R. Langer, D.G. Anderson, Lipid-based nanotherapeutics for siRNA delivery, J. Intern. Med. 267 (2010) 9–21.
[7] H. Xing, K. Hwang, Y. Lu, Recent developments of liposomes as nanocarriers for theranostic applications, Theranostics 6 (2016) 1336–1352.
[8] K. Tatpati, S. Sau, S. Kashaw, A. Iyer, siRNA delivery strategies: a comprehensive review of recent developments, Nanomaterials 7 (2017) 77.
[9] G. Turturici, R. Tinnirello, G. Sconzo, F. Geraci, Extracellular membrane vesicles as a mechanism of cell-to-cell communication: advantages and disadvantages, Am. J. Physiol. Cell Physiol. 306 (2014) C621–C633.
[10] S. Rani, T. Ritter, The exosome - a naturally secreted nanoparticle and its application to wound healing, Adv. Mater. 28 (2016) 5542–5552.
[11] D. Ha, N. Yang, V. Nadithe, Exosomes as therapeutic drug carriers and delivery vehicles across biological membranes: current perspectives and future challenges, Acta Pharm. Sin. B 6 (2016) 287–296.
[12] S. Kamerkar, V.S. LeBleu, H. Sugimoto, S. Yang, C.F. Ruivo, S.A. Melo, J.J. Lee, R. Kalluri, Exosomes Facilitate Therapeutic Targeting of Oncogenic KRAS in Pancreatic Cancer, Nature, advance online publication, 2017.
[13] L. Alvarez-Erviti, Y. Seow, H. Yin, C. Betts, S. Lakhal, M.J. Wood, Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes, Nat. Biotechnol. 29 (2011) 341–345.
[14] S.J. Gardai, K.A. McPhillips, S.C. Frasch, W.J. Janssen, A. Starefeldt, J.E. Murphy-Ullrich, D.L. Bratton, P.A. Oldenborg, M. Michalak, P.M. Henson, Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte, Cell 123 (2005) 321–334.
[15] S. Ohno, M. Takanashi, K. Sudo, S. Ueda, A. Ishikawa, N. Matsuyama, K. Fujita, T. Mizutani, T. Ohgi, T. Ochiya, N. Gotoh, M. Kuroda, Systemically injected exosomes targeted to EGFR deliver antitumor microRNA to breast cancer cells, Mol. Ther. 21 (2013) 185–191.
[16] A. Mizrak, M.F. Bolukbasi, G.B. Ozdener, G.J. Brenner, S. Madlener, E.P. Erkan, T. Strobel, X.O. Breakefield, O. Saydam, Genetically engineered microvesicles carrying suicide mRNA/protein inhibit schwannoma tumor growth, Mol. Ther. 21 (2013) 101–108.
[17] Y. Takahashi, M. Nishikawa, H. Shinotsuka, Y. Matsui, S. Ohara, T. Imai, Y. Takakura, Visualization and in vivo tracking of the exosomes of murine melanoma B16-BL6 cells in mice after intravenous injection, J. Biotechnol. 165 (2013) 77–84.
[18] Y.S. Lee, S.H. Kim, J.A. Cho, C.W. Kim, Introduction of the CIITA gene into tumor cells produces exosomes with enhanced anti-tumor effects, Exp. Mol. Med. 43 (2011) 281–290.
[19] W. Yin, S. Ouyang, Y. Li, B. Xiao, H. Yang, Immature dendritic cell-derived exosomes: a promise subcellular vaccine for autoimmunity, Inflammation 36 (2013) 232–240.
[20] Y.H. Tian, S.P. Li, J. Song, T.J. Ji, M.T. Zhu, G.J. Anderson, J.Y. Wei, G.J. Nie, A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy, Biomaterials 35 (2014) 2383–2390.
[21] J.L. Munoz, S.A. Bliss, S.J. Greco, S.H. Ramkissoon, K.L. Ligon, P. Rameshwar, Delivery of functional anti-mir-9 by mesenchymal stem cell-derived exosomes to glioblastoma multiforme cells conferred chemosensitivity, Mol. Ther. Nucleic Acids 2 (2013) e126.
[22] M. Katakowski, B. Buller, X. Zheng, Y. Lu, T. Rogers, O. Osobamiro, W. Shu, F. Jiang, M. Chopp, Exosomes from marrow stromal cells expressing miR-146b inhibit glioma growth, Cancer Lett. 335 (2013) 201–204.
[23] R.W. Yeo, R.C. Lai, B. Zhang, S.S. Tan, Y. Yin, B.J. Teh, S.K. Lim, Mesenchymal stem cell: an efficient mass producer of exosomes for drug delivery, Adv. Drug Deliv. Rev. 65 (2013) 336–341.
[24] T.S. Chen, F. Arslan, Y. Yin, S.S. Tan, R.C. Lai, A.B.H. Choo, J. Padmanabhan, C.N. Lee, D.P.V. de Kleijn, S.K. Lim, Enabling a robust scalable manufacturing process for therapeutic exosomes through oncogenic immortalization of human ESC-derived MSCs, J. Transl. Med. 9 (2011).
[25] W. Zhu, L. Huang, Y. Li, X. Zhang, J. Gu, Y. Yan, X. Xu, M. Wang, H. Qian, W. Xu, Exosomes derived from human bone marrow mesenchymal stem cells promote tumor growth in vivo, Cancer Lett. 315 (2012) 28–37.
[26] B.C. Melnik, S.M. John, G. Schmitz, Milk: an exosomal microRNA transmitter promoting thymic regulatory T cell maturation preventing the development of atopy? J. Transl. Med. 12 (2014) 43.
[27] F. Aqil, J. Jeyabalan, A.K. Agrawal, A.H. Kyakulaga, R. Munagala, L. Parker, R.C. Gupta, Exosomal delivery of berry anthocyanidins for the management of ovarian cancer, Food Funct. 8 (2017) 4100–4107.
[28] R. Munagala, F. Aqil, J. Jeyabalan, R.C. Gupta, Bovine milk-derived exosomes for drug delivery, Cancer Lett. 371 (2016) 48–61.
[29] F. Aqil, H. Kausar, A.K. Agrawal, J. Jeyabalan, A.-H. Kyakulaga, R. Munagala, R. Gupta, Exosomal formulation enhances therapeutic response of celastrol against lung cancer, Exp. Mol. Pathol. 101 (2016) 12–21.
[30] S. Mishra, A.R. Horswill, Heparin mimics extracellular DNA in binding to cell surface-localized proteins and promoting Staphylococcus aureus biofilm formation, mSphere (2017) 2.
[31] H. Kausar, J. Jeyabalan, F. Aqil, D. Chabba, J. Sidana, I.P. Singh, R.C. Gupta, Berry anthocyanidins synergistically suppress growth and invasive potential of human non-small-cell lung cancer cells, Cancer Lett. 325 (2012) 54–62.
[32] S. Akhtar, I.F. Benter, Nonviral delivery of synthetic siRNAs in vivo, J. Clin. Invest. 117 (2007) 3623–3632.
[33] F. Pi, D.W. Binzel, T.J. Lee, Z. Li, M. Sun, P. Rychahou, H. Li, F. Haque, S. Wang, C.M. Croce, B. Guo, B.M. Evers, P. Guo, Nanoparticle orientation to control RNA loading and ligand display on extracellular vesicles for cancer regression, Nat. Nanotechnol. 13 (2018) 82–89.
[34] Q. Pan, V. Ramakrishnaiah, S. Henry, S. Fouraschen, P.E. de Ruiter, J. Kwekkeboom, H.W. Tilanus, H.L. Janssen, L.J. van der Laan, Hepatic cell-to-cell transmission of small silencing RNA can extend the therapeutic reach of RNA interference (RNAi), Gut 61 (2012) 1330–1339.
[35] S. El Andaloussi, S. Lakhal, I. Mager, M.J. Wood, Exosomes for targeted siRNA delivery across biological barriers, Adv. Drug Deliv. Rev. 65 (2013) 391–397.
[36] F. Aqil, R. Munagala, J. Jeyabalan, A.K. Agrawal, R. Gupta, Exosomes for the enhanced tissue bioavailability and efficacy of curcumin, AAPS J. 19 (2017) 1691–1702.
[37] A.K. Agrawal, F. Aqil, J. Jeyabalan, W.A. Spencer, J. Beck, B.W. Gachuki, S.S. Alhakeem, K. Oben, R. Munagala, S. Bondada, R.C. Gupta, Milk-derived exosomes for oral delivery of paclitaxel, Nanomedicine 13 (2017) 1627–1636.
[38] D. Sun, X. Zhuang, X. Xiang, Y. Liu, S. Zhang, C. Liu, S. Barnes, W. Grizzle, D. Miller, H.G. Zhang, A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes, Mol. Ther. 18 (2010) 1606–1614.
[39] S. Kamerkar, V.S. LeBleu, H. Sugimoto, S. Yang, C.F. Ruivo, S.A. Melo, J.J. Lee, R. Kalluri, Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer, Nature 546 (2017) 498–503.