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In vitro evaluation of endothelial exosomes as carriers for small interfering ribonucleic acid delivery

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Abstract: Exosomes, one subpopulation of nanosize extracellular vesicles derived from multivesicular bodies, ranging from 30 to 150 nm in size, emerged as promising carriers for small interfering ribonucleic acid (siRNA) delivery, as they are capable of transmitting molecular messages between cells through carried small noncoding RNAs, messenger RNAs, deoxyribonucleic acids, and proteins. Endothelial cells are involved in a number of important biological processes, and are a major source of circulating exosomes. In this study, we prepared exosomes from endothelial cells and evaluated their capacity to deliver siRNA into primary endothelial cells. Exosomes were isolated and purified by sequential centrifugation and ultracentrifugation from cultured mouse aortic endothelial cells. Similar to exosome particles from other cell sources, endothelial exosomes are nanometer-size vesicles, examined by both the NanoSight instrument and transmission electron microscopy. Enzyme-linked immunosorbent assay analysis confirmed the expression of two exosome markers: CD9 and CD63. Flow cytometry and fluorescence microscopy studies demonstrated that endothelial exosomes were heterogeneously distributed within cells. In a gene-silencing study with luciferase-expressing endothelial cells, exosomes loaded with siRNA inhibited luciferase expression by more than 40%. In contrast, siRNA alone and control siRNA only suppressed luciferase expression by less than 15%. In conclusion, we demonstrated that endothelial exosomes have the capability to accommodate and deliver short foreign nucleic acids into endothelial cells.

Keywords: extracellular vesicles, exosomes, gene delivery, siRNA, endothelium

Introduction

Extracellular nanosize membrane-bound vesicles are produced by almost all types of cells in mammals. Their existence has been known for over three decades, but only recently have these particles drawn attention, due to their diagnostic relevance and therapeutic potential.[1] Exosomes are a subpopulation of extracellular vesicles derived from multivesicular bodies, ranging from 30 to 150 nm in size. One of the main functions of exosomes is to transmit cell-to-cell molecular messages through small noncoding ribonucleic acid (RNAs), messenger RNAs, deoxyribonucleic acids, and proteins.[2] They possess a highly variable cargo composition depending on the information they carry and a great variety of ligands on the membrane surface specific for the cells to which they are delivering the molecular message. Exosomes from different types of cells may have different compositions and functions.[3]

Exosomes derived from endothelial cells (endothelial exosomes) are currently under intensive investigations to better understand their role in pathological processes, such as vascular inflammation and atherosclerosis.[4,5] Besides studying the

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contribution of endothelial exosomes to disease formation, it would be beneficial to explore their potentials as therapeutic delivery vehicles, as the vascular endothelium maintains an extensive communication network within a large variety of cells/tissues in the body and has been a key target of gene therapy. Further, adaptation to a continuously changing environment, such as that resulting from hypoxia and inflammation, etc, and connection with other cells occur widely through exosomes.[4,5]

In this work, we isolated and characterized exosomes from primary endothelial cells. Endothelial exosomes were tested for interaction with primary endothelial cells and further studied in vitro for accommodation and delivery of extrinsic oligonucleotides for gene silencing of luciferase in transfected endothelial cells.

Materials and methods

Cell culture

Primary endothelial cells were isolated from the aorta of C57BL/6 ApoE$^{-/-}$ mice and grown as described previously.[6] Cells were initially maintained at 37°C at 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific). For exosome isolation, cells were cultured in media supplemented with 2% exosome-depleted FBS. FBS was depleted of bovine exosomes by ultracentrifugation at 4°C and 120,000× *g* for 120 minutes. Viability of cells was tested by the propidium iodide assay.

Isolation, purification, and analysis of exosomes

The supernatant of primary endothelial cells grown in triple flasks was harvested when cell confluence reached 90%. Exosomes were isolated from the supernatant using standard serial centrifugations and filtration through 0.45–0.2 μm polyvinylidene difluoride filters followed by ultracentrifugation at 120,000× *g* to pellet exosomes. Exosomes were washed in phosphate buffered saline (PBS) and centrifuged twice at 120,000× *g* before the final exosome pellet was resuspended in PBS, and aliquots were used immediately or stored at −80°C for further analysis.

To determine exosome production per cell, endothelial cells with an initial density of 6.75×10^6 in 75 mL medium were grown and purified as described in the previous paragraph. The concentration of particles was determined by the NanoSight NS300 instrument, as described in the following section.

Size-distribution analysis of exosomal particles

Real-time high-resolution particle detection, counting, and sizing were performed on the NanoSight NS300 following manufacturer protocols (Malvern Instruments, Malvern, UK). Particle concentration (particles/mL) was calculated by the NanoSight system. The Nanoparticle Tracking Analysis system was also used to compare changes in concentrations and sizes before and after electroporation and ultracentrifugation of exosomes.

Classic and cryogenic transmission electron microscopy of exosomes

Exosomes in PBS were fixed in a final concentration of 2% paraformaldehyde, mounted on copper-mesh formvar grids (Electron Microscopy Sciences, Hatfield, PA, USA) and negatively stained by 2% uranyl acetate. Samples were observed using a JEOL 1230 transmission electron microscope (JEOL, Tokyo, Japan) at the University of Virginia Advanced Microscopy Facility and a Tecnai F20 Twin transmission electron microscope (FEI, Hillsboro, OR, USA). Sample preparations for cryo-transmission electron microscopy (TEM) imaging of exosomes were based on a previously established protocol.[7] In brief, an aliquot of concentrated exosomes (~3.5 μL) was applied to a glow-discharged, perforated carbon-coated grid (2/2-3C C-Flat; Protochips, Raleigh, NC, USA), manually blotted with filter paper, and rapidly plunged into liquid ethane. The grids were stored in liquid nitrogen, then transferred to a Gatan 626 cryospecimen holder (Gatan, Warrrendale, PA, USA) and maintained at −180°C. Low-dose images were collected at a nominal magnification of 29,000× on the Tecnai F20 Twin transmission electron microscope operating at 120 kV. Digital micrographs were recorded on a Gatan US4000 charge-coupled device camera.

ELISA of endothelial exosomes

The presence of tetraspanin CD63 and CD9, which are exosomal protein markers, was confirmed by using an enzyme-linked immunosorbent assay (ELISA) kit (System Biosciences, Mountain View, CA, USA). Briefly, standard exosomes provided in the kit or purified endothelial exosomes were incubated in duplicates with a primary antibody (rabbit) to CD63 or CD9 and then a horseradish peroxidase enzyme-linked secondary antibody (goat anti-rabbit). A colorimetric substrate was used for the assay read-out. The results were quantitated by a SpectraMax® 190 (Molecular Devices, Sunnyvale, CA, USA) plate reader at 450 nm. Intensity

values of endothelial exosomes were compared to those of exosome standards to give the concentration of CD63 or CD9, as well as the concentration of the exosomes based on these markers (particles/mL).

Fluorescence microscopy and flow-cytometry analysis

Exosomes were labeled with lipophilic green fluorescent dye (DiO) and incubated with the endothelial cells for fluorescence microscopy study and flow-cytometry analysis. For labeling with DiO, purified endothelial exosomes (10^9 CD63-positive particles calculated as earlier) were incubated with Fast DiO green fluorescent membrane dye (Invitrogen) at a final concentration of 2 μg/mL for 1 hour at room temperature. Labeled exosomes were diluted with PBS and spun at 120,000× *g* for 90 minutes to sediment labeled exosomes and remove unbound dye. The purification process of washing and ultracentrifugation was repeated twice before the labeled exosome pellet was resuspended in PBS.

For microscopic analysis, endothelial cells grown on cover glass were incubated with DiO-labeled exosomes in Opti-MEM at 37°C for 1 hour. After incubation, cells were washed with PBS to remove unbound labeled exosomes and subsequently imaged with a fully motorized Zeiss upright AxioImager Z1 microscope (Carl Zeiss Microscopy, Thornwood, NY, USA) equipped with Apotome 2 to produce confocal-like images. An Axio Cam HRm digital monochromatic camera was used for image acquisition.

For flow-cytometry analysis, primary endothelial cells were seeded in six-well plates 5×10^5/well and grown overnight. Prior to treatment with DiO-labeled exosomes, cells were washed with PBS, and then the medium was replaced with 900 μL Opti-MEM. DiO-labeled exosomes (5×10^8) in 100 μL PBS/well (CD63-positive, calculated as earlier) were added and incubated at 37°C for 1 hour. Cells stained directly with 1 μL/well DiO (1 μg/mL) served as a positive control, and unstained cells as a negative control. Cells were removed from plastic by trypsin, centrifuged, and resuspended in 500 μL PBS. Fluorescence-activated cell sorting (FACS) analysis was performed at the University of Virginia Flow Cytometry Core Facility using the FACSCalibur with a 530/30 filter and FlowJo Collectors' Edition Acquisition software. Each experimental group was performed in triplicate. To confirm that the fluorescence intensities were due to DiO-labeled vesicles and not to residual dye in the supernatant during the preparation of DiO-labeled exosomes, 1 mL washed buffer from the first and second ultracentrifugations, respectively, were incubated with cells and analyzed by flow cytometry along with the experimental groups.

In vitro evaluation of siRNA-delivery function of endothelial exosomes

Endothelial cells grown to 50% confluence in a T75 cm^2 flask were transfected with 100 μL FuGENE® 6 transfecting agents (Promega, Fitchburg, WI, USA) and 50 μg pGL2 plasmid (Promega) expressing luciferase under the control of an SV40 promoter. After 6 hours of transfection, the medium was supplemented with 5% FBS/DMEM. At 24 hours following transfection, cells were washed with PBS, removed with trypsin, and seeded on 24-well plates at an initial density of 2×10^4/well 1 day prior to the treatment with exosomes. Luciferase expression was evenly distributed on multiwell plates, which was achieved by the aforementioned bulk transfection of the cells followed by stepwise plating.

Purified endothelial exosomes were diluted in PBS to a concentration of 6×10^7 particles/100 μL (based on CD63-positive particles). Exosomes were incubated with 0.5 nmol/mL siRNA against luciferase (siRNA[luc], silencer firefly luciferase siRNA), or nonsilencing control siRNA (siRNA[cont] Ambion; Thermo Fisher Scientific) on ice for 10 minutes. Electroporation was applied with 400 mV and 200 μF in a 0.4 cm gap chamber in 100 μL volume using the BTX ECM 600 electroporation system. Electroporated fractions of the same group were pooled. Both electroporated and nonelectroporated (exosomes mixed with siRNA but not electroporated) exosomes were diluted in PBS and subjected to ultracentrifugation at 120,000× *g* for 90 minutes to sediment exosomes and remove excess siRNA. Sedimented exosomes/siRNA(luc), exosomes/siRNA(cont), and nonelectroporated exosomes were resuspended in PBS and incubated with luciferase-expressing primary endothelial cells in 200 μL Opti-MEM to evaluate the gene-silencing effect. Prior to treatment with exosomes/siRNAs, luciferase-expressing cells were washed twice with PBS and serum-deprived in Opti-MEM for 2 hours. Cells treated directly with naked siRNA(luc) 100 nM served as an additional control. Expressed luciferase activity was read 3 days posttreatment by a 20/20n luminometer (Promega). Each experimental group was represented in quadruplicates.

Lastly, Oligofectamine™, a standard carrier of siRNA, complexed with siRNA(luc) was used as a positive control for the gene-silencing effect in comparison with exosomes/siRNA(luc). Luciferase-expressing endothelial cells seeded on 24-well plates (prepared as earlier) were transfected with a final concentration of 100 nM siRNA(luc) or siRNA(cont)

complexed with 1.5 µL/well Oligofectamine (Thermo Fisher Scientific). Prior to treatment, cells were washed twice with PBS and incubated with serum-free Opti-MEM for 2 hours. Oligofectamine alone (1.5 µL/well) and 100 nM naked siRNA(luc) served as additional controls. Cells received DMEM with 10% FBS 4 hours following transfection. Luciferase activity was measured 3 days posttreatment as described earlier. Each experimental group was represented in quadruplicates.

Statistical analysis

Significant differences were determined by Student's *t*-test; values of $P<0.05$ were considered to be significant, unless stated otherwise. Error bars represent standard deviation.

Results

Characterization of endothelial exosomes

Exosomes were isolated from primary endothelial cells by the combination of filtration and ultracentrifugation. They were characterized by analyses for particle size, distribution, concentration, and presence of well-established exosomal protein markers. The mean particle diameter was 92±38 nm, with a mode of 81 nm (Figure 1A). Particles were also characterized under cryo- and classic TEM. Negative staining of classic TEM demonstrated cup-shaped, round particles (Figure 1B). Cryo-TEM imaging provided a more detailed anatomy: 0.2 micron-filtered preparations showed spherical vesicles, with unevenly distributed dense material inside (Figure 1C). The presence of exosomal markers was also investigated by CD9 and CD63 ELISAs. The results confirmed abundant CD63 and CD9 expression (the concentrations of particles positive for CD63 and CD9 were 1.56×10^{10} and 2.27×10^{9} particles/mL, respectively).[8]

Dead bodies from decomposing cells can contaminate exosome preparation. In order to determine the proportion of dead cells, a viability assay was conducted. Dead cells were present in 0.68%±0.12% in the endothelial culture. This suggested that organelles from dead cells are minimally present as contaminant particles. Finally, exosome production per cell was investigated: one endothelial cell can produce 60–70 particles in 36 hours.

Interaction of exosomes with primary endothelial cells

In order to evaluate the interaction between endothelial exosomes and cultured primary endothelial cells, exosomes were labeled with DiO and incubated with the endothelial cells for fluorescence microscopy and flow cytometry. Fluorescence microscopy images of the treated cells exhibited green spots or small patches (Figure 2A, upper panel). In contrast, the endothelial cells treated with DiO showed a homogeneous green fluorescence staining (Figure 2A, lower panel).

DiO-labeled exosomes incubated with endothelial cells were also analyzed by flow-cytometry assay to provide quantitative measurement of the above interaction (Figure 2B). Cells treated with DiO-labeled exosomes (DiO exosomes) produced lower fluorescence intensity compared to cells stained with DiO only (DiO direct) (Figure 2B). Residual dye was substantially eliminated after a second wash, suggesting that the signal from DiO exosomes was from the DiO associated with exosomes.

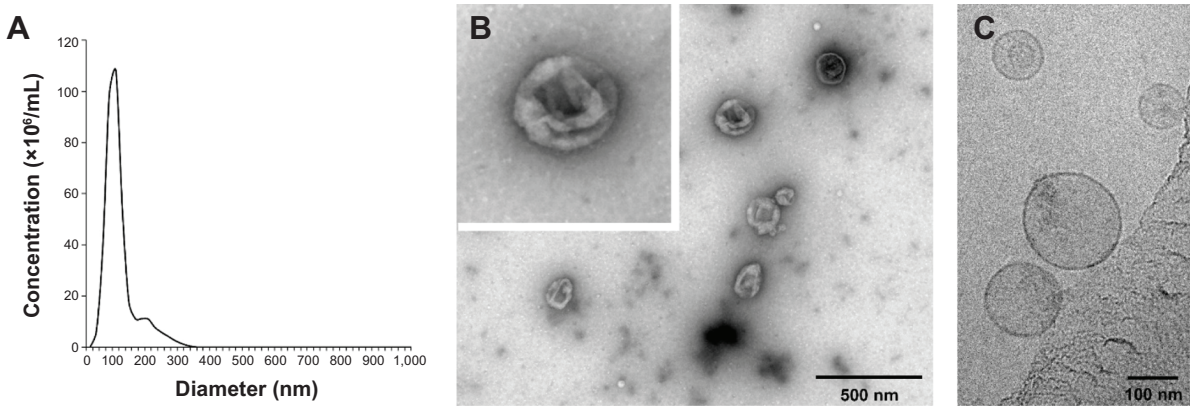

Figure 1 Characterization of exosomes isolated from cultured primary endothelial cells.
Notes: The graph represents size distribution of nanoparticles by NanoSight particle-tracking analysis (**A**). Classic transmission electron microscopy depicts multiple cup-shaped, shrunken vesicles (inset shows a collapsed exosome) (**B**). Cryogenic transmission electron microscopy image represents membrane bound vesicles (**C**).

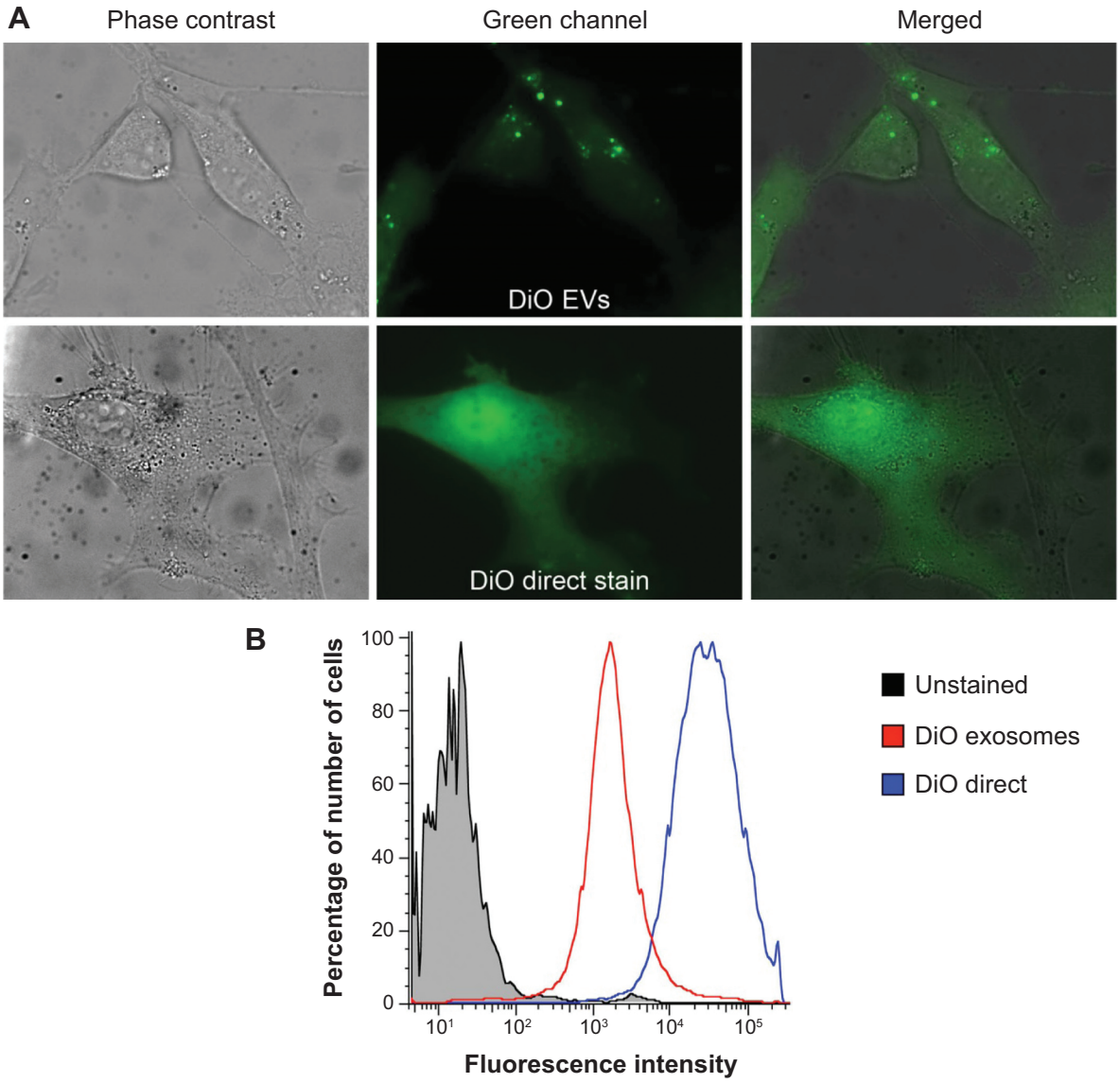

Figure 2 (**A**) Phase contrast and fluorescence microscopy images demonstrated interaction of green fluorescent dye (DiO)-labeled endothelial exosomes with primary endothelial cells (upper panels) and the distribution of fluorescence in primary endothelial cells labeled directly with DiO (lower panels). An oil-immersion objective of 100× was used for image acquisition. (**B**) Flow cytometry analysis of primary endothelial cells treated with DiO-labeled exosomes and DiO only.
Abbreviation: EVs, exosome vesicles.

Function of endothelial exosomes as delivery vehicles of foreign nucleic acids

The ability of the endothelial exosomes to accommodate and deliver small exogenous nucleic acids to endothelial cells for gene silencing was evaluated. Primary endothelial cells were transiently transfected with pGL2, a luciferase-encoding vector to generate the luciferase-expressing endothelial cells. siRNA(luc) designed to reduce luciferase activity by inactivating homologous sequences of the messenger RNA transcribed from pGL2 was introduced into exosomes by electroporation. The gene-silencing effect was evaluated by incubating luciferase-expressing endothelial cells with exosomes loaded with siRNA(luc) (exosomes/siRNA[luc]), exosomes loaded with siRNA(contr) (exosomes/siRNA[contr]), and siRNA(luc) alone (Figure 3A).

Also, Oligofectamine, complexed with siRNA(luc) was used as a positive control for a gene-silencing effect in comparison with exosome/siRNA(luc). As shown in Figure 3, A and B, the exosomes/siRNA(luc) exhibited a comparable inhibition effect to siRNA(luc) complexed with Oligofectamine. The exosomes/siRNA(luc) resulted in a significantly lower level (by 40%) of luciferase expression compared to the control groups, including exosomes/siRNA(cont), and naked siRNA(luc) ($P<0.05$), while the nonelectroporated siRNA

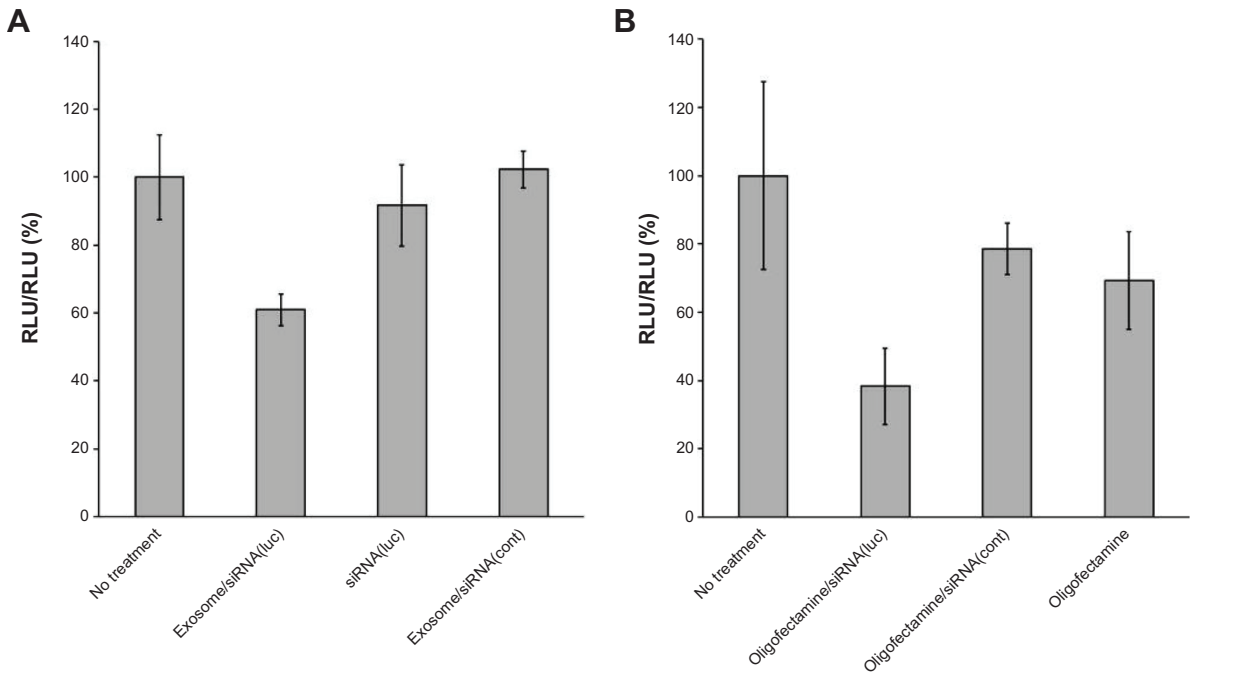

Figure 3 The in vitro gene-silencing effect of exosomes loaded with small interfering ribonucleic acid (siRNA).
Notes: The graphs depict the blocking effect of electroporated endothelial exosomes with siRNA(luc) (**A**) and siRNA(luc) complexed with Oligofectamine (**B**) on primary endothelial cells expressing luciferase ($P<0.05$, n=4).
Abbreviations: siRNA(luc), siRNA against luciferase; siRNA(cont), nonsilencing control siRNA; RLU, relative luminescence units.

exosomes (siRNA mixed with exosomes) only inhibited luciferase expression as much as the siRNA(luc) alone (not shown). All the results demonstrated that endothelial exosomes can accommodate and deliver extrinsic siRNAs.

Discussion

In order to achieve effective and efficient siRNA delivery, it is critical that siRNAs are encapsulated within or conjugated to delivery vehicles (eg, nanocarriers). However, existing viral, bacterial, bacteriophage, and synthetic lipid-based delivery methods have not to date achieved expected transfection efficiency, and all the expectations and hope surrounding initial preclinical studies are yet to be matched with clinical efficacy. This highlights the need to harness a natural delivery mechanism that is safe and efficient. Exosomes may prove to be the ideal siRNA carriers to fulfill this role, as their natural role is to transmit molecular messages between cells without invoking an immune response.

Other investigators have shown that exosomes can be generated from immune cells, blood plasma, mesenchymal stem cells, and brain endothelial cells.[9–12] However, only a few were used for drug or short-nucleic-acid delivery.[13–17] We hereby presented the isolation and function characterization of exosomes from endothelial cells to deliver siRNA to endothelial cells. As exosomes from different types of cells may have different compositions and functions, endothelial cells were chosen to produce exosomes, as these exosomes may not have other unnecessary components from other cell sources to be delivered to the target endothelial cells. There are also some advantages of choosing endothelial cells as the therapy target. First, the vascular endothelium has been a key target of gene therapy for the following reasons: diseases of the vasculature, such as atherosclerosis, diabetic angiopathy, and autoimmune vasculitis, are devastating and affect a large portion of the population, yet do not have a definitive therapy; endothelial cells in vasculature are readily accessible for direct contact;[18] and lastly endothelial cells and plasma are easily obtained and the generation of exosomes could be conveniently scaled up. Numerous reports are available about exosomes of nonendothelial origin, describing their effect on the endothelium, but very little is known about endothelial exosomes or their potential as delivery vehicles for exogenous agents.[4,19]

In this study, we demonstrated that cultured primary endothelial cells isolated from aorta can be used to produce exosomes. Primary endothelial exosomes share features common to those derived from other cells. These features include spherical membrane-bound particles by TEM and expression of CD9 and CD63 markers.[8,20] Although the intracellular fate of exosomes is unclear, the fluorescence dye-labeled exosomes show uneven distribution in cells, suggesting that exosome uptake by cells is mediated by a particular unknown

pathway. The results of the gene-silencing study shown in this paper demonstrated that exosomes loaded with siRNAs against luciferase were able to achieve significant reduction of the luciferase expression in parent cells compared to controls. We demonstrated that exosomes were able to take up the siRNAs through electroporation, and were then able to deliver the cargo to the cells, where siRNA took effect. Further investigation is needed, however, to improve the efficiency of gene silencing and to test the effect of engineered endothelial exosomes in in vivo conditions, including the interaction of exosomes with the endothelial lining of the vessels and identification of receptor-ligand connection(s), which makes exosome attachment/internalization to cells possible.

In this initial study, we used electroporation to load the siRNA into exosomes. Electroporation is a widely used method to introduce nucleic acids and drugs into membrane-bound structures like eukaryotic and prokaryotic cells.[21,22] However, electroporation may not be the best technique to load siRNA into exosomes.[23] First, the loading efficiency is relatively low, at 15%–25% from our study (data not shown). Second, electroporation resulted in some degree of damaged particles and fused/aggregated vesicles. It is a well-known phenomenon that the optimal electric field strength required to open big-enough pores in the membrane will lyse part of the sample, especially with the use of exponential decay wave pulse generators.[22,24] Although further optimization of the electroporation may improve the loading yield and minimize the morphological alteration of exosomes, ie, use of square-wave pulse generators, other molecular engineering methods and loading technologies are needed and are under active investigation in our laboratory.

Conclusion

We successfully isolated exosomes from primary endothelial cells by conventional ultracentrifugation methods and confirmed their identities with microscopy and protein-marker analysis. The initial in vitro study in luciferase-transfected cells demonstrated the potential to deliver siRNA into cells to silence the target gene. Further investigations on the engineering of exosomes to improve targeting and siRNA delivery in vivo are warranted.

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Disclosure

The authors report no conflicts of interest in this work.

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