Bioengineered Extracellular Membranous Nanovesicles for Efficient Small-Interfering RNA Delivery: Versatile Platforms for Stem Cell Engineering and In Vivo Delivery

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Naturally derived nanovesicles secreted from various cell types and found in body fluids can provide effective platforms for the delivery of various cargoes because of their intrinsic ability to be internalized for intercellular signal transmission and membrane recycling. In this study, the versatility of bioengineered extracellular membranous nanovesicles as potent carriers of small-interfering RNAs (siRNAs) for stem cell engineering and in vivo delivery has been explored. Here, exosomes have been engineered, one of the cell-derived vesicle types, to overexpress exosomal proteins fused with cell-adhesion or cell-penetrating peptides for enhanced intracellular gene transfer. To devise a more effective delivery system with potential for mass production, a new siRNA delivery system has also been developed by artificially inducing the outward budding of plasma membrane nanovesicles. Those nanovesicles have been engineered by overexpressing E-cadherin to facilitate siRNA delivery to human stem cells with resistance to intracellular gene transfer. Both types of engineered nanovesicles deliver siRNAs to human stem cells for lineage specification with negligible cytotoxicity. The nanovesicles are efficient in delivering siRNA in vivo, suggesting feasibility for gene therapy. Cell-derived, bioengineered nanovesicles used for siRNA delivery can provide functional platforms enabling effective stem cell therapeutics and in vivo gene therapy.

1. Introduction

The delivery of DNA or small RNAs, including small-interfering RNAs (siRNAs), for the regulation of gene expression has opened new avenues in gene therapy and cellular engineering for a wide range of diseases.\textsuperscript{[1,2]} In recent decades, a variety of delivery materials have been developed for gene therapy, the genetic engineering of stem cell therapy, and the reprogramming of cells to pluripotency or transdifferentiation.\textsuperscript{[1,3,4]} Most gene delivery methods have relied on viral vectors, but safety issues remain a major concern.\textsuperscript{[5,6]} Several types of synthetic carriers, such as polymer/lipid nanoparticles, liposomes, and inorganic nanoparticles, have been proposed as safer alternatives,\textsuperscript{[7]} but few of those vectors have been successfully applied because their gene-delivery efficiencies in primary cells, including stem cells, or in vivo applications are not high enough. In addition, some cause substantial cellular toxicity or immunogenicity.\textsuperscript{[8,9]} Therefore, a fundamental and clinically relevant engineering challenge for gene and cell therapies lies in the in vitro and in vivo development of safe and effective delivery vehicles.\textsuperscript{[10,11]}

Recently, naturally derived membranous vesicles have shown their utility as drug\textsuperscript{[12–15]} and gene delivery\textsuperscript{[16,17]}, as alternatives to viral vectors and synthetic materials for effective, biocompatible, gene delivery. In particular, exosomes, naturally occurring transport nanovesicles with a diameter of 40–100 nm that are secreted by most cell types, have been attested to serve as potent vehicles for gene delivery\textsuperscript{[16,18–20]} because of their intrinsic roles in intercellular signaling and membrane fusion in both biological and pathological settings.\textsuperscript{[21,22]} For instance, Andaloussi et al. reported exosome-mediated siRNA delivery both in vitro and in vivo.\textsuperscript{[18,23]} In addition, nanovesicles derived from cells were validated for DNA delivery\textsuperscript{[24]} and chemotherapeutic delivery for cancer treatment.\textsuperscript{[14,15]} Given the current limitations of viral and nonviral synthetic systems, the utilization of natural materials such as cell-derived exosomes and nanovesicles might provide an untapped source of attractive gene delivery strategies.\textsuperscript{[23]} There are still a number of challenges to the therapeutic application of exosomes and nanovesicles, however, including needs for further improvement in gene-delivery efficiency, specific targeting, and large-scale production.\textsuperscript{[25]}

We propose two types of bioengineered extracellular membranous nanovesicles as efficient siRNA delivery platforms for in vitro stem cell engineering and in vivo delivery: surface-engineered exosomes and plasma membrane-derived nanovesicles (PMNVs). To facilitate improved target-cell attachment and intracellular uptake, we engineered exosome membranes to...
display multiple cell-adhesion peptides (Arg-Gly-Asp; RGD) or a cell-penetrating peptide (CPP). We also obtained PMNVs by chemically inducing membrane vesicle formation, mimicking the outward budding process of microvesicles. Compared with exosomes in terms of preparation and potential for mass production, PMNVs are relatively simple to make in only a few steps and can be obtained in larger quantities at lower cost. The engineered exosomes and PMNVs showed high siRNA transfection efficiency and negligible cytotoxicity in human adipose-derived stem cells (hADSCs) and human neural stem cells (hNSCs) compared with commercial transfection reagents. They also promoted the direct differentiation of human stem cells into desired cell lineages by mediating the efficient delivery of target siRNAs for osteogenesis and neurogenesis. We further engineered the PMNVs by overexpressing E-cadherin to facilitate endocytosis-mediated uptake into target stem cells expressing E-cadherin. The enhanced cadherin interaction of the PMNVs improved siRNA delivery to human induced pluripotent stem cells (hiPSCs). We showed that the engineered exosomes and PMNVs had high siRNA delivery efficacy and marginal toxicity in vivo.

2. Results and Discussion

2.1. Characterization of Exosomes and PMNVs

We prepared cell-derived exosomes and PMNVs as potential siRNA carriers. For PMNV preparation, we first collected giant plasma membrane vesicles (GPMVs) formed by human embryonic kidney 293 (HEK293) cells (Figure 1A and Figure S1A, Supporting Information), which are commonly used as a model to study the lipids and proteins between coexisting fluid–fluid phases in compositionally complex membranes.[26] Chemically induced GPMVs have been utilized in various biochemical and biophysical studies for characterization of plasma membrane lipid composition,[27] physical properties,[28] permeability to various molecules,[29,30] and structural and biochemical properties of a membrane protein.[31] We sonicated the GPMVs for 3 min and then physically extruded them through a membrane filter with 100 nm pores to obtain a population of nanovesicles that were homogeneous in size. The size of PMNVs can be controlled using filter membranes with different pore sizes (Figure S1B, Supporting Information). To isolate exosomes secreted from HEK293 cells, we cultured the cells in

![Diagram of exosome and PMNV generation](image)

**Figure 1.** Characterization of exosomes and PMNVs. A) Schematic illustration of the generation of PMNVs. B) Transmission electron microscopy images of exosomes and PMNVs (scale bars = 500 nm). C) Size and zeta potential of exosomes and PMNVs analyzed by dynamic light scattering \((n = 3)\). D) Size distribution of exosomes and PMNVs analyzed by nanoparticle tracking analyzer (NTA; \(n = 3\)). E) The number of nanovesicles produced from a single cell analyzed by NTA \((n = 3)\). F) The number of nanovesicles produced from a single cell per hour in preparation \((n = 3)\). G) Uptake assay of Cy5-siRNA transfected with exosomes and PMNVs into HeLa cells (scale bar = 200 μm).
exosome-free medium for 48 h. We collected and ultracentrifuged the medium, and then we washed and concentrated the resulting exosomes in a buffer as previously described.\[18\]

Characterization of the prepared exosomes and PMNVs revealed that they had biophysical properties suitable for siRNA delivery. To characterize the exosomes and PMNVs, we visualized them by transmission electron microscopy (Figure 1B). The vesicles were circular and appeared physically homogenous. The size of the vesicles measured by microscopy was ≈100–150 nm, which matched the average hydrodynamic diameter measured by dynamic light scattering (exosome: 137 nm and PMNV: 139 nm, Figure 1C). Nanoparticle tracking analysis of the size distributions revealed that the modal diameter of the largest populations of exosomes and PMNVs was 112 nm and 138 nm, respectively (Figure 1D). Those data confirmed that the size of the vesicles was within a range of 100–150 nm, which is suitable for efficient cellular uptake.

PMNVs provide comparative advantages over exosomes with respect to preparation as a novel delivery platform capable of mass production and high productivity. The average number of exosomes and PMNVs produced from a single cell was ≈475 and 14 333, respectively (Figure 1E). The exosomes were collected from the culture medium incubated with cells for 48 h, and the PMNVs were collected after 3–4 h incubation with vesicle-formation solution. Thus, compared with exosomes, the PMNVs were produced in larger quantities within a shorter incubation period. On average, a single cell yielded 9.89 exosomes in 1 h, whereas a single cell yielded 4095 PMNVs in the same amount of time, indicating more than 400-fold higher production yield whereas a single cell yielded 4095 PMNVs in the same amount of time. On average, a single cell yielded 9.89 exosomes in 1 h, whereas a single cell yielded 4095 PMNVs in the same amount of time, indicating more than 400-fold higher production yield whereas a single cell yielded 4095 PMNVs in the same amount of time.

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superior biocompatibility even with high doses of siRNA. Thus, the nanovesicles could be useful in applications requiring high doses or multiple transfections of siRNA.

2.3. Improved siRNA Delivery into Human Stem Cells by Bioengineered Nanovesicles

The siRNA transfection efficiency and cytotoxicity of the engineered exosomes and PMNVs were further examined in hADScs and hNSCs, which are known to be resistant to exogenous gene transfer. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis showed that the delivery to hADScs of an siRNA targeting the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using engineered exosomes and PMNVs achieved GAPDH knockdown comparable to that achieved using LF2000 (Figure 2D, left panel). We also checked whether the type of cells used to generate the nanovesicles could affect the cell-type specificity of siRNA delivery. Nanovesicles derived from HEK293 cells and hADScs, respectively, were tested for GAPDH siRNA transfection of hADScs. The hADSc-derived nanovesicles induced slightly higher GADPH knockdown than the HEK293-derived nanovesicles in hADScs, but the difference was not statistically significant (Figure 2D, left panel). Therefore, we produced all the vesicles used in further studies using HEK293 cells. All of the delivery systems showed low cytotoxicity with more than 90% hADSc viability, suggesting high biocompatibility (Figure 2C–E). Those features are especially attractive to those interested in the genetic modification of stem cells and primary cells for...
applications including lineage specification, paracrine enhancement, and reprogramming, because an efficiency-cytotoxicity dilemma is often encountered in the genetic engineering of cells. The engineered nanovesicles were efficient in the siRNA knockdown of reporter (GFP) and housekeeping (GAPDH) genes, so we tested them for use in stem cell engineering to silence factors that inhibit osteogenesis and neurogenesis. To enhance the neuronal differentiation of hNSCs and the osteogenic differentiation of hADSCs, we used the nanovesicles to deliver siRNAs against repressor element-1 silencing transcription factor (REST) and guanine nucleotide-binding protein (G-protein) alpha-stimulating activity polypeptide (GNAS).

2.4. Enhancement of hADSC Osteogenic Differentiation via Delivery of GNAS siRNA by Bioengineered Nanovesicles

We used the engineered nanovesicles to deliver GNAS siRNA (siGNAS) to promote the osteogenic differentiation of hADSCs. GNAS is a complex imprinted gene that encodes multiple transcripts through the use of multiple promoters and first exons that splice onto a common set of downstream exons. One of the main transcripts includes the stimulatory G-protein alpha subunit (Gs-α), a key component of many signal transduction pathways. The expression of Gs-α inhibits osteogenic differentiation by means of the proteolytic degradation of core-binding factor alpha, a transcription factor for the differentiation of stem or precursor cells into osteoblasts. Therefore, several studies have reported that the inactivation or downregulation of GNAS led to increased osteogenic differentiation.

We transfected hADSCs with nanovesicles containing siGNAS and then cultured them in osteogenic-induction medium for 14 and 21 d. Two days after transfection, the cells transfected with Exo-R11 and PMNV had lower levels of GNAS expression than no treated cells and cells transfected with Exo or Exo-RGD (Figure 3A). The engineered exosomes (Exo-R11 and Exo-RGD) exhibited greater knockdown efficiency than Exo, suggesting that the modification of exosomal

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**Figure 3.** Enhanced osteogenic differentiation of hADSCs induced by delivery of siGNAS by engineered nanovesicles. A) GNAS mRNA expression after 2 d of siGNAS transfection with nanovesicles (n = 3; *p < 0.01 vs NT; ##p < 0.01 vs LF2000; +++p < 0.01 vs Exo). B) Gene expression levels of osteogenic markers COL1A2 and OPN in the differentiated hADSCs at d 14 and 21 of culture in osteogenic differentiation medium (n = 3; *p < 0.05 and **p < 0.01 vs NT). C) Quantification of Ca²⁺ deposition (n = 6–10; *p < 0.05 and **p < 0.01 vs NT; ##p < 0.01 vs LF2000; +++p < 0.01 vs Exo). D) Representative images from Alizarin S staining of hADSCs with siGNAS transfection using nanovesicles 21 d after differentiation (scale bar = 500 μm). E) Immunostaining analysis of hADSCs after siGNAS transfection using nanovesicles for osteogenic markers COL1A2 and OPN after 21 d of differentiation (scale bars = 100 μm).
membrane protein with RGD and R11 can enhance siRNA delivery (1.00 ± 0.30 GNAS/GAPDH for no transfection (NT), 0.53 ± 0.21 for Exo, 0.21 ± 0.02 for Exo-R11, and 0.37 ± 0.13 for Exo-RGD; Figure 3A).

The reduction of GNAS transcription in the transfected hADSCs was accompanied by enhanced osteogenic differentiation, as assessed by qRT-PCR, immunostaining, and Alizarin Red S staining (Figure 3B–E). At days 14 and 21, the expression of the differentiation markers collagen type I (COL1A2) and osteopontin (OPN) was upregulated at similar levels in all of the transfected cells compared with those in cells that were not transfected (Figure 3B). Among the treatment groups, the cells transfected with Exo-R11 showed the highest COL1A2 and OPN expression levels 14 d after osteogenesis induction (Figure 3B). At day 21, the cells transfected with Exo-R11 had the highest COL1A2 expression level (Figure 3B). We performed Alizarin Red S staining to detect mineralization after 21 d of culture (Figure 3C,D). Cells transfected with nanovesicles containing siGNAS generally had more extensive mineralization and calcium deposition compared with cells that were not transfected (Figure 3C,D). We observed substantially higher mineralization intensities with the presence of red nodules in differentiated hADSCs transfected with engineered exosomes (Exo-RGD, Exo-R11) and PMNVs compared to the cells transfected with LF2000 and unmodified exosomes (Figure 3D). Immunofluorescence staining with COL1A2 and OPN revealed higher fluorescence intensities of both markers in cells transfected with engineered exosomes or PMNV (Figure 3E), which is consistent with the findings from the qRT-PCR and Alizarin Red S staining. Furthermore, the hADSCs transfected with Exo-RGD, Exo-R11, or PMNV showed morphological changes similar to those of osteogenic cells, which are flatter and more spread out, whereas the cells that were not transfected or were transfected with LF2000 or unmodified exosomes showed fibroblast-like morphologies (Figure 3E). Overall, those results demonstrate that the delivery of siGNAS by bioengineered nanovesicles is effective in reducing the GNAS expression and thus enhancing the osteogenic differentiation of hADSCs.

### 2.5. Promotion of Neuronal Differentiation of hNSCs by the Knockdown of REST via siRNA Delivery Using Bioengineered Nanovesicles

We manipulated the neuronal differentiation of hNSCs derived from human fetal brain tissue by transfecting the cells with engineered nanovesicles containing REST siRNA (siREST). REST is known to function as a transcriptional repressor that suppresses neuronal genes by binding to a DNA sequence element called the neuron-restrictive silencer element. The repressor is highly expressed in undifferentiated neuronal progenitor cells and is thought to act as a master negative regulator of neurogenesis. By lowering the REST expression in hNSCs, progenitor cells have been made to differentiate further into neuronal lineages. We observed the knockdown of REST expression at the transcript level following siREST delivery by nanovesicles in all transfected cells 2 d after transfection (Figure 4A). Among the delivery systems, Exo-RGD was the most efficient in reducing REST gene expression (Figure 4A, 0.39 ± 0.03 REST/GAPDH).

The REST knockdown by nanovesicle-mediated siREST delivery significantly promoted the neuronal differentiation of hNSCs, as confirmed by qRT-PCR and immunostaining of several neural markers. The hNSCs were cultured for 5 and 9 d after transfection and analyzed for differentiation. At day 5, the changes in expression levels of the NSC marker Nestin were not significant, but all the siREST-treated cells at day 9 showed reduced expression of Nestin compared with untreated cells, indicating that the REST knockdown accelerated differentiation (Figure 4B). The expression of glial fibrillary acidic protein (GFAP), a glial cell marker, was significantly reduced in all the nanovesicle-transfected cells at day 9 (0.34 ± 0.06 for Exo-R11, 0.32 ± 0.04 for Exo-RGD, and 0.29 ± 0.03 for PMNV), which implies inhibition of hNSC differentiation to the glial lineage (Figure 4B). The expression of class III beta-tubulin (TuJ1), a phenotypic marker for early neuronal cells, and that of microtubule-associated protein 2 (MAP2), a later neuronal marker, were upregulated in cells transfected with siREST-containing nanovesicles at all time points (Figure 4B). The cells transfected with Exo-RGD or PMNV showed the most enhanced levels of both neuronal markers compared with other cells including those transfected with LF2000 (Figure 4B). Meanwhile, the expression of oligodendrocyte lineage transcription factor 2 (Olig2), a marker for motor neurons, was significantly upregulated at day 9 in all the cells transfected with nanovesicles (Figure 4B). Those results confirm that siREST delivery using engineered nanovesicles rapidly decreased the stemness of hNSCs and substantially accelerated hNSC differentiation to neuronal lineages rather than to glial cells. TuJ1-positive and MAP2-positive neuronal cells in all the siREST-transfected cells exhibited morphologically longer and highly extended neurite outgrowth compared with untreated cells, and the differences in morphological changes became more apparent at day 9 (Figure 4C). All the cells transfected with siREST showed longer average neurite length compared with untreated cells (Figure 4D). The hNSCs transfected with Exo-RGD and PMNV had significantly longer neurites than those transfected with LF2000 (103.7 ± 28.9 μm for LF2000, 133.9 ± 47.1 μm for Exo-RGD, and 128.6 ± 41.0 μm for PMNV). Those results confirm that REST knockdown using engineered nanovesicles effectively induced early neuronal differentiation in hNSCs and led to a shift of differentiation toward more mature neuronal lineages.

### 2.6. Potential Mechanism of Improved siRNA Delivery by the Modified PMNVs

After demonstrating the proficiency of bioengineered exosomes and PMNVs for delivering siRNA into human stem cells and directing lineage differentiation in vitro, we sought to explore the engineering of PMNVs to further improve siRNA delivery in human pluripotent stem cells and also to investigate potential mechanisms by which the engineered PMNVs were taken up into the cells. To do that, we generated PMNVs from HEK293 cells with E-cadherin overexpressed in the plasma membrane after transfection with an E-cadherin-encoding plasmid. E-cadherin is a membrane-spanning protein of adherent junctions that plays a key role in cell-cell contact formation.
We confirmed the incorporation of E-cadherin into PMNVs (PMNV-Ecad) by western blot (Figure 5A). E-cadherin is highly expressed in pluripotent stem cells including embryonic stem cells and iPSCs, and has been shown to regulate pluripotency by mediating cell–cell adhesion and interaction.\(^{43}\) hiPSCs are known to have low gene-transfection efficiency due partly to their low cloning efficiency.\(^{44}\) We hypothesized that PMNVs engineered to overexpress E-cadherin (PMNV-Ecad) could exhibit enhanced gene-transfection efficiency because of increased affinity interactions with iPSC surfaces via enhanced cadherin-cadherin interaction. A previous study reported that anchoring E-cadherin and fibronectin on inorganic nanocrystals enhances the efficacy of gene delivery into embryonic stem cells.\(^{44}\) hiPSCs transfected with PMNV-Ecad displayed greater GAPDH knockdown than hiPSCs transfected with unmodified PMNVs or LF2000 (0.75 ± 0.04 for LF2000, 0.75 ± 0.02 for PMNV, and 0.55 ± 0.07 for PMNV-Ecad; Figure 5C), which was confirmed by the determination of GAPDH expression relative to β-actin expression in the transfected cells. Ecad-modified PMNVs were also more effective than unmodified PMNVs or LF2000 for GAPDH siRNA transfection of hADSCs (0.58 ± 0.04 for LF2000, 0.43 ± 0.11 for PMNV, and 0.37 ± 0.10 for PMNV-Ecad; Figure 5D). Those results indicate that PMNV-Ecad can be useful for siRNA transfection of hard-to-transfect cells.

We conducted uptake pathway-inhibition studies to explore the mechanisms by which engineered PMNVs (PMNV-Ecad) and exosomes (Exo-R11) are internalized. Several studies have suggested that exosomes are internalized through clathrin-mediated endocytosis and macropinocytosis. We tested those two processes as potential uptake mechanisms.\(^ {45–47}\) We first examined the clathrin-dependent endocytosis of PMNV-Ecad (Figure 5E–G) and Exo-R11 (Figure S3, Supporting Information).
Information) by inhibiting the process with K+ depletion and the knockdown of clathrin heavy chain (CHC). We labeled Exo-R11 and PMNV-Ecad with the lipophilic dye DiI, allowed them to be taken up by hADSCs, and detected the internalized nanoparticles by confocal microscopy. The depletion of K+ inhibits the formation of clathrin-coated pits by dissociating the clathrin lattices at the inner leaflet of the plasma membrane.[48] Treatment with K+ depletion buffer partially inhibited PMNV-Ecad and Exo-R11 uptake (Figure 5E and Figure S3, Supporting Information). CHC knockdown by siRNA blocked the uptake of PMNV-Ecad and Exo-R11 (Figure 5F and Figure S3, Supporting Information). Those results indicate that the uptake of modified exosomes and PMNVs (Exo-R11 and PMNV-Ecad) involves clathrin-mediated endocytosis.

We assessed macropinocytosis as another pathway for PMNV-Ecad internalization (Figure 5G), because previous studies reported that exosomes derived from glioblastoma[45] and PC12 cells[46] are taken up via macropinocytosis as well as via clathrin-mediated endocytosis. To abolish macropinocytosis, we treated hADSCs with ethyl-isopropyl amiloride (EIPA), an Na+-H+ exchanger inhibitor, or with LY294002, a phosphoinositide 3-kinase (PI3K) inhibitor, before adding PMNV-Ecad vesicles. The uptake was notably reduced, indicating that macropinocytosis is involved in PMNV-Ecad uptake (Figure 5G). We confirmed the significant reduction in the rate of vesicle uptake and the number of internalized vesicles by fluorescent image-based quantification analysis (the graphs in Figure 5E–G). Taken together, our results indicate that the uptake of Exo-R11 and PMNV-Ecad occurs through both clathrin-mediated endocytosis and macropinocytosis.

Native exosomes and PMNVs without surface modifications also seem to be internalized into cells via the same uptake mechanisms elucidated with modified exosomes (Exo-R11) and PMNVs (PMNV-Ecad). The cellular uptake mechanisms of native exosomes have been widely studied by several groups demonstrating that exosomes enter cells via clathrin-mediated endocytosis[46,49–51] and macropinocytosis.[46] We have already observed that modified exosomes (Exo-R11) also enter cells...
through the same pathways (Figure S3, Supporting Information). Uptake inhibition studies on native PMNVs without surface modification revealed the significant decrease in siRNA uptake upon K⁺ depletion and EIPA and LY294002 treatments (Figure S4, Supporting Information), which confirmed that native PMNVs are also internalized into cells via the same pathways (clathrin-mediated endocytosis and macropinocytosis) as modified PMNVs (PMNV-Ecad). Considering that GPMVs are formed by outward protrusion of the plasma membrane, it is assumed that the composition of GPMV membrane is similar to that of cellular plasma membrane. In addition, it has been known that GPMVs do not contain cytosolic organelles or nuclear fragments. Indeed, proteomics analysis to examine the protein contents of GPMVs revealed that 93% of GPMV membrane proteins is derived from plasma membrane proteins and the rest is composed of intracellular membrane proteins. Since PMNVs are prepared by reducing the size of GPMVs through sonication and extrusion, we can predict that PMNVs possess similar membrane composition as GPMVs. Therefore, although intracellular uptake of engineered nanovesicles could be improved through specific binding and interactions between targeting peptides or proteins of engineered nanovesicles and interacting ligand molecules, it is thought that exosomes and PMNVs are basically internalized into cells via similar cellular uptake mechanisms, irrespective of the presence of targeting moieties.

2.7. In Vivo Systemic Delivery of siRNA by Nanovesicles

We conducted injection studies in animals to determine the feasibility of using exosomes and PMNVs as effective siRNA carriers in vivo. We first monitored the in vivo biodistribution of siRNA encapsulated within Exo-R11 and PMNVs by fluorescence imaging of the whole body or the organs of mice that were intravenously injected with fluorescently labeled siRNA (Alexa 647-tagged). The biodistribution images of mice 15 and 150 min after injection indicated strong fluorescence signals throughout the whole body at 15 min and signals localized to specific regions with diminished fluorescence at 150 min (Figure 6A). The quantified intensity of the fluorescent siRNA signals in both treatment groups (Exo-R11 and PMNV) was decreased 150 min after injection (Figure 6B). The distributions of the siRNA-containing nanovesicles were also confirmed by ex vivo optimal imaging of excised organs from experimental and negative-control animals at 150 min post injection (Figure 6C,D). The highest fluorescence intensity was accumulated in the liver and also in the kidneys (Figure 6C). Quantification of the fluorescence intensity in the retrieved organs indicated that the nanovesicles were delivered mostly to the liver but also to the lung in case of Exo-R11 (Figure 6D). We assumed that the unloaded free siRNA was cleared rapidly through the kidneys and excreted in the urine. Considering that the strong fluorescent signals detected in bladder 15 min after intravenous injection of nanovesicle formulation was mainly derived from unloaded free siRNA (Figure 6A), which probably excreted in the urine, it is thought that unloaded free siRNA did not contribute to silencing of target gene expression.

After identifying the liver as a major target organ for siRNA delivery by the nanovesicles, we investigated gene knockdown in the liver using nanovesicles containing siRNA against apolipoprotein B (APOP; Figure 6E). APOB is an essential protein, predominantly expressed in the liver, for the formation of low-density lipoproteins (LDLs) in the metabolism of dietary and endogenous cholesterol and is a ligand for the LDL receptor. Serum levels of APOB, LDL, and cholesterol correlate significantly with increased risk of coronary artery disease. The lowering of serum cholesterol and LDL levels is a predominant clinical strategy for the management of coronary artery disease and has been achieved by endogenously silencing APOB gene expression. We administered Exo, Exo-R11, and PMNV containing APOB siRNA (sAPOP) intravenously to mice and analyzed the knockdown of APOB expression in the liver by qRT-PCR 2 d after administration (Figure 6E). The APOB transcript levels in the livers transfected with Exo-R11 (0.23 ± 0.11) and PMNV (0.17 ± 0.04) were significantly lower than those in livers injected with phosphate buffered saline (PBS) or transfected with corresponding negative controls, which consisted of Exo-R11 and PMNV loaded with scrambled siRNA (siSCR; 0.93 ± 0.26 for Exo-R11/siSCR and 1.02 ± 0.18 for PMNV/siSCR). The results validated the hypothesis that Exo-R11 and PMNV can act as efficient siRNA carriers in vivo. We analyzed the hepatoxic effects of the nanovesicles by measuring the serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and bilirubin 12 h before and 12 and 36 h after the injection of nanovesicles containing siSCR (Figure 6F and Table S1, Supporting Information). The serum AST level increased slightly 12 h after the injections but decreased back to the level of the PBS controls 36 h after the injections (Figure 6F). There were no significant differences in the levels of ALT and AST at 12 and 36 h post injection between the mice injected with the nanovesicles and those injected with PBS (Figure 6F). Furthermore, we detected no changes in the level of bilirubin before and after injection with the nanovesicles (Table S1, Supporting Information), indicating that Exo and PMNV exhibit insignificant hepatotoxicity. Taken together, our results indicate the siRNA-containing Exo and PMNV successfully mediated siRNA delivery, predominantly to the liver, and target-gene silencing with no significant hepatoxicity. Those results suggest the potential applicability of the engineered exosomes and PMNVs for siRNA-based gene therapeutics.

3. Conclusion

The clinical translation of gene therapy, stem cell engineering, and reprogramming has relied heavily on the development of gene carriers that can effectively deliver target genes into cells or tissues without safety concerns. Our results confirmed that nanovesicles of bioengineered exosomes and PMNVs exhibit high siRNA transfection efficiencies in various types of stem cells and in vivo siRNA delivery to liver, while showing no detectable toxicities. Therefore, the engineered extracellular membranous nanovesicle-based platform might provide a valuable siRNA delivery method for in vitro stem cell engineering for lineage specification and in vivo siRNA-based therapeutic applications. To the best of our knowledge, our study is the first report demonstrating the utilization of cell-derived nanovesicles as siRNA carriers for engineering various types of stem cells.
Our naturally derived nanovesicles have several advantages over currently established delivery systems. Both viral and nonviral delivery approaches have attractive features for gene delivery; however, potential toxicity and economical scale-up for production are the challenging issues that remain unsolved. Our results demonstrate that exosomes and PMNVs have no detectable cytotoxicity, can be engineered for improved delivery efficiency and directed targeting, and can be economically generated with high yield in a few relatively simple steps. The production process involves ultracentrifugation and sonication followed by extrusion. Large-scale manufacture could be achieved with PMNVs. The size of the PMNVs can also be readily controlled. Furthermore, our systems show high transfection efficiency in hard-to-transfect cells compared with that of recently reported biomimetic nanoparticles. Our approach has great therapeutic potential in clinical settings, considering that the nanoparticles can be derived from patients’ own cells, allowing the issue of immunogenicity to be circumvented.

In conclusion, our findings provide great promise for cell-derived nanovesicles as a new class of small RNA delivery system for gene therapy and stem cell engineering. In future work, further optimization of the delivery efficiency via...
dose-dependent and multiple dose tests and targeting strategies might need to be extensively studied with a focus on lowering the dose regimens and ensuring long-term efficacy and safety.

4. Experimental Section

Cell Culture: HEK293 cells, HeLa cells, GFP-HeLa cells, and primary embryonic mouse fibroblasts were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL) and 1% penicillin-streptomycin (Gibco BRL). hADSCs were purchased from Invitrogen (Carlsbad, CA, USA) and cultured in low-glucose DMEM (Gibco BRL, Gaithersburg, MD, USA). In this study, unloaded free siRNA was defined as siRNA that was not encapsulated in nanovesicles after electroporation. Because unloaded free siRNA was not removed from nanovesicles after electroporation, and siRNA dose stated in this study indicates the amount of total siRNA used for encapsulation, including unloaded free siRNA. To measure the efficiency of the siRNA encapsulation into the nanovesicles, the RiboGreen assay (Invitrogen) was performed following a protocol from a previous study.[61] This assay is based on the fluorescence of RiboGreen upon intercalation with siRNA. Briefly, the formulations of exosomes or PMNVs were incubated with RNA-binding dye RiboGreen and measured with a microplate reader (Tecan Group Ltd., Männedorf, Switzerland) for the fluorescent signals before and after treatment of Triton X-100 (Sigma). Triton X-100 (Sigma) is a surfactant that lyses lipids. Thus, in the absence of the detergent, signal comes from unloaded free siRNA, whereas in the presence of the detergent, signal comes from total siRNA (loaded and unloaded free siRNA). The siRNA concentrations were obtained from the fluorescence using a standard curve prepared with standard siRNA solutions of known concentrations. The fluorescence of nanovesicles alone was subtracted from that of siRNA-encapsulating nanovesicles in the presence of Triton X-100 in order to exclude any contribution from the nanovesicles themselves to fluorescent signals. The encapsulation efficiency was calculated with a following equation: [(siRNA in the presence of Triton X-100 – total siRNA used for encapsulation) / total siRNA used for encapsulation] × 100 (%). GFP-HeLa cells were seeded (2.5 × 10^5 cells cm^{-2}) and transfected with 1.25 μg siRNA cm^{-2} siRNAs (SCR-siRNA (siSCR), GFP-siRNA (siGFP), and red-fluorescence-labeled siRNA (Cy5-siRNA), ST Pharm, Seoul, Korea) using nanovesicles one day after the seeding. LF2000 (Invitrogen) served as a control reagent in all in vitro transfection experiments. After 4 h incubation at 37 °C, the medium was replaced with fresh medium to remove untransfected siRNA. After 2 d of transfection, the silencing of GFP expression and the uptake of Cy5-siRNA into the cells were observed using a fluorescent microscope (IX71, Olympus, Tokyo, Japan). To evaluate the transfection efficiency in the GFP-HeLa cells, GFP-positive cells were quantified by flow cytometry analysis using a fluorescence-activated cell sorting (FACS) caliber flow cytometer (BD Biosciences, San Jose, CA, USA). The transfection efficiency in human stem cells (hADSCs, hNSCs, and hiPSCs) treated with GAPDH-siRNA (siGAPDH, ST Pharm) was measured by RT-qPCR with GAPDH primer, with the expression of GAPDH normalized to that of β-actin. The viability of the transfected cells was evaluated by measuring the mitochondrial metabolic activity of the cells using an MTT assay (Sigma). The viability and GAPDH knockdown efficiency of the transfected cells were normalized to those of untreated cells. The sequences of siRNAs used for the transfection experiments (siGFP, siSCR, and siGAPDH) are listed in the Supporting Information Table S3.

In Vivo Transfection and Osteogenic Differentiation of hADSCs: hADSCs (1.5 × 10^5 cells cm^{-2}) were transfected with siRNA against GNAS (1.25 μg siGNAS cm^{-2}, GE Dharmacon, Lafayette, CO, USA)
encapsulated in nanovesicles one day after seeding. After 4 h incubation at 37 °C, the medium was replaced with osteogenic-induction medium to induce osteogenic differentiation. The differentiation medium was exchanged twice per week. The osteogenic-induction medium was prepared by adding 100 × 10^−6 M desamethasone (Sigma), 50 μg mL^−1 Lascidic acid (Sigma), and 10 × 10^−6 M β-glycerophosphate (Sigma) to DMEM containing 10% (v/v) FBS (Gibco BRL) and 1% (v/v) penicillin-streptomycin (Gibco BRL). At 14 and 21 d after the induction, the cells were analyzed by qRT-PCR, immunostaining, and Alizarin S staining to check calcium deposition and the expression of osteogenic markers.

_in vitro Transfection for Neural Differentiation of hNSCs: _hNSCs_ were seeded at a density of 1.25 × 10^5 cells cm^−2 and treated with nanovesicles loaded with siRNA against REST (1.25 μg siREST cm^−2), GE Dharmacon) 1 day after seeding. The nanovesicles were removed after 4 h incubation by replacing the transfection medium with fresh medium (DMEM/F12 with N-2 supplement). The expression of REST in the cells was examined by qRT-PCR 2 d after transfection. After 5 or 9 d, the cells were analyzed by qRT-PCR and immunostaining assays to check the expression of neuronal markers.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR): The gene expression profiles of cells were quantified using qRT-PCR. At each time point, RNA was extracted from the cells using the RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA), and cDNA was synthesized using the PrimeScript II first strand cDNA synthesis kit (Takara, Shiga, Japan). qRT-PCR was performed using the TaqMan Fast Universal PCR Master Mix System (Applied Biosystems, Foster City, CA, USA) and the StepOnePlus Real-Time PCR System (Applied Biosystems). The gene expression was quantified using TaqMan gene expression assays (Applied Biosystems) for each target (human genes—GAPDH: Hs02758991_g1, β-actin: Hs01060665_g1).

**Immunocytochemistry:** Samples were fixed in 10% (v/v) formalin (Sigma), permeabilized with 0.1% (v/v) Triton X-100 in PBS, and then blocked with 5% (v/v) bovine serum albumin for 1 h. The samples were then sequentially incubated with primary antibodies overnight at 4 °C and secondary antibodies for 1 h at room temperature. The primary antibodies used for the staining were as follows: mouse anti-Tuj1 (1:200, Cell Signaling, Danvers, MA, USA), rabbit anti-αMAP2 (1:200, Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-COL1A2 (1:50, Millipore, Temecula, CA, USA), and mouse anti-OPN (1:100, Santa Cruz Biotechnology). The secondary antibodies used were as follows: Alexa-Fluor 488 goat anti-mouse IgG antibodies (1:200, Invitrogen) and Alexa-Fluor 594 donkey anti-rabbit IgG antibodies (1:200, Life Technologies). Nuclei were counterstained with 4’,6-diamidino-2-phenylindole (DAPI, Sigma) and imaged by confocal microscopy (LSM 700, Carl Zeiss, Jena, Germany).

**Alizarin Red S Staining:** Osteogenic differentiation was analyzed by visualizing mineralization using Alizarin Red S staining. Differentiated hADSCs were fixed in 2.5% glutaraldehyde (Sigma) in PBS for 15 min at 37 °C. As a control, cells were cultured in the buffer supplemented with 10 × 10^−3 M KCl. Dlabeled Exo-R11 and PMNV-Ecad (5 μg cm^−2) were incubated with cells at 37 °C for 2 h for the inhibitor studies and for 3 h for the K− depletion study. To knockdown CHC, siRNA against CHC (Invitrogen) was transfected using LF2000 2 d before incubation with nanovesicles. Dlabeled Exo-R11 and PMNV were incubated with the cells at 37 °C for 2 h. After the cells were washed with PBS twice, fixed by 10% formalin, and incubated with DAPI for 10 min, the images were imaged by confocal microscopy (Carl Zeiss, Jena, Germany). All settings used for the imaging and processing were kept constant.

**In Vivo Imaging:** All animal experiments were performed in accordance with the Korean Food and Drug Administration guidelines. Experimental protocols for in vivo imaging and siRNA delivery were reviewed and approved by the Yonsei Laboratory Animal Research Center (Permit No: IACUC-201306-305-01). The biodistribution of the siRNA-containing nanovesicles was investigated using the Optix MX3 optical molecular imaging system (Advanced Research Technologies, Quebec, Canada). Briefly, Alexa-Fluor 647-conjugated siRNA (AF647-siRNA, Dharmacon) was encapsulated into Exo-R11 or PMNV by electroperoration, and 250 μL solution of the siRNA-containing nanovesicles (50 μg siRNA/mouse) was then intravenously administered to hairless mice (SHK1-1, 7 weeks old, Orient Bio, Sungnam, Korea). The fluorescence images of the whole body distribution were acquired at 670 nm excitation 15 and 150 min after injection. Two days after administration, 10 × 10^3 μL of blood. PBS-perfused livers were collected and homogenized in Trizol (Life Technologies, Carlsbad, CA, USA) and analyzed by real-time polymerase chain reaction (qRT-PCR) using the TaqMan gene expression assays. The APOB expression level in liver samples was determined with 95% (p < 0.05) and 99% (p < 0.01) confidence intervals.

**In Vivo APOB Silencing:** To induce in vivo silencing of APOB expression in mouse liver, BALB/c mice (female, 6–7 weeks old, Narabio, Pyungtaek, Korea) received 250 μL solution of nanovesicles (Exo, Exo-R11, and PMNV) loaded with siAPOB (2.75 mg nanovesicles kg^−1) via tail-vein injection. Two days after administration, the mice were anesthetized and the liver was perfused with PBS to remove the blood. PBS-perfused livers were collected and homogenized in Trizol reagent (Ambion, Austin, TX, USA) by tissuesyuler (Qiagen) according to the manufacturer’s instructions. The APOB expression level in each treatment group was determined by qRT-PCR with TaqMan gene expression assays.

**In Vivo Hepatotoxicity Evaluation:** To test the hepatotoxicity of the nanovesicles, mice were intravenously administered nanovesicles loaded with scrambled siRNA, and serum was taken through retro-orbital bleeding before and after siRNA administration. Exosomes were isolated from HEK293 cells, and PMNVs were isolated from primary embryonic mouse fibroblasts. The levels of AST, ALT, and bilirubin were determined for each serum sample using DRI-CHEM 4000i (Fujifilm, Tokyo, Japan) according to the manufacturer’s instructions.

**Statistical Analysis:** Quantitative data were expressed as the mean ± standard deviation. Significance was calculated using Student’s t-tests (GraphPad Software, La Jolla, CA, USA). Statistical significance was determined with 95% (p < 0.05) and 99% (p < 0.01) confidence intervals.
Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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