Implantation of rAAV-treated mitochondria as an effective and safe protein replacement therapy to target human osteoarthritic articular chondrocytes

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INTRODUCTION: rAAV-mediated gene therapy has gained increasing attention, being involved in more than 350 clinical trials worldwide [1] and providing strong approaches to treat human osteoarthritis [2,3]. Despite its promise, the use of this vector class still faces important limitations such as the neutralization of the viral particles by naturally existing anti-AAV capsid antibodies and the risk of integration of the recombinant viral material in the host genome [3]. Mitochondria, primarily known as the energy supplier of eukaryotic cells, host ribosomes that coordinate protein translation and assembly [4]. The aim of this study was to explore the feasibility and safety of a strategy based on the implantation of mitochondria genetically modified by rAAV as an innovative gene therapy for human osteoarthritis [4,5], taking advantage of the mitochondria's inherent protein translation capability.

METHODS: rAAV vectors were packaged, purified, and titrated as previously described [6,7]. rAAV-RFP carries the *Discosoma* sp. red fluorescent protein (RFP) controlled by the CMV-IE promoter/enhancer [7]. Human osteoarthritic articular cartilage biopsies (n = 5; 6-mm diameter; Mankin score = 7-9) were randomly collected from the femoral condyle of patients undergoing total knee arthroplasty and primary human osteoarthritic articular chondrocytes were isolated using established protocols [6]. Mitochondria were immediately extracted from freshly isolated the chondrocytes (10^7 cells) using the Mitochondria Isolation Kit (Thermo Scientific™) [8] to obtain one unit that was subsequently resuspended for 1 h at 37°C in 110 µl of serum-free DMEM medium and rAAV (40 µl). The samples were then completed with 150 µl of DMEM, 10% FBS for another incubation of 24 or 48 h at 37°C. rAAV-treated mitochondria (1 unit) were transplanted in the chondrocytes (5 x 10^4 cells in 6-well plates) for 24 h at 37°C. Transgene (RFP) expression was monitored under fluorescence microscopy, including histomorphometric analyses [7]. Cell viability was measured using the Cell Proliferation Reagent WST-1 (Sigma-Aldrich, Merck), with absorbance values proportional to the cell numbers and with % viability calculated as previously described [7]. Cell apoptosis was estimated with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method (Invitrogen™), with apoptotic indices calculated as previously described [6]. Each condition was performed in triplicate in three independent experiments. The t-test was employed, with $P < 0.05$ considered statistically significant.

RESULTS: *Efficacy of rAAV-mediated gene transfer and overexpression in mitochondria*: Successful rAAV-mediated gene transfer was noted in mitochondria extracted from primary human osteoarthritic articular chondrocytes as seen by the effective, robust detection of a live red fluorescence signal when providing rAAV-RFP to the mitochondria relative to the control treatment (no vector condition) (**Fig. 1a**), with significant levels of rAAV gene transfer achieved over time (~55% and ~80% of RFP^+ mitochondria with rAAV-RFP after 24 h and 48 h *versus* always < 2% in the controls, i.e. ~28- and ~40-fold difference, respectively, always $P \leq 0.05$) (**Fig. 1b**). *Efficacy of implantation of rAAV-treated mitochondria in primary human osteoarthritic chondrocytes*: Successful implantation of rAAV-treated mitochondria was noted in primary human osteoarthritic articular chondrocytes as seen by the effective, robust detection of a live red fluorescence signal when providing rAAV-RFP-treated mitochondria (1 unit) to the cells relative to the control treatment (mitochondria without vector) (**Fig. 2a**), with significant levels of mitochondria-guided rAAV gene transfer achieved over time (~75% and ~80% of RFP^+ cells with rAAV-RFP-treated mitochondria after 24 h and 48 h *versus* always < 2% in the controls, i.e. ~38- and ~40-fold difference, respectively, always $P \leq 0.05$) (**Fig. 2b**). *Safety of implantation of rAAV-treated mitochondria in primary human osteoarthritic articular chondrocytes*: Implantation of mitochondria in primary human osteoarthritic articular chondrocytes was well tolerated as seen by the results of a TUNEL assay (**Fig. 3a**), with optimal protective (anti-apoptotic) effects achieved when providing high amounts of mitochondria (~3.4-fold decrease with either 1 or 2 units mitochondria after 24 h *versus* control treatment without mitochondria, $P \leq 0.05$) (**Fig. 3b**). Safe implantation of rAAV-treated mitochondria (1 unit) was noted in primary human osteoarthritic articular chondrocytes as seen by the results of a WST-1 assay, with protective (survival) effects on the cells (~1.7- and ~1.9-fold increase after 24 h and 48 h *versus* control treatment without mitochondria, always $P \leq 0.05$, without significant difference *versus* the implantation of untreated mitochondria, always $P \geq 0.05$) (**Fig. 3c**).

DISCUSSION: The current study reveals the benefits of implanting mitochondria to guide the safe and effective overexpression of genetic sequences in human osteoarthritic articular chondrocytes via rAAV gene transfer.

SIGNIFICANCE/CLINICAL RELEVANCE: This evaluation reports the potential of mitochondria implantation as a promising rAAV-mediated protein replacement therapy for human osteoarthritis.

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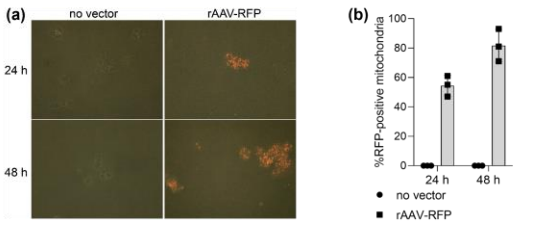

Fig. 1. Efficacy of rAAV-mediated gene transfer in mitochondria from primary human osteoarthritic articular chondrocytes. Detection of transgene (RFP) expression in mitochondria treated with rAAV-RFP (**a**) (representative data) with a semi-quantitative estimation of the % of RFP^+ mitochondria (**b**).

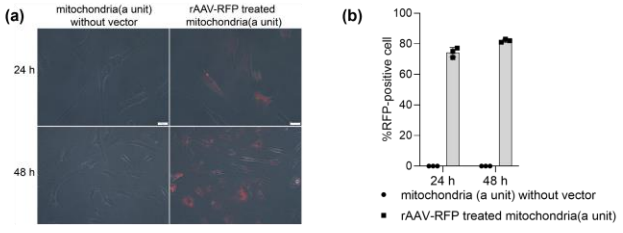

Fig. 2. Efficacy of implantation of rAAV-treated mitochondria in primary human osteoarthritic articular chondrocytes. Detection of transgene (RFP) expression in human osteoarthritic articular chondrocytes modified with rAAV-RFP-treated mitochondria (**a**) (representative data) with a semi-quantitative estimation of the % of RFP^+ cells (**b**).

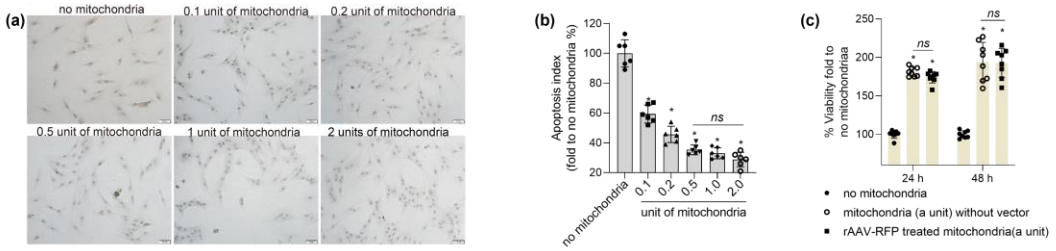

Fig. 3. Safety of implantation of mitochondria and of rAAV-treated mitochondria in primary human osteoarthritic articular chondrocytes. Detection of apoptosis in human osteoarthritic articular chondrocytes modified with mitochondria by TUNEL assay (**a**) (representative data; 24 h) with a semi-quantitative estimation of the apoptosis indices (**b**). Detection of cell viability in human osteoarthritic articular chondrocytes modified with rAAV-RFP-treated mitochondria by WST-1 assay (**c**).