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Cancer-derived exosomes as a delivery platform of CRISPR/Cas9 confer cancer cell tropism-dependent targeting

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ABSTRACT

An intracellular delivery system for CRISPR/Cas9 is crucial for its application as a therapeutic genome editing technology in a broad range of diseases. Current vehicles carrying CRISPR/Cas9 limit *in vivo* delivery because of low tolerance and immunogenicity; thus, the *in vivo* delivery of genome editing remains challenging. Here, we report that cancer-derived exosomes function as natural carriers that can efficiently deliver CRISPR/Cas9 plasmids to cancer. Compared to epithelial cell-derived exosomes, cancer-derived exosomes provide potential vehicles for effective *in vivo* delivery *via* selective accumulation in ovarian cancer tumors of SKOV3 xenograft mice, most likely because of their cell tropism. CRISPR/Cas9-loaded exosomes can suppress expression of poly (ADP-ribose) polymerase-1 (PARP-1), resulting in the induction of apoptosis in ovarian cancer. Furthermore, the inhibition of PARP-1 by CRISPR/Cas9-mediated genome editing enhances the chemosensitivity to cisplatin, showing synergistic cytotoxicity. Based on these results, tumor-derived exosomes may be very promising for cancer therapeutics in the future.

1. Introduction

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated endonuclease (Cas) 9 system is a powerful tool for genomic editing. CRISPR/Cas9, which originated from the acquired immune system in bacteria and archaea, is composed of RNA-guided DNA endonuclease Cas9 and a chimeric single guide RNA (sgRNA) [1,2]. The sgRNA contains two naturally occurring RNA species: CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) [3]. The sgRNA binds to the Cas9 and directs it to the specific genomic locus of interest, the complementary DNA adjacent to the protospacer adjacent motif (PAM), which consists of either an NGG or an NAG [4]. Numerous studies have demonstrated that CRISPR/Cas9-mediated approaches enable efficient gene disruption and gene modification in various cell types and organisms, respectively. Thus, the concept of suppressing protein expression *via* CRISPR/Cas9-mediated gene editing may become a powerful tool in cancer therapeutics.

Currently, the most adequate *in vivo* delivery vehicles are limited to polycationic liposomes and viral vectors [5–7]. Until now, viral vectors have been considered ideal for encapsulating plasmids or mRNA owing to their high loading capacity. However, there are still concerns about safety and stability issues [8]. Thus, to overcome the toxicity and immunogenicity in the body, proper *in vivo* delivery carriers should be developed for genome editing therapy. Recently, exosomes have emerged as delivery vesicles capable of therapeutic applications because they are composed of nanospherical membrane-type structures with a bilayer of lipids [9] similar to that of cell membranes and liposomes. Exosomes are natural nano-vesicles produced by numerous cell types, including immune cells, epithelial cells and tumor cells. These endogenous vehicles, ranging from 30 nm to 120 nm in diameter, are known to originate by budding from the internal vesicles of multivesicular bodies (MVBs) and are released into the extracellular environment. They were considered as small organelles to discard unwanted molecules and cell debris. However, recently it has been discovered that exosomes take part in multiple biological processes including immune modulation, signal transduction, and tolerance development. Furthermore, these vesicles are thought to function as important mediators of cell-to-cell communication. Numerous proteomic and transcriptomic profiles have revealed that exosomes contain various proteins, messengers, long noncoding RNAs (IncRNAs) and miRNA, and they are emerging in many research fields as novel regulators in intracellular communication [10–12]. Thus, their ability to

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carry various molecules prompts the use of exosomes with high biocompatibility and low immunogenicity as delivery vehicles, especially for cancer therapy [13,14].

Poly (ADP-ribose) polymerase-1 (PARP-1), a member of the 17 PARP enzyme members, has been rising as a potential therapeutic target in cancer treatment [15,16]. The PARP proteins are involved in a broad range of cellular processes including DNA damage response, chromatin remodeling, cell cycle regulation and cell death [17]. PARP-1 is the most abundant in various cells and functions in the repair of single-strand DNA breaks (SSBs) by catalyzing the NAD^{+}-dependent addition of poly (ADP-ribose) units [18]. Clinically, PARP is a promising target for cancer treatment, particularly for cancers such as BRCA1/2 deficient breast and ovarian cancers [19,20]. The trapping of PARP to the sites of DNA damage causes cell death; thus, the development of PARP inhibitors enables killing cancer cells. However, the potency in trapping PARP differs among the PARP inhibitors in cancer therapy, and resistance of PARP inhibitor therapy has been identified [21,22]. Additionally, PARP inhibitors for clinical use are still unable to discriminate between individual PARP isoforms [23]. Thus, there should be careful considerations before developing PARP inhibitors.

Herein, to develop an efficient cancer therapy, plasmid-encapsulating exosomes were created to introduce cas9 and sgRNA in cancer cells. We established the *in vivo* delivery vehicle for the disruption of PARP-1 expression by tumor-derived exosomes loaded with CRISPR/Cas9 against PARP-1 (Fig. 1). Inhibition of PARP-1 *via* the efficient delivery system of the genome editing tool enables a synergistic cytotoxicity effect on ovarian cancer cells when combined with platinum-based chemotherapy.

2. Materials and methods

2.1. Materials

Cas9 and sgRNA targeting PARP-1 expression plasmid vectors were prepared by Toolgen (Korea). Primers were synthesized by Macrogen (Korea). A QIAamp DNA mini kit was purchased from Qiagen (USA). The T7 endonuclease I enzyme was purchased from New England Biolabs (USA). DMEM, FBS, and penicillin and streptomycin solutions were purchased from Wellgen (Korea). Primers were purchased from Macrogen (Korea).

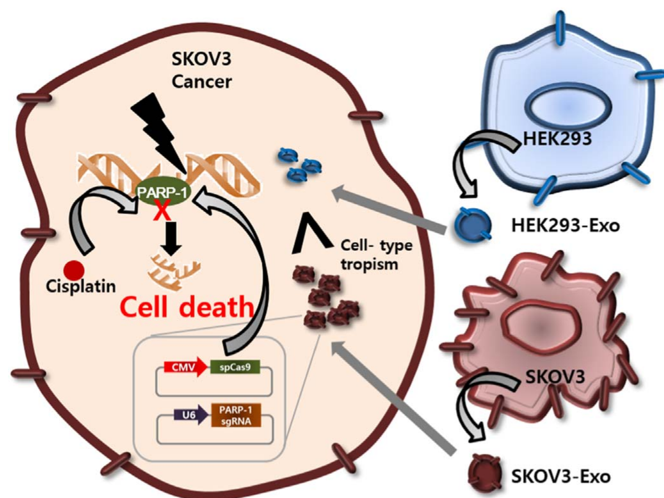

Fig. 1. Scheme of a combination therapy in ovarian cancer cells. Tumor-derived exosomes are loaded with CRISPR/Cas9 targeting PARP-1 enhanced inhibition of cancer cell propagation when combined with cisplatin. To load Cas9-NLS and PARP-1 sgRNA into exosomes, an electroporation-based approach was conducted. In addition, tumor-derived exosomes (SKOV3-Exo) accelerate their accumulation within SKOV3 tumor cells compared to epithelial cell-derived exosomes (HEK293-Exo) because of their cell tropism.

2.2. Cell culture

The ovarian cancer cell line SKOV3 and HEK293 were obtained from the Korean Cell Line Bank (KCLB). Cells were maintained in RPMI1640 for human ovarian cancer cell line SKOV3 and in DMEM for human epithelial cell line HEK293, both of which were supplemented with 10% FBS and 1% penicillin and streptomycin solutions. For exosome isolation, cells were cultured in media depleted of FBS. Mycoplasma contamination was checked for every month.

2.3. In vitro T7E1 assay

Following the manufacturer's instructions, Cas9 and sgRNAs were loaded into SKOV3 cells *via* electroporation or a transfection reagent, LF2000 (Invitrogen), for 48 h. After loading, their genomic DNA was isolated using a QIAamp DNA mini kit (QIAGEN). Each sgRNA genomic target site was prepared using a PCR amplicon with specific primers: forward strand (5′-TGTTCGGTGGCGGCT-3′) and reverse strand (5′-ATGGTACCAGCGGTCAAT-3′). PCR amplicons were purified, and 100 ng was reannealed using a thermocycler and then digested with T7 endonuclease I (T7E1) according to the manufacturer's instructions. Digested DNA was analyzed using the Gel-Doc (Bio-Rad) system and band intensities were quantified using ImageLab software (Bio-Rad).

2.4. Exosome purification

For the isolation of exosomes from HEK293 and SKOV3 cells, each culture medium was harvested and initially centrifuged at 300*g* and 4 °C for 10 min to remove the dead cells. Supernatants were subsequently centrifuged at 3000*g* and 4 °C for 10 min and then filtered through 0.22 μm filters (Merck Millipore) to remove apoptotic cells and microvesicles. Clarified supernatants were pelleted using ExoQuick™ according to the manufacturer's instructions (System Biosciences). The pellets were resuspended in PBS solution and purified using an Amicon Ultra-15 Centrifugal Filter (100 kDa) to concentrate the pure exosomes.

2.5. Characterization of exosomes

To analyze the size distribution of exosomes, the Zetasizer Nano ZS90 (Malvern) was used. For measurement, purified exosomes were diluted 100-fold. To obtain TEM images, each 5 μL of exosomes isolated from the media of HEK293 and SKOV3 cells and fixed in 2% paraformaldehyde was transferred onto a carbon-coated grid. The samples were fixed in 2.5% glutaraldehyde solution for 20 min at room temperature by dropping. After washing three times with PBS buffer, 2% uranyl acetate was used to stain for 10 min. Finally, isolated exosomes were visualized using TEM (TECNAI, Phillips) to observe their morphologies and structures.

To confirm the homogenous morphology of purified exosomes, an AFM analysis was carried out. In brief, one drop of exosomes was absorbed on freshly cleaved mica and then air-dried. Micrometer-scale imaging was obtained in contact mode with XE-100 AFM (Park Systems) and processed using a PARK system XEI software program.

To detect exosome-specific markers on isolated exosomes, purified exosome pellets were lysed with RIPA buffer and proteinase inhibitors (Sigma-Aldrich). The protein concentrations were determined using a NanoDrop (Thermo Scientific), and proteins were analyzed with an SDS-PAGE gel and detected using an LAS3000 imager. To detect exosomal proteins, samples were incubated with primary anti-CD63 (Santa Cruz, 1:100) or anti-TSG antibodies (Santa Cruz, 1:100), followed by HRP-conjugated anti-rabbit IgG antibody (Bethyl, 1:5000).

2.6. Cellular uptake of exosomes

To label the two types of exosomes, exosomes were incubated with 10 μM of Dio (green) or Did (red), a lipophilic fluorescent dye

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