Targeted Delivery of IL-1Ra mRNA using Cationic Peptide Modified Exosomes for Cartilage Repair

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INTRODUCTION: Osteoarthritis (OA) is a chronic joint degenerative disease associated with pain, inflammation, and cartilage degradation[1]. Interleukin-1 receptor antagonist (IL-1Ra) is a potent disease-modifying agent that competitively binds with IL-1 and hampers its degradative effect on cartilage[2]. OA gene therapy is based on using adeno-associated viral vectors that cause joint inflammation and detrimental side effects. Non-viral carriers for mRNA utilize synthetic lipid nanoparticles (LNPs) or polymeric micelles, but safety and immunogenicity concerns constrain their long-term use[3]. In addition, targeted delivery of mRNA to chondrocytes in the deeper cartilage layers is limited[3]. Exosomes (Exo), on the other hand, are native LNPs that possess intrinsic therapeutic potential and are less immunogenic[4], however, large size (40-200 nm) and negatively charged lipid bilayer hinder their penetration into the deep zone of negatively charged cartilage. Based on the negative fixed charge density of cartilage, our previous research has developed an arginine-rich short-length cationic peptide carrier with a net charge of +14 (CPC+14R) that resulted in superior chondrocyte uptake, full-thickness cartilage penetration, and retention[5]. To overcome drug transport barriers with exosomes, we harness electrostatic interactions with negatively charged proteoglycans by surface-modifying exosomes using CPC+14R to impart cationic charge (**Fig.1A**). In this work, we used eGFP-mRNA as a proof-of-concept to evaluate the efficacy of cationic exosomes (Exo-CPC+14R) as a carrier for gene delivery. Further, we used IL-1Ra mRNA as a disease-modifying gene by means of Exo-CPC+14R and demonstrated their effectiveness in ameliorating cartilage degradation.

METHODS: Hydrophobic DSPE of DSPE-PEG (2000)-Azide (DPA) was inserted in the lipid bilayer of exosomes while hydrophilic PEG (2000) remained outside with an azide group. CPC+14R [RRRR(NNRRR)$_3$R] was conjugated to DPA using DBCO-NHS ester linker via click chemistry. To assess *in vivo* cartilage transport, fluorescently labeled Exo and Exo-CPC+14R were intra-articularly (IA) injected in mice knee joints 9 weeks after DMM (Destabilization of medial meniscus) surgery (n=5/group). The sections of tibial and femur bones were imaged on day 1 post-IA injection using confocal microscopy. eGFP-mRNA was loaded in Exo and Exo-CPC+14R by a simple incubation using Lipofectamine 2000. Human chondrocytes were treated with Exo and Exo-CPC+14R for 4 h and were imaged 18 h post-transfection using confocal microscopy (n=3). Targeted delivery of eGFP-mRNA using Exo-CPC+14R was assessed *in vivo* using the same DMM mouse model (n=5/group). eGFP expression in tibial and femur bones of mice was evaluated at 24 h post-IA injection using confocal microscopy. eGFP-mRNA delivery was also evaluated in IL-1α treated human talus cartilage explants (3mm×1mm) using confocal microscopy after 48 h treatment for eGFP expression, and chondrocyte nuclei were stained with Hoechst (n=4/group). Bioactivity of *in vitro* transcribed IL-1Ra mRNA was determined by stimulating human chondrocytes with IL-1β and oncostatin, followed by mRNA treatment for 4 h (n=3). IL-1Ra protein production was measured from culture media and cell lysate using ELISA. IL-1Ra mRNA delivery was assessed in a similar cytokine-challenged human cartilage model, as discussed above. Following 48 h treatment, the IL-1Ra expression in chondrocytes was confirmed using RNAScope ISH technique.

RESULTS: Surface charge on Exos was successfully reversed from -25.4 ± 1.3 to -2.5 ± 1.5 mV by the conjugation of the cationic motif, CPC+14R (**Fig. 1B**) with no evident change in the size of exosomes. Exo-CPC+14R exhibited high fluorescence in the full-thickness of the medial femoral cartilage of DMM mice joints, whereas unmodified Exo did not show fluorescence (**Fig. 1C**), confirming the deep penetration of cationic exosomes in cartilage. Higher eGFP expression in human chondrocytes was observed in the Exo-CPC+14R group, indicating successful delivery of eGFP-mRNA *in vitro* (**Fig.1D**). The eGFP-mRNA loaded Exo resulted in minimal eGFP expression. Whereas Exo-CPC+14R facilitated superior eGFP expression in deep cartilage tissue in the OA mouse model (**Fig. 1E**) and IL-1α treated human cartilage explants (**Fig. 1F**). Dose-dependent increased levels of IL-1Ra protein secretion in the media at 18 h post-transfection confirmed the bioactivity of IL-1Ra mRNA (**Fig. 1G**). Preliminary RNAscope demonstrated increased numbers of chondrocytes with positive IL-1Ra signal within explants treated with Exo-CPC+14R (38%) compared to Exo (17%) (**Fig. 1H**).

DISCUSSION: We surface-engineered exosomes by conjugating cationic peptide carrier and demonstrated its full-thickness cartilage penetration in an OA mouse model leveraged by the electrostatic interactions with negatively charged proteoglycans. Of note, our study successfully showed the potential of cationic exosomes for an effective mRNA delivery using eGFP/IL-1Ra mRNA in mice joints and OA-conditioned human cartilage explants. Ongoing work evaluates the disease-modifying effect of IL-1Ra mRNA in OA-conditioned human cartilage by measuring the levels of prostaglandin E2 and other catabolic markers. The efficacy of IL-1Ra mRNA-loaded cationic exosomes will be evaluated in a medial meniscus transection (MMT) rat model of post-traumatic OA.

SIGNIFICANCE: Cationic exosomes can load large nucleic acids and enable targeted delivery of eGFP/IL-1Ra mRNA to chondrocytes residing in deep cartilage layers. Hence, cationic exosomes hold the potential to be an ideal non-viral carrier for any disease-modifying gene.

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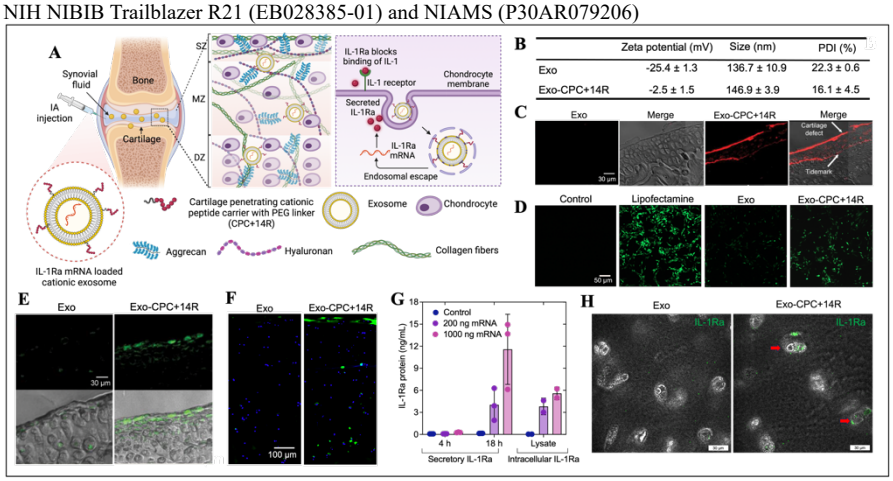

Fig.1A. Schematics representing cationic exosome-mediated delivery of IL-Ra mRNA to chondrocytes residing in the deep zone of cartilage. **B.** Size, zeta potential and PDI of Exo and Exo-CPC+14R. **C.** Exo and Exo-CPC+14R distribution in cartilage of DMM mice joints on day 1 post IA injection. **D.** *In vitro* eGFP expression in human chondrocytes. mRNA loaded Exo and Exo-CPC+14R induced GFP expression in (**E**) cartilage of DMM mice joints and (**F**) in IL-1α treated human talus cartilage explants. **G.** IL-1Ra protein production in human chondrocytes. **H**. *In vitro* IL-1Ra expression in IL-1α treated human talus cartilage explants. Data are presented as mean ± SD. P values are estimated by two-way ANOVA with Tukey's multiple comparisons test. **** vs Control, P<0.0001, *vs control, P<0.05 and n.s – not significant.