Molecular Therapy
Original Article

Autologous transplantation of mitochondria/rAAV IGF-I platforms in human osteoarthritic articular chondrocytes to treat osteoarthritis

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Despite various available treatments, highly prevalent osteoarthritis (OA) cannot be cured in patients. In light of evidence showing mitochondria dysfunction during the disease progression, our goal was to develop a novel therapeutic concept based on the transplantation of mitochondria as a platform to deliver recombinant adeno-associated virus (rAAV) gene vectors with potency for OA. For the first time, to our best knowledge, we report the successful creation of a safe mitochondria/rAAV system effectively promoting the overexpression of a candidate insulin-like growth factor I (IGF-I) by administration to autologous human osteoarthritic articular chondrocytes versus control conditions (reporter mitochondria/rAAV *lacZ* system, rAAV-free system, absence of mitochondria transplantation; up to 8.4-fold difference). The candidate mitochondria/rAAV IGF-I system significantly improved key activities in the transplanted cells (proliferation/survival, extracellular matrix production, mitochondria functions) relative to the control conditions (up to a 9.5-fold difference), including when provided in a pluronic F127 (PF127) hydrogel for reinforced delivery (up to a 5.9-fold difference). Such effects were accompanied by increased levels of cartilage-specific SOX9 and *Mfn-1* (mitochondria fusion) and decreased levels of *Drp-1* (mitochondria fission) and proinflammatory tumor necrosis factor alpha (*TNF-α*; up to 4.5-fold difference). This study shows the potential of combining the use of mitochondria with rAAV as a promising approach for human OA.

INTRODUCTION

Osteoarthritis (OA) is a highly prevalent human chronic, serious disease of the joints associated with impaired functions and disability in patients, representing a considerable socio-economic burden for which there is no definitive cure to date.[1–6] OA is associated with a progressive and irreversible degradation of the articular cartilage, with changes in the subchondral bone structure and the formation of osteophytes, together with inflammation and synovitis,[7–9] overall impairing the quality of life of affected individuals. Despite the availability of a variety of treatment options, among which include pharmacological regimens and surgical interventions, none of them allow the full regeneration of damaged tissues in the joint of patients with OA,[5,10–14] demonstrating the critical need for new, effective therapies.

Interestingly, OA progression has been associated with mitochondria dysfunction in the articular chondrocytes, affecting several key processes involved in the maintenance of the cartilage integrity and leading to increased oxidative stress, inflammation, and matrix catabolism and altered levels of cell vitality[15–21] due to the critical roles of these organelles in the energy supply, metabolism, and survival of eukaryotic cells.[22,23] Such dysfunction has been linked to mutations in the mitochondrial DNA (mtDNA) as genetic variants (haplogroups), altering the metabolic and physiological processes in OA chondrocytes.[24–30] In this regard, gene therapy (including genome editing techniques) that offers attractive tools to correct gene mutations, in particular for the treatment of monogenic disorders,[31–35] may be applied against mitochondria-associated pathologies[36–47] using either the nucleus/cytoplasmic pathway (indirect complementation with mitochondrial translocation of the gene product) or the intrinsic (direct) mitochondria transcription/translation pathway.[37,48–51] Yet, due to the complexity, heteroplasmy, and high variability of the haplogroups and mutations in the mtDNA between patients with OA[26,27,52,53] and challenges in genetically targeting the mitochondria in a specific, effective, and durable manner,[48,49,54] gene therapy that aims at addressing such pathological mtDNA diversity in OA remains elusive.[37,55]

An alternative strategy to tackle mitochondria dysfunction in OA is to manipulate these central organelles in order to generate therapeutic cell-based systems as prospective carriers for OA gene therapy in light of evidence showing the feasibility and benefits of mitochondria transplantation in patients[56–60] and in preclinical models of joint disorders *in vitro* and *in vivo* via direct knee intra-articular injection.[61,62] The goal of the present study was to

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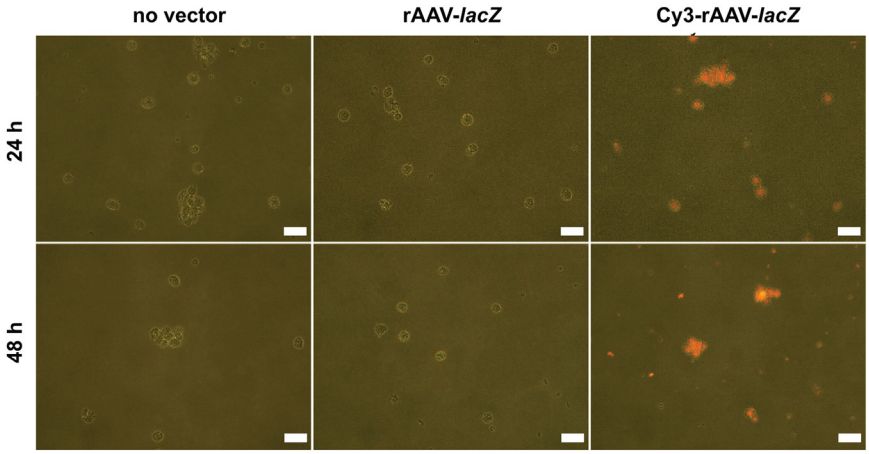

Figure 1. rAAV delivery to hOAC mitochondria
Isolated hOAC mitochondria were treated with a Cy3-labeled rAAV-*lacZ* vector (Cy3-rAAV-*lacZ*) or control conditions (unlabeled rAAV-*lacZ* vector: rAAV-*lacZ*; 10% sucrose: no vector), and Cy3 labeling was detected at the denoted time points (magnification ×40; overlay; scale bars: 40 μm).[80]

combine the use of mitochondria isolated from human primary OA articular chondrocytes (the ultimate target for therapy) with gene transfer vectors based on the adeno-associated virus (AAV) as an innovative therapeutic platform amenable to cell transplantation in order to correct mitochondria dysfunction in autologous OA cells.[56] Recombinant AAV (rAAV) vectors were selected as highly effective gene carriers for OA,[63–66] especially serotype 2,[63,65] compared with less effective or more deleterious vector classes (nonviral, adenoviral, retro-/lentiviral constructs)[50,54,67–70] and due to evidence of their ability to rectify mtDNA mutations by indirect nucleus/cytoplasmic complementation[71] or via direct mitochondria targeting.[72] Such an approach based on the transplantation of mitochondria/rAAV platforms might be more advantageous than the direct use of rAAV vectors by maximizing the rescue of the mitochondrial functions in OA cells using additional, exogenous mitochondria.[56,61] The insulin-like growth factor I (IGF-I) was chosen as the therapeutic candidate to address OA mitochondria dysfunction in light of its major roles in mitochondria protection, enhancing cell survival and metabolism.[68,73–76] The current results show that competent, safe transplantation of therapeutic mitochondria/rAAV (IGF-I) autologous platforms stimulates the biological activities of human OA chondrocytes (hOACs; proliferation/survival, extracellular matrix production, mitochondria functions) as a free system or in a biocompatible thermosensitive hydrogel (pluronic F127 [PF127], approved by the US Food and Drug Administration [FDA]) that can form by sol-gel transition at 37°C for improved delivery in biomedical applications,[77–79] allowing, in particular, for minimally invasive injection in articular cartilage lesions *in vivo*,[78] showing the value of this strategy for a future treatment of human OA.

RESULTS

Efficacy of rAAV delivery to isolated hOAC mitochondria

The feasibility of delivering rAAV vectors to isolated hOAC mitochondria was first tested using Cy3-labeled rAAV vectors versus control conditions (no-vector treatment, unlabeled rAAV vectors).

The successful delivery of rAAV-*lacZ* to hOAC mitochondria was achieved as observed by the strong detection of a live fluorescent signal when using a Cy3-labeled rAAV-*lacZ* vector ("Cy3-rAAV-*lacZ*") after 24 and 48 h (83% and 73% of fluorescent mitochondria, respectively) relative to the control conditions (no-vector treatment: "no vector"; unlabeled rAAV-*lacZ* vector: "rAAV-*lacZ*"), where no signal was detected (Figure 1).

Efficacy of rAAV-treated mitochondria transplantation in hOACs

The feasibility of transplanting rAAV-treated mitochondria in hOACs was tested using the reporter rAAV-RFP (red fluorescent protein) and the candidate rAAV-hIGF-I vectors versus respective control conditions.

Successful transplantation of reporter (RFP) rAAV-treated mitochondria in hOACs was achieved as observed by the robust detection of a live (red) fluorescent signal when providing rAAV-RFP-treated mitochondria to the cells ("mitochondria/rAAV-RFP") and co-localizing with the mitochondria as seen using the MitoTracker Green FM relative to the control condition (mitochondria without vector: "mitochondria/no vector"; Figure 2A), with significantly attained levels of RFP expression (∼75% and ∼80% of RFP^+ hOACs after 24 and 48 h, respectively, versus always <2% in the corresponding controls, i.e., ∼38- and ∼40-fold differences, respectively, $p \leq 0.0001$; Figure 2B).

Successful transplantation of candidate (IGF-I) rAAV-treated mitochondria in hOACs was also achieved as observed by significant IGF-I expression levels assessed by immunocytochemical analysis (Figures 2C and 2D) and ELISA (Figure 2E) when providing rAAV-hIGF-I-treated mitochondria to the cells ("mitochondria/rAAV-hIGF-I") relative to the control conditions (absence of mitochondria transplantation: "no mitochondria"; mitochondria without vector: mitochondria/no vector; rAAV-*lacZ*-treated mitochondria: "mitochondria/rAAV-*lacZ*"; up to 2.2- and 8.4-fold differences after 24 h by immunocytochemical analysis and ELISA, respectively; $p \leq 0.0001$).

Safety of rAAV-treated mitochondria transplantation in hOACs

The safety of transplanting rAAV-treated mitochondria in hOACs was tested using the reporter rAAV-RFP vector versus control conditions (absence of mitochondria transplantation, mitochondria without vector).

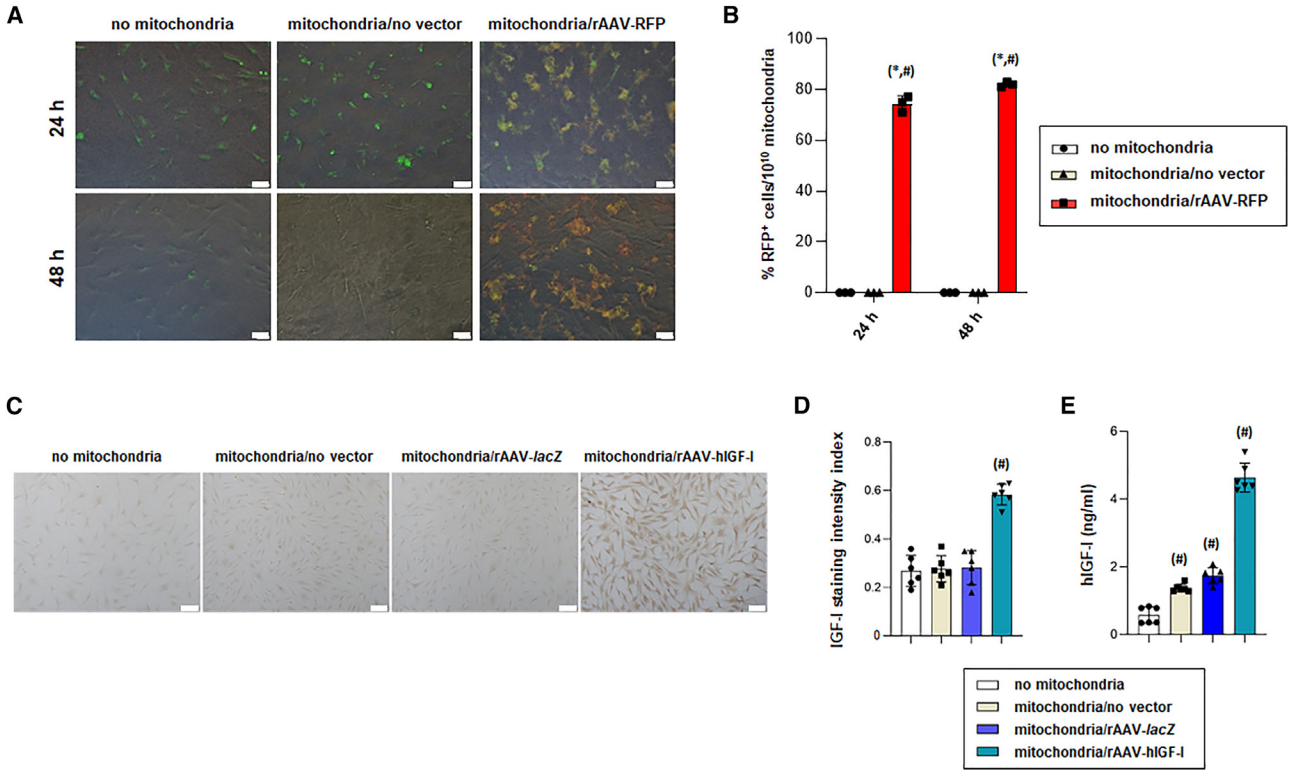

Figure 2. rAAV-treated mitochondria transplantation in hOACs

hOAC mitochondria treated with rAAV vectors (rAAV-RFP: mitochondria/rAAV-RFP; rAAV-*lacZ*: mitochondria/rAAV-*lacZ*; rAAV-hIGF-I: mitochondria/rAAV-hIGF-I) or 10% sucrose (mitochondria/no vector) were transplanted in hOACs, using a control condition without mitochondria (no mitochondria). Detection of (A and B) RFP expression in the presence of MitoTracker Green FM at the denoted time points (magnification ×20; overlay) and (C–E) IGF-I expression after 24 h (magnification ×10) (scale bars: 100 μm).[63,81] Data are given as means ± SD. Statistically significant relative to *the mitochondria/no-vector treatment and #the no-mitochondria treatment.

The safe, protective (proliferative) transplantation of rAAV-treated mitochondria in hOACs was achieved as observed by significantly increased levels of cell viability when providing rAAV-RFP-treated mitochondria to the cells (mitochondria/rAAV-RFP) relative to the control condition in the absence of mitochondria transplantation (no mitochondria; 1.8- and 2-fold differences after 24 and 48 h, respectively; $p \leq 0.0001$; Figure 3A). Importantly, the presence of rAAV in the mitochondria (mitochondria/rAAV-RFP) exerted no deleterious effects on the cells relative to the control condition when mitochondria were provided without a vector (mitochondria/no vector; $p \geq 0.986$; Figure 3A). The safe, protective (anti-apoptotic) transplantation of rAAV-treated mitochondria in hOACs was confirmed, with significantly decreased levels of apoptosis when providing rAAV-RFP-treated mitochondria to the cells (mitochondria/rAAV-RFP) relative to the control conditions (absence of mitochondria transplantation: no mitochondria; mitochondria without vector: mitochondria/no vector; 3- and 1.8-fold differences after 24 h, respectively; $p \leq 0.041$; Figures 3B and 3C).

Effects of rAAV-hIGF-I-treated mitochondria transplantation in hOACs on the biological activities of the cells

The effects of transplanting candidate rAAV-treated mitochondria in hOACs on the biological activities of the cells were next tested using the candidate rAAV-hIGF-I vector versus control conditions (absence of mitochondria transplantation, mitochondria without vector, and rAAV-*lacZ*-treated mitochondria).

Transplantation of rAAV-hIGF-I-treated mitochondria in hOACs (mitochondria/rAAV-hIGF-I) favorably impacted the viability of the cells, with significant increases in the levels of viability and the DNA contents relative to the control conditions (absence of mitochondria transplantation: no mitochondria; mitochondria without vector: mitochondria/no vector; rAAV-*lacZ*-treated mitochondria: mitochondria/rAAV-*lacZ*; up to 1.9- and 1.8-fold differences after 24 h in cell viability and DNA contents, respectively; $p \leq 0.0001$; Figures 4A and 4B) and significant decreases in the levels of cell apoptosis (up to 4.2-fold difference after 24 h; $p \leq 0.0001$; Figures 4C and 4D).

Transplantation of rAAV-hIGF-I-treated mitochondria in hOACs (mitochondria/rAAV-hIGF-I) further advantageously triggered the production of preferable extracellular matrix compounds, with significant increases in the proteoglycan deposition and contents and in type II collagen deposition relative to the control conditions (no mitochondria, mitochondria/no vector, and mitochondria/rAAV-*lacZ*; up

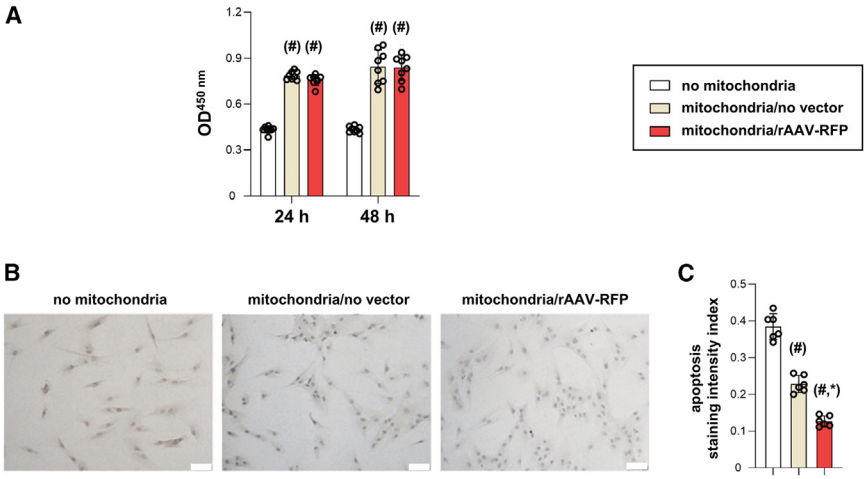

Figure 3. Safety of rAAV-treated mitochondria transplantation in hOACs

hOAC mitochondria treated with rAAV-RFP (mitochondria/rAAV-RFP) or 10% sucrose (mitochondria/no vector) were transplanted in hOACs, using a control condition without mitochondria (no mitochondria). Detection of (A) cell viability at the denoted time points and (B and C) apoptosis after 24 h (magnification ×20; scale bars: 100 μm).[63,80,81] Data are given as means ± SD. Statistically significant relative to #the no-mitochondria treatment and *the mitochondria/no-vector treatment.

to 2-, 9.5-, and 1.7-fold differences after 24 h in the proteoglycan deposition and contents and type II collagen deposition, respectively; $p \leq 0.0001$; Figures 4E–4I).

The transplantation of rAAV-hIGF-I-treated mitochondria in hOACs (mitochondria/rAAV-hIGF-I) also beneficially restrained the production of undesirable extracellular matrix compounds, with significant decreases in osteogenic type I collagen and hypertrophic type X collagen deposition relative to the control conditions (no mitochondria, mitochondria/no vector, and mitochondria/rAAV-*lacZ*; up to 2.5-fold difference after 24 h in type I and type X collagen deposition; $p \leq 0.0011$; Figures 4H, 4J, and 4K).

Such effects of rAAV-hIGF-I-treated mitochondria transplantation in hOACs (mitochondria/rAAV-hIGF-I) were accompanied by significantly increased levels of cartilage-specific SOX9 expression relative to the control conditions (no mitochondria, mitochondria/no vector, and mitochondria/rAAV-*lacZ*; up to 3.7-fold difference after 24 h; $p \leq 0.0001$; Figures 4H and 4L).

Effects of rAAV-hIGF-I-treated mitochondria transplantation in hOACs on the mitochondria functions of the cells

The effects of transplanting candidate rAAV-treated mitochondria in hOACs on the mitochondria functions of the cells were then tested using the candidate rAAV-hIGF-I vector versus control conditions (absence of mitochondria transplantation, mitochondria without vector, and rAAV-*lacZ*-treated mitochondria).

The transplantation of rAAV-hIGF-I-treated mitochondria in hOACs (mitochondria/rAAV-hIGF-I) valuably targeted the mitochondria functions of the cells, with significant increases in the ATP contents and mitochondrial membrane potential (ΔΨM) relative to the control conditions (absence of mitochondria transplantation: no mitochondria; mitochondria without vector: mitochondria/no vector; rAAV-*lacZ*-treated mitochondria: mitochondria/rAAV-*lacZ*; up to 1.6- and 5.7-fold differences after 24 h in the ATP contents and ΔΨM, respectively; $p \leq 0.0006$; Figures 5A–5C) and significant decreases in the reactive oxygen species (ROS) levels (up to 3.2-fold difference after 24 h; $p \leq 0.0001$; Figure 5D).

Such effects of rAAV-hIGF-I-treated mitochondria transplantation in hOACs (mitochondria/rAAV-hIGF-I) were accompanied by significantly decreased levels of *Drp-1* (dynamin-related protein 1) expression (a marker of mitochondria fission), significantly increased levels of *Mfn-1* (mitofusin-1) expression (a marker of mitochondria fusion), and significantly decreased levels of proinflammatory tumor necrosis factor alpha (*TNF-α*) expression relative to the control conditions (no mitochondria, mitochondria/no vector, and mitochondria/rAAV-*lacZ*; up to 2.1-, 2-, and 4.5-fold differences after 24 h in *Drp-1*, *Mfn-1*, and *TNF-α* expression, respectively; $p \leq 0.0001$; Figures 5E and 5F).

PF127-guided rAAV-treated mitochondria co-culture in hOACs

The feasibility of using PF127 hydrogels to guide the delivery of candidate rAAV-treated mitochondria to hOACs was finally tested versus the absence of mitochondria administration as the most significant control condition relative to the other control treatments tested earlier (Figures 2, 4, and 5).

The successful delivery of PF127-guided candidate (IGF-I) rAAV-treated mitochondria to hOACs was achieved as seen by significant IGF-I expression levels assessed by immunocytochemical analysis (Figures 6A and 6B) and ELISA (Figure 6C) when providing PF127-guided rAAV-hIGF-I-treated mitochondria to the cells ("mitochondria/rAAV-hIGF-I in PF127") relative to the control condition (absence of mitochondria administration: no mitochondria; 4- and 4.9-fold differences after 24 h by immunocytochemical analysis and ELISA, respectively; $p \leq 0.0001$).

The delivery of PF127-guided rAAV-hIGF-I-treated mitochondria to hOACs (mitochondria/rAAV-hIGF-I in PF127) favorably impacted the viability of the cells, with significant increases in the levels of cell viability relative to the control condition (no mitochondria; 2.5-fold difference after 24 h; $p \leq 0.0001$; Figure 7A) and significant

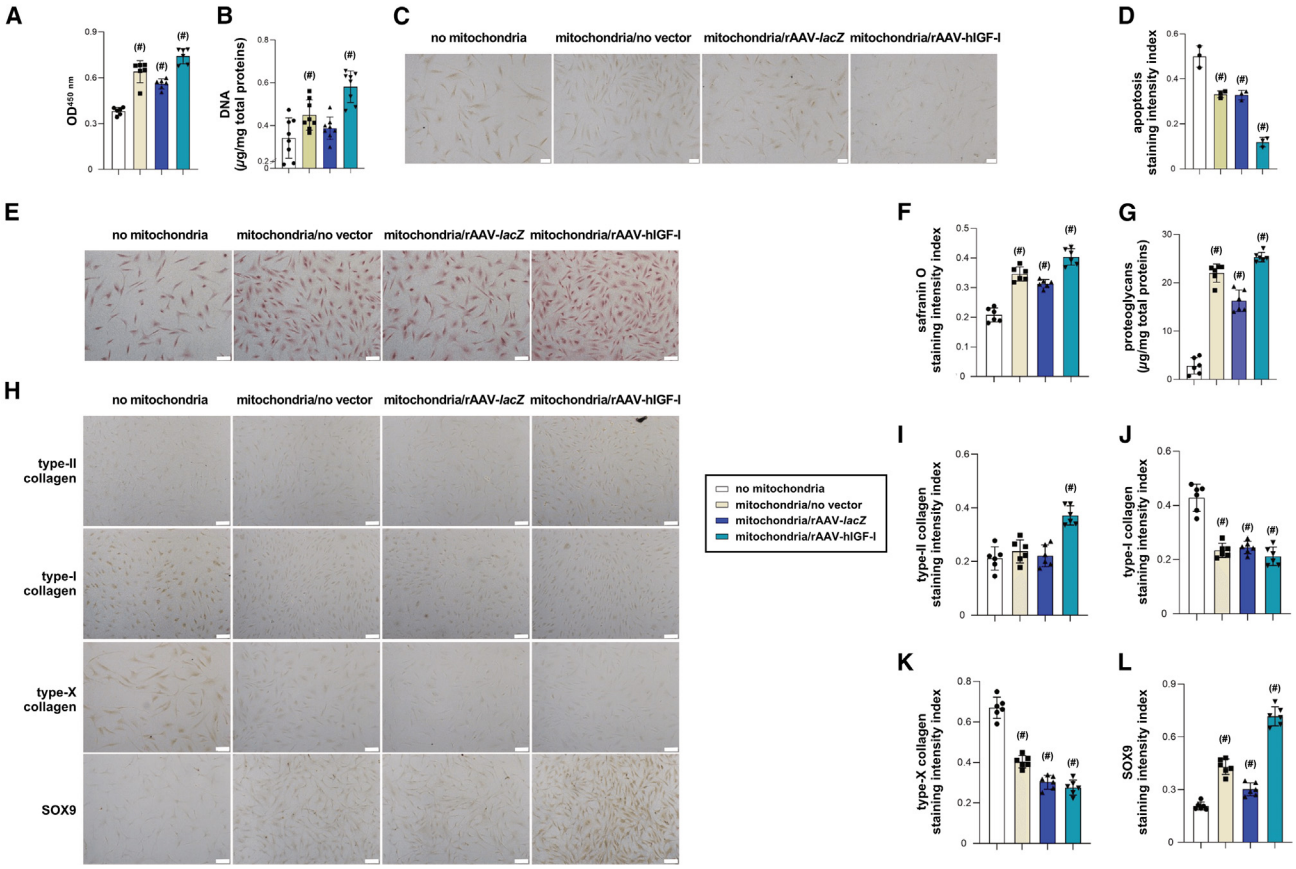

Figure 4. Biological activities of hOACs transplanted with rAAV-hIGF-I-treated mitochondria

hOAC mitochondria treated with rAAV vectors (rAAV-*lacZ*: mitochondria/rAAV-*lacZ*; rAAV-hIGF-I: mitochondria/rAAV-hIGF-I) or 10% sucrose (mitochondria/no vector) were transplanted in hOACs, using a control condition without mitochondria (no mitochondria). Detection of (A) cell viability, (B) the DNA contents, (C and D) apoptosis (magnification ×20), (E and F) proteoglycan deposition (magnification ×20), (G) the proteoglycan contents, and (H–L) type II, I, and X collagen deposition and SOX9 expression (magnification ×10) after 24 h (scale bars: 100 µm).[63,80,81] Data are given as means ± SD. Statistically significant relative to #the no-mitochondria treatment.

decreases in the levels of cell apoptosis (3.8-fold difference after 24 h; $p \leq 0.0002$; Figures 7B and 7C). The delivery of PF127-guided rAAV-hIGF-I-treated mitochondria to hOACs (mitochondria/rAAV-hIGF-I in PF127) further advantageously triggered the production of preferable extracellular matrix compounds, with significant increases in the proteoglycan deposition and contents and type II collagen deposition relative to the control condition (no mitochondria; 1.9-, 3.9-, and 2.2-fold differences after 24 h in the proteoglycan deposition and contents and type II collagen deposition, respectively; $p \leq 0.0001$; Figures 7D–7H). The delivery of PF127-guided rAAV-hIGF-I-treated mitochondria to hOACs (mitochondria/rAAV-hIGF-I in PF127) also beneficially restrained the production of undesirable extracellular matrix compounds, with significant decreases in osteogenic type I collagen and hypertrophic type X collagen deposition relative to the control condition (no mitochondria; up to 2.1-fold difference after 24 h in type I and type X collagen deposition, respectively; $p \leq 0.0001$; Figures 7G, 7I, and 7J). Such effects of PF127-guided rAAV-hIGF-I-treated mitochondria administration in hOACs (mitochondria/rAAV-hIGF-I in PF127) were accompanied by significantly increased levels of cartilage-specific SOX9 expression relative to the control condition (no mitochondria; 2.6-fold difference after 24 h; $p \leq 0.0013$; Figures 7G and 7K).

The delivery of PF127-guided rAAV-hIGF-I-treated mitochondria to hOACs (mitochondria/rAAV-hIGF-I in PF127) valuably targeted the mitochondria functions of the cells, with significant increases in the ATP contents and $\Delta\Psi M$ relative to the control condition (no mitochondria; 1.5- and 5.9-fold differences after 24 h in the ATP contents and $\Delta\Psi M$, respectively; $p \leq 0.050$; Figures 8A–8C) and significant decreases in the ROS levels (2.2-fold difference after 24 h; $p \leq 0.0001$; Figure 8D). Such effects of PF127-guided rAAV-hIGF-I-treated mitochondria administration in hOACs (mitochondria/rAAV-hIGF-I in PF127) were accompanied by significantly decreased levels of *Drp-1* expression, significantly increased levels of *Mfn-1* expression, and significantly decreased levels of *TNF-α* expression relative to the control condition (no mitochondria; 2-, 1.1-, and 4.1-fold differences after 24 h in *Drp-1*, *Mfn-1*, and *TNF-α* expression, respectively; $p \leq 0.047$; Figures 8E and 8F).

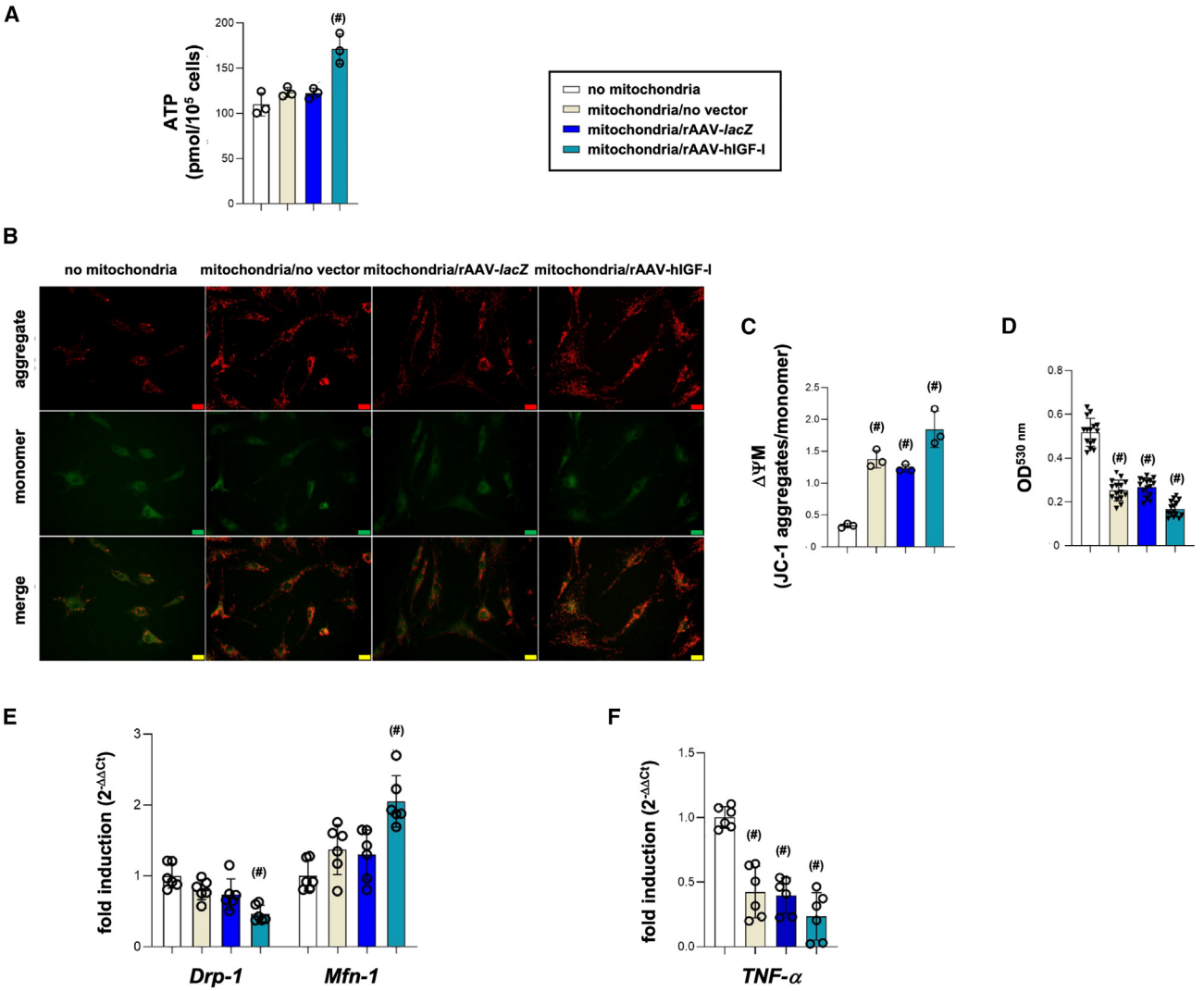

Figure 5. Mitochondria functions in hOACs transplanted with rAAV-hIGF-I-treated mitochondria

hOAC mitochondria treated with rAAV vectors (rAAV-*lacZ*: mitochondria/rAAV-*lacZ*; rAAV-hIGF-I: mitochondria/rAAV-hIGF-I) or 10% sucrose (mitochondria/no vector) were transplanted in hOACs, using a control condition without mitochondria (no mitochondria). Detection of (A) the ATP contents, (B and C) the mitochondrial membrane potential (ΔΨM) (magnification ×20; scale bars: 100 μm), (D) the ROS levels, and (E and F) the *Drp-1*, *Mfn-1*, and *TNF-α* gene expression profiles relative to the no-mitochondria treatment after 24 h.[81–84] Data are given as means ± SD. Statistically significant relative to #the no-mitochondria treatment.

DISCUSSION

Gene therapy[31–35] offers valuable tools to treat OA,[67,69,70] a progressive and irreversible disorder for which there is no reliable cure to date.[3–5,12,13] While mtDNA genetic variants (haplogroups) occurring in OA may be corrected by gene therapy, their complexity and variability[24,25,27–30,53] make them relatively difficult to tackle in the widely affected human population. The goal of this study was to rather propose and investigate a new avenue of therapeutic research based on the comprehensive development of transplantable, regenerative mitochondria gene therapy platforms for human OA.[56,60–62] This system was generated as a means to deliver highly effective rAAV vectors[64–66] coding for the potent reparative IGF-I agent[63,65,68,73–76] in autologous primary hOACs in order to address and counteract OA-associated mitochondria dysfunction mechanisms.[15–21]

For the first time, to our best knowledge, the present results show the successful, convenient delivery of rAAV vectors to isolated hOACs mitochondria, while more complex, related work has commonly been performed thus far by complementing (nucleus/cytoplasmic pathway) or targeting the mitochondria in the entire cell using rAAVs to rectify mtDNA mutations.[71,72] The data next reveal, also for the first time, to our best knowledge, that hOACs are successfully and safely amenable to transplantation with mitochondria/rAAV reporter (RFP) and candidate (IGF-I) systems relative to their respective

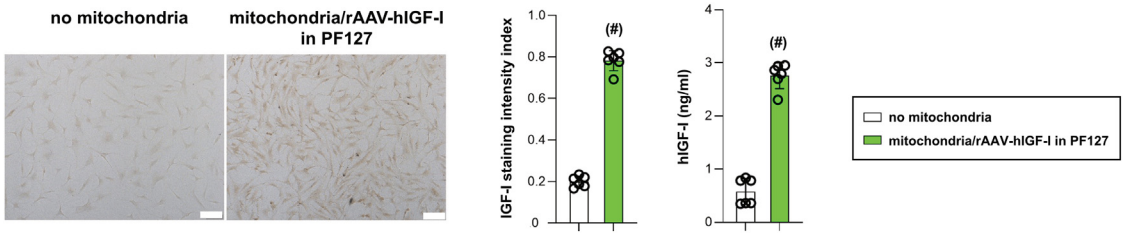

Figure 6. PF127-guided rAAV-hIGF-I-treated mitochondria delivery to hOACs

hOAC mitochondria treated with rAAV-hIGF-I and formulated in PF127 hydrogels (mitochondria/rAAV-hIGF-I in PF127) were applied to hOACs, using a condition without mitochondria as the most significant control (Figures 2, 4, and 5) (no mitochondria). Detection of IGF-I expression (magnification ×10; scale bars: 100 μm) after 24 h.[63,81] Data are given as means ± SD. Statistically significant relative to #the no-mitochondria treatment.

control conditions (up to ∼80%) in a protective and anti-apoptotic manner for up to 48 h, the longest time point evaluated as proof of concept. Remarkably, the candidate mitochondria/rAAV (IGF-I) system was capable of enhancing the levels of cell viability and the deposition of preferable extracellular matrix components (cartilage-specific proteoglycans, type II collagen) in transplanted hOACs while reducing those of unwanted compounds (osteogenic type I collagen, hypertrophic type X collagen) versus control treatments, probably due to increased expression levels of the pro-anabolic SOX9 transcription factor.[63] These results are in good agreement with the properties of IGF-I[85,86] and earlier findings where the same rAAV-hIGF-I vectors were directly applied to hOACs.[63] Interestingly, the addition of mitochondria without rAAVs also increased the deposition of proteoglycans, in good agreement with the chondroinductive effects of these organelles in chondrocytes.[87] In contrast, the administration of mitochondria with rAAV-*lacZ* decreased the proteoglycan contents, an observation also reported by others,[88] possibly due to innate immune sensing of rAAV vectors by Toll-like receptors (TLRs),[89] an effect that was likely compensated for by the overexpression of pro-anabolic IGF-I. Furthermore, the candidate mitochondria/rAAV (IGF-I) system advantageously exhibited mitoprotective activities in transplanted hOACs, enhancing the levels of ATP, the $\Delta\Psi M$, and the expression of *Mfn-1* (mitochondria fusion) while reducing the levels of ROS and the expression of *Drp-1* (mitochondria fission) and inflammatory *TNF-α* relative to the control treatments, again concordant with the activities of IGF-I.[68,73,74,76] Similar results were noted when providing the candidate mitochondria/rAAV (IGF-I) system to hOACs via a biocompatible PF127 hydrogel for reinforced delivery,[77–79] reaching beneficial levels in the range of those achieved in the hydrogel-free approach and expanding work using other types of hydrogels in the absence of a gene therapy component.[90,91]

In light of such encouraging, preliminary observations at an early time point as a proof of concept of the current newly described system, work is now ongoing to monitor the potential of transplanting the therapeutic mitochondria/rAAV (IGF-I) platform in hOACs over extended periods of time. In the event of favorable, sustained, and safe outcomes, the approach will be translated in articular cartilage lesions *in vitro* (human osteochondral defect model)[80] and preclinical *in vivo* models of the disease,[61,64–66] adapting the amounts of autologous mitochondria and/or the dose of rAAV vector for transplantation *in vivo* in the unlikely case of detrimental reactions or moderate effects. Alternatively, autologous mitochondria may be isolated from other cell sources, including progenitor cells, such as mesenchymal stromal cells (MSCs),[91,92] with a regenerative potency in OA.[93,94] Overall, the current technology shows the value of rAAV-associated mitochondria transplantation as a promising therapeutic ("rescue") strategy for human OA.

MATERIALS AND METHODS

Reagents

All reagents are from Sigma (Munich, Germany), including the cell proliferation reagent WST-1 and the Fluorometric Intracellular ROS kit (MAK144), unless otherwise indicated. Transwell plates (#3414) were purchased at Corning (Merck, Darmstadt, Germany). The Cy3 Ab Labeling Kit was purchased from Amersham/GE Healthcare (Munich, Germany). The MitoTracker Green FM (M7514) is from Invitrogen (Darmstadt, Germany). PF127 was kindly provided by BASF (Ludwigshafen, Germany). The JC-1 fluorescent dye (CBIC2) is from MedChem Express (BIOZOL, Eching, Germany). The Mitochondria Isolation Kit, ATP Determination Kit, and BCA Protein Assay are from Thermo Fisher Scientific (Karlsruhe, Germany). The anti-IGF-I (AF-291-NA) antibody is from R&D Systems (Bio-Techne, Wiesbaden, Germany), the anti-type II collagen (II-II6B3) antibody is from DSHB (Iowa City, IA, USA), the anti-type I collagen (QF-5610) antibody is from Acris (Hiddenhausen, Germany), the anti-type X collagen (SAB4200800) antibody is from Sigma, and the anti-SOX9 (C-20) antibody is from Santa Cruz Biotechnology (Heidelberg, Germany). Biotinylated secondary antibodies and the ABC kit are from Vector Laboratories (Grünberg, Germany). The human IGF-I ELISA (DG100B) is from R&D Systems. The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) kit (C10619) is from Invitrogen.

Cell culture

Human OA cartilage was obtained from the joints of patients undergoing total knee arthroplasty (n = 8, Mankin score: 7–9). The study was approved by the Ethics Committee of the Saarland Physicians Council (*Ärztekammer des Saarlandes*, reference no. Ha06/08), and

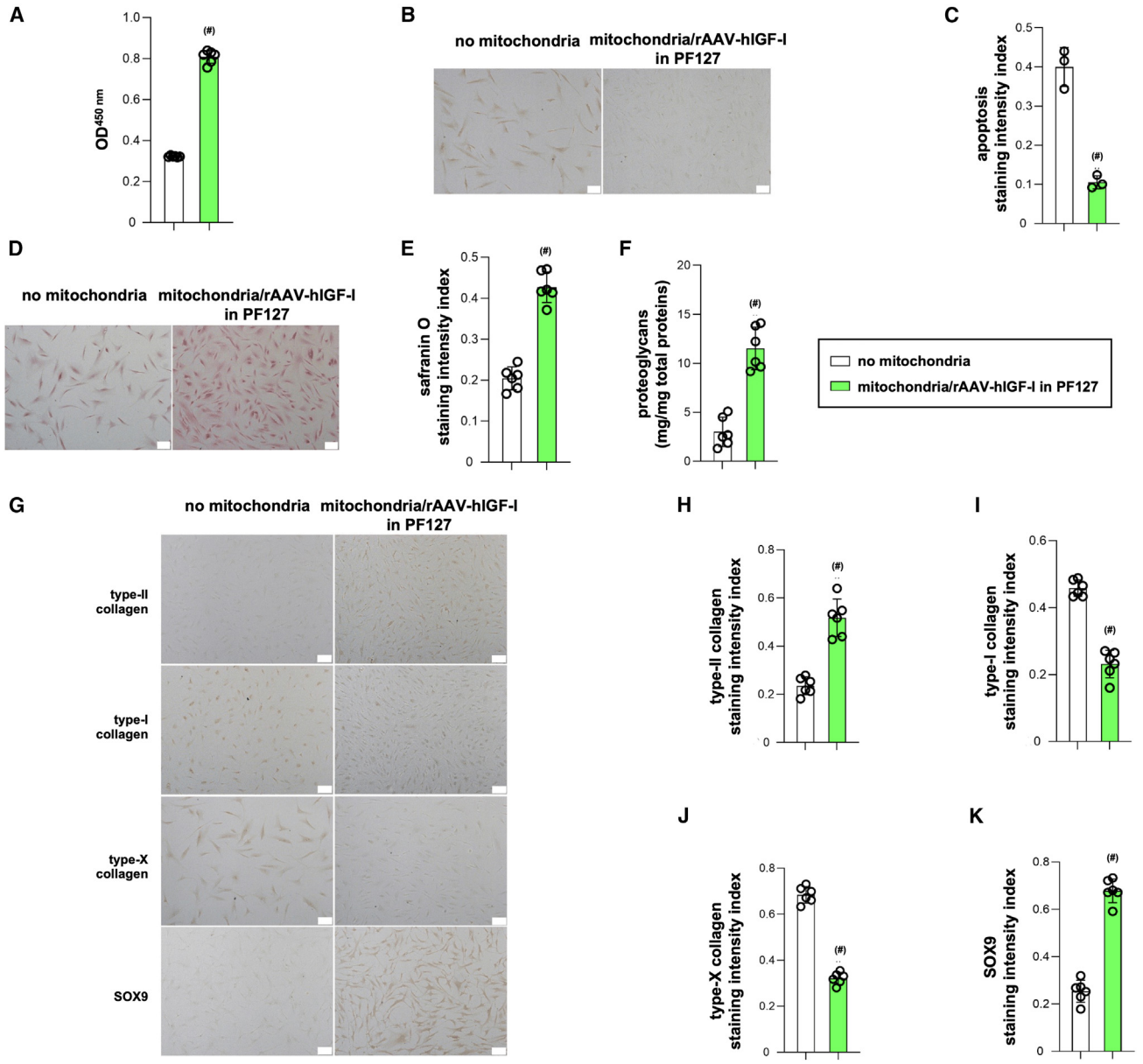

Figure 7. Biological activities of hOACs treated with PF127-guided rAAV-hIGF-I-treated mitochondria

hOAC mitochondria treated with rAAV-hIGF-I and formulated in PF127 hydrogels (mitochondria/rAAV-hIGF-I in PF127) were applied to hOACs, using a condition without mitochondria as the most significant control (Figures 2, 4, and 5) (no mitochondria). Detection of (A) cell viability, (B and C) apoptosis (magnification ×20), (D and E) proteoglycan deposition (magnification ×20), (F) the proteoglycan contents, and (G–K) type II, I, and X collagen deposition and SOX9 expression (magnification ×10) after 24 h (scale bars: 100 μm).[63,80,81] Data are given as means ± SD. Statistically significant relative to #the no-mitochondria treatment.

all procedures were in accordance with the Helsinki Declaration. Informed consent was obtained from all participants. hOACs were isolated as previously described[63,80,81] and used not later than passage 3. Cells were incubated in DMEM with 10% fetal bovine serum (complete medium) in the presence of 100 U/mL penicillin G and 100 μL/mL streptomycin as a final growth medium. Cells were plated in T75 flasks and maintained at 37°C in a humidified atmosphere with 5% CO_2.

Plasmids, rAAV vectors, and Cy3 vector labeling

The constructs were derived from pSSV9, an AAV-2 genomic clone.[95,96] rAAV-RFP carries the *Discosoma* sp. RFP gene, rAAV-*lacZ*; the *E. coli* β-galactosidase (*lacZ*) gene; and rAAV-hIGF-I, a human IGF-I cDNA, all controlled by the CMV-IE promoter/enhancer (Figure S1).[63,80,81] The vectors were packaged as conventional (not self-complementary) vectors using a helper-free, two-plasmid transfection system in the 293 packaging cell line

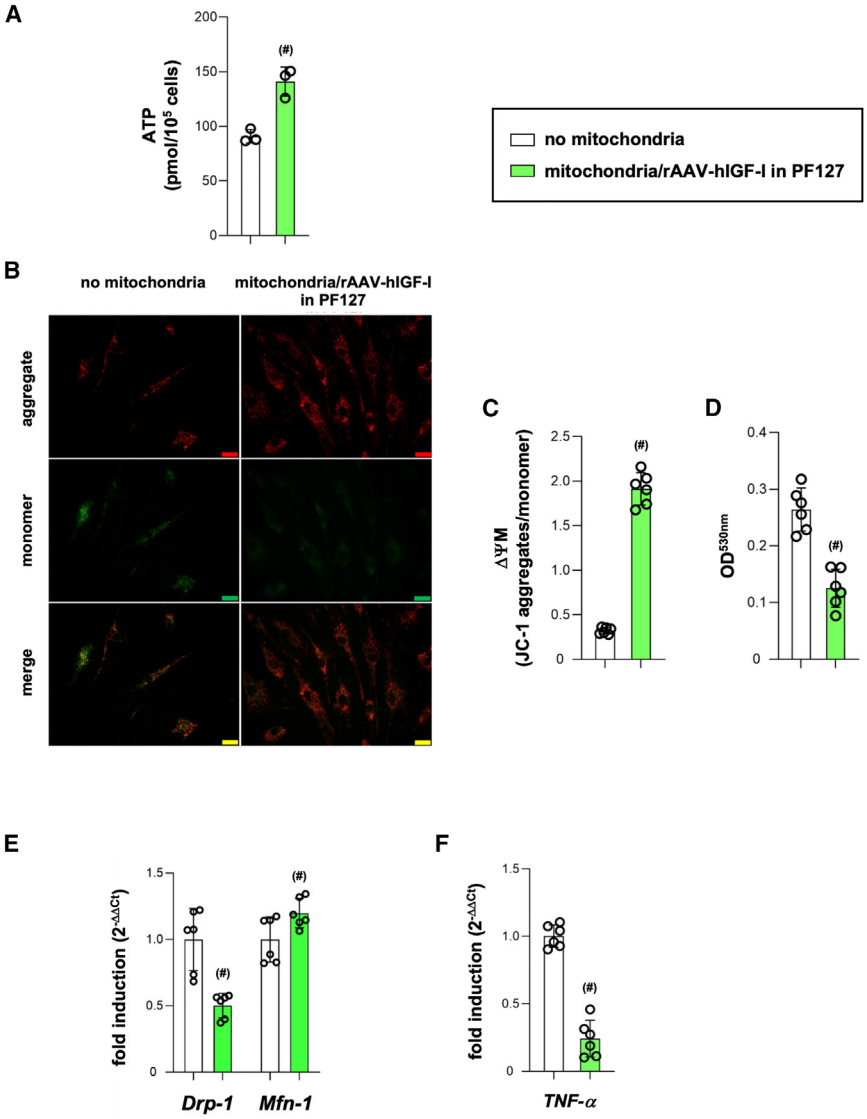

Figure 8. Mitochondria functions in hOACs treated with PF127-guided rAAV-hIGF-I-treated mitochondria

hOAC mitochondria treated with rAAV-hIGF-I and formulated in PF127 hydrogels (mitochondria/rAAV-hIGF-I in PF127) were applied to hOACs, using a condition without mitochondria as the most significant control (Figures 2, 4, and 5) (no mitochondria). Detection of (A) the ATP contents, (B and C) the mitochondrial membrane potential ($\Delta\Psi M$) (magnification ×20; scale bars: 100 μm), (D) the ROS levels, and (E and F) the *Drp-1*, *Mfn-1*, and *TNF-α* gene expression profiles relative to the no-mitochondria treatment after 24 h.[81–84] Data are given as means ± SD. Statistically significant relative to #the no-mitochondria treatment.

(an adenovirus-transformed human embryonic kidney cell line) with the packaging plasmid pXX2 and the adenovirus helper plasmid pXX6 as previously described.[80,81] The vector preparations in 10% sucrose were placed in a 10,000 molecular weight cutoff dialysis cassette (#66380, Thermo Fisher Scientific) and purified by dialysis for 24 h at 4°C against 10% sucrose (250 mL).[80,81] The dialyzed vector preparations (2 μL) were then titered by amplification with real-time PCR using the Brilliant SYBR Green QPCR Master Mix (Stratagene, Agilent Technologies, Waldbronn, Germany) on the Mx3000P QPCR operator system (Stratagene) as follows: initial incubation (95°C, 10 min) and amplification for 50 cycles (denaturation at 95°C, 15 s; annealing at 67°C, 5 s; extension at 72°C, 12 s).[80,81] The primers (Invitrogen) used for rAAV quantification were F-SV40pA (5′-CCA CATTTGTAGAGGTTTACTTGCT-3′) and R-SV40pA (5′-AGCTG CAATAAACAAGTTAACAACAAC-3′) (both 400 nM final concentration).[80,81] Titers averaged 10^{10} vector genomes/mL (~1/500 functional recombinant viral particles, i.e., 2×10^7 functional vector genomes/mL).[80,81,97]

Some rAAV vectors were labeled with the Cy3 Ab Labeling Kit as previously described.[80] Briefly, rAAV preparations (1 mL) were incubated with sodium carbonate/sodium bicarbonate buffer (pH 9.3) for 30 min at room temperature and then with Cy3 solution, followed by extensive dialysis against 20 mM HEPES (pH 7.5)/150 mL NaCl. Labeling was evaluated in the specific conditions by fluorescent microscopy using a 568 nm filter (Olympus CKX41; Hamburg, Germany).[80]

Mitochondria isolation, rAAV and PF127 treatments, and transplantation

Mitochondria were immediately extracted from freshly isolated hOACs (10^7 cells) using the Mitochondria Isolation Kit[82,83,98,99] to generate one unit of mitochondria (10^{10} mitochondria).

For the rAAV-treated mitochondria transplantation strategy, one unit of mitochondria was resuspended in a mixture of serum-free DMEM (110 μL) with rAAV (40 μL, i.e., 8×10^5 functional vector genomes)[81] (or with 40 μL of 10% sucrose for the no-vector control treatment) for 90 min at 37°C, followed by the addition of complete medium (150 μL) for 24 or 48 h at 37°C. The rAAV-treated mitochondria were centrifuged, resuspended in 2 mL growth medium, and transplanted in patient-matched hOACs (5×10^4 cells in 6-well plates) by direct administration to the cultures at 37°C[99] prior to the various evaluations performed at early time points for proof of concept. In some experiments, MitoTracker Green FM (100 nM) was added to the cultures for 45 min at 37°C to detect mitochondria according to the manufacturer's recommendations.

For the PF127-guided rAAV-treated mitochondria delivery strategy, a mixture of serum-free DMEM (60 μL) with rAAV (40 μL,

i.e., 8×10^5 functional vector genomes) (or with 40 μL of 10% sucrose for the no-vector control treatment) was incubated with one unit of mitochondria for 90 min at 37°C, followed by the addition of serum-free DMEM (100 μL) for 48 h at 37°C. PF127 hydrogels (24% w/v in 10% sucrose; 200 μL) prepared at 4°C[78] were then added to the rAAV-treated mitochondria for 30 min on ice. The rAAV-treated mitochondria in PF127 hydrogels (400 μL) were administered in patient-matched hOACs (5×10^4 cells in the lower chamber of a transwell in 6-well plates) by direct administration to the upper chamber of the transwell at 37°C[98] prior to the various evaluations performed at early time points for proof of concept.

Detection of transgene expression

Transgene (RFP) expression was monitored by fluorescent microscopy using a 568 nm filter, while detection of mitochondria with MitoTracker Green FM was performed using a 490 nm filter (Olympus CKX41).[63,81] Transgene (IGF-I) expression was assessed by immunocytochemical analysis using a specific antibody, a biotinylated secondary antibody, and the ABC method with diaminobenzidine (DAB) as the chromogen.[63,81] Samples were examined under light microscopy (Olympus BX 45). To control for secondary immunoglobulins, samples were processed with omission of the primary antibody.[63,81] Transgene (IGF-I) expression was also monitored by ELISA, as previously described, using a GENios spectrophotometer/fluorometer (Tecan, Crailsheim, Germany).[63,81]

Biochemical assays

Cell viability was monitored with the Cell Proliferation Reagent WST-1, with OD_{450} being proportional to the cell numbers as previously described.[63,80,81] Cell apoptosis was monitored with the TUNEL kit.[63] The DNA contents were assessed with the Hoechst 33258 assay and the proteoglycan contents by binding to the dimethylmethylene blue (DMMB) dye relative to the total protein contents measured using the BCA Protein Assay.[63,81] All measurements were performed using a GENios spectrophotometer/fluorometer (Tecan).[63,80,81]

Mitochondria functional tests

The ATP contents in the supernatants of hOAC cultures and the intracellular ROS levels in hOAC cultures were measured using the ATP Determination Kit and the Fluorometric Intracellular ROS Kit, respectively, as previously described.[82,83] All measurements were performed using a GENios spectrophotometer/fluorometer (Tecan).[82,83] The $\Delta\Psi M$ was measured using the JC-1 fluorescent dye as previously described.[82,84]

Histological and immunocytochemical analyses

The cultures were harvested and fixed in 4% paraformaldehyde. The deposition of proteoglycans was monitored by histological safranin O staining.[63,80,81] Expression of type I, II, and X collagens and SOX9 was evaluated by immunocytochemical analysis using specific primary antibodies, biotinylated secondary antibodies, and the ABC method with DAB as the chromogen.[63,80,81] Samples were examined under light microscopy (Olympus BX45). To control for secondary immunoglobulins, samples were processed with omission of the primary antibody.

Histomorphometric analyses

The percentage of fluorescent mitochondria using Cy3-labeled rAAV vectors ($Cy3^+$ mitochondria relative to the total numbers of mitochondria in culture), the percentage of RFP-stained cells (RFP^+ cells relative to the total numbers of cells on immunocytochemically stained cultures), the IGF-I, apoptotic, safranin O, type I, II, and X collagens, and SOX9 staining intensity indices (ratio of positively stained area to the total area evaluated on immunocytochemically stained cultures), and the $\Delta\Psi M$ (number of JC-1 aggregates to the number of monomers) were measured at four standardized sites with replicate samples[63,80–82,84] using ImageJ 1.53K (ImageJ, Bethesda, MD, USA).[100]

Real-time RT-PCR analysis

Total cellular RNA was extracted from the cultures using the RNeasy Protect Mini Kit with an on-column RNase-free DNase treatment (Qiagen, Hilden, Germany). RNA was eluted in 30 μL RNase-free water. Reverse transcription was carried out with 8 μL eluate using the 1st Strand cDNA Synthesis kit for RT-PCR (AMV; Roche Applied Science, Sigma).[81] An aliquot of the cDNA product (2 μL) was amplified with real-time PCR using the Brilliant SYBR Green QPCR Master Mix (Stratagene)[81] on the Mx3000P QPCR operator system (Stratagene) as follows: initial incubation (95°C, 10 min), amplification for 55 cycles (denaturation at 95°C, 30 s; annealing at 55°C, 1 min; extension at 72°C, 30 s), denaturation (95°C, 1 min), and final incubation (55°C, 30 s).[81] The primers (Invitrogen) used were *Drp-1* (a marker of mitochondria fission; forward 5′-TCAACCTCCGCG TCTACTC-3'; reverse 5′-GATCTGGAACTCGATGTCGGG-3′), *Mfn-1* (a marker of mitochondria fusion; forward 5′-GAGGTGC TATCTCGGAGACAC-3′; reverse 5′-GCCAATCCCACTAGGGA GAAC-3′), *TNF-α* (proinflammatory marker; forward 5′-AGAAC CCCCTGGAGATAACC-3′; reverse 5′-AAGTGCAGCAGGCAG AAGAG-3′), and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; forward 5′-GAAGGTGAAGGTCGGAGTC-3′; reverse 5′-GAAGATGGTGATGGGATTTC-3′) (all 150 nM final concentration).[81] Control conditions included reactions using water and non-reverse-transcribed mRNA. The specificity of the products was confirmed by melting curve analysis. The threshold cycle (Ct) value for each gene of interest was measured for each amplified sample using MxPro QPCR software (Stratagene), and values were normalized to GAPDH expression by using the $2^{-\Delta\Delta Ct}$ method.[81]

Statistical analysis

Each condition was performed in quintuplicate in four independent experiments. Data are given as the mean ± standard deviation (SD). Statistical analysis and representation were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). One-way analysis of variance with Tukey's least significant difference (LSD) or Games-Howell post hoc test was applied to evaluate differences between groups. Any $p \leq 0.05$ was considered statistically significant.

DATA AND CODE AVAILABILITY

All data needed to evaluate the conclusions in the paper are present in the paper and/or the supplemental information. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

G.Z. and M.C. designed the work. G.Z., W.L., J.K.V., and D.W. contributed to the acquisition of the data (cell culture: G.Z.; plasmids, rAAV vectors, and Cy3 vector labeling: G.Z., W.L., and J.K.V.; mitochondria isolation, rAAV and PF127 treatments, transplantation, and detection: G.Z., W.L., J.K.V., and D.W.; detection of transgene expression: G.Z.; biochemical assays: G.Z.; mitochondria functional tests: G.Z.; histological and immunocytochemical analyses: G.Z., W.L., J.K.V., and D.W.; histomorphometric analyses: G.Z., W.L., and J.K.V.; real-time RT-PCR analysis: G.Z., W.L., and J.K.V.; statistical analysis: G.Z. and W.L.; and documentation: G.Z., W.L., J.K.V., and D.W.). G.Z., W.L., J.K.V., D.W., H.M., and M.C. analyzed and interpreted the data. M.C. supervised the work. G.Z., H.M., and M.C. acquired funding. G.Z. and M.C. drafted the work. All authors revised the work, approved the submitted version of the work, and agreed to both be personally accountable for their own contributions and ensure that questions related to the accuracy or integrity of any part of the work, even ones in which they were not personally involved, are appropriately investigated and resolved and the resolution documented in the literature.

DECLARATION OF INTERESTS

The authors have no conflicts of interest to declare.

SUPPLEMENTAL INFORMATION

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