αvβ3-Targeted sEvs for Efficient Intracellular Delivery of Proteins Using MFG-E8

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Abstract

Background: Small extracellular vesicles (sEvs) are nanometer-sized membranous particles shed by many types of cells and can transfer a multitude of cargos between cells. Recent studies of sEvs have been focusing on their potential to be novel drug carriers due to natural composition and other promising characteristics. However, there are challenges in sEvs-based drug delivery, one of which is the inefficient loading of drugs into sEvs, especially for large biomolecules.

Results: In this study, we proposed a membrane-associated protein milk fat globule–epidermal growth factor 8 protein (MFG-E8) to produce αvβ3-targeted sEvs with high delivery efficiency of interested protein. MFG-E8 is a secreted protein with NH2-terminal epidermal growth factor (EGF)–like domains, containing an Arg-Gly-Asp sequence that binds αvβ3 and αvβ5 integrins, and COOH terminal domains C1 and C2, which can bind to lipid membrane with strong affinity. Firstly, we transiently expressed MFG-E8 in HEK293F cells and found that this protein could be secreted and adhere to the cell membrane. The recombinant MFG-E8 is also secreted into sEvs and located at the outer membrane of sEvs. Then we generated engineered sEvs by expressing high levels of the EGFP fused to MFG-E8 in HEK293F cells and showed that MFG-E8 could increase the delivery efficiency of EGFP into sEvs. Further delivery of Gaussia luciferase（GL） by fusion expression with MFG-E8 in donor cells demonstrated that target proteins fused with MFG-E8 still kept their activity. Finally, we identified the sEvs' target to αvβ3 by comparing the transfection efficiency with MFG-E8 loaded sEvs(Exo-MFG-E8) in αvβ positive cells and αvβ3 negative cells. Analysis showed higher target protein could transfect into αvβ positive cells with Exo-MFG-E8 than with sEvs only with EGFP(Exo-EGFP), meaning the engineered sEvs with MFG-E8 not only could target the αvβ positive cells, but also could increase the delivery of target protein into sEvs.

Conclusion: this study suggests that recombinant MFG-E8 is an ideal protein to increasingly deliver the drug into sEvs and give sEvs the ability to target the αvβ positive cells.

Background

Small extracellular vesicles (sEvs) are membranous vesicles with 40-120nm in diameter released by a variety of cells. They are thought to play a key role in cell-to-cell communication by transporting a multitude of cargos between cells, including mRNAs, proteins, microRNA (miRNA), non-coding RNAs, and DNA, impacting on many physiological and pathological cellular processes, such as immune response, inflammation, cancer progression, and et al.[1–8]. sEvs have also been detected as diagnostic, prognostic, and treatment monitoring biomarkers[9, 10]. In recent years, sEvs have been studied as potential therapeutic agents and viable vaccines in clinical immunotherapy[6, 10–13].

sEvs also have the potential to function as drug delivery vehicles because of their natural composition. Comparing with other nanoparticles such as liposomes or polymeric nanoparticles, sEvs are superior in that: 1. they have low immunogenicity due to small size and the same bilayer cellular membrane as human cells; 2. they have high permeability to migrate through various biological barriers, such as mucosal and blood-brain barrier. 3. They are more stable than artificial nanoparticles in the circulation system because they can bypass complement activation to avoid phagocytosis and degradation. 4. Furthermore, the loading of hydrophobic compounds into sEvs was found to be higher than in liposomes[14–17].

sEvs have been exploited for therapeutic drug delivery as seen in Curcumin[18–20], and Tumor chemotherapeutic agents including doxorubicin (Dox) or paclitaxel (PTX)[21, 22]. The capability of delivering exogenous RNAs, especially siRNA, has also been under several investigations[23–25]. Currently, there are two different approaches for loading drugs into sEvs: exogenous (i.e. after sEvs isolation) and endogenous loading (i.e. during sEvs biogenesis)[26]. For exogenous loading of sEvs, different techniques have been employed, including incubation at room temperature, permeabilization with saponin, freeze-thaw cycles, sonication, or extrusion[27–29]. However, these techniques could result in the aggregation of sEvs or their cargo and even alteration of their physicochemical or morphological characteristics[30]. Moreover, these aforementioned technics are less promising for functional proteins because of their larger molecular weight[31]. On the other hand, the endogenous approach is more suitable for protein loading, where sEvs can be loaded during biogenesis via direct transfection of a recombinant vector with genes of interested protein. After synthesized, the recombinant protein is sorted into sEvs with other cytosolic constituents.

Because the sorting mechanism of cytosolic protein into sEvs is poorly understood, a strategy of efficient loading is to fusion the therapeutic protein with proteins enriched in sEvs, such as CD63, CD9 and et al[31–34]. In this report, we proposed a membrane-associated protein milk fat globule–epidermal growth factor 8 protein(MFG-E8) to deliver proteins into sEvs. MFG-E8 is a secreted protein with three functional domains: NH2-terminal epidermal growth factor (EGF)–like domains, which contain an Arg-Gly-Asp sequence that binds αvβ3 and αvβ5 integrins, and COOH terminal domains C1 and C2, which can bind to lipid membrane with strong affinity[35, 36]. MFG-E8 was found to be abundant in sEvs secreted by many kinds of cells[37, 38]. We sought to transient express the exogenous proteins by fusion it with MFG-E8 in HEK293 cells and to dress exogenous proteins onto sEvs with the C1C2 domain of MFG-E8. Meanwhile, the (EGF)–like domains could target the sEvs to cells with overexpression of αvβ3 and αvβ5 integrins.

Methods

Cell culture

For the suspension culture, HEK293F cells (ATCC, ACS-4500™) were cultivated in 50-mL Tubespin containing 10 mL ProCHO5 medium (Lanza Co.) at a density of 0.5×10^6cells/mL. Cultures were maintained in a shaking incubator at 37°C with stirring speed at 180 rpm. For adherent cell culture, HEK293T cells (ATCC, CRL-11268™) were incubated in 5mL DMEM medium containing 10% fetal calf serum (Gibco) in an incubator at 37°C and cells were passaged twice a week. The cell density and viability were determined by the Trypan Blue exclusion method.

Plasmids

All plasmids were constructed with plasmid pCDNA3.4 (Invitrogen co.), including pCDNA 3.4/MFG-E8, pCDNA3.4/MFG-E8-EGFP, pCDNA3.4/EGFP, pCDNA3.4/MFG-E8-Gaussia luciferase(GL), and pCDNA3.4/CD9-GL. After digestion with KpnI and XhoI, the open reading frame (ORF) of MFG-E8, EGFP, MFG-E8-GL or CD9-GL was inserted into the multiple cloning site (MCS) of pCDNA3.4. For fusion expression, a flexible linker (GGGGSGGGGSGGGGS) was used to link DNA sequences of two genes.

Transient expression of protein in HEK293F cells

One day prior to transfection, HEK 293F Cells were seeded in fresh ProCHO5 medium at a density of 2×10^6 cells/mL. On the day of transfection, cells were centrifuged at 800 rpm for 5 min and resuspended in 2mL RPMI1640 media at the indicated cell density in TubeSpins. The plasmid DNA and 25 kDa linear polyethyleneimine (PEI, Polysciences, Warrington, PA) were mixed and stood for 10min, and then added to the culture. The transfected culture was first incubated for 3h at 37°C with 5% CO2, 85% humidity, and agitation at 180 rpm, followed by adding EX-Cell HEK293 medium (Sigma) to 10mL.

sEvs isolation.

Cell culture medium was collected and centrifuged at 3,000g for 15 min to remove cellular debris, and the supernatants were transferred to an appropriate vessel for the MF-600 ultracentrifuge (Hanil Science Industrial, Incheon, Korea) according to the method described by Théry C[46]. Successive centrifugations at increasing speeds were performed to throw the pellet away (300g for 10 min- 2000g for10 min–10,000g for 30 min). In the last step, the supernatant was collected and centrifuged one more time at 100,000g for 70 min and only the pellets were kept. The pellet was washed in a large volume of PBS for three times to eliminate contaminating proteins, and centrifuged one last time at the same high speed. The final sEvs pellets were resuspended in 100 ml PBS and filtered through a syringe filter (0.2 mm, Sartorius). The morphology of sEvs was observed by Transmission Electronic Microscopy (FEI co., CZ). The number and size of sEvs particles were measured by nanoparticle-tracking analysis with a Nanosight NS300 (Malvern Instruments).

Western Blotting.

Cells were cultured for up to 72 or 96h post-transfection before the supernatant was collected. Cell lysates and isolated sEvs were subjected to 12% SDS-PAGE and western blotting according to standard protocols. Western blots were incubated at 4℃ for 16 h with the indicated primary antibodies against MFG-E8, EGFP, CD9, CD63, luciferase or αvβ3 (Invitrogen, Cat.No. PA5-82036; Proteintech, Cat.No. 66002-1-Ig; 60232-1-Ig; 67605-1-Ig; 67293-1-Ig; Abcam, Cat.No. ab190147) and then washed for three times in Tris-buffered saline T (TBS-T), followed by 1 h incubation with Goat Anti-Mouse IgG(H + L) (Proteintech, Cat.No. SA00001-1) at room temperature.

Confocal microscopy

Cells were successively incubated with 4% paraformaldehyde, 0.25% TritonX-100, and 1% BSA. At the end of each step, cells were centrifuged and washed with PBS buffer. In the end, cells were incubated with anti-M8 antibodies solution for 12h at 4℃, followed by incubation with Goat Anti-Mouse IgG H&L (FITC) (Abcam, Cat.No. ab6785) and DAPI solution respectively, then observed by Zeiss laser confocal microscopy.

Transmission Electronic Microscopy (TEM) analysis with gold nanoparticles labeled sEvs

The preparation of gold nanoparticles (AuNPs) and gold nanoparticles labeled mAb (AuNPs-mAb) was done according to reference [47]. Briefly, 1 mL of 1% HAuCl4 was quickly added to the 50mL boiled ultrapure water and 1.2 mL trisodium citrate dihydrate (10 mg/mL) was added after a few seconds. The mixture was heated for 10 min, and then diluted with ultrapure water to 50 mL.

For preparation of AuNPs-antibody, 1mL of AuNPs (0.02mg/mL) was adjusted to pH 8.5 with 0.25M K_2CO_3, and 10μL anti-MFG-E8 antibody was diluted with 1×TBST to 100μL. Then the antibody was quickly added to the AuNPs solution. The mixture was rotated for 15min and kept still for 15 min at room temperature. Subsequently, 100 μL of 10% BSA were added to cover the unconjugated site, and rotated for another 15 min and then kept still for 15 min. Finally, the mixture was centrifuged at 12000 rpm for 30 min and the precipitate was resuspended in 50–100 μL PBS, followed by incubation with isolated sEvs overnight at 4℃. 10μL of labeled sEvs was dropped on GRID,and applied to TEM after air-drying.

Gaussia Luciferase (GL) activity analysis

The GL-loaded sEvs were rinsed with 100μL/well of 1X DPBS buffer, followed by cell lysis by adding 50–100μL/well of 1X Cell Lysis Buffer and shaking for 15-30min.

Gaussia Luciferase (GL) activity was analyzed according to the protocol Pierce™ Gaussia Luciferase Glow Assay Kit (Thermo Scientific). In brief, the Working Solution was prepared by adding 50μL of 100X Coelenterazine to 5mL of Gaussia Glow Assay Buffer firstly. 10–20μL/well of cell lysate was added to an opaque 96-well plate, then 50μL of Working Solution was added to each well. After 10 minutes for signal stabilization, we detect the light output in a Luminometer (Berthold, Bad Wildbad, Germany).

Flow cytometry

To confirm whether recombinant MFG-E8 adhered to the cell membrane, cells were adjusted in advance and cultured in an incubator at 37°C with 5% CO_2. Then $1*10^6$ cells were placed in a 1.5 ml tube. The cells were centrifuged at 1000*g for 5 mins and washed thrice with 1x PBS. Next, the cells were resuspended in 100μl PBS., 5μl of the anti-MFG-E8 antibody was added to the cell suspension. The sample was mix thoroughly at 37 ℃ in the dark for 30 mins. Then the sample was centrifuged at 1000*g for 5 mins and washed thrice with 1x PBS. Finally, 5μl anti-Mouse IgG H&L(FITC) was added to the cell suspension, and the sample was mix again at 37 ℃ in the dark for 30 mins. The sample was centrifuged at 1000*g for 5 mins and washed thrice with 1x

PBS. The cells were resuspended in 500 μL of PBS and examined using flow cytometry. To demonstrate sEvs with MFG-E8 had αvβ 3 targeting, cells were placed in a 1.5 ml tube after incubating with MFG-E8-EGFP-sEvs of EGFP-sEvs. The cells were centrifuged at 1000*g for 5 mins and washed thrice with 1x PBS. Then, the cells were resuspended in 500 μL of PBS and examined using flow cytometry.

Results

recombinant MFG-E8 secreted from host cells but retained outside of cell

The recombinant plasmid with an MFG-E8 protein-coding gene and a signal peptide sequence was constructed and transfected into HEK293F cells to transiently express the recombinant MFG-E8. On day 4 of post-transfection, cell culture was harvested and analyzed by Western Blot. MFG-E8 could be expressed in 293F cells successfully(Fig. 1A). However, most of the protein existed in the cell debris with few found in the supernatant of cell culture, meaning few proteins secreted outside the host cells despite MFG-E8 has a signal peptide in the N-terminal (Fig. 1B).

Because MFG-E8 contains C1 and C2 domains which can bind to the lipid of cell membrane[35, 36], we sought to confirm if recombinant MFG-E8 adhered to the cell membrane. We incubated the cells with anti- MFG-E8 antibody and anti-Mouse lgG H&L(FITC)(green) as a second antibody, while using DIL for cell membrane(red). We analyzed the mixture by flow cytometry and laser confocal microscope. It was found that MFG-E8 protein (green) was located outside the cell membrane under a confocal microscope (Fig. 1C) and about 40 % of the cells were FITC-positive indicated by flow cytometry analysis (Fig. 1D). Results above showed that the recombinant MFG-E8 was secreted and attached to the outside of the cell membrane.

MFG-E8 could enter sEvs and linked to outside of sEvs membrane

The sEvs were isolated from the supernatant of cell culture by successive ultracentrifugation at increasing speeds, followed by multiple times of washing to further eliminate the contaminating proteins.

Nanoparticle-tracking analysis (NTA) showed that most of the vesicles in precipitate had a size of 120nm approximately, corresponding to the range of described sEvs (Fig. 2A). The existence of sEvs was further confirmed by exosomal protein marker CD9 in these vesicles. We also observed MFG-E8 in these sEvs (Fig. 2B). Although MFG-E8 was an exosomal protein, the concentration of it in sEvs from blank HEK 293F cells is lower than that in sEvs secreted from donor cells with overexpression of MFG-E8.

In order to investigate the location of MFG-E8 in sEvs, we examined sEvs under transmission electron microscopy (TEM) after incubating sEvs with anti-MFG-E8 antibodies labeled AuNPs (anti-MFG-E8-AuNPs-mAb). Both the control sEvs and sEvs with MFG-E8 exhibited cup-shaped bilayer membranes and were measured about 100nm in diameters. Additionally, the anti-MFG-E8-AuNPs-mAb was shown as the black dots under TEM. Because the anti-MFG-E8-AuNPs-mAb was too large to enter into sEvs, it could bind with MFG-E8 only when it outside the membrane of sEvs. We found that the black dots were existed in the sEvs, indicating that MFG-E8 should be located in the outside of the sEvs (Fig. 2C).

Fusion of MFG-E8 to Target Proteins Results in Efficient Loading into sEvs

In order to investigate if MFG-E8 could address other proteins into sEvs, we transfected HEK293F cells with pCNDA3.4/MFG-E8-EGFP to express this fusion protein, meanwhile using pCNDA3.4/ EGFP as a control. sEvs were isolated at day 4 of post-transfection as the aforementioned way. Both MFG-E8 and EGFP were found in sEvs secreted from HEK293 cells transfected with pCNDA3.4/MFG-E8-EGFP and pCNDA3.4/ EGFP. The concentration of EGFP in sEvs from pCNDA3.4/MFG-E8-EGFP transfected cells was much higher than that in sEvs from pCNDA3.4/ EGFP cells (Fig. 3A). The mechanism of protein sorting into sEvs remained unclear, but MFG-E8 could address more EGFP into sEvs by fusion expression.

Since the exosomal membrane has the same component as the cell membrane, sEvs can automatically fuse with the membrane of target cells to transfect its contents. sEvs were thought to be a good natural transfection reagent and drug carrier because of their high transfection efficiency and good biocompatibility. To confirm this, HEK 293T cells were incubated with sEvs loaded with MFG-E8-EGFP. At 4-6h of post-transfection, the fluorescence of EGFP was found in recipient cells under confocal microscopy (Fig. 3B).

The results above showed MFG-E8 could address other proteins into sEvs, and then could mediate protein delivery to recipient cells by sEvs transfection.

The target protein delivered into sEvs by MFG-E8 remained its activity.

Although MFG-E8 could carry the target proteins into sEvs in a protein fusion manner, whether the proteins in sEvs were active or not could not be demonstrated by the above-mentioned experiment. Therefore, we proceeded to test if proteins remained active after delivered into sEvs. Gaussia Luciferase (GL) was chosen to be a reporter protein because it could catalyze its substrate to emit fluorescence only when luciferase is active. To achieve this goal, the plasmid pCDNA3.4/MFG-E8-GL was constructed, while pCDNA3.4/CD9-GL was used as a positive control because CD9 is a known exosomal protein that usually served as a protein carrier into sEvs.

HEK293F cells were transfected with these two plasmids respectively and sEvs were isolated in an aforementioned way. MFG-E8-GL(M8-GL) and CD9-GL were confirmed in these two kinds of sEvs using Gaussia luciferase antibodies (Fig. 4A and B). With CD63 as a reference protein of sEvs, we compared relative GL protein concentration in sEvs (GL/CD63). The average value of M8-GL/CD63 (7.52) was significantly higher than CD9-GL/CD63 (3.2) ($p < 0.01$), indicating MFG-E8 had higher efficiency to deliver target proteins into sEvs (Fig. 4C). Finally, we detected the luciferase activity based on the catalytic activity of its substrate Coelenterazine. It was shown that both M8-GL and CD9-GL in sEvs were active to catalyze Coelenterazine, the activity of M8-GL was higher than CD9-GL (Fig. 4D). All above demonstrated the target protein delivered into sEvs by MFG-E8 remained its activity.

sEvs with MFG-E8 had αvβ 3 targeting

There is an Arg-Gly-Asp sequence in NH2-terminal epidermal growth factor (EGF)–like domains of MFG-E8, which could bind with αvβ3 and αvβ5 integrins that are usually overexpressed in some tumor cells. MFG-E8 was demonstrated in our study above to exist outside exosomal membrane, so we identified whether the sEvs with MFG-E8 could target αvβ3 and αvβ5 integrins.

We screened several types of cells to find αvβ3-positive and αvβ3-negative cells. It was shown that A549, human lung adenocarcinoma cells have the highest αvβ3 expression; and human lymphoblastoid cells Raji and Daudi are αvβ3-negative (Fig. 5A). A549 cells and Raji cells were chosen as αvβ3-positive and αvβ3-negative cells, respectively. These two kinds of cells were transfected by sEvs with MFG-E8-EGFP(MFG-E8-EGFP-sEvs), using sEvs with EGFP(EGFP-sEvs) as control. Analysis by flow cytometry showed about 9.7% of A549 cells were EGFP-positive while only 1.04% of Raji cells were EGFP-positive. Compared to the cells transfected with EGFP-sEvs, those transfected by MFG-E8-EGFP-sEvs had more EGFP transfected into the cells (Fig. 5B). The above results indicated that sEvs not only could carry target proteins into sEvs, but also make sEvs could target αvβ 3 in recipient cells.

Discussion

Therapeutic proteins and polypeptides, such as enzymes, cytokines and antibodies, are available for treating various human diseases. However, these protein-based drugs usually are sensitive to changes in temperature, solvent and pH, posing significant challenges in achieving the best therapeutic outcomes. Moreover, the majority of clinically available biopharmaceutical drugs are limited to the extracellular environment because of their poor membrane permeation [34, 39]. With a small size and the same bilayer cellular membrane as human cells, sEvs are a promising drug carrier although their application was hindered by lacking efficient methods of cargo loading. For protein-based therapeutics, endogenously loading has been exploited in several reports by fusion or interaction with proteins enriched in sEvs, one of which is MFG-E8[37, 38].

MFG-E8, also called lactadherin, was originally identified as a component of milk fat globules that bud from the mammary epithelial and later on was found in many kinds of cells[40]. MFG-E8 has an epidermal growth factor (EGF)–like domains at NH2 –terminal, which contain a conserved arginine-glycine-aspartate (RGD) motif that can bind with αvβ3 and αvβ5 integrins, and C1C2 domain at COOH terminal domains, which could bind with phospholipids, especially phosphatidylserine[41]. MFG-E8 can act as a bridge between apoptotic cells and macrophage by binding with the PS of apoptotic cells through its C1C2 domain and also attaching to the αvβ3/αvβ5-integrin expressed on activated macrophages through the RGD motif [42]. In our study, we found that MFG-E8 could be secreted from cells and attached to the cell membrane, but not into the media.

Because of its adhesion to the membrane, the C1C2 domain can be used to target other protein or peptide onto sEvs [36, 43].When C1C2 domain was fused with other proteins such as interleukin 2 (IL-2) or granulocyte macrophage colony-stimulating factor (GM-CSF) instead of EGF-like domain, the fusion proteins were found in sEvs secreted by cells[43]. In our study, fusion with the whole MFG-E8 also addressed other proteins to the sEvs. By TEM analysis, we found the fluorescence of EGFP (fusion expressed with MFG-E8) circled the outside of sEvs, meaning the fusion protein locating on the surface of sEvs. Zeelenberg IS et al reported that sEvs with tumor antigens addressed by fusion with C1C2 domain could induce efficient antitumor immune responses[36]. All of these indicated that fusion of peptides to the MFG-E8 or C1C2 domain could be used to display peptides or proteins on the surface of sEvs. However, the activity of the addressed protein or peptides by MFG-E8 or C1C2 domain may be inhibited because of its close association with membrane, so we further identified the protein activity by fusion expression of MFG-E8 with luciferase. Unlike EGFP, there was fluorescence emitted only when luciferase catalyzes its substrate. It was found the protein fused with MFG-E8 could remain its activity.

Besides sEvs' unique possibilities for the cargo loading, sEvs may also offer beneficial features for drug delivery in terms of targeting. In contrast with synthetic lipid nanoparticles, whose stability would be affected with the addition of targeting peptides and whose synthesis is complicated, displaying targeting ligands on sEvs is relatively simple because peptide ligands can be genetically fused to the extra-exosomal termini of exosomal membrane proteins[33]. sEvs targeting specifically to neurons, microglia, oligodendrocytes in the brain have been achieved by engineering dendritic cells and HEK293 cells to fusion express the neuron-specific rabies viral glycoprotein(RVG) peptide and an exosomal membrane protein Lamp2b[23, 44]. Similarly, the RGD peptide was another targeting peptide that was used to engineer the sEvs to target breast cancer cells via αvβ3 integrin by fusing to the N terminus of Lamp2b[45]. Because there is an RGD motif in MFG-E8, we supposed that the engineered sEvs with MGF-E8 would have the capability of targeting αvβ3 integrin. Our study demonstrated that sEvs engineered with MFG-E8 could deliver more protein of interest into receptor cells, and more proteins could transfect into αvβ3-positive cells than αvβ3-negative cells by engineered sEvs transfection, meaning MFG-E8 engineering could facilitate sEvs αvβ3 integrin targeting.

In our study, we chose to transfect HEK293F cells with MFG-E8-expressing plasmid to acquire an engineered sEvs. Through fusion expression of MFG-E8 and the proteins of interest, we demonstrated that MFG-E8 is not only a suitable delivery protein that can address other proteins to sEvs, but also able to confer sEvs the targeting capabilities to high integrin cells such as some tumor cells. It has also been reported MFG-E8 may play a positive role in the membrane secretion to improve the sEvs budding[37, 38], although in our study, transfection of HEK293 cells with MFG-E8 only increased sEvs slightly ,but not significantly. In summary, MFG-E8 is a suitable protein with both abilities of cargo loading and sEvs targeting integrin.

Conclusions

According to the results of the present study, Recombinant MFG-E8 could be secreted and adhere to the outside of cell membrane in HEK293F cells. Then, the engineered sEvs by expressing of protein fused to MFG-E8 show that MFG-E8 could transport protein to recipient cells by sEvs and increase the the delivery efficiency of protein into sEvs. Furthermore, MFG-E8 could not only encapsulate active protein in sEvs, but also make sEvs could target αvβ 3 in recipient cells.

Abbreviations

sEvs: Small extracellular vesicles; MFG-E8: milk fat globule–epidermal growth factor 8 protein; EGF: epidermal growth factor; TEM: transmission electron microscopy; NTA: nanoparticle-tracking analysis; GL: Gaussia Luciferase;

Declarations

Ethics approval and consent to participate

Not application.

Consent for publication

Not application.

Availability of data and materials

The datasets supporting the conclusions of this article are included with in the article. All strain materials were obtained from Jinan University, Guangzhou, China.

The sequences used during the current study are available in the NCBI repository https://www.ncbi.nlm.nih.gov/nuccore/NM_005928.4,https://www.ncbi.nlm.nih.gov/nuccore/LC006266.1,https://www.ncbi.nlm.nih.gov/nuccore/AH006868.3,

Competing interest

The authors have declared no conflict of interest exists.

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Author's contribution

MJX, WK and LCXX conceived and designed the experiments. MJX, WK and LCXX performed the experiments. MJX wrote the manuscript. All authors reviewed and approved the manuscript.

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Not application.

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Figures

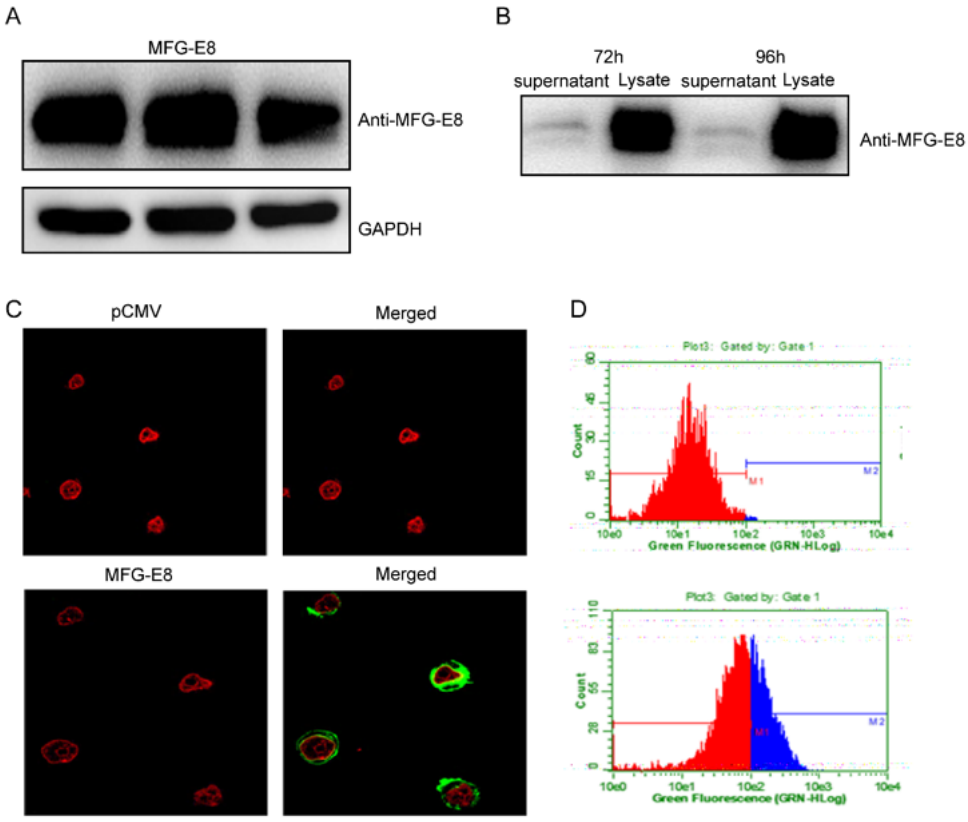

Figure 1

Transient expression of MFG-E8 in HEK293F cells. HEK 293F cells were transfected with pCDNA 3.4/MFG-E8 and the expression of recombinant MFG-E8 was confirmed by Western blot (A). After centrifugation of cell culture, the recombinant protein was found in precipitate but not supernatant (B). The recombinant MFG-E8 was further confirmed to link with outside of cell membrane with anti-MFG-E8 antibody by laser confocal microscope(C) and flow cytometry (D).

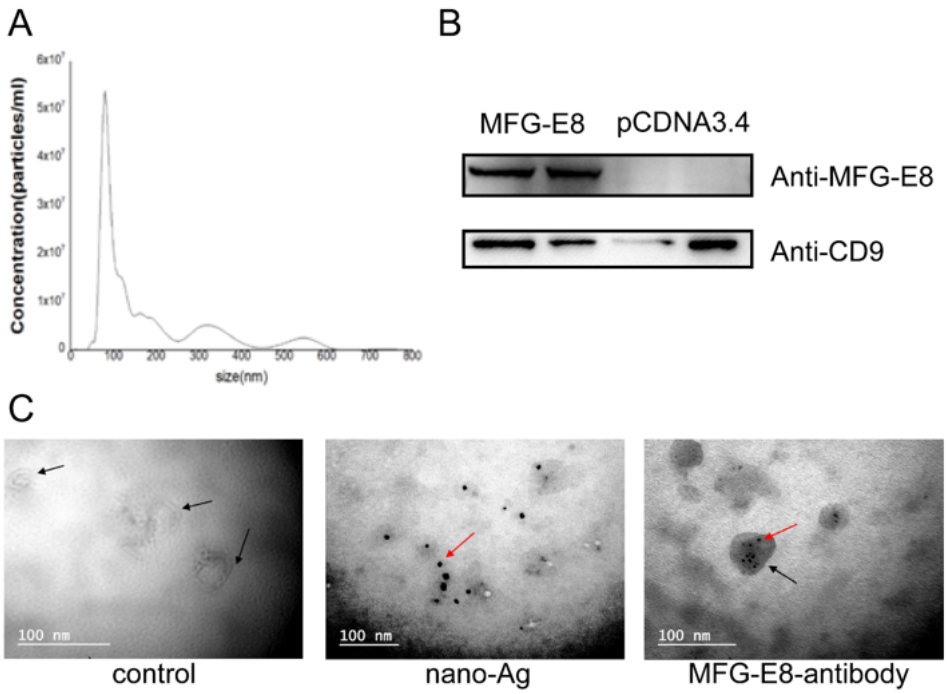

Figure 2

Isolation of sEvs and confirm the MFG-E8's present outside the sEvs. sEvs were isolated from cell culture at day 4 after transient transfection of recombinant MFG-E8 and were analyzed by NTA analyzer(A), and Western blot(B) to confirm the existence of MFG-E8 in sEvs. Under the transmission electron microscopy, MFG-E8 was showed by anti-MFG-E8-AuNPs-mAb.Black dots refer to anti-MFG-E8-AuNPs-mAb, black arrows refer to sEvs and red arrows refer to MFG-E8 (C) (Scale bar = 100 nm).

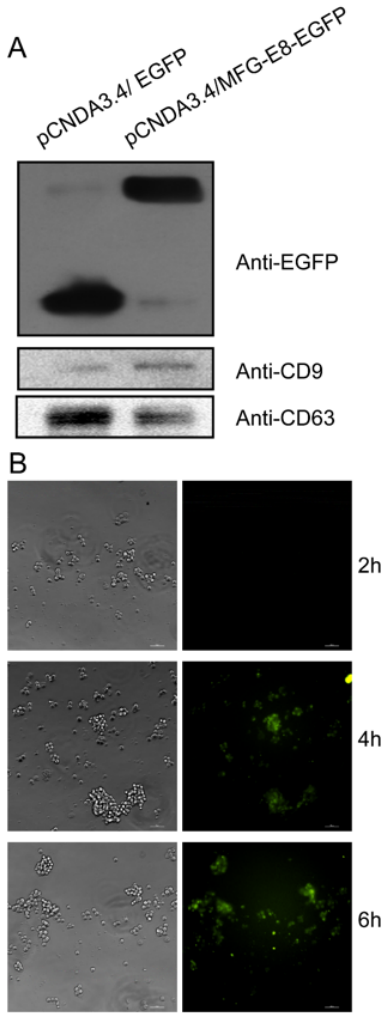

Figure 3

Delivery of EGFP into sEvs by MFG-E8 fusion expression. Compared with the sEvs secreted from pCNDA3.4/EGFP, the concentration of EGFP in sEvs with E8-EGFP was much more (A), The recipient cells HEK293T cells were transfected with sEvs with MFG-E8-EGFP for 6h, cells were investigated under confocal microscopy(B).

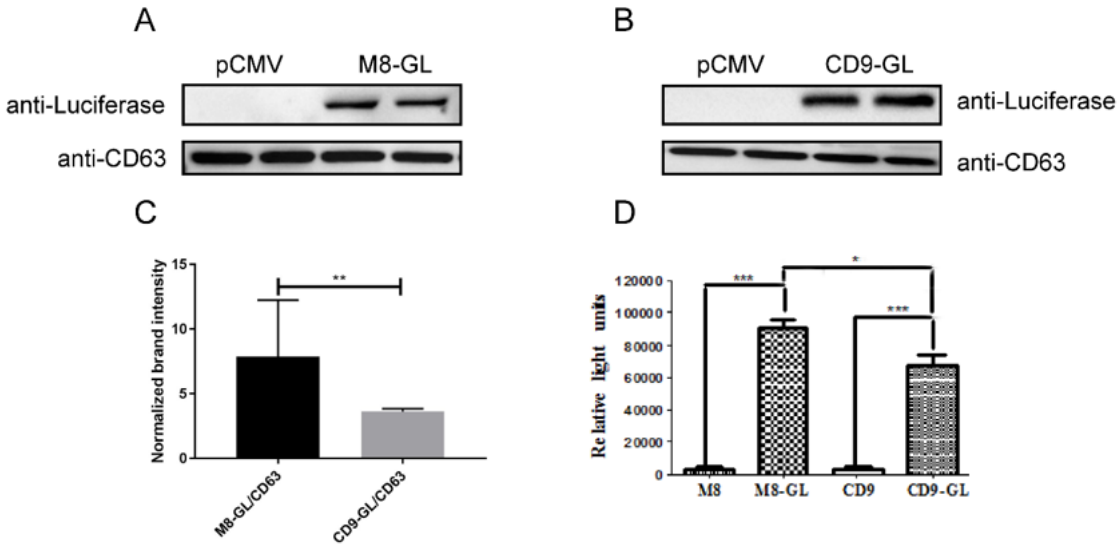

Figure 4

Delivery of Gaussia Luciferase (GL) into sEvs by fusion expression. HEK293F cells were transfected with pCNDA3.4/MFG-E8-GL and pCDNA3.4/CD9-GL respectively and sEvs were isolated. Both M8-GL(A) and CD9-GL(B) were found in the sEvs by Western blot analysis. The relative GL concentration in sEvs was analyzed by comparing M8-GL/CD63 and CD9-GL/CD63(C). And the luciferase activity in two sEvs was compared by analyzing the catalytic activity of the enzyme (D).

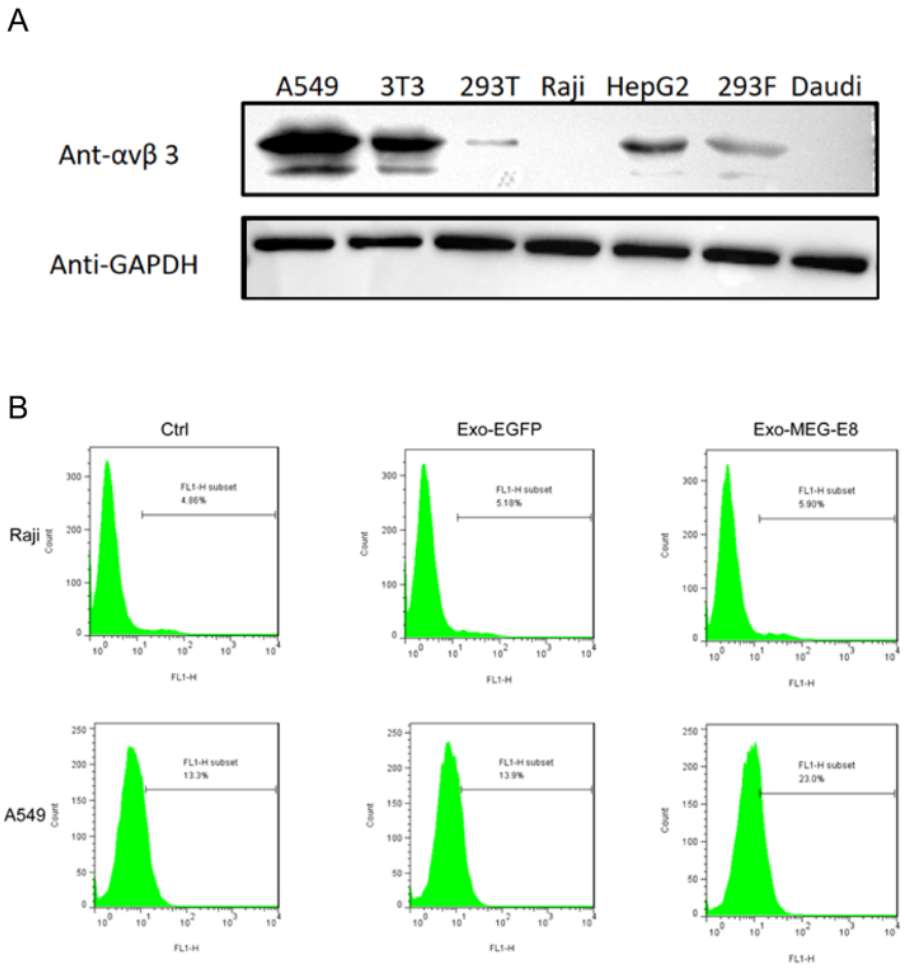

Figure 5

The αvβ 3 targeting of sEvs with MFG-E8. The αvβ3 expression of several kinds of cells was analyzed by Western Blot (A). After sEvs transfection, EGFP concentration was analyzed in A549 cells and Raji cells by flow cytometry (B).

Supplementary Files

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