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Cardiac-specific delivery by cardiac tissue-targeting peptide-expressing exosomes

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ABSTRACT

Naturally occurring RNA carriers such as exosomes might be an untapped source of effective delivery vehicles. However, if exosomes are to be exploited for therapeutic applications, they must target specific tissues or cell types to avoid off-target effects. This study evaluated whether genetic modification of exosomes could enhance exosome delivery to heart cells and heart tissue without toxicity. Exosomes expressing cardiac-targeting peptide (CTP)-Lamp2b on the exosomal membrane (CTP-Exo) were generated by introducing vectors encoding CTP-Lamp2b into HEK 293 cells. The expression of CTP-Lamp2b peptide on exosomes was stabilized by attaching glycosylation sequences. Exosomes expressing only Lamp2b on exosomal membranes (CTL-Exo) were generated as a control. The *in vitro* and *in vivo* uptake of CTL-Exo and CTP-Exo was evaluated in cell lines and mice. Both exosomes were delivered to HEK 293 and H9C2 cells. The delivery of the exosome was not different between CTP-Exo and CTL-Exo in HEK 293 cells, whereas the delivery of CTP-Exo was 16% greater than that of CTL-Exo in H9C2 cells ($P = 0.047$). Cell viability was maintained at almost 100% with different dosages of both CTL-Exo and CTP-Exo. Moreover, compared with CTL-Exo, the *in vivo* delivery of exosomes to the hearts of mice was increased by 15% with CTP-Exo ($P = 0.035$). The delivery to livers and spleens was not different between the two exosomes. Genetic modification of exosomes by expressing CTP-Lamp2b on the exosomal membrane enhanced exosome delivery to heart cells and the heart tissue. These results suggested that CTP-Exo might be used as a therapeutic tool for heart disease.

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1. Introduction

Cell therapy became a potential therapeutic approach to repair infarcted hearts. Despite exciting opportunities for heart repair of stem cells [1], recent studies suggest that, in addition to cellular replacement, paracrine factors released by injected cells are important for cell-based tissue restoration [2]. These paracrine factors may be secreted directly from the transplanted cells or released as cargo in extracellular vesicles (EVs) [2–4]. Exosomes are the smallest EVs, with a diameter of 30–150 nm, and they are secreted by numerous cell types [5,6]. Exosomes can be taken up by endocytosis or fusion with the recipient cell plasma membrane, actions determined by membrane-bound protein interactions, followed by the release of genetic and protein material within the exosomes into the cells [7,8].

Exosomes carry mRNAs, miRNAs and proteins from their parental cells representing an important component of paracrine signaling and cell-to-cell communication [9]. The interest of scientists in exosomes has expanded rapidly because of their diverse pathological and therapeutic effects [10]. However, if exosomes are to be used for therapeutic tools, it will be necessary to target specific tissues or cell types to avoid off-target effects.

Moreover, although the delivering technique of siRNA was recently advanced, targeting specific tissues or cell types while avoiding nonspecific delivery to other organs, especially to the liver, remains challenging. Another major barrier is immunogenicity of

Abbreviations: CTP, Cardiac-targeting peptide; RVG, Rabies virus glycoprotein; EV, Extracellular vesicles; TFF, Tangential flow filtration; NTA, Nanoparticle tracking analysis; MSP, Muscle specific peptide.

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the siRNA or delivery vehicle, especially if repeated dosing is needed to treat chronic or degenerative diseases [11]. Recently, by engineering cells to express Lamp2b, an exosomal membrane protein, fused to the neuron-specific RVG (rabies virus glycoprotein) peptide [12], researchers were able to target the brain [13]. The peptide sequence APWHLSSQYSRT, termed cardiac-targeting peptide (CTP), has been used to deliver therapeutic peptides, proteins, and nucleic acid specifically to the heart [14]. However, exosomes targeting the heart have not been developed.

Accordingly, we generated exosomes expressing CTP-Lamp2b on exosomal membranes through gene modification. Further, we characterized the morphology and contents of CTP-Exo using a multi-instrumental approach and evaluated whether the genetically modified exosomes enhanced exosome delivery to heart cells and heart tissue.

2. Materials and methods

2.1. Cloning

pcDNA GNSTM-3-FLAG-10-Lamp2b-HA was a gift from Joshua Leonard (Addgene plasmid #71293). CTP (APWHLSSQYSRT) nucleotides (5′-GCCCCCTGGCACCTGTCCTCCCAGTACTCCCGGACC-3′) were inserted into this plasmid using a standard PCR method and the primers 5′- ACT ATG GGC AGT GGA GCC CCC TGG CAC CTG TCC TCC CAG TAC TCC CGG ACC GAC TAT AAA GAT GAC-3′ and 5′- GTC ATC TTT ATA GTC GGT CCG GGA GTA CTG GGA GGA CAG GTG CCA GGG GGC TCC ACT GCC CAT AGT-3′. The construct was confirmed by DNA sequencing (Fig. 1).

2.2. Cell culture and plasmid

To generate exosomes expressing CTP-Lamp2b, we fused the CTP peptide to the extra-exosomal N terminus of murine Lamp2b protein by introducing the GNSTM-FLAG-Lamp2b-HA plasmid into HEK 293 cells. Exosomes expressing only Lamp2b on exosomal membranes were generated as a control. We transfected the mCherry-CD81-10 plasmid, which was a gift from Michael Davidson (Addgene plasmid #55012), into HEK 293 cells to track the delivery of exosomes. Adding the GNSTM glycosylation motif protected N-terminal peptides from degradation [15]. To validate whether FLAG-Lamp2b-HA and mCherry-CD81 were transfected successfully into HEK 293 cells, we analyzed the levels of mCherry fluorescence 48 h after transfection using a confocal microscope [16].

HEK 293 cells (Korean Cell Line Bank, Seoul, Korea) and H9C2 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM; Welgene) containing 10% fetal bovine serum (FBS; Young In Frontier) and 1% penicillin-streptomycin (Gibco). Cells were cultured in a humidified incubator at 37 °C with 5% CO_2.

2.3. Exosome isolation and characterization

Exosomes were prepared after Lipofectamine-based transfection of semiconfluent HEK 293 cells seeded at 5×10^6 in a 15-cm dish. The cell culture medium was replaced with fresh serum-free DMEM supplemented with Glutamax (1% final concentration; Gibco). After 2 h, 8 μg of plasmid encoding mCherry-CD81-10 was co-transfected with 8 of μg Lamp2b or CTP-Lamp2b plasmid. Imaging of exosomes with fluorescently labeled proteins enabled tracing of the exosomes. The supernatants were harvested after 48 h of culture for exosome isolation by differential centrifugation.

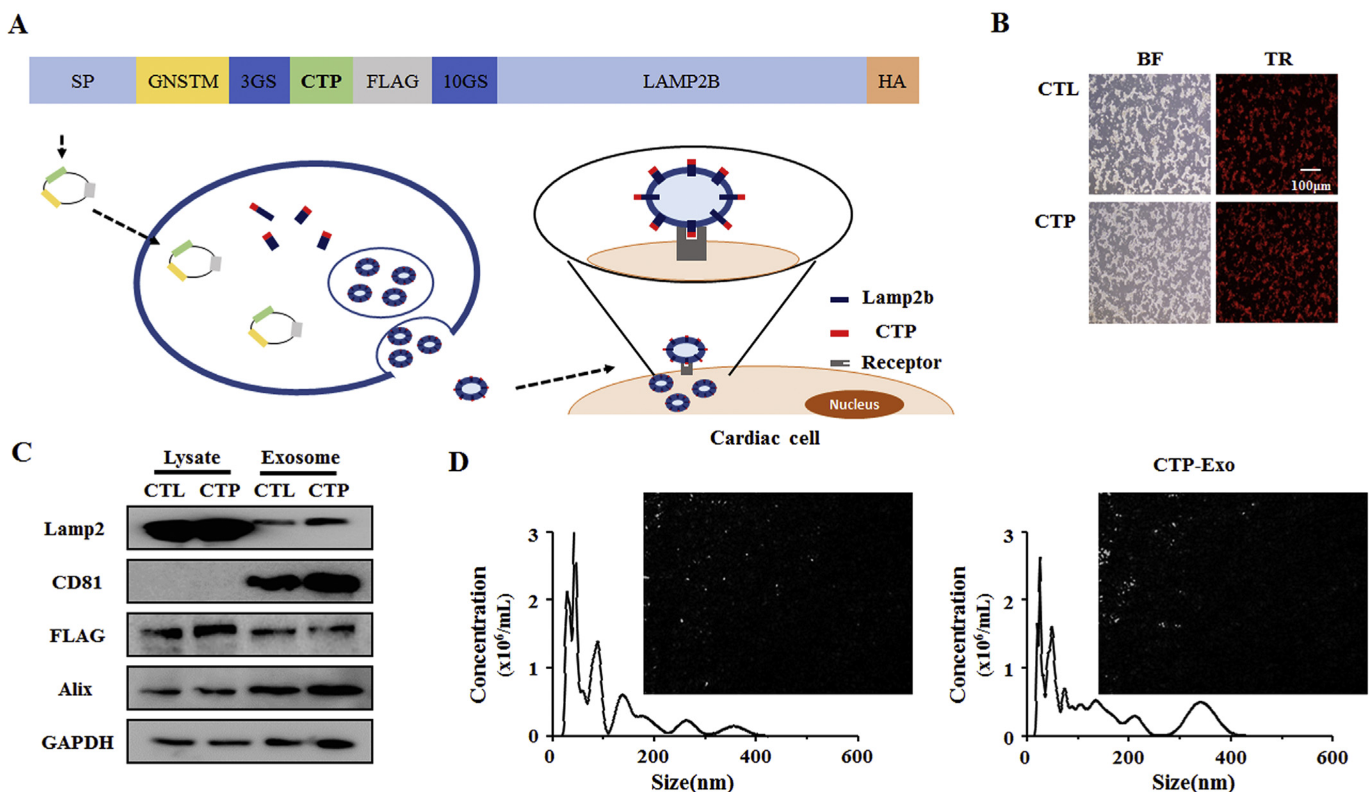

Fig. 1. Characterization of exosomes. A, Schematic of cardiac-targeting exosomes in cardiac cells. B, Expression of CTP-Lamp2b tagged with CD81-mCherry. C, Western blot analysis of transfected HEK 293 cells and isolated exosomes. D, NTA analysis of exosomes showing the number and size distribution of particles.

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