Original Article



# Delivery of vascular endothelial growth factor (VEGFC) via engineered exosomes improves lymphedema

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**Background:** Lymphedema is a chronic disease results from impaired flow of the lymphatic system. Therefore, reconstruction of lymphatic system is crucial to treat limb lymphedema. Vascular endothelial growth factor (VEGFC) has been reported to be an important regulator involved in the growth and differentiation of lymphatic endothelial cells; however; the application of exosomes with VEGFC in the treatment of lymphedema has been rarely reported.

**Methods:** From the membrane-based fusion technology, we constructed engineered exosomes that overexpress CD63-VEGFC fusion protein (CD63-VEGFC/exos). We examined the *in vitro* effects of CD63-VEGFC/exos on the proliferation, migration, and tube formation of human dermal lymphatic endothelial cells (HDLECs) by MTT assay, migration assay, and tube formation assay, respectively. CD63-VEGFC/exos were embedded in sodium alginate hydrogel and their effect on lymphedema was evaluated by a mouse model.

**Results:** VEGFC could be successfully delivered to lymphatic endothelial cells via engineered CD63-VEGFC/exos. Treatment with CD63-VEGFC/exos resulted in a significant increase in the proliferation, migration, and tube formation of lymphatic endothelial cells. Using CD63-VEGFC/egos in sodium alginate hydrogel enabled a sequenced release of exosomes and markedly improved lymphedema in a mouse model.

**Conclusions:** Our findings supply a novel adipose tissue-derived stem cell (ADSC)-exo-based strategy that delivers target proteins to lymphatic endothelial cells and thus enhances the treatment of lymphedema.

**Keywords:** Lymphedema; engineered exosomes; vascular endothelial growth factor (VEGFC); CD63; delivery system

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## Introduction

Lymphedema is a common postoperative complication of cancer or a result of a parasitic infection (1-3). Lymphedema is caused by the aberrant accumulation of interstitial fluid, which results in swelling and disability, mostly in the extremities (4). There are 140–250 million patients with limb lymphedema worldwide. Rebuilding the lymphatic duct is suggested to be crucial to treat lymphedema. Lymphatic venous anastomosis and vascularized lymph

node transfer are considered the critical approaches for lymphedema (5). However, lymphatic venous anastomosis often results in lymphatic occlusion, and rebuilding of the communication between the new lymph node and the original lymphatic system remains a significant challenge (6-9). Therefore, novel strategies still must improve the therapy of lymphedema.

Mesenchymal stem cells (MSCs) are adult stem cells with multidirectional differentiation and self-renewal and have been widely used in the treatment of many diseases (10-12). It has been reported that adipose tissue-derived stem cells (ADSCs) play an essential role in lymphangiogenesis (13,14). However, treatment with MSCs often causes a series of cell-related side effects, including changes in disease phenotypes and uncontrolled implanted cells, thus limiting its clinical application. Exosomes are a group of small membrane-bound vesicles (30–120 nm in diameter) secreted by multiple cell types. Exosomes are necessary communication tools between cells, which transfer mRNAs, miRNAs, and proteins to recipient cells and thus modulate their activities (15,16). Some note that exosomes derived from MSCs that can mimic the biological functions of MSCs and have a series of therapeutic effects on tissue regeneration, immune regulation, and reduction of tissue degeneration (10-13,16). MSCs derived-exosomes have been applied to research on myocardial infarction, stroke, limb ischemia, hypoxic-ischemic brain injury, kidney injury, and cartilage injury (15-19). However, the effect of MSC exosomes on lymphedema is still unknown.

Vascular endothelial growth factor (VEGFC) plays a critical role in the growth of lymphatic endothelial cells and is proposed to be a potential target for lymphedema treatment (20-23). However, how to deliver VEGFC to target cells remains a major issue. Engineered exosomes as endogenous delivery carriers exhibit low immunogenicity, can protect the activity and stability of proteins or nucleic acid molecules, and prolong the half-life of drugs compared with other carriers (15-19). In this study, we developed a strategy to produce engineered ADSC-exos for VEGFC delivery. To improve the loading of VEGFC in exosomes, VEGFC was fused with CD63, and the fusion construct was transfected into ADSCs. Engineered exosomes were then isolated, and the effects of CD63-VEGFC-exos on the proliferation, migration, and tube formation of lymphatic endothelial cells and lymphedema were investigated.

We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/atm-20-6605).

## Methods

Experiments were performed under a project license (SYXK-2020-0233) granted by The First Affiliated Hospital of Sun Yat-sen University, in compliance with Chinese national or institutional guidelines for the care and use of animals.

### *Isolation of ADSCs and cell culture*

Human adipose tissue-derived stem cells were purchased from Guangzhou Cyagen Biology. ADSCs are cultured in DMEM-low glucose, holding 10% fetal bovine serum (FBS). Human dermal lymphatic endothelial cells (HDLECs) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and kept in DMEM medium, holding 10% fetal FBS. All cells are cultured at 37 ℃ with 5% $CO_2$.

### *Extraction and characterization of ADSC exosomes*

DMEM/F12 medium was centrifuged at 100,000 g for 16 hours to prepare the exosome-free medium. ADSCs were cultured in an exosome-free medium for 48 hours. After infection, with lentivirus expressing different constructs, the conditioned medium was collected for exosome extraction. The medium was centrifuged at 3,000 g for 15 minutes, 2,000 g for 30 minutes, and 100,000 g for 70 minutes. The pellet was washed once with PBS and centrifuged again at 100,000 g for another 70 minutes. After fixation with 3% glutaraldehyde for 2 hours, exosomes were stained with 2% uranyl acetate for 30 seconds. The morphology of the exosome was analyzed by the transmission electron microscopy (TEM, Zeiss, Jena, Germany).

### *The uptake of exosomes*

The exosomes were labeled with PKH67 to assess the uptake of exosomes by HDLECs (Sigma, USA). Then, 2 μg of PKH67-stained exosomes were added to 6-well plates holding $1.5\times10^5$ HDLECs. Twenty-four hours later, cells were incubated with 4% paraformaldehyde for 20 minutes, and nuclei were stained with DAPI. The uptake of exosomes was visualized by a confocal microscope (Zeiss, Jena, Germany).

 

### *CCK-8 assay*

The CCK-8 assay was used to determine the effect of exosomes on the proliferation of HDLECs. Cells were seeded in 96-well plates and culture overnight. Cells were treated with ADSC-exos (10 μg/mL). After culturing for 24, 48, and 72 hours, a CCK-8 reagent was added to the plates. Then, the OD value was measured at a wavelength of 450 nm on a microplate reader.

### *Migration assay*

HDLECs in 100 μL medium were seeded in the upper chamber of 24-well Transwell plates to observe the effect of ADSC-exos on HDLEC migration (Corning, USA). Then, ADSC-exos (10 μg/mL) in 600 μL medium containing 1% FBS were added at the lower chamber. After incubation for 24 hours, cells on the top of the filter were swabbed off, and cells on the bottom were fixed in 4% paraformaldehyde and then stained with 0.05% crystal violet. Three random fields were selected, and the number of cells was counted.

### *Quantitative RT-PCR (qRT-PCR) analysis*

HDLECs were treated with 10 μg/mL ADSC-exos for 48 hours, and then total RNA of HDLECs was extracted using Trizol reagent (Life science, USA). RNA was transcribed to be cDNA using the Thermo Scientific RevertAid, First Strand, cDNA Synthesis Kit (Thermo), and PCR was performed using SYBR Green (Tiangen Biotech, Beijing, China). The PCR conditions were: 95 ℃ for 30 seconds, 40 cycles of 95 ℃ for 15 seconds, and 60 ℃ for 30 seconds. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serves as an internal control. The relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method.

### *Immunofluorescence assay*

Cells were fixed with 4% PFA and permeabilized with 0.5% Triton X-100 diluted in PBS for 10 minutes. Then, the cells were stained with DAPI and observed under a fluorescence microscope (Zeiss, Jena, Germany).

### *Construction of ADSC-exos-alginate*

Sodium alginate powder was dissolved in PBS to prepare a 2% (w/v) sodium alginate solution. Then 2% sodium alginate solution containing ADSC-exos (8 μg/mL) is mixed with 2% (w/v) calcium chloride solution and stirred gently to form ADSC-exos-alginate gels (control/exos-alginate, vector/exos-alginate, VEGFC-exos-alginate, and CD-63-VEGFC-exos-alginate).

### *The release of ADSC-exos from alginate hydrogel*

The release ratio of ADSC-exos in alginate hydrogel was tested with the fluorescent signal, as described previously (24). Briefly, ADSC-exos were stained with ExoGlow-RNA EV Labeling kit (System Biosciences). Then, 100 μL alginate hydrogels with 80 μg ADSC-exos were plated in a 96-well plate holding 100 μL PBS. After incubation at 37 ℃ for 0, 1, 3, 6, 12, and 24 hours, the supernatant was collected, and the fluorescence of the supernatant was detected. A standard curve of exosome concentration was set up for the gradient concentration of labeled ADSC-exos from 0.15–10 μg in 100 μL PBS. Then the fluorescent signal determines the released exosome according to the standard curve.

### *Establishment of mouse tail lymphedema model*

The mouse tail lymphedema model was established as described before (25). Intraperitoneal injection anesthetized the 6-week-old C57BL/6J female mouse with 8% chloral hydrate. After anesthesia, a circular incision was made at 1 cm from the base of the tail. Then, the skin of the tail was removed (~2 mm in deep). The major lymphatic trunks were found through a distal intradermal injection of 5 μL of methylene blue. Under an operating microscope, the blue-stained lymphatic vessels were removed, and the cut ends, and the skin of the wound margin was cauterized to prevent the lymphatic vessels from re-canalizing.

### *Immunohistochemistry*

Sections were prepared from the injured distal tissues of mice, incubated with 3% hydrogen peroxide in methanol for 30 minutes to block the endogenous peroxidase, and boiled in a citrate buffer for antigen retrieval. After blocking with 10% goat serum, the sections were incubated with the primary antibody at 4 ℃ overnight. Then the sections were incubated with biotinylated goat-anti-rabbit secondary antibody for 30 minutes at room temperature. A horseradish peroxidase-streptavidin reagent (ImmunoCruz system, Santa Cruz Biotechnology) was used to develop the color. Then the sections were counter-stained with hematoxylin to visualize the nuclei.

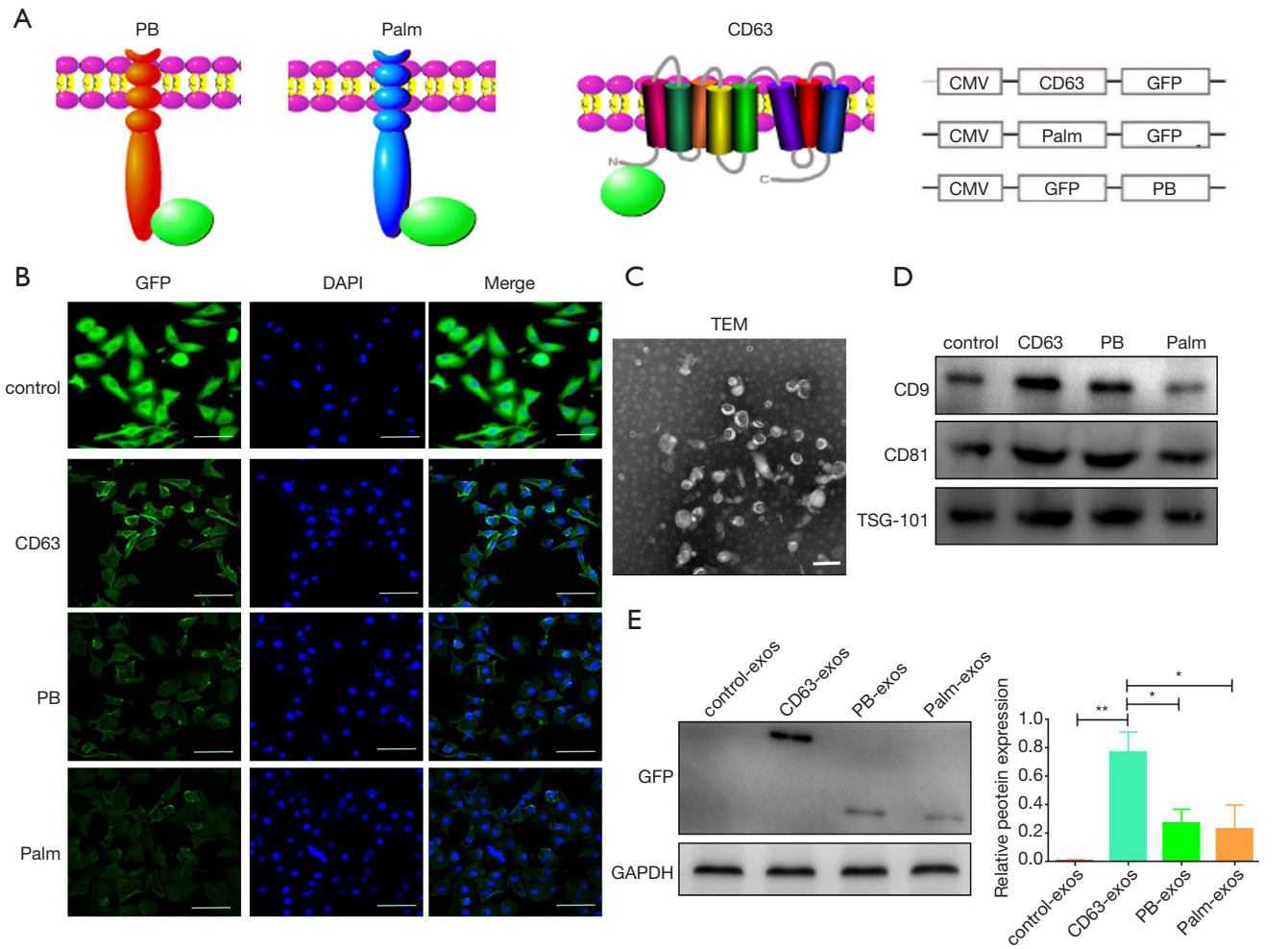


**Figure 1** Comparison of loading efficiency of three different engineered exosomes. (A) Schematic diagram of the construction of three vectors expressing GFP fusion protein. (B) The expression of GFP in 293T transfected with three different GFP fusion constructs was determined by immunofluorescence staining (200×). (C) Exosomes extracted from 293T were characterized by transmission electron microscopy. (D) A Western blot test detected the expression of exosome markers. (E) A Western blot test determined the expression of GFP in different engineered exosomes. *, P<0.05, **, P<0.01. ADSCs, adipose tissue-derived stem cells.

### *Statistical analysis*

All data were presented as mean ± SD. A comparison among distinct groups was performed using the one-way ANOVA. Statistical significance was considered the P value lower than 0.05.

## Results

### *CD63 improves GFP expression on the membrane*

To find the ideal elements that could improve the loading of target molecules, GFP, a green fluorescent protein, was the endogenous cargo target. Two membrane proteins (PB, Palm) and a transmembrane protein (CD63) were cloned into the lentiviral vector pLVX-GFP to produce fused proteins CD63-GFP, GFP-PB, and Palm-GFP (*Figure 1A*). 293T cells are transduced with lentivirus expressing different fused constructs. Immunofluorescence staining showed that the density of GFP on the membrane was highest in the CD63 group, followed by PB and Palm groups (*Figure 1B*). Then, exosomes were isolated from 293T cells transduced with different lentiviruses (*Figure 1C,D*). Western blot analysis showed that CD63/exos had the highest level of GFP, followed by PB/exos and Palm/exos

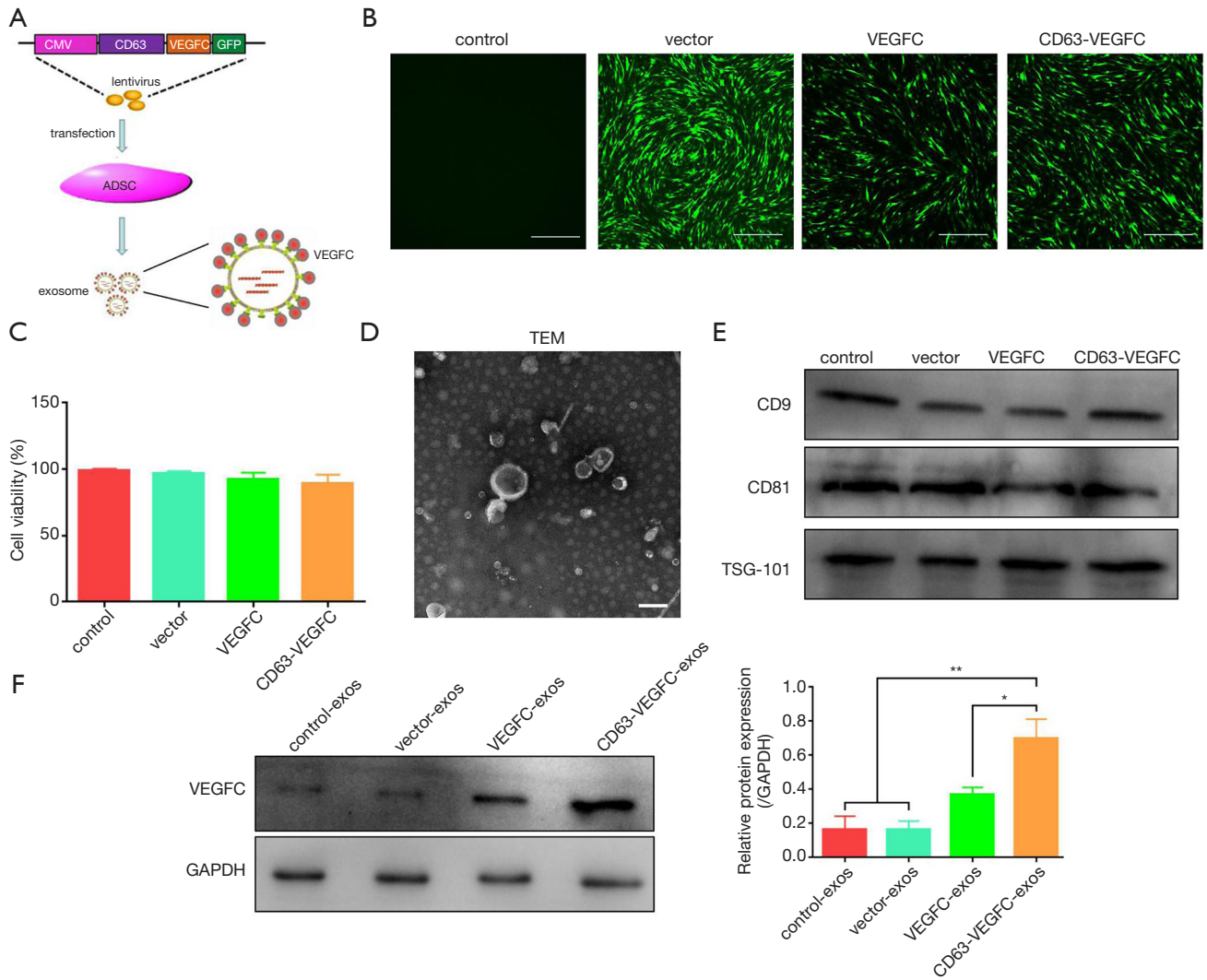


**Figure 2** Characterization of engineered ADSC exosomes. (A) Schematic diagram of the construction of CD63-VEGFC-exos. (B) The expression of VEGFC in ADSCs transduced with different lentiviruses is determined by immunofluorescence staining (100×). (C) The CCK-8 assay examined the viability of ADSCs. (D) The morphology of exosomes from ADSCs is characterized by transmission electron microscopy. (E) A Western blot test detected the expression of exosome markers. (F) A Western blot test examined the expression of VEGFC in exosomes from ADSCs. *, P<0.05, **, P<0.01. ADSC, adipose tissue-derived stem cell; VEGFC, vascular endothelial growth factor.

(*Figure 1E*). Therefore, CD63 was selected as the element for further construction of exosomes.

### *Engineered exosomes enhance the loading of VEGFC*

VEGFC is a critical regulator in lymphedema (20-23). To efficiently deliver VEGFC to the target cells via exosomes, we fused VEGFC to the C-terminal of CD63 and then subcloned to the pLVX-GFP vector (*Figure 2A*). ADSCs stably expressing CD63-VEGFC were set up by transduction with lentivirus (*Figure 2B*). A CCK-8 assay showed that lentivirus infection did not affect the viability of ADSCs (*Figure 2C*). The morphology of exosomes extracted from ADSCs was verified by TEM (*Figure 2D*). Also, the expression of exosome markers (CD9, CD91, TSG-101) was confirmed by a Western blot test (*Figure 2E*). When examining the loading of VEGFC in exosomes, we found the protein level of VEGFC was significantly greater in CD63-VEGFC/exos compared with the control groups (control/exos, vector/exos, and VEGFC/exos) (*Figure 2F*).

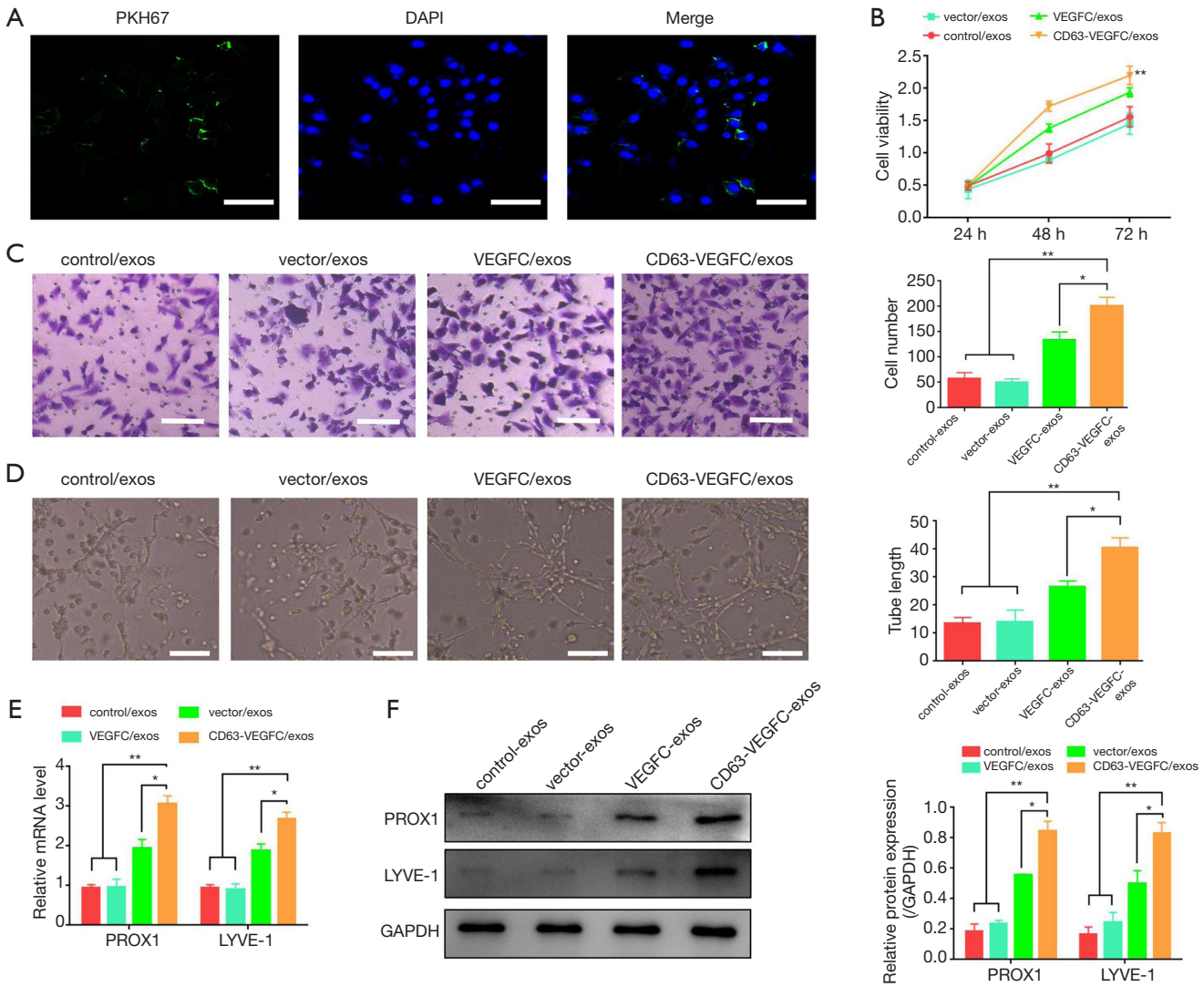


**Figure 3** Engineered exosomes promote the proliferation, migration, and tube formation of lymphatic endothelial cells. (A) The uptake of exosomes by lymphatic endothelial cells is determined by immunofluorescence imaging (200×). (B) The CCK-8 assay determined the effect of CD63-VEGFC-exos on HDLEC viability. (C) The Transwell assay determined the effect of CD63-VEGFC-exos on HDLEC migration. (D) The effect of CD63-VEGFC-exos on HDLEC tube formation was determined by tube formation assay. (E,F) The expression of lymphatic vessel markers (PROX1, LYVE-1) was determined by qRT-PCR and Western blot, respectively. *, P<0.05, **, P 0.01. VEGFC, vascular endothelial growth factor; HDLEC, human dermal lymphatic endothelial cell; qRT-PCR, quantitative RT-PCR.

Together, these data showed that engineered exosomes could effectively increase the loading of VEGFC.

### *Engineered exosomes promote the proliferation, migration, and tube formation of lymphatic endothelial cells*

First, we examined whether HDLECs could take up ADSC-exos. ADSC-exos were labeled with PKH67 dye and then incubated with HDLECs. Immunofluorescence staining showed that several green spots were observed in HDLECs (*Figure 3A*), suggesting uptake of ADSC-exos. Viability analysis revealed that treatment with CD63-VEGFC/exos resulted in a significant increase in the growth of HDLECs compared with the controls (control/exos, vector/exos, and VEGFC/exos) (*Figure 3B*). A Transwell migration assay also showed CD63-VEGFC/exos remarkably elevated the

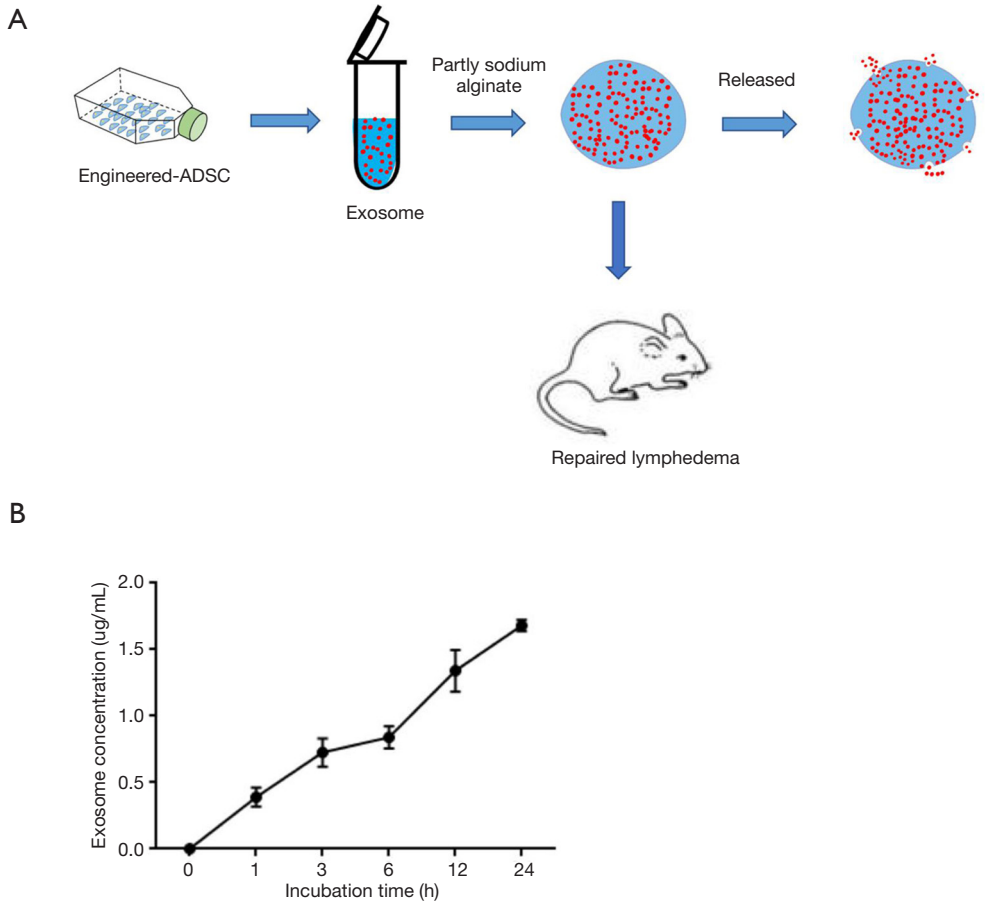


**Figure 4** Incorporation of ADSC-exos with alginate hydrogel. (A) Schematic diagram of the construction of ADSC-exos-alginate. (B) The release rate of exosomes from ADSC-exos-alginate. ADSC, adipose tissue-derived stem cell.

migration of HDLECs, which was followed by VEGFC/exos, vector/exos, and control/exos (*Figure 3C*). HDLECs treated with CD63-VEGFC/exos formed more tubes compared with the controls (control/exos, vector/exos, and VEGFC/exos) (*Figure 3D*). The mRNA and protein expressions of lymph tube markers (PROX1, LYEV-1, ERK and p-ERK) were greatly increased after treatment with CD63-VEGFC/exos compared with the controls (control/exos, vector/exos, and VEGFC/exos) (*Figure 3E,F*, Figure S1). Together, these results showed that CD63-VEGFC/exos have a positive effect on lymphatic endothelial cell proliferation, migration, and tube formation.

### *Engineered exosomes improve lymphedema in a mouse model*

To immobilize the engineered exosomes to the injury area, we first embedded the CD63-VEGFC/exos into the alginate hydrogel (*Figure 4A*). CD63-VEGFC/exos were labeled with the ExoGlow RNA probe, and the number of exosomes was determined by fluorescence density. The result showed that the incubating time increased, CD63-VEGFC/exos gradually released from the alginate hydrogel (*Figure 4B*).

We then investigated whether CD63-VEGFC/exos embedded in alginate hydrogel had a beneficial effect on lymphedema. An acquired mouse tail lymphedema model was set up via microsurgical ablation. Engineered exosomes embedded in alginate hydrogel were injected into the subcutaneous edema area of the mouse tail. Forty-two days after the operation, the diameter of the mouse tail was measured. The result showed that treatment with CD63-VEGFC/exos-alginate markedly reduced the diameter of the mouse tail compared with the controls (*Figure 5A*). The mRNA and protein levels of lymph tube markers (PROX1

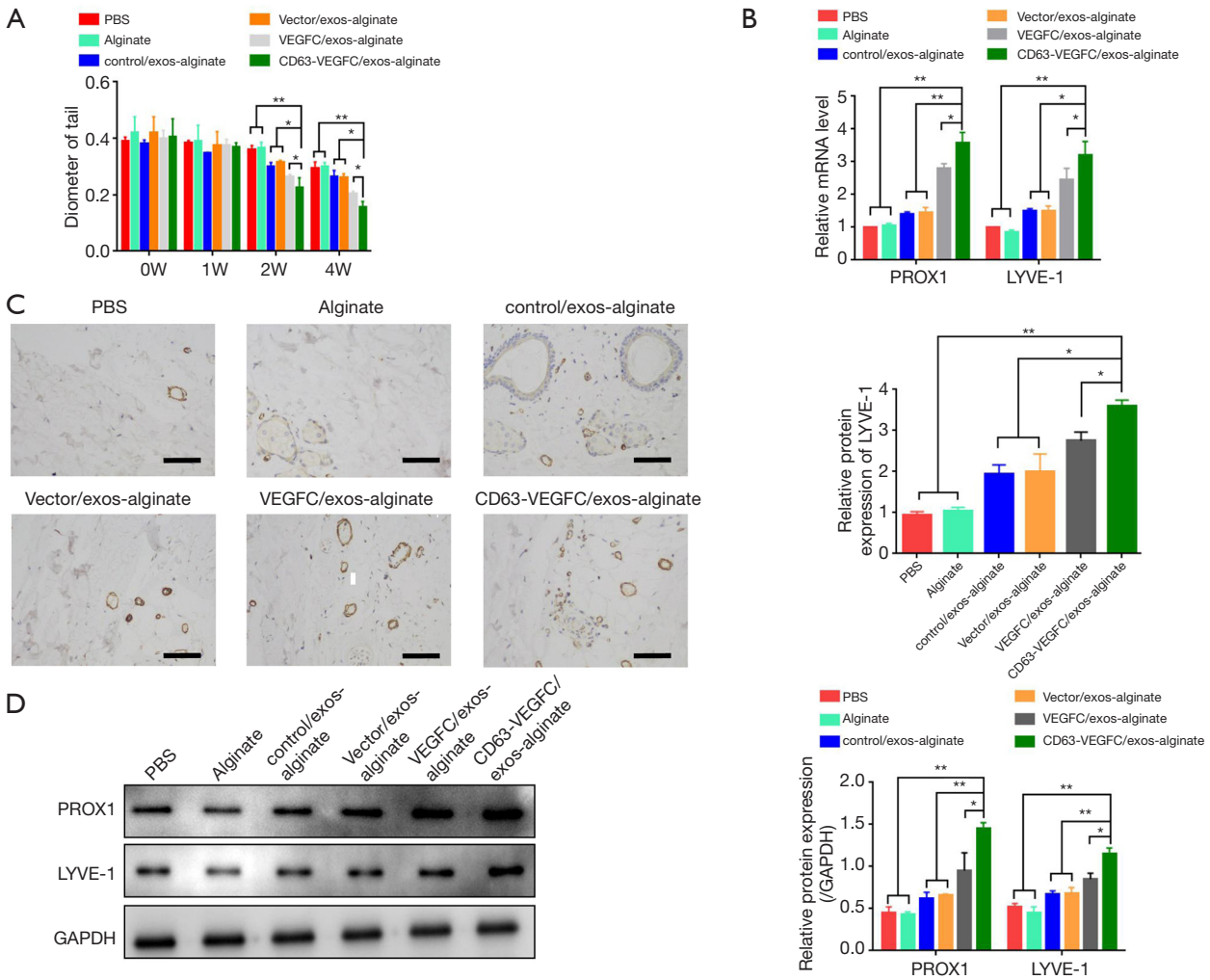


**Figure 5** The effect of CD63-VEGFC-exos-alginate on mouse lymphedema. (A) The diameter of the injured area of the tail in mice treated with ADSC-exos-alginate. (B) qRT-PCR analysis of the expression of lymphatic vessel markers (PROX1, LYVE-1) in the tail tissues of mice treated with ADSC-exos-alginate. (C) Immunohistochemical analysis of LYVE-1 expression in the tail tissues of mice treated with ADSC-exos-alginate. (D) Western blot analysis of the expression of lymphatic vessel markers (PROX1, LYVE-1) in the tail tissues of mice treated with ADSC-exos-alginate. *, P<0.05, **, P<0.01, ***, P<0.001. VEGFC, vascular endothelial growth factor; ADSC, adipose tissue-derived stem cell; qRT-PCR, quantitative RT-PCR.

and LYEV-1) were also significantly increased in the CD63-VEGFC/exos-alginate group compared with the control groups, as showed by qRT-PCR, immunohistochemistry, and Western blot (*Figure 5B,C,D*). Collectively, these data showed that engineered CD63-VEGFC/exos could improve lymphedema *in vivo*.

## Discussion

Reconstruction of the structure and function of the endogenous lymphatic system is vital for the effective treatment of lymphedema. However, strategies that can improve the reconstruction are still lacking. In the present study, we developed a strategy to produce engineered exosomes, which simultaneously deliver VEGFC to improve lymphatic reconstruction and thus enhance the treatment of lymphedema.

Lymphatic vessel formation is a complicated process that involves the proliferation, migration, and tube formation of lymphatic endothelial cells. Several chemokines, growth

factors, adhesion molecules, and extracellular matrix regulators are implicated in this process. Among them, VEGFC is considered the primary modulator. It has been shown that VEGFC promotes the differentiation of lymphatic endothelial cells and thus the formation of lymphatic vessels (20-23). Animal studies have also shown that lentivirus or adenovirus-mediated overexpression of VEGFC improves lymphedema (26). However, the instability/degradation of VEGFC, low delivery efficiency, and the short residence rate, limiting the efficacy. Therefore, developing an efficient delivery strategy to transport VEGFC to target cells is significant in lymphatic vessel regeneration.

Exosomes derived from MSCs are suggested to be a valuable tool for target delivery and display therapeutic potential in many diseases. For example, it has been reported that ADSC-secreted exosomes with polypyrrole titanium significantly enhance osteogenesis *in vivo* (27). In a diabetic model, the delivery of SPION-conjugated BAY55-9837 by exosomes significantly increases the secretion of insulin and improves the treatment of type 2 diabetes mellitus (28). These results show that exosomes have potential applications as transport carriers for targeted therapies. However, cargo loading is still a significant challenge for exosome-based delivery. There are two significant ways of loading drugs/targets to exosomes: electroporation/ultrasound and exosome formation (29). Exosomes from different cell types have drug/target selectivity. Thus, only overexpressing specific molecules may not improve the loading. Many studies have revealed that exosome membranes are originated from cell membranes. Thus, co-overexpression of membrane proteins, including CD9, CD63, Lamp2, could positively enrich RNA and proteins in exosomes and improve the delivery efficiency (30-32). Consistently, we showed that fusing VEGFC with CD63 could significantly increase the expression of VEGFC in ADSC-exos. CD63-VEGFC-exos could significantly enhance the proliferation, migration, and tube formation of lymphatic endothelial cells.

Although exosomes play a vital role in drug delivery, low retention rates and short half-life remain significant challenges. Incorporation of exosomes in hydrogel appears as a promising strategy against these problems. Alginate is a type of natural linear polysaccharide extracted from brown algae and has been widely used in drug delivery and tissue engineering because of its biocompatibility and similarity with the extracellular matrix (33,34). For instance, the delivery of MSC-derived small extracellular vesicles (sEVs) embedded in alginate hydrogel highly improves cardiac function (35). Sequential delivery of VEGF-A (165), followed by PDGF-BB with alginate hydrogels, improves angiogenic effect (36). In agreement with these studies, we found that incorporating CD63-VEGFC/exos in sodium alginate hydrogel reduced mouse lymphedema substantially. Therefore, incorporation of exosomes carrying VEGFC in alginate hydrogel might offer a novel strategy for the therapy of lymphedema. However, the stability and safety of CD63-VEGFC/exos are still needed to be clarified in future studies before further applications.

## Conclusions

In this study, we reported, for the first time, the effect of engineered ADSC-exos in the treatment of lymphedema. Our data showed that CD63-VEGFC/exos remarkably promoted the proliferation, migration, and tube formation of lymphatic endothelial cells. Incorporation of CD63-VEGFC/exos in sodium alginate hydrogel significantly improved lymphedema in mice. Our findings supply a novel ADSC-exo-based strategy for treating lymphedema.

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## Footnote

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*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related

 

to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The procedure was proved by the Research Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University. All the volunteers provided informed consent. Experiments were performed under a project license (SYXK-2020-0233) granted by The First Affiliated Hospital of Sun Yat-sen University, in compliance with Chinese national or institutional guidelines for the care and use of animals.



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