Cartilage-Targeting mRNA-loaded Exosomes for Osteoarthritis Gene Therapy

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INTRODUCTION: Cartilage degradation associated with osteoarthritis (OA) remains unaddressed due to a lack of an effective targeting drug/gene delivery system[1]. Receptor antagonist of interleukin-1 (IL-1Ra) is a potent disease-modifying agent that effectively blocks IL-1 from binding to its cell receptors and reduces inflammatory cues[2]. Clinical approaches for IL-1Ra gene delivery in OA use adeno-associated viral vectors or synthetic lipid- or polymer-based carriers providing robust transgene expression[3]. However, immunogenicity risks limit their prolonged use, and effective nuclear/cytosolic gene delivery to chondrocytes remains challenging[4]. Exosomes (Exo), on the contrary, are natural, cell-derived lipid vesicles with lower immunogenicity[5]. Yet, their large size (30-200 nm) and negatively charged lipid bilayer impede their diffusion into deep zones of anionic cartilage matrix. To circumvent delivery barriers, we recently surface-engineered Exos by leveraging the negative fixed charge density (FCD) of cartilage using an arginine-rich, short-length cationic peptide carrier (CPC) with a net charge of +14, to confer a cationic charge at the nanoscale[6,7]. These CPC-engineered exosomes (Exo-CPC) exhibit electrostatic interactions with negatively charged glycosaminoglycans (GAGs), enabling full-thickness cartilage penetration (**Fig. 1A**) and effective delivery of eGFP mRNA to deep-resident chondrocytes in a post-traumatic mouse model of OA[6]. In this work, we leverage these cartilage-targeting Exo-CPC as a carrier to deliver IL-1Ra mRNA (**Fig. 1A**) and demonstrate the intra-tissue depot effect of gene-loaded Exo-CPC with a single dose to restore IL-1-induced cartilage degradation.

METHODS: Exo-CPC was synthesized by a lipid insertion method using DSPE-PEG(2000)-azide lipid conjugated to CPC [RRRR(NNRRR)$_3$R] via copper-free click chemistry[6]. *In vitro* transcribed (IVT) IL-1Ra mRNA was loaded into Exos (Exo$_{IL\text{-}1RamRNA}$/Exo-CPC$_{IL\text{-}1RamRNA}$) using Lipofectamine Messenger Max (LMM). mRNA loading was quantified by Qubit; size and ζ-potential were measured using DLS. To evaluate bioactivity, IL-1β (2 ng/mL)-challenged human chondrocyte micromass cultures were treated with 500 ng Exo-CPC$_{IL\text{-}1RamRNA}$. An equivalent-dose of mRNA-LMM control was used. Media (n=4/group) were collected at 48-h to quantify IL-6 and PGE2 using ELISAs. Targeted gene delivery was assessed in IL-1α-treated human talus cartilage explants (Collin grade 0, 3mm×1mm, n=4/group). After 48 h treatment with 500 ng Exo$_{IL\text{-}1RamRNA}$ and Exo-CPC$_{IL\text{-}1RamRNA}$, IL-1Ra and PGE2 RNA expression in chondrocytes was assessed by RNAScope. Intra-tissue depot effect of Exo-CPC$_{IL\text{-}1RamRNA}$ was confirmed in human cartilage tissues collected from patients undergoing total knee arthroplasty (TKA; 68-y.o. female-KL grade 3, and 72-y.o. male-KL grade 2). TKA explants (3mm×1mm) received a single dose of 1 μg mRNA containing Exo and Exo-CPC on day-0 with a continuous IL-1β-stimulation (2 ng/mL) every two days. Cumulative GAG release (n=8-10 explants/group) was analyzed over 8 days from culture media and explants. Cartilage retention of IL-1Ra protein was analyzed on day 4 using ELISA. Gene expression (n=3 explants/group) on day 8 was measured using qPCR. Data are presented as mean ± SD. A Kruskal-Wallis with Dunn's multiple comparison test was used. * indicates $p< 0.05$.

RESULTS: Net negative charge of Exos was modified from -21.2 ± 0.8 to -2.4 ± 0.75 mV after surface-conjugation with CPC motifs, with an IL-1Ra mRNA loading efficiency of ~ 57 ± 8.7 % (**Fig. 1B**). Consistent with our previous study[6], the size and ζ-potential of Exo and Exo-CPC were unaffected post mRNA-loading. Exo-CPC-mediated delivery of IL-1Ra mRNA in human chondrocyte micromass culture downregulated IL-6 (**Fig. 1C-i,** $^{\#\#}p< 0.01$) and PGE2 levels (**Fig. 1C-ii,** $^{\#\#}p<0.0001$) relative to IL-1-stimulated cells and LMM condition. RNAScope imaging showed a higher fraction of IL-1Ra-positive chondrocytes in human cartilage treated with Exo-CPC$_{IL\text{-}1RAmRNA}$ (45%) than with Exo$_{IL\text{-}1RAmRNA}$ (22%) (**Fig. 1D-i**). Consistently, PGE2 signal was lower in chondrocytes following Exo-CPC$_{IL\text{-}1RAmRNA}$, implying cartilage-targeted delivery (**Fig. 1D-ii**). A single dose of Exo-CPC$_{IL\text{-}1RAmRNA}$ rescued TKA cartilage from significant GAG loss and restored GAG levels to those of control tissue, whereas Exo$_{IL\text{-}1RAmRNA}$ failed to suppress GAG release, similar to IL-1β condition (**Fig. 1E**). On day 4, IL-1Ra protein levels were higher in TKA cartilage treated with Exo-CPC$_{IL\text{-}1RAmRNA}$ than with Exo$_{IL\text{-}1RAmRNA}$, confirming its cartilage depot effect (**Fig. 1F**, $^{\ddagger}p< 0.05$). By day 8, Exo-CPC$_{IL\text{-}1RAmRNA}$ suppressed *MMP-13* gene expression compared with Exo$_{IL\text{-}1RAmRNA}$, IL-1β , and control conditions (**Fig. 1G**).

DISCUSSION: This study demonstrates cartilage-targeting of CPC-engineered exosomes, which leverage negative FCD in cartilage and electrostatic interactions with anionic GAGs, to deliver IL-1Ra mRNA effectively to deep-resident chondrocytes than native exosomes. CPC-engineered exosomes created intra-tissue depots and, with a single mRNA dose, mitigated IL-1-induced catabolism of human TKA cartilage over an 8-day culture period. Ongoing work includes further validation of disease-modifying effects in additional human TKA donor tissues, immunohistochemistry, and probing inflammatory signaling by RNA-seq. *In vivo* efficacy of IL-1Ra mRNA-loaded CPC-engineered exosomes will be evaluated in a medial meniscus transection rat model of OA.

SIGNIFICANCE: A single dose of CPC-engineered exosomes enables targeted IL-1Ra mRNA delivery to chondrocytes and prevents cartilage degradation. Therefore, CPC-engineered exosomes hold potential to serve as a non-viral, intra-tissue depot-based platform for sustained disease-modifying RNA therapy.

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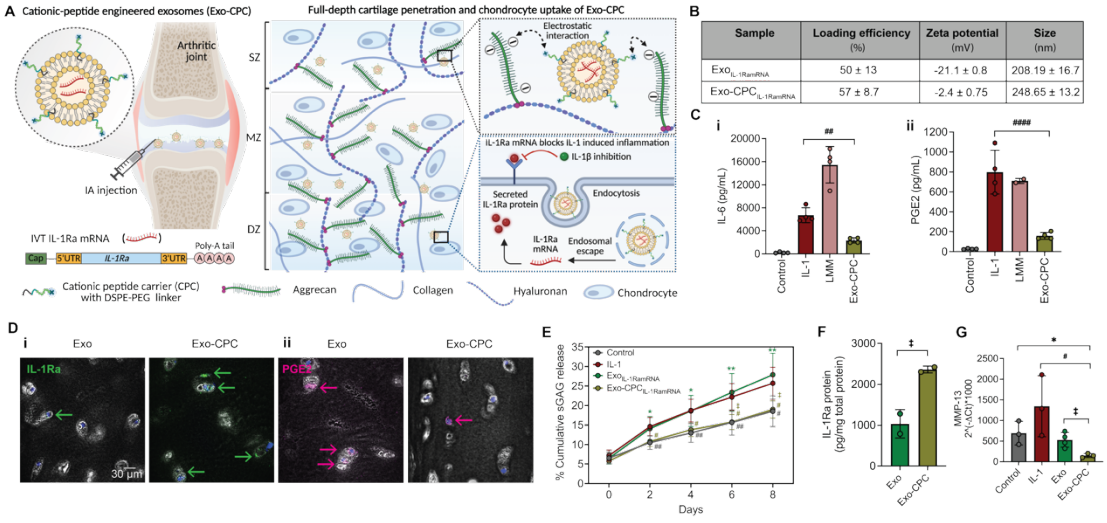

Fig. 1A. Schematics representing cartilage-targeting of CPC-engineered exosomes and the efficient delivery of IL-1Ra mRNA to deep-residing chondrocytes. SZ: superficial zone; MZ: middle zone; DZ: deep zone. **B.** mRNA loading, size, and ζ-potential of mRNA-loaded Exo and Exo-CPC. **C.** IL-6 and PGE2 levels in IL-1β-induced human chondrocyte micromass cultures. **D.** In vitro **(i)** IL-1Ra (green arrows) and **(ii)** PGE2 (magenta arrows) RNA expression in IL-1-treated human talus cartilage. **E.** Percent cumulative GAG released to media from IL-1β-induced human TKA cartilage over 8 days after a single-dose treatment of Exo$_{IL\text{-}1RamRNA}$ and Exo-CPC$_{IL\text{-}1RamRNA}$. **F.** IL-1Ra protein levels retained in IL-1β-induced TKA cartilage tissues on day-4. **G.** Catabolic MMP-13 gene expression in IL-1β-induced TKA cartilage on day 8. **** $p< 0.0001$, ** $p< 0.01$, * $p< 0.05$. * vs control, # vs IL-1, ‡ vs Exo.