Functional exosome-mimic for delivery of siRNA to cancer: *in vitro* and
in vivo evaluation

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Abstract

Exosomes, the smallest subgroup of extracellular vesicles, have been recognized as extracellular organelles that contain genetic and proteomic information for long distance intercellular communication. Exosome-based drug delivery is currently a subject of intensive research. Here, we report a novel strategy to produce nanoscale exosome-mimics (EMs) in sufficient quantity for gene delivery in cancer both *in vitro* and *in vivo*. Size-controllable EMs were generated at a high yield by serial extrusion of non-tumorigenic epithelial MCF-10A cells through filters with different pore sizes. siRNA was then encapsulated into the EMs by electroporation. Biosafety and uptake efficiency of the EMs were evaluated both *in vitro* and *in vivo*. The mechanism underlying their cellular endocytosis was also studied.

Keywords: Exosome, siRNA, electroporation, MCF-7, endocytosis

Introduction

Unrestrained cell growth and disturbed cell-cycle regulation are hallmarks of human cancers. Studies have revealed that cyclin-dependent kinases (CDKs), a group of serine/threonine kinases, are critical regulators that drive cell cycle transition.[1, 2] Among them, CDK4 plays a critical role in regulation of cell G1-S transition. Dysregulation of CDK4 has been reported in most cancer cells, including breast, lung, leukemia, and liver cancers.[3-5] Thus, targeting of the CDK4 pathway has been a major focus in the development of cancer therapies. RNA interference (RNAi)-based agents, including small interfering RNAs (siRNAs), microRNAs (miRs) and anti-microRNAs (anti-miRs), have been extensively exploited for selective gene regulation in different diseases.[6-10] Due to their limited stability in the biological environment, various nanotechnologies have been used to facilitate the delivery of nucleic acid drugs into the target cells. However, inefficient cellular uptake and undesired side effects greatly hinder their applications in clinic. Therefore, development of novel delivery systems for efficient and safe RNAi therapy is essential for future cancer treatment.

Exosomes, the smallest subgroup of extracellular vesicles, are natural nanoscale vesicles (40-150 nm) secreted constitutively by many cell types. Studies have shown that exosomes are present in different biofluids including blood, saliva, urine, and breast milk. They are rich in important biomolecules such as proteins (CD9, CD63, CD81), mRNAs, and miRNAs, thereby they play critical roles in many physiological and pathological processes, for example, cell proliferation, differentiation, tumorigenesis, T cell immune response, and virus spreading. Moreover, recent studies have shown that exosomes are intrinsically implicated in de novo transcriptional and translational processes in the recipient cells.[11-15] In this regard, exosomes have been recognized as extracellular organelles that contain genetic and proteomic information for long distance communication between cells.

Because of their nanoscale size, excellent biocompatibility, and specific expression of cell surface receptors, exosomes have been recognized as viable and natural vehicles for drug delivery applications. For instance, exosomes loaded with exogenous

miR-155 mimic or miR-155 inhibitor have shown to successfully regulate genes in recipient cells.[16] In contrast to the commonly-used synthetic vehicles, the high biocompatibility of exosomes makes them ideal for drug delivery without inducing the undesirable pro-inflammatory reactions and immune response. However, the limited quantity of exosomes with desirable RNA and/or protein content that can be secreted by mammalian cells is a major impediment for their applications in nanomedicine. Tumor cells can produce large quantities of exosomes. However, they have potential safety issues. Therefore, alternative methods to generate a large quantity of homogenous exosomes carrying specific cargos are of great interest.

In the present study, we adopted a novel strategy to produce nanoscaled EMs for gene delivery to cancer cells. Size-controllable EMs at a high yield were generated by serially extruding non-tumorigenic epithelial MCF-10A cells through filters with different pore sizes. siRNA was then loaded into the EMs by electroporation. These EMs were evaluated for safety and efficacy both *in vitro* and *in vivo*. The mechanism of their cellular endocytosis was also studied.

Materials and Methods

Materials

Dulbecco's Modified Eagle Medium (DMEM) media, fetal bovine serum (FBS), trypsin-EDTA, and penicillin-streptomycin were obtained from Invitrogen (Grand Island, NY, USA). CellTiter 96 AQueos One Solution Cell Proliferation (MTS) assay kit was purchased from Promega (Madison, WI, USA). Cy5-labeled control siRNA (Cy5-siRNA), FAM-labeled control siRNA (FAM-siRNA), CDK4 siRNA and the negative control siRNA were obtained from Applied Biosystems (Austin, TX, USA). Sucrose, filipin III and Ly29004 were purchased from Sigma Aldrich (St. Louis, MO, USA). All other reagents and chemicals were of analytical grade and used without further purification.

Cell Culture

MCF-10A and MCF-7 cells purchased from American Type Culture Collection (ATCC)(Manassas, VA) were grown in DMEM supplemented with 10%

heat-inactivated FBS (Invitrogen, Carlsbad, CA) and 1% antibiotic (Invitrogen, Carlsbad, CA). All cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.^[17]

Preparation and Purification of Exosomes.

MCF10A cells were cultured in FBS-free DMEM medium for 48 h. The supernatant was collected and then centrifuged at 1,500 x g for 5 min to remove cells and 10,000 x g for 20 min at 4°C to deplete residual debris. Afterward, the exosomes were pelleted by ultracentrifugation at 150,000 x g for 2 h at 4°C and resuspended in PBS. The protein concentration was measured on a NanoDrop spectrophotometer and adjusted to a concentration of 1 µg/µl with PBS for the further usage. Step gradient ultracentrifugation was used to purify the exosomes. Briefly, 50% iodixanol (iodixanol:PBS = 1:1) was placed at the bottom of the ultracentrifuge tube, and the same volume of 10% iodixanol was placed on top with the samples. The samples were centrifuged at 100,000 x g for 2 h at 4°C and the exosomes were obtained from the interface of the 50% and 10% iodixanol layers.

Preparation and Purification of EMs

MCF10A cells were washed twice with cold PBS and harvested by a cell scraper. The cell suspensions were sequentially extruded through 10 µm, 5 µm, and 1 µm polycarbonate membrane using a mini-extruder. The obtained EMs were further purified by gradient ultracentrifugation as described above. **Here, we defined the EMs as the nano-sized vesicles mainly composed of plasma membrane collected from the same layer as exosome by gradient ultracentrifugation, which have the similar size, zeta potential, the surface protein markers as exosomes.**

Particle Size and Zeta-Potential Measurements

The size distribution of exosomes and EMs was obtained by dynamic light scattering goniometry (Brookhaven Instruments Corp., Holtsville, NY). The wavelength of the laser was set at 632.8 nm and the detection angle at 90°. The measurement was taken at 20°C; the mean diameters from three independent experiments were reported in the form of mean ± standard deviation.

The zeta-potential of exosomes and EMs were measured using a ZetaPALS zeta

potential analyzer (Brookhaven Instruments Corp., Holtsville, NY). Samples were diluted in 20 mM HEPES buffer. Three measurements, each composed of 10 runs, were carried out at 20°C. The zeta potential values were obtained using the Smoluchowski model and presented as mean $\pm$ standard deviation (n=3).

Preparation of siRNA-loaded EMs

To standardize loading conditions of EMs to achieve satisfactory and repeatable outcome, the loading conditions by electroporation were optimized as follows. A Neon electroporation system was used. The EMs obtained were mixed with the Neon electroporation buffer at a 1:1 ratio. Cy5 labeled siRNA was added to the mixture at a final concentration of Cy5-siRNA:EM protein = 100 pmol : 1 μ g/ml. Electroporation was then performed at various voltages per manufacturer's instructions with the pulse width set at 10 ms. The effect of pulse time was also assessed at the optimized voltage. After electroporation, one unit of RNase A was added to the mixture to eliminate free siRNA outside the EMs. To reduce the undesirable electroporation-induced siRNA precipitation during the loading process, EDTA was added[18]. The encapsulation efficiency of siRNA in the EMs was determined by measuring siRNA by the RiboGreen assay (Invitrogen, Carlsbad, CA) [8] .

Plasma Stability Assay

The siRNA loaded exosomes or EMs were incubated in plasma at 37 °C, and various time points, the samples were collected and the siRNA content were measured by the RiboGreen assay described before[8].

Fluorescence Microscopy

The electroporation effect was evaluated by the co-localization of EMs and Cy5-siRNA using fluorescence microscopy. Briefly, 200 μ l Cy5-siRNA and EMs mixture after electroporation was mixed gently with 500 μ l Diluent C solution, and incubated on ice for 5 min. Meanwhile, 1 μ l PKH dye was diluted with 500 μ l Diluent C solution in another tube. The two solutions were mixed and incubated on ice for 5 min. 1% BSA solution was used to stop the staining. The Cy5-siRNA-loaded EMs were pelleted by ultracentrifugation and resuspended in 20 μ l PBS. They were then

visualized with a Nikon fluorescence microscope (Nikon Elipse Ti) [19] . The co-localization percentage was calculated by equation $\% = \text{Cy5 containing EMs} / \text{total EMs labeled by PKH dye}$. The siRNA loading efficiency was further calculated by method described before[8, 20].

Cytotoxicity Assay

MCF-7 cells were seeded at a density of 1×10^4 cells/well in a 96-well plate 24 h prior to transfection. Cells were washed three times with serum-free media and incubated with various concentrations of siRNA-containing EMs or exosomes. At 4 h post-transfection, the media was replaced with fresh cell culture media and the cells were incubated at 37°C for an additional 20 h. Cell viability was then analyzed by MTT assay per manufacturer's instructions. Briefly, 10 μ l of the MTT reagent (Roche, Germany) was added to each well. After incubation of the microplate in a humidified atmosphere (5% CO₂, 37°C) for 4 h, 100 μ l of the solubilization solution was added to each well. The microplate was incubated at 37°C overnight and the spectrophotometrical absorbance was measured using a microplate reader. The measurement wavelength was set at 580 nm while the reference wavelength was 650 nm. Cell survival was reported as a percentage of the untreated control.

Confocal Microscopy

After incubation with free FAM-siRNA or FAM-siRNA-loaded EMs for 4 h, the MCF-7 cells were washed twice with cold PBS and fixed with 4% formaldehyde in PBS for 30 min. Cell nuclei were stained with 5 mg/ml of Hoechst 33342 for 5 min at room temperature. Green fluorescence of Cy5-siRNA and blue fluorescence of Hoechst were visualized and recorded on an Olympus FV1000 Filter Confocal Microscope (Olympus Optical Co., Tokyo, Japan)[21].

Quantitative Real Time Reverse Transcription PCR (qRT-PCR) Analysis Total RNAs were obtained using TRIzol reagent (Invitrogen) per manufacturer's instructions. Reverse transcription of equal amounts of RNA was carried out using a first-strand cDNA synthesis kit (Invitrogen) with random hexamers as primers. The expression levels of CDK4 and GAPDH genes were measured by qRT-PCR using the SYBR Green PCR Master Mix (ABI, Foster City, CA).[22] The primers used for the

amplification reactions were as follows: CDK4, forward: 5'-CTACCTCTCGATATGAGCCAGT-3', and reverse: 5'-CATCTGGTAGCTGTAGATTCTG-3'; GAPDH, forward: 5'-CTGGTAAAGTGGATATTGTTGCCAT-3' and reverse: 5'-TGGAATCA TATTGGAACATGTAAACC-3'. All results were normalized relative to that of GAPDH to correct for differences in template input.

Western Blotting

Western blotting analysis was performed as described previously.[20] Briefly, the samples were denatured in a 5x sample buffer. Equal amounts of proteins were loaded and electrophoresed on a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The transferred blots were blocked with 5% non-fat milk in Tris-buffered saline (TBS, 150 mM NaCl, 20 mM Tris-HCl, pH=7.4) and incubated with primary antibodies at 4°C overnight. After washing, the blots were incubated with peroxidase-conjugated secondary antibodies for 45 min and developed using the ECL detection system. Image J. software was used to calculate the band intensity

Analysis of DNA Content by Flow Cytometry

MCF-7 cells were seeded at a density of 5×10^5 cells/well in a 6-well plate 24 h prior to the experiment. Cells were treated with either free siRNA or EMs for 48 h. After trypsinization, cells were washed three time with cold PBS (pH=7.4) and fixed in 70% ethanol. After fixation, cells were incubated with 100 U/ml RNase A at 37°C for 20 min, followed by resuspension and hypotonic lysis in 1 ml of PI buffer (0.5 mg/ml propidium iodide, 0.1% sodium citrate, 0.05% Triton X-100). The percentages of cells in the G1, S, and G2-M phases were determined by analysis with the Multicycle computer program provided by Phoenix Flow Systems[23].

Animal Study

BALB/C nu/nu mice 4-5 weeks old were purchased from Beijing Vital River Laboratory Animal Co., Ltd. (Beijing Laboratory Animal Research Center) and kept in isolator cages in a pathogen-free facility. The experimental protocols involving mice were approved by Scientific Investigation Board of Science & Technology of Jilin Province, and were conducted in accordance with the National Institute of Health

Guide for the Care and Use of Laboratory Animals[24].

In vivo Targeting and Biodistribution in the Tumor Xenograft Mouse Model by IVIS Imaging

BALB/C-nu mice were used to study the *in vivo* targeting and biodistribution of siRNA loaded EMs. Mice were engrafted subcutaneously with 1×10^6 MCF-7 cells in the right flank, and the tumor volume was calculated as $(\text{width})^2 \times (\text{length})/2$. Around 1 month after inoculation, the mice with a palpable tumor $\sim 1 \text{ cm}^3$ in size were chosen for this study. Cy5 labeled control siRNA were loaded into EMs under the optimum conditions described above. Free siRNA or EMs loaded with Cy5-siRNA were injected intravenously through the tail vein at a dosage of 1.5 mg/kg. At 6 h after injection, the Cy5 fluorescence in the tumor bearing mice was captured by Xenogen IVIS-200 optical *in vivo* Imaging system (Caliper Life Sciences, Hopkinton, MA). Mice were then sacrificed, and major organs including liver, spleen, lung, heart, kidney, and tumor were collected. The fluorescence signals of Cy5 were recorded and analyzed.

Tumor Xenograft Studies in Mice

After the xenografts reached the size around 100 mm^3 , animals were injected with CDK siRNA loaded EMs, Scramble siRNA loaded EMs, free siRNA or PBS every three days. The tumors were measured every 3 days and their sizes were calculated using the formula $L \times W^2 \times 0.5$. The weight of mice was measured every 3 days during the experiment and no obvious decrease in the body weight of any mouse was detected after drug treatment. Upon the completion of treatment for 18 days, tumor grafts were harvested and tumor weights were measured.

Histology and Immunohistochemistry (IHC) Analysis.

For IHC, all slides were deparaffinized in xylene 3 times and rehydrated through graded ethanol. Antigen retrieval and immunostaining was performed as described previously and a Vector M.O.M. Basic Kit was used[25]. Briefly, antigen retrieval was carried out using 10 mM citrate buffer (pH=6.0). Slides were incubated in 0.3% hydrogen peroxide for 30 min to quench the activity of endogenous horseradish peroxidase and then blocked with TBST/5% normal goat serum. The primary

antibody against CDK4 was used at 1:1000 dilution.

Statistical Analysis

Data are presented in the form of mean $\pm$ SE. Statistical analysis was performed using a two-tailed Student's t-test, and differences with a P value less than 0.05 were designated statistically significant.

Results

Production of EMs from MCF-10A Cells

To obtain a pool of safe cell-derived EMs for gene therapy, MCF-10A, a non-tumorigenic human breast epithelial cell line was sequentially extruded through 10 μ m, 5 μ m, and 1 μ m polycarbonate membranes using a mini-extruder as shown in Fig. 1A. These EMs and exosomes were purified by OptiPrep density gradient ultracentrifugation. In our study, 379.3 μ g EMs were obtained using 1×10^7 MCF-10A cells compared with only 2.5 μ g exosomes from the same number of cells (Fig 1B). The yield of total proteins from EMs was 150 folds higher than that of cell-secreted exosomes. In terms of vesicle numbers, 3.0×10^{11} EMs were produced while only 2.6×10^9 exosomes were produced from the same number (1×10^7) of cells as shown in Fig 1C. Our EM approach significantly increased the yield of the EM number and total protein compared to cell secreted exosomes. The size distributions of exosome and EMs are comparable as shown in Fig 1. The mean diameters by volume are 144.9 ± 15.6 nm for exosomes and 131.0 ± 20.5 for EMs (Fig. 1D & 1E). The zeta potentials are -14.5 ± 3.2 mV for the exosomes and -12.6 ± 2.2 mV for the EMs (Table 1). Moreover, the detection of exosomal marker protein CD63, CD9, CD81 and TSG101 on both exosomes and EMs further indicates that both vesicle preparations contain CD63 exosome-marker protein on the surface though the exosomes showed a higher expression of such exosomal markers (Fig. 1F). Plasma stability experiments showed that the EMs loaded with siRNA can protect the payload from enzyme digestion (half life > 48 h) while the free siRNA has the half-life less than 6 h (Fig 1G). The exosome can also protect the siRNA from degradation. All these data suggest that the EMs obtained by our method have the similar composition

and performance as the naturally produced exosomes.

Figure 1

Figure 1. Generation and characterization of exosome and EMs from MCF-10A cells.

(A) Schematic demonstration for the generation of siRNA loaded EMs by electroporation. (B, C) The yield of EMs and Exo measured as the total protein and particle number from 1×10^7 MCF-10A cells ($n=3$). (D, E) Size distribution of EMs and Exo by DLS. (F) Western blot analysis of Exo and EM marker protein CD63, CD9, CD81, and TSG101 (total protein = 15 μ g). EM: Extracellular vesicle-Mimic; Exo: exosome. (G) Plasma stability of siRNA in EMs and Exo. ** vs Control free

siRNA group, $P < 0.01$.

Table 1. Characterization of the exosome and EMs before and after electroporation. N=3, mean $\pm$ SE.

	Exosomes		EMs	
	Before	After	Before	After
Particle Size (nm)	144.9 $\pm$ 15.6	151.3 $\pm$ 17.9	131.0 $\pm$ 20.5	128.9 $\pm$ 27.6
Zeta Potential (mV)	-14.5 $\pm$ 3.2	-15.7 $\pm$ 4.7	-12.6 $\pm$ 2.2	-13.5 $\pm$ 4.1

Optimization of siRNA Loading to EMs

Efficient loading of siRNA is a key to turn EMs into viable nanocarriers for siRNA delivery. Electroporation was adopted to permeabilize the EMs for siRNA loading. To achieve high efficiency, the voltage of electroporation was optimized for loading of siRNA. We used Cy5 labeled-siRNA as a payload to check the colocalization of siRNA with EMs after electroporation. The EMs were stained with PKH67, and a series of different voltages (1000 -1400 V) were tried. Free Cy5-siRNA attached to the surface of EMs after electroporation was removed by incubating the samples with RNase A for 1 h. As shown in Fig. 2 and Table 2, the incorporation of siRNA into EMs was voltage sensitive since the colocalization percentage of cy5-siRNA and EMs was significantly affected by the electroporation voltage. At 1000 V, the colocalization percentage was around 0. With an increase in voltage, the colocalization percentage increased sharply to $13.7 \pm 6.0\%$ at 1100 V, and $49.7 \pm 6.03\%$ at 1200 V as shown in Fig 2A & 2B. When the voltage was further increased from 1300 V to 1400 V, no significant change of the colocalization percentage was observed. The introduction of siRNA into EMs at various voltages was further evaluated by siRNA loading efficiency. Consistent with the colocalization data, the siRNA loading efficiency increased from around 0% at 1000 V to a maximum of $\sim 16\%$ at 1300 V. Interestingly, when the voltage increased from 1300 to 1400 V, the loading efficiency was slightly decreased, though the colocalization percentage was slightly increased. Based on these data, 1300 V was chosen as the optimal voltage for EM electroporation.

We next optimized the pulse number on the loading efficiency with the voltage fixed at 1300 V. With an increase in the electroporation frequency, both colocalization

percentage and loading efficiency decreased sharply. When the pulse time was 2, there was 70% decrease in both colocalization percentage and siRNA loading efficiency.

Figure 2

Figure 2. Optimization of electroporation condition for siRNA loading into EMs. (A) Representative fluorescence images of EMs or Exo after electroporation. Green: PKH-dye labeled EMs or Exo. Red: Cy5-siRNA. (B) Percentage of EMs or Exo with Cy5-siRNA fluorescence under various conditions. * $P < 0.05$, vs Mixture, ** $P < 0.01$,

vs Mixture (C) Percentage of EMs with Cy5-siRNA at electroporation voltage = 1300 V. X1: 1 pulse; X2: 2 pulse; and X3: 3 pulse. n=3, arrows show the colocalization of siRNA with EMs (or Exo). **** P<0.01, vs X1**

The colocalization percentage and siRNA loading efficiency were further reduced to 5.2% and 2.5%, respectively, when 3 pulses were used (Fig. 2C and Table 2). According to these results, the optimal electroporation conditions for this study were 1300 V and 1 pulse. In all these experiments, the pulse duration was set at 10 ms.

Table 2. Colocalization percentage and siRNA loading efficiency at various electroporation conditions. N=3, mean $\pm$ SE.

Electroporation condition (V)	Colocalization Percentage (%)	Loading efficiency (%)
1000	0.6 $\pm$ 1.0	0.1 $\pm$ 0.4
1100	13.7 $\pm$ 6.0	3.7 $\pm$ 3.3
1200	49.7 $\pm$ 6.3	8.9 $\pm$ 5.6
1300	86.3 $\pm$ 4.9	15.6 $\pm$ 4.2
1400	90.1 $\pm$ 7.6	13.7 $\pm$ 7.1
1300 x 2	25.6 $\pm$ 1.7	5.2 $\pm$ 1.6
1300 x 3	18.0 $\pm$ 9.1	2.5 $\pm$ 1.8

Uptake of EMs in MCF-7 cells

In order to investigate the uptake of siRNA-loaded EMs by MCF-7 cells, confocal microscopy was performed 4 h after transfection of cells with FAM-siRNA loaded EMs. Results showed extensive internalization and accumulation of fluorescently labeled FAM-siRNA in the cytosol in the EM group. Similar results were observed in siRNA loaded exosome group, while the free siRNA group showed almost no accumulation in the cytosol (Fig. 3A). This result was further verified by flow cytometry of MCF-7 cells. As shown in Fig. 3B & 3C, the cells exhibited markedly increased fluorescence intensity in siRNA-loaded EM group compared to those treated with free siRNA or siRNA/EM mixture groups. The siRNA loaded exosome group showed a similar fluorescence intensity to the EMs group. This data suggests that the incorporation of siRNA into EMs significantly improved the cellular uptake of the siRNA. More importantly, it demonstrated that the EMs had high delivery efficiency, comparable to the same number of exosomes.

Specific Down-regulation of CDK4 Target Genes and Induction of Cell Cycle Arrest by EM-mediated siRNA Delivery

To evaluate the down-regulation of specific target genes by EM-mediated delivery of

Figure 3. Cellular uptake and intracellular location of EMs in MCF-7 cells. (A) Intracellular localization of EMs by confocal microscopy. Blue: DAPI staining; Green: FAM-siRNA. **Scale bar: 10μm** (B) MCF-7 cells were treated with various groups at 37°C for 4 h, and cellular fluorecence was recorded by a FACSCalibur flow

cytometer. X-axis: cellular fluorescence intensity; Y-axis: cell counts. (C) Fluorescence intensity measured for different treatment groups. 1: PBS only; 2: Free siRNA; 3: Exo+siRNA mixture, 4: EM + siRNA mixture; 5: siRNA-loaded Exo; 6: siRNA-loaded EM, n=3. **** P<0.01 vs free siRNA**

siRNA, we investigated the delivery of an siRNA that targets CDK4 which is an attractive target for cancer therapy. Scramble siRNA was used as a negative control for CDK4 siRNA. qRT-PCR analysis revealed that the mRNA level of CDK4 was dramatically decreased when treated with siRNAs delivered by EMs relative to the level of untreated cells, while a similar treatment with free siRNA did not induce measurable gene downregulation (Fig. 4A). Consistent with the cellular uptake results, the delivery of the siRNA/EM mixture showed only a slight decrease in CDK4 gene expression. The siRNA loaded exosomes showed a similar gene downregulation effect as compared with the EMs. The gene downregulation effect was further verified by determining the CDK4 protein level using Western blot. As shown in Fig. 4B, the CDK4 protein level was decreased by 76.2% in the EM group. A similar significant downregulation effect was observed in the siRNA loaded exosomes. Inhibition of CDK4 is expected to induce cell cycle arrest at G1 since it is an essential factor for cell cycle progression. By flow cytometry, we observed significant G1 cell cycle arrest by EMs compared with the untreated group (Fig. 4C). The cell population arrest in G1 was increased to 73.12% in the EM group, and 67.08% in the exosome group. On the other hand, the untreated group showed only 50.60% of cells in the G1 stage. No obvious difference in cell cycle distribution was detected between free siRNA and untreated groups. Taken together, our results indicate that delivery of CDK4 siRNA by EMs can effectively downregulate CDK4 gene expression and inhibit cancer cell growth.

Cellular Entry Pathways of EM-mediated siRNA Delivery

To further elucidate the mechanism of EM mediated cellular uptake and trafficking, MCF-7 cells were treated with Cy5-siRNA loaded EMs and labeled with various endocytic pathway markers (green): clathrin mediated endocytosis with A488-Tf, caveolae-mediated endocytosis with A488-CT-B, or macropinocytosis with

FITC-dextran. As shown in Fig. 5A, siRNA co-localized strongly with A488-Tf and A488-CT-B, suggesting that the entry of EMs was via both clathrin and caveolae mediated endocytosis. Inhibition of the clathrin-mediated endocytosis by sucrose reduced the cellular uptake of siRNA by 52.5%. Treatment with filipin, a lipid

Figure 4

Figure 4. EMs alters apoptosis-related signaling molecules in MCF-7 cells. (A) qRT-PCR analysis of CDK4 mRNA expression level after different treatments(n=3).

(B) The expression levels of the apoptosis signaling proteins CDK4 examined using Western blot. 15 μ g total protein was used for each lane. (C) Flow cytometry analysis of the cell cycle of MCF-7 cells at 48 h after transfection with EMs(n=3). ** $p<0.01$, vs Untreat.

raft/caveolae-mediated endocytosis inhibitor, decreased the cellular uptake by 42.7%. The entry of siRNA into MCF-7 cells was reduced by 16.0% by macropinocytosis inhibitor wormannin (Fig. 5B). Taken together, our data suggest that all three endocytic pathways are involved in the EM mediated siRNA delivery while clathrin and caveolae-mediated endocytosis take leading roles in this process.

Figure 5

Figure 5. Cellular entry pathways of EMs in MCF-7 cells. (A) Intracellular location of EMs in MCF-7 cells. EMs co-localized with A488-Tf (endosomes), FITC-dextran (macropinosomes), and A488-CT-B (caveosomes) separately after 1 h incubation. The Cy5-siRNA is shown in red, the cellular markers are shown in green, and overlay

of Cy3- siRNA and markers is shown in yellow. (B) Influence of endocytosis inhibitors on EMs cellular uptake. Mean fluorescent intensity of Cy5-siRNA after incubation with various inhibitors. Each bar is the mean of three experiments, scale bar = 10 μ m, * $p < 0.05$, vs Ctrl, ** $P < 0.01$, vs Ctrl.

Effect of EMs on Cellular and Systemic Toxicity

Next, we investigated the safety of obtained EMs, both *in vitro* and *in vivo*. As shown in Fig. 6A, no significant cytotoxicity was detected in the experimental groups. Cell viability was maintained at over 90% across all the groups tested ($P > 0.05$). We further studied whether the siRNA loaded EMs can induce systemic toxicity in the immune competent mice. Serum analysis results showed no significant liver/kidney toxicity in mice treated with siRNA loaded EMs or exosomes (Fig. 6B). Normal serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were recorded in mice treated with EMs, exosomes, or PBS, suggesting negligible liver damaging effect of these EMs and exosomes. Furthermore, comparable serum blood urea nitrogen (BUN) and creatinine levels indicated no kidney damage occurred in these mice. Taken together, these data suggest that the obtained EMs had no significant cellular toxicity or systemic toxicity.

Figure 6

Figure 6. Effect of EMs on cellular and systemic toxicity. (A) Viabilities of MCF-7 cells treated with various complexes were measured by the MTT assay (n=6). (B) *In vivo* toxicity assay by measuring serum ALT, AST, BUN and Creatinine level 48 h after i.v. PBS, EM, or Exosome in wild type mice, n=5.

***In vivo* Targeting of EMs to Tumors in a Murine Xenograft Model**

We evaluated the tumor targeting effect of EMs *in vivo* using a tumor xenograft mouse model, BALB/C-nu mice. As shown in Fig. 7A, strong accumulation of Cy5 fluorescence was observed by whole-mouse imaging in the tumor area for the EM group. In contrast, the free siRNA group showed weak fluorescence in the corresponding area. The tumor targeting effect of the EMs on siRNA biodistribution was also evaluated. As shown in Figs. 7B&C, the free siRNA strongly accumulated in both liver and spleen, while the other organs studied, including heart, lung, kidney and tumor, showed very low accumulation. On the other hand, the fluorescence

Figure 7

Figure 7. *In vivo* fluorescent images of MCF-7 tumor bearing mice after i.v. injection of EMs. The fluorescent images of whole body imaging (A) for MCF-7 tumor bearing mice with EMs. Tissue distribution (B) of EMs after intravenous administration, tissues of EMs were harvested and then measured (C), n=6.

intensity was dramatically increased at the tumor site in the EM group (2201.5 ± 404.9 vs. 564.5 ± 154.2 for EMs vs. free siRNA, $p < 0.01$). Meanwhile, the siRNA accumulation in liver and spleen was significantly reduced. Interestingly, a slight increase of fluorescence intensity in heart, lung, and kidney compared to free siRNA group was observed though there was no statistical difference. All these data suggest that EMs can efficiently target siRNA to the tumor site while reducing its accumulation in liver and spleen.

Anti-tumor Effect of EMs in Xenograft Mouse Model

The *in vivo* anti-tumor effect of CDK4 siRNA loaded exosome mimics (EMs) was assessed by tail i.v. injection of free or EMs (siRNA 1.5 mg/kg) in subcutaneous MCF-7 tumor xenograft mice. Each mouse was injected with PBS, free CDK4 siRNA, scramble siRNA loaded EMS, or CDK4 siRNA loaded EMs every three days as described before. As shown in Fig. 8A, the free CDK4 siRNA had no observable effect on tumor growth compared with the PBS control group. On the other hand, the CDK4 siRNA loaded EM group greatly slowed down tumor growth. No significant body weight loss was observed across different groups during the experimental period (Fig. 8B). The sizes of tumors at day 18 were ranked as follows: EM-siRNA < EM-NC-siRNA (Negative Control siRNA) $\approx$ Free siRNA $\approx$ Control, as shown in Figs. 8C&D. The tumor tissues were further evaluated through IHC staining. The H&E staining showed that the control group displayed a high density of tumor cells with intact structures, and the free siRNA treatment group showed a similar pattern (Fig. 8E). On the other hand, the tumor cells were much sparser and appeared separated from each other in the EM-siRNA group. The expression of CDK4 protein was also significantly reduced in the EM-siRNA group as compared with the control group. Meanwhile, free siRNA or EM-NC siRNA showed no effect on CDK4 expression by immunohistochemistry assay. This CDK4 downregulation effect was further verified

by PCR since the CDK4 mRNA level was reduced by almost 60% in the tumor tissue (Fig. 8F). Our results suggest that EM-CDK4 siRNA can significantly downregulate the CDK4 expression at the tumor site, inhibiting tumor growth *in vivo*.

Figure 8

Figure 8. Tumor xenograft studies in MCF-7 tumor bearing mice after i.v. injection of EMs. (A) Tumor volume growth curves (n=6). (B) The mice weight change

curve(n=6). (C) The appearance of tumor change (n=6). (D) The tumor weight change curve (n=6). (E) Immunohistochemical analysis of tumor CDK4 changes. (F) Quantitation of CDK4 mRNA change. *:P< 0.05 vs. control.

Discussion

RNA interference (RNAi)-based oligonucleotides, including small interfering RNAs (siRNAs), microRNAs (miRs) and anti-microRNAs (anti-miRs), have been extensively exploited for selective gene regulation in different diseases.[6-8, 26, 27] Due to their unstable characteristic in the biological environment, nanoparticles have been used to facilitate the delivery of the oligonucleotide drugs into target cells.[28-31] However, inefficient cellular uptake and undesirable side effects hinder their clinical translation. Thus, safe delivery systems are needed. In this study, we presented the production and therapeutic evaluation of novel EMs for siRNA delivery *in vitro* and *in vivo*. By serial extrusion of non-tumorigenic epithelial MCF-10A cells, we obtained a good yield of nano-sized vesicles, called exosome-mimics (EMs). Compared with commonly used artificial liposomes and nanoparticles employed for siRNA delivery, exosomes have been proven to achieve more efficient delivery after systemic administration.[32] Moreover, in contrast to the commonly-used synthesized components for nanoparticles, the natural characteristics of exosomes makes them ideal for specific drug delivery without inducing undesired pro-inflammatory reactions and immune response. Our study proved that the EMs have similar characteristics as exosomes. **Compared with naturally excreted exosome, the yield of EMs is significantly high, which is around 150 folds of exosome from the same cells. Meanwhile, the biosafety *in vivo* study revealed that EMs did not induce any pro-inflammatory reactions or immune response in mice. We also conducted a direct comparison between our novel EMs and conventional exosomes harvested from the same cells for the siRNA delivery, and found that they have similar effects in treating tumors. This result is also consistent with previous findings obtained in other cell lines.[33, 34] These data suggest that, regardless of whether excreted by cells naturally or formed by extrusion artificially, these nano-sized vesicles may contain**

similar surface proteins and are able to deliver the payload safely and efficiently with excellent targeting to the tumor cells. Interestingly, in the cellular uptake experiments, we noticed that the mixture of siRNA with either exosomes, or EMs, could also increase the siRNA uptake, compared to siRNA alone. Since the major components of either exosomes or EMs are lipids such as cholesterol, it is quite possible that siRNA may be protected from enzyme digestion by simply forming a mixture with cholesterol. Another possibility is that the entry of siRNA is facilitated by such mixture due to the similar component of exosomes(EMs) with cell plasma membrane. More experiments are required to verify these hypotheses. It is generally believed that the specific protein on the exosome surface is responsive for its tumor targeting effect and recent studies indeed supported this notion by showing that removal of the surface proteins from these nano-vesicles by trypsinization led to the loss of the tumor targeting effects, both *in vitro* and *in vivo*. Recently, using the similar cell-derived nanovesicles, Jang *et al* successfully target doxorubicin to tumor cells *in vivo*[34]. All these results suggest that the EMs may be used as a novel nanocarrier for gene delivery.

To our knowledge, this is the first study on the application of electroporation for the EM loading with siRNA. The electroporation conditions, including voltage and pulse time, were optimized for the siRNA loading. Interestingly, the siRNA/EMs colocalization percentage was increased sharply from <20% to >80% when the voltage was increased from 1100 to 1300 V, indicating a possible voltage threshold in the siRNA loading process. The loading efficiency was actually decreased slightly when the electroporation voltage was changed from 1300 to 1400 V, and then decreased dramatically at 1500 V. Meanwhile, with the increase of the number of electroporation pulses, the loading efficiency was decreased. The decreased efficiency could have been caused by the breakup of EMs at high voltage or multiple pulses. Our data suggest that the siRNA loading conditions could be precisely controlled for these EMs. Under optimal conditions, more than 15% of the siRNA was successfully incorporated into EMs. To reduce the undesirable electroporation-induced siRNA precipitation during the loading process, EDTA was added[18]. To rule out the

possibility that the observed fluorescence was from siRNA attached to the surface rather than loaded inside the EMs, we used RNase A to digest the EM surface siRNA after electroporation.

Many studies have revealed that exosomes can transport various biological cargos such as mRNAs, miRNAs and proteins to target cells, however, the detailed molecular mechanism underlying exosome mediated delivery has not yet been systemically evaluated. Recently published studies[35, 36] showed that endocytosis plays a key role in exosome based delivery, but the detailed uptake pathway may vary in different cell types. For example, Tian *et al*[36] found that clathrin-mediated endocytosis and macropinocytosis pathways were involved in the cellular uptake of PC12 cell-derived exosomes. Nanbo *et al*[35] revealed that exosomes derived from Epstein-Barr virus-infected cells were mediated by caveolin-dependent endocytosis. In contrast, caveolin-1 was found to negatively regulate the uptake of exosomes from human GBM cells[37]. Thus, it is hypothesized that the uptake mechanism of exosomes through endocytosis may depend on the receptors of recipient cells. In our study, at least two kinds of endocytosis, clathrin-mediated and caveolin-mediated endocytosis, are involved in the uptake of EMs in recipient cells based on the selective endocytosis inhibitors and specific endocytosis markers. We also observed the contribution of macropinocytosis endocytosis in this process, though its effect is not as obvious as the two other endocytosis pathways. However, our data do not exclude the possibility that other endocytosis pathways may be involved in the EM uptake process, and further studies should be carried out on this topic.

Conclusion

In conclusion, our *in vitro* and *in vivo* data have provided proof of concept for the functional EMs as natural nanocarriers for the delivery of RNA interference drugs to cancer cells. The CDK4 siRNA loaded EMs can significantly downregulate the CDK4 mRNA and protein expression *in vitro* and *in vivo*. EMs from relevant cell types may also provide excellent targeting for delivery.

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