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Innovative treatments for human osteoarthritis based on therapeutic rAAV vector gene transfer and controlled release systems

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Declaration

I hereby declare that this thesis is my own original work and effort. All experiments, except for those specified, were exclusively performed by me. Except for the publications written by myself listed in the publication list, the data presented here have not been submitted elsewhere for any award. Where other sources of information and help have been used, they have been indicated and acknowledged.

Homburg, 22.08.2024

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CONTENT

List of Abbreviations

AAV	adeno-associated virus
AC	adenylyl cyclase
ACAD9	acyl-CoA dehydrogenase protein 9
ACAN	aggrecan
ACLT	anterior cruciate ligament transection
ADAMTS	a disintegrin-like and metalloproteinase with thrombospondin motifs
AIFM1	apoptosis-inducing factor mitochondrion-associated 1
ATF3	activating transcription factor 3
ATG7	autophagy related 7
ATP	adenosine triphosphate
β-gal	β-galactosidase
BGP-15	O-(3-piperidino-2-hydroxy-1-propyl)nicotinic amidoxime
BMP-2	bone morphogenetic protein 2
BPTES	bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CISD2	CDGSH iron sulfur domain 2
CMC	critical micellar concentration

CMV-IE	cytomegalovirus immediate-early (promoter)
c-myc	cellular myelocytomatosis
COL2A1	type-II collagen (gene)
CoQ	coenzyme Q
COVID-19	coronavirus disease 2019
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats/CRISPR-associated 9
Ct	threshold cycle
DAB	diaminobenzidine
DAMPs	damage-associated molecular patterns
DddA	double-stranded DNA deaminase
DMEM	Dulbecco's modified Eagle's medium
DMMB	dimethylmethylene blue
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DRP-1	dynamin-related protein 1
Drp-1	dynamin-related protein 1 (gene)
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ER	endoplasmic reticulum
ERK	extracellular regulated kinase

ETC	electron transport chain
ETHE1	ethylmalonic encephalopathy protein 1
FAD	flavin adenine dinucleotide
FBS	fetal bovine serum
FDA	U.S. Food and Drug Administration
FGF-2	basic fibroblast growth factor
FIS1	fission protein-1
FMN	flavin mononucleotide
g	gram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase (gene)
GlcN	D-glucosamine
GLS	glutaminase
Grp75	glucose-regulated protein 75
GTPase	guanosine triphosphate hydrolase
h	hour(s)
H&E	hematoxylin and eosin
HIF-1α	hypoxia-inducible factor alpha
hIGF-I	human insulin-like growth factor I
hIPSCs	human induced pluripotent stem cells
HIV	human immunodeficiency virus
HLA-DRα	class II major histocompatibility complex antigen

HMG	high mobility group box
hMSCs	human mesenchymal stromal cells
hOACs	human osteoarthritic chondrocytes
HSV	herpes simplex virus
ICRS	International Cartilage Repair Society
IGF-I	insulin-like growth factor I
IL-1β	interleukin 1 beta
IL-1β	interleukin 1 beta (gene)
IL-1Ra	interleukin-1 receptor antagonist
IMM	inner mitochondrial membranes
IMs	intermembranous spaces
IP3R/ITPR2	inositol 1,4,5-trisphosphate receptor 2
JNK	c-jun NH2-terminal kinase
kb	kilobase(s)
KUNB31	(4-hydroxy-7-isopropylbenzo[d]isoxazol-5-yl)(isoindolin-2-yl)methanone
lacZ	β-galactosidase (gene)
LPL	lipoprotein lipase
MAMs	mitochondria-associated endoplasmic reticulum membranes
MAPK	mitogen-activated protein kinase
MCU	mitoCa^{2+} uniporter holocomplex

MFF	mitochondrial fission factor
Mfn-1	mitofusin 1
Mfn-1	mitofusin 1 (gene)
Mfn-2	mitofusin 2
mg	milligram
MH	maximal height
MHD	maximal horizontal distance
micro-CT	micro-computed tomography
microRNA	micro-ribonucleic acid
MICU1	mitochondrial calcium uptake 1
MiD49	mitochondrial dynamics protein-49
MiD51	mitochondrial dynamics protein-51
min	minute(s)
Miro1	mitochondrial Rho 1
MitoQ	mitoquinone
ml	milliliter
µl	microlitre
mm	millimeter
mM	mmol/l, millimoles per liter
µm	micrometer
MMPs	matrix metalloproteinases

MNGIE	mitochondrial neurogastrointestinal encephalomyopathy
mRNA	messenger ribonucleic acid
MSCs	mesenchymal stromal cells
mtDNA	mitochondrial deoxyribonucleic acid
mTOR	mammalian target of rapamycin
MVD	maximal vertical distance
MW	maximal width
n.a.	not applicable
NAD^+	nicotinamide adenine dinucleotide
NaSS	4-styrenesulfonic acid sodium salt hydrate
NEAT1	nuclear enriched abundant transcript 1
NF1	neurofibromatosis-1
NF-κB	nuclear factor kappa B
NIH	National Institutes of Health
NLRP3	nucleotide oligomerization domain-like receptor protein 3
NOD	nucleotide oligomerization domain
n.s.	not significant
NSAIDs	non-steroidal anti-inflammatory drugs
OA	osteoarthritis
OMMs	outer mitochondrial membranes
OXPHOS	oxidative phosphorylation

PAMPs	pathogen-associated molecular patterns
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PF127	pluronic® F-127
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3
PGC-1α	peroxisome proliferator activated receptor gamma coactivator 1 alpha
PGE2	prostaglandin E2
PI3K/Akt	phosphatidylinositol 3-kinase/protein kinase B
Pim-1	proviral insertion in murine 1
PKA	protein kinase A
PKB	protein kinase B
PRRs	pattern recognition receptors
PUM2	pumilio2
rAAV	recombinant adeno-associated viral vector
RFP	red fluorescent protein
rIL-1Ra	recombinant interleukin-1 receptor antagonist
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
s	second(s)

safranin O	safranin orange/fast green
SARS	severe acute respiratory syndrome
SARS-CoV	severe acute respiratory syndrome coronavirus
SAS	subarticular spongiosa
scAAV	self-complementary adeno-associated virus
SD	standard deviation
SIRT	sirtuin
SMA	spinal muscular atrophy
SOD	superoxide dismutase
SOX9	sex-determining region Y-type high-mobility group box 9
sox9	sex-determining region Y-type high-mobility group box 9 (gene)
STAT3	signal transducer and activator of transcription 3
TALENs	transcription activator-like effectors nucleases
TCA	tricarboxylic acid
TCM	traditional Chinese medicine
TGF-β	transforming growth factor beta
TNF-α	tumor necrosis factor alpha
TNF-α	tumor necrosis factor alpha (gene)
TPP	triphenylphosphonium
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

TYMP	thymidine phosphorylasethymine phosphorylase
U	units
UPRmt	mitochondrial unfolded protein response
VDAC	voltage-dependent anion channel
VOI	volume of interest
v/v	volume/volume
w/v	weight/volume
ZFNs	zinc-finger nucleases

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1. ABSTRACT

Osteoarthritis (OA) is a progressive, degenerative joint disorder commonly occurring in middle-aged and elderly individuals. This disease that imposes a huge socio-economic burden on the society has currently no effective cure to prevent or reverse its progression, showing the critical need for alternative treatments. A hallmark of OA is the occurrence of pathological phenotypic (cellular, homeostatic, mitochondrial) changes in the OA chondrocytes forming the articular cartilage. Recombinant adeno-associated virus (rAAV)-mediated gene therapy is a potential therapeutic strategy to durably repair joint damage, offering a strong tool to manage human OA. However, the use of rAAV vectors *in vivo* may be affected by the presence of natural neutralizing anti-AAV capsid antibodies in the joints. Remarkably, this hurdle may be counteracted when providing the vectors via biocompatible materials in a controlled delivery manner, more particularly when using hydrogels that are highly compatible with the articular cartilage structure and functions. Of further note, there is substantial evidence that mitochondria, the cellular powerhouses, are compatible with the use of rAAV vectors. The goal of the present study was therefore to test whether primary human OA chondrocytes (hOACs) are amenable to rAAV vector-based gene transfer in order to restore their altered phenotypic changes using two elaborate strategies: (1) the autologous implantation of mitochondria as platforms of delivery of therapeutic rAAV vectors and (2) the hydrogel-based controlled delivery of rAAV vectors with anti-inflammatory drugs *in vitro*. The results of the present work demonstrate that (1) mitochondria can provide rAAV vector delivery platforms capable of effectively and safely improving the viability, homeostasis, and mitochondrial functions in implantable autologous hOACs via rAAV-mediated overexpression of the therapeutic insulin-like growth factor I (IGF-I) gene sequence (including when applied using a biocompatible material (pluronic® F-127 - PF127) hydrogel). Our results also demonstrate that (2) a type-I collagen hydrogel can provide rAAV vector delivery systems capable of effectively and safely improving the viability, homeostasis, and mitochondrial functions in hOACs via rAAV-mediated overexpression of a therapeutic cartilage-specific transcription factor sex-determining region Y-type high-mobility group box 9 (SOX9) candidate with a recombinant anti-inflammatory interleukin-1 receptor antagonist (rIL-1Ra). These highly innovative technologies supporting the effective and safe delivery of rAAV vectors and drugs are valuable translational tools for future human OA therapy.

2. ZUSAMMENFASSUNG

Die Arthrose ist eine fortschreitende, degenerative Gelenkerkrankung, die häufig bei Menschen mittleren und höheren Alters auftritt. Für diese Krankheit, die eine enorme sozioökonomische Belastung für die Gesellschaft darstellt, gibt es derzeit kein wirksames Heilmittel, um ihr Fortschreiten zu verhindern oder umzukehren, was den dringenden Bedarf an innovativen Behandlungen zeigt. Ein Kennzeichen der Arthrose ist das Auftreten pathologischer phänotypischer (zellulärer, homöostatischer, mitochondrialer) Veränderungen in den Chondrozyten, die den Gelenkknorpel bilden. Die durch rekombinante adeno-assoziierte virale (rAAV) Vektoren vermittelte Gentherapie ist eine potenzielle therapeutische Strategie zur dauerhaften Behebung von Gelenkschäden und bietet ein wirksames Instrument zur Behandlung der menschlichen Arthrose. Die Verwendung von rAAV-Vektoren in vivo kann jedoch durch das Vorhandensein natürlicher neutralisierender Anti-AAV-Kapsid-Antikörper in den Gelenken beeinträchtigt werden. Bemerkenswerterweise kann diese Hürde überwunden werden, wenn die Vektoren über biokompatible Materialien in einer kontrollierten Verabreichungsform bereitgestellt werden, insbesondere durch die Verwendung von Hydrogelen, die mit der Struktur und den Funktionen des Gelenkknorpels hoch kompatibel sind. Darüber hinaus gibt es zahlreiche Hinweise darauf, dass Mitochondrien, die Kraftwerke der Zellen, mit der Verwendung von rAAV-Vektoren kompatibel sind. Ziel der vorliegenden Studie war es daher, zu testen, ob primäre humane arthrotische Chondrozyten (hOACs) für einen rAAV-Vektor-basierten Gentransfer geeignet sind, um ihre veränderten phänotypischen Veränderungen mit Hilfe von zwei neuartigen Strategien wiederherzustellen: (1) die autologe Implantation von Mitochondrien als Plattformen für die Verabreichung von therapeutischen rAAV-Vektoren und (2) die kontrollierte Verabreichung von rAAV-Vektoren mit fortschrittlichen Therapeutika auf Hydrogelbasis *in vitro*. Die Ergebnisse der vorliegenden Arbeit zeigen, dass (1) Mitochondrien rAAV-Vektor-Transportplattformen darstellen können, die in der Lage sind, die Lebensfähigkeit, die Homöostase und die mitochondrialen Funktionen in implantierbaren autologen hOACs durch rAAV-vermittelte Überexpression einer therapeutischen insulinartigen Wachstumsfaktor I (IGF-I)-Gensequenz wirksam und sicher zu verbessern (auch bei Verwendung eines biokompatiblen Materials (pluronic® F-127 - PF127) Hydrogel). Die Ergebnisse der vorliegenden Arbeit zeigen auch, dass (2)

ein Typ-I-Kollagen-Hydrogel rAAV-Vektorverabreichungssysteme bereitstellen kann, die in der Lage sind, die Lebensfähigkeit, die Homöostase und die mitochondrialen Funktionen in hOACs durch die rAAV-vermittelte Überexpression eines therapeutischen knorpelspezifischen Transkriptionsfaktors der sex-determining region Y-type high-mobility group box 9 (SOX9) mit einem rekombinanten entzündungshemmenden Interleukin-1-Rezeptor-Antagonisten (rIL-1Ra) wirksam und sicher zu verbessern. Diese hochinnovativen Technologien, die die wirksame und sichere Verabreichung von rAAV-Vektoren und Medikamenten unterstützen, stellen wertvolle translationale Werkzeuge für künftige therapeutische Anwendungen bei menschlicher Arthrose dar.

3. INTRODUCTION

3.1. Osteoarthritis

Osteoarthritis (OA) is the most common chronic disease of large joints, such as the knee, hip, and hand joints, that mostly occurs in elderly individuals, with an incidence of greater than 60% in individuals over 65 years of age and that may further rise within the next 30 years (Hunter, March, Chew, & Lancet Commission on, 2021; Quicke, Conaghan, Corp, & Peat, 2022). OA does not only shatter joint functions thus physical abilities but also induces considerable pain in patients, often at long-term (Jones, Togashi, Wilson, Heckmann, & Vangsness, 2019). The risk factors for OA include primary (gender, genetic inheritance, obesity, hormonal changes) and secondary (trauma, mechanical stress, metabolic changes) components (Martel-Pelletier et al., 2016). The features of OA include the articular cartilage erosion, synovial inflammation, synovial hyperplasia, abnormal angiogenesis, ligaments and tendons instability, subchondral bone disturbance, and joint capsule fibrosis (Kapoor, Martel-Pelletier, Lajeunesse, Pelletier, & Fahmi, 2011; Liu-Bryan & Terkeltaub, 2015; Loeser, Collins, & Diekman, 2016; Martel-Pelletier et al., 2016; L. Sharma, 2021) (**Figure 1**).

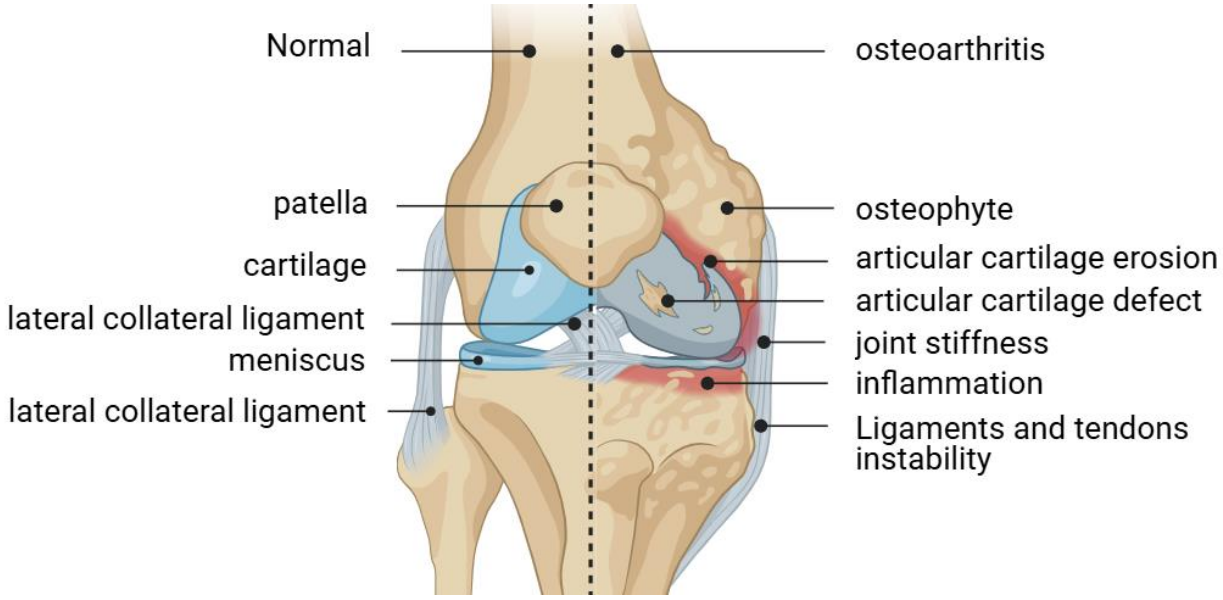


Figure 1. Phenotypes of a normal and osteoarthritic knee joint (created with BioRender).

The healthy articular cartilage consists of a layer of hyaline cartilage devoid of vascularization, lymphatic system, and innervation, covering the end of bones in the joint to resist shocks and withstand mechanical loading and impact (Hunter & Eckstein, 2011). Under pathological conditions in OA, the chondrocytes undergo phenotypic changes and alterations in their homeostasis in response to an accumulation of detrimental pro-inflammatory mediators (interleukin-1 beta - IL-1β, tumor necrosis factor alpha - TNF-α, nitric oxide - NO, prostaglandin E2 - PGE2, reactive oxygen species - ROS, etc.) and matrix-degrading enzymes (matrix metalloproteinases - MMPs, a disintegrin-like and metalloproteinase with thrombospondin motifs - ADAMTS, etc.), leading to the loss of extracellular matrix elements (proteoglycans, type-II collagen) (D. Chen et al., 2017; Zhong, Madry, & Cucchiarini, 2022) (**Figure 2**).

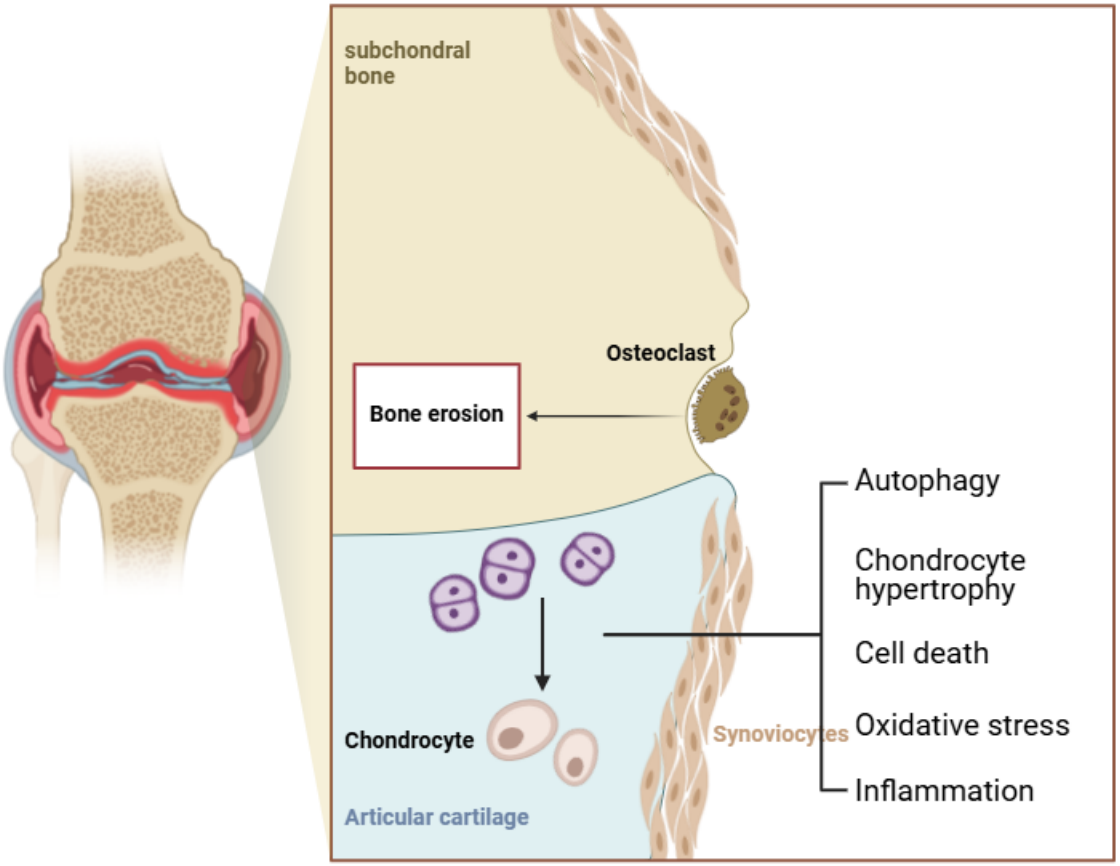


Figure 2. Pathological features of osteoarthritis (created with BioRender).

A treatment for OA should be based on individual circumstances and disease progression, including a combination of treatment options. In the early stages of the disease, treatments such as education, exercise, weight loss, and physical therapy are mainly used (Hunter & Felson, 2006; H. Madry & Cucchiarini, 2013). Yet, when the disease becomes noticeable, patients are often already in middle OA stages, a period when drug treatments need to be applied. The most commonly used drug treatment regimens are non-steroidal anti-inflammatory drugs (NSAIDs) to reduce inflammation and relieve joint pain (da Costa et al., 2021). However, their side effects including gastrointestinal discomfort, cardiovascular problems, bleeding problems, and liver and kidney damage brought a huge haze to the treatment of OA (Cooper et al., 2019; Zhong, Long, Chen, & Yu, 2021). Other treatments include joint lubricants (hyaluronic acid) to reduce frictions on damaged cartilage surfaces (Jones et al., 2019) but their efficacy remains controversial (Bijlsma, Berenbaum, & Lafeber, 2011). For severe and end-stage OA cases, surgical procedures may be attempted (osteotomy and joint replacement) but may lead to a loss of quality of life because of associated problems apart from the economical burden (Hunter & Bierma-Zeinstra, 2019). These findings (**Figure 3**) show the crucial need to identify novel pathomechanisms of the disease that may lead to the generation of improved, more effective therapeutic options for OA.

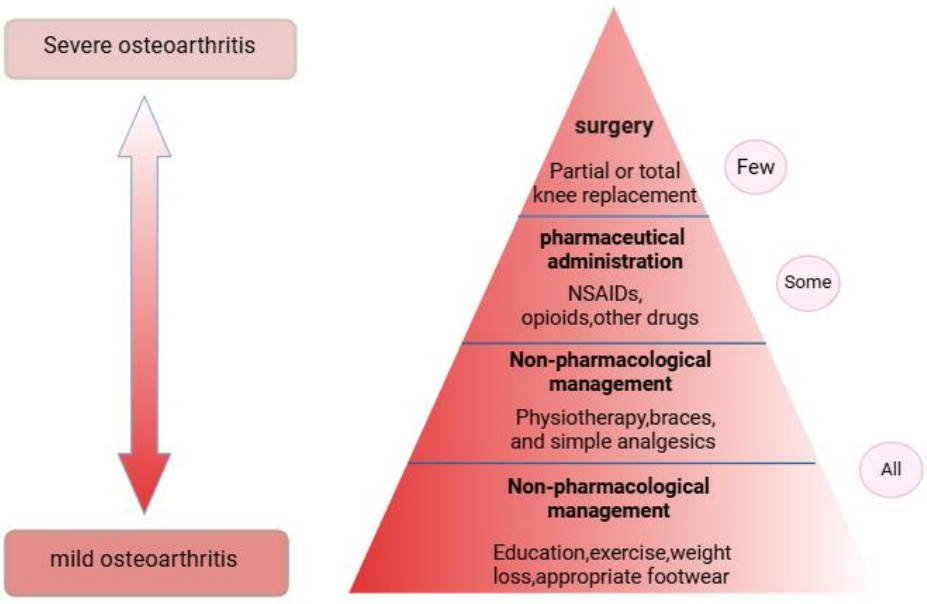


Figure 3. Treatments of osteoarthritis (created with BioRender).

3.2. rAAV vector gene delivery for the treatment of osteoarthritis

In contrast to gene therapy for hereditary conditions that seeks to replace altered genes with healthy counterparts, gene therapy for OA takes on a different, more nuanced role (X. Li, Shen, Deng, & Huang, 2023). Its purpose is to recalibrate the delicate metabolic equilibrium within OA cartilage, achieved by either directly or indirectly amplifying the expression of therapeutic genes or tempering the activity of detrimental ones (H. Madry & Cucchiarini, 2013). Unlike conventional pharmaceutical interventions, gene-based treatments provide a controlled, precise, and enduring means to deliver therapeutic agents to the affected site (C. Evans, J. Gouze, E. Gouze, P. Robbins, & S. Ghivizzani, 2004). Consequently, gene therapy stands as a promising option to address the gradual and irreversible nature of conditions like OA (Henning Madry & Cucchiarini, 2016).

The potential of gene therapy for OA finds its validation in a series of preclinical and clinical trials. A noteworthy milestone in this journey was achieved by Pelletier *et al*. (Pelletier et al., 1997) who demonstrated for the first time *in vivo* that transplanting synoviocytes engineered to express the gene for an interleukin 1 receptor antagonist (IL-1Ra) via intra-articular injection significantly delayed the progression of OA in a mature mongrel dogs model induced by early anterior cruciate ligament transection (ACLT). Recently, a phase I trial was developed to apply chondrocytes modified to produce transforming growth factor beta 1 (TGF-β1) through intra-articular injection in patients with advanced OA, showcasing the expanding horizon of gene-based therapeutic approaches (Ha, Noh, Choi, & Lee, 2012). In recent decades, the scientific community underscored a recurring challenge: the dense matrix enveloping chondrocytes in their native environment presents a formidable obstacle to gene transfer within the cartilage (C. H. Evans, J. N. Gouze, E. Gouze, P. D. Robbins, & S. C. Ghivizzani, 2004; Trippel, Ghivizzani, & Nixon, 2004). Even in advanced cases of OA, the depletion of this matrix may not be sufficient to expose the relatively small population of chondrocytes introduced into the joint space (C. H. Evans et al., 2004). Nevertheless, with ongoing advancements in gene transfer techniques (**Table 1**), the prospect of efficiently targeting chondrocytes embedded in their matrix became increasingly achievable. Indeed, the basic science of gene transfer in the articular cartilage is currently making significant progress. For example, highly efficient

gene vectors were identified that can directly transduce chondrocytes in the human OA cartilage (C. Evans et al., 2004; Henning Madry & Cucchiarini, 2016). It became therefore possible to directly deliver cartilage-specific therapeutic factors (such as transcription factors and intracellular signaling molecules) that are neither secreted nor taken up by cells.

Table 1. Gene transfer vectors

Classes	Types	Benefits	Shortcomings	Integration
nonviral vectors	lipoplex, polyplex, polyethylenimine, chitosan polymer	non-infectious, low toxicity, easy to produce, large capacity, controllability	relatively low efficiency, short-term transgene expression, large particles	no
viral vectors	adenoviral vectors	very high efficiency	potential replication competence, toxicity, immunogenicity, short-term transgene expression	no
	retroviral, lentiviral vectors	high efficiency, long-term transgene expression	potential replication competence, risk of insertional mutagenesis, biotoxicity	yes
	herpes simplex viral (HSV) vectors	high-efficiency, large capacity	relative cytotoxicity, short-term transgene expression	no
	rAAV vectors	high-efficiency, long-term transgene expression, low immunogenicity, use of scAAV	relative difficulty to produce, small loading ability, potential serotype-restricted cell specificity, expensive	mostly episomal

From the insights presented in **Table 1**, rAAV vectors display many key advantages over competing systems and became a preferred tool for experimental OA settings *in vivo* (Goodrich et al., 2013; Santangelo & Bertone, 2011; Watson et al., 2013) and for clinical applications (Asokan, Schaffer, & Samulski, 2012; Evans, Ghivizzani, & Robbins, 2013; Grieger & Samulski, 2012; Mingozzi & High, 2011). Particularly, the rAAV vector, a genetically modified version of the wild-type human adeno-associated virus (AAV), emerged as a prominent candidate in the realm of OA gene vectors (Flotte, 2004). rAAV vectors are constructed upon a non-pathogenic, replication-defective human parvovirus (AAV), rendering them less immunogenic than adenoviral vectors and less toxic than herpes simplex viral (HSV) vectors (Mueller & Flotte, 2008; Naso, Tomkowicz, Perry III, & Strohl, 2017). These vectors are carefully engineered through the removal of all viral gene coding sequences (Daya & Berns, 2008). One of the remarkable strengths of rAAV vectors is their capacity to maintain stable, episomal transgenes *versus* integrative retroviral and lentiviral vectors that may lead to insertional mutagenesis. These transgenes can be expressed with remarkable efficiency, both in nondividing chondrocytes (reaching up to 95% efficiency) (Arai et al., 2000; H Madry, Cucchiarini, Terwilliger, & Trippel, 2003) and dividing cells like mesenchymal stromal cells (MSCs) (with efficiency ranging from 65% to 92%) (Cucchiarini, Ekici, Schetting, Kohn, & Madry, 2011; Stender et al., 2007; J. K. Venkatesan et al., 2012), sustaining their activity over extended periods of time relative to less effective nonviral and adenoviral vectors. For instance, an rAAV-2 vector carrying the β-galactosidase (*lacZ*) marker gene has demonstrated longevity *in situ* (H Madry et al., 2003), with expression persisting for at least 150 days, even within the dense cartilage extracellular matrix (Goater et al., 2000; H Madry et al., 2003). However, it is important to note that while improvements have been made, such as the introduction of self-complementary AAV (scAAV) to bypass the step-limiting conversion of single-stranded into double-stranded DNA, and the development of trans-splicing systems, these vectors still face a limitation in terms of the size of the transgene they can carry (McCarty, Monahan, & Samulski, 2001). This size constraint poses challenges when delivering larger sequences, such as those encoding essential extracellular matrix components. Furthermore, not all AAV serotypes exhibit equal proficiency in transducing their intended targets. Therefore, from this perspective, rAAV-mediated gene therapy has shortcomings for chronic diseases such as progressive OA.

3.3. Mitochondria

The symbiotic state of mitochondria and eukaryotic cells traces back to a "survival of the fittest" event that occurred about 1.5 billion years ago when a prokaryotic cell engulfed an alpha-probacteria and retained it as a functional component, an organelle (Lane & Martin, 2010). Mitochondria inherit the structural and genetic basis of their bacterial ancestors, consisting of two separate, functionally distinct outer mitochondrial membranes (OMMs) and inner mitochondrial membranes (IMMs) that enclose intermembranous spaces (IMs) and a stromal compartment possessing a partially independent circular genome - the mitochondrial DNA (mtDNA) - reduced during evolution by gene transfer to the nucleus (Gabaldon & Huynen, 2004; Lecrenier, Van Der Bruggen, & Foury, 1997; Valeria Tiranti et al., 1997). During a long period of natural adaptation and evolution, newly acquired intracellular symbionts produced new specialized aerobic respiration that efficiently extracts energy from glucose through the electron transport chain (ETC) and oxidative phosphorylation (OXPHOS) to produce ATP, the main "currency" that provides energy metabolism in life (Friedman & Nunnari, 2014). This overwhelms the original energy operating rules of eukaryotic cells, becoming the main energy pathway, and is supported by the bacteriophage-related mitochondrial maintenance systems, supported by the observation that the closest relatives of many mtDNA modifying enzymes (such as mtDNA polymerases) are bacteriophage proteins (Valeria Tiranti et al., 1997).

The long-term intracellular symbiont state facilitates in-depth communication and compromise between mitochondria and eukaryotic cells that allows the intracellular grinding into a dynamic interconnected network tightly bound to other cell compartments. Mitochondria, the central node in the operation of this system, are not only the cell powerpacks but also perform a smooth shuttle with the nucleus, making the mitochondria-nucleus information delivery crucial for the maintenance of the intracellular metabolism in physiological conditions otherwise affected in pathologies like aging, oxidation, inflammation, immunological diseases, metabolic syndromes, obesity, cancer, and degenerative disorders. The mitochondrial retrograde signaling affects the decision-making of the nucleus accomplished by retrograde signal-mediated protein activation of nuclear gene expression or by altering its epigenetics through DNA methylation and post-

translational modification of histones (Butow & Avadhani, 2004; Desai et al., 2020). The nucleus responds to signals from the mitochondria to assist cellular metabolic crises under various pathological conditions as listed above. In addition to having a strong network with the nucleus, mitochondria are equally tightly connected with other organelles and the membrane contact sites between them are critical for lipid and ion exchange, membrane dynamics, and signal transduction (Murley & Nunnari, 2016). Mitochondria and lysosomes cooperate to complete autophagy (Yoo & Jung, 2018). Mitochondrial binding to the endoplasmic reticulum (ER) via mitochondria-associated ER membranes (MAMs) (Barazzuol, Giamogante, & Cali, 2021) controls the transport of calcium, a regulator of the overall mitochondrial membrane potential ($\Delta\Psi m$) and an important second messenger that transmits external or cytoplasmic signals to the mitochondria, affected in neurodegenerative diseases and aging (Fink, Rauckhorst, Taylor, Yu, & Sivitz, 2022; Lewis, Uchiyama, & Nunnari, 2016). Mitochondria play an important role in the normal cell functions either as energy supply station or as metabolic information hub. Mitochondrial dysfunction is implicated in major human diseases (Nunnari & Suomalainen, 2012; Suomalainen & Battersby, 2018; Vafai & Mootha, 2012) (**Figure 4**) such as aging, oxidative disorders, inflammatory diseases, mitochondrial diseases, and cancer.

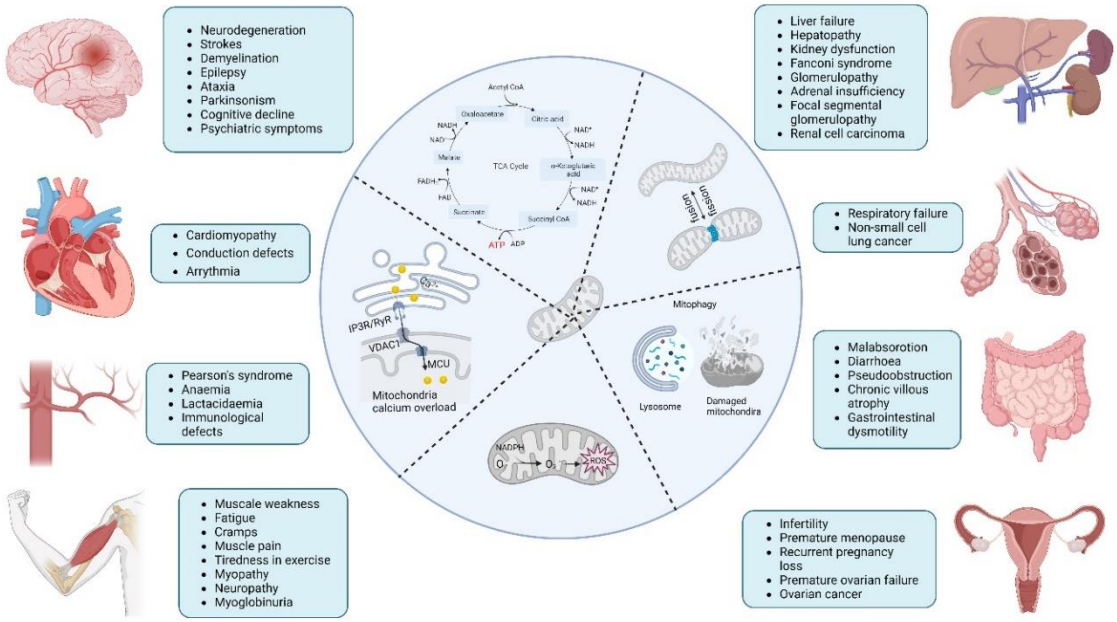


Figure 4. The centrality of mitochondria in human diseases (created with BioRender).

In OA, mitochondrial abnormalities also contribute to the pathological progression of the disease. Independently from the oxygen blood supply, the chondrocytes were traditionally perceived as highly glycolytic cells, obtaining energy from anaerobic respiration of glucose, a notion explaining that - until recently - mitochondria were long neglected in the pathophysiology of OA (Gavriilidis, Miwa, von Zglinicki, Taylor, & Young, 2013; R. B. Lee & Urban, 2002). Yet, up to 25% of the articular cartilage energy needs to be met by high-energy electron transfer across the inner mitochondrial membrane during oxidative phosphorylation, a percentage pulled up under the high energy demand of the tissue (Heer & Brenner, 2020). Changes in the mitochondrial behavior regarding ATP production, aerobic respiration efficiency, ROS levels, and calcium signals are well documented and matched with processes leading to OA cartilage degradation such as oxidative stress, inflammation storm, and chronic inflammation-related matrix loss (L. Zheng, Zhang, Sheng, & Mobasheri, 2021). Compared with healthy chondrocytes, mitochondria of OA chondrocytes display abnormal features, showing an increase in the mitochondrial mass yet with decreased activities of the respiratory complexes II and III (Blanco & Rego-Perez, 2018) while mitochondrial electron transfer is incomplete, resulting in excessive ROS production consuming high-energy electrons and greatly reducing ATP production (Martin et al., 2012). The accumulated ROS subsequently damages both the mtDNA and the mitochondrial membrane proteins and poses great challenges to the metabolism of the nucleus and of the entire chondrocytes (Bolduc, Collins, & Loeser, 2019; Lepetsos & Papavassiliou, 2016). Additionally, mitochondrial dysfunction is accompanied by a decline of antioxidant capacity endorsed by antioxidant enzymes (superoxide dismutase - SOD, glutathione - GSH) while changes in the levels of these enzymes show a dependence on the severity of OA relative to normal cartilage tissue (Lepetsos & Papavassiliou, 2016). Such evidence strongly suggests that mitochondrial dysfunction and metabolic disorders provide a reasonable framework for the pathogenesis of OA.

Furthermore, mtDNA is a direct contributor to the mitochondrial behavior and metabolism in OA as mtDNA mutations are linked to the disease pathology (Blanco, Valdes, & Rego-Perez, 2018; Gorman et al., 2015) (**Figure 5**), with a minimum prevalence rate of 20 per 100,000 (Blanco et al., 2018; Gorman et al., 2015). Chang *et al*. (Chang et al., 2005), conducting systematic genetic screening of OA cases, also evidenced age-dependent gene deletions in the 4.977-bp mtDNA related to the process of idiopathic knee

joint OA. Overall, these findings reveal that mtDNA mutations contribute to the degeneration of chondrocytes in OA and that repairing the integrity of mtDNA or complementing the altered mitochondrial functions may reverse this process.

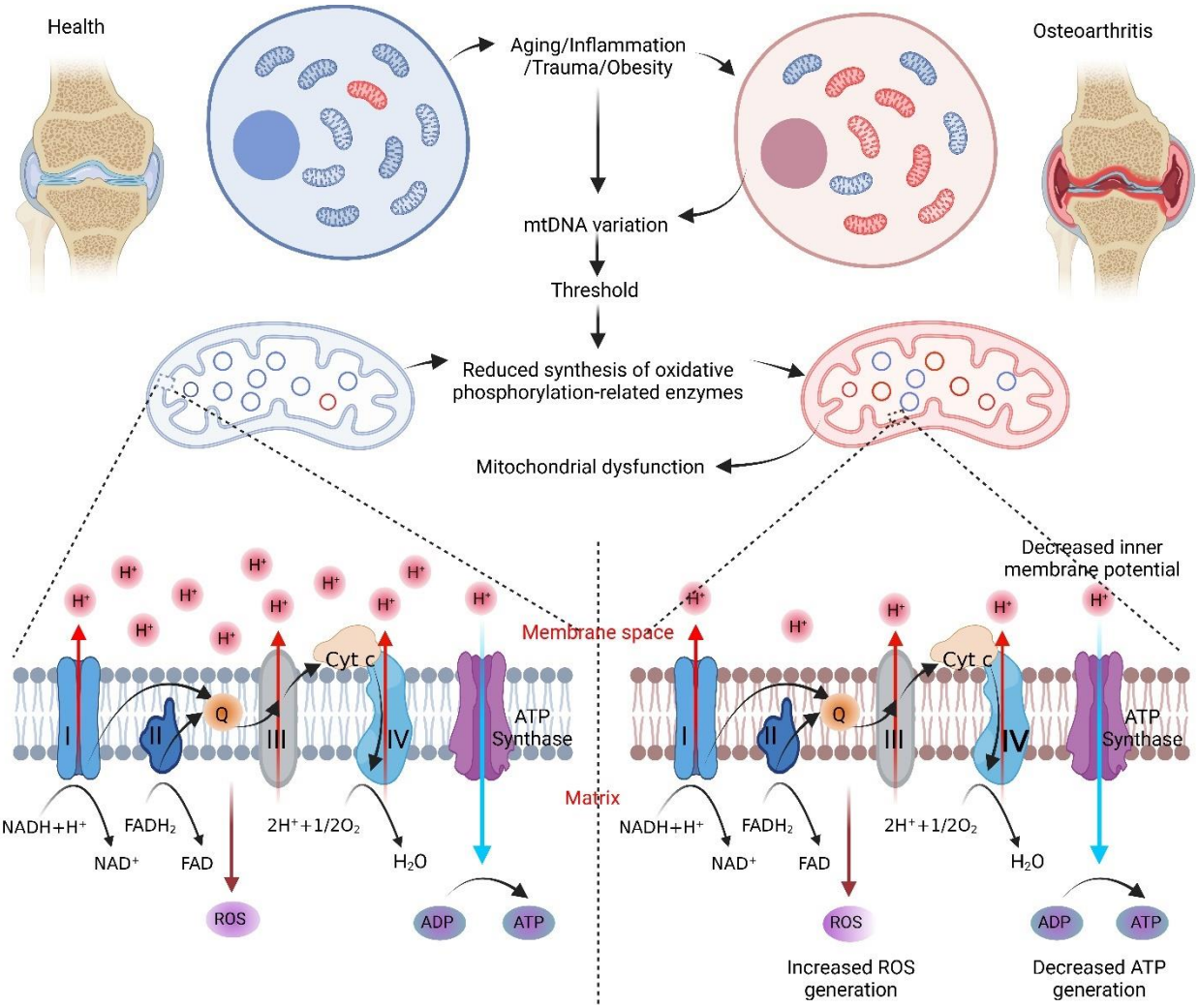


Figure 5. Influence of mtDNA heterogeneity and mitochondrial functions in osteoarthritis. Under the influence of various risk factors, mtDNA mutations are observed in OA, affecting the synthesis of oxidative phosphorylation-related enzymes. Reduced synthesis of these enzymes leads to a poor transfer of high-energy electrons, which, in turn, reduces the production of ATP, increases the production of ROS and reduces the mitochondrial membrane potential. Increased ROS can subsequently aggravate the oxidative damage of chondrocytes, leading to cartilage degradation (created with BioRender).

3.4. Mitochondria-based disease treatments

3.4.1. Strategies targeting the mitochondria in aging

Aging is by far the greatest risk factor for a variety of common human diseases including cardiovascular disorders, arthritis, coronary heart disease, and neurodegenerative diseases.

Mitochondria have long been implicated in aging, with a time-dependent accumulation of mtDNA variants and an imbalance of ROS (Harman, 1956). Most of these aging-related disorders are first associated with shattered energy-demanding organs like the myocardium, brain, and muscle, where energy is supplied by the mitochondria performing the OXPHOS pathway to conduct electrons in the ETC for a conversion of electrical potential energy into bioenergy that supports the cellular activities (Hatefi, 1985). In senescent cells, the electron transport in the ETC is blocked and leaks in the mitochondrial matrix (Cadenas & Davies, 2000). This undesired electron robs the mitochondrial IM of protons, depleting the $\Delta\Psi m$, which is extremely important for electron transport in the ETC and reacts with oxygen to generate ROS (Balaban, Nemoto, & Finkel, 2005) causing irreversible damage to DNA (mtDNA and nuclear DNA), proteins, and biological membranes (Apel & Hirt, 2004).

Due to their role as multifaceted regulators of aging and cellular senescence (**Figure 6**), mitochondria have therefore been targeted to generate anti-aging treatments by balancing mitochondrial metabolism and mitophagy (the "quality control" mechanism of mitochondria breaking down damaged mitochondria and removing dysfunctional and undesirable mitochondrial components and by-products), by maintaining the mitochondrial calcium (mitoCa^{2+}) homeostasis, and by modulating mitochondrial dynamics (**Table 2**).

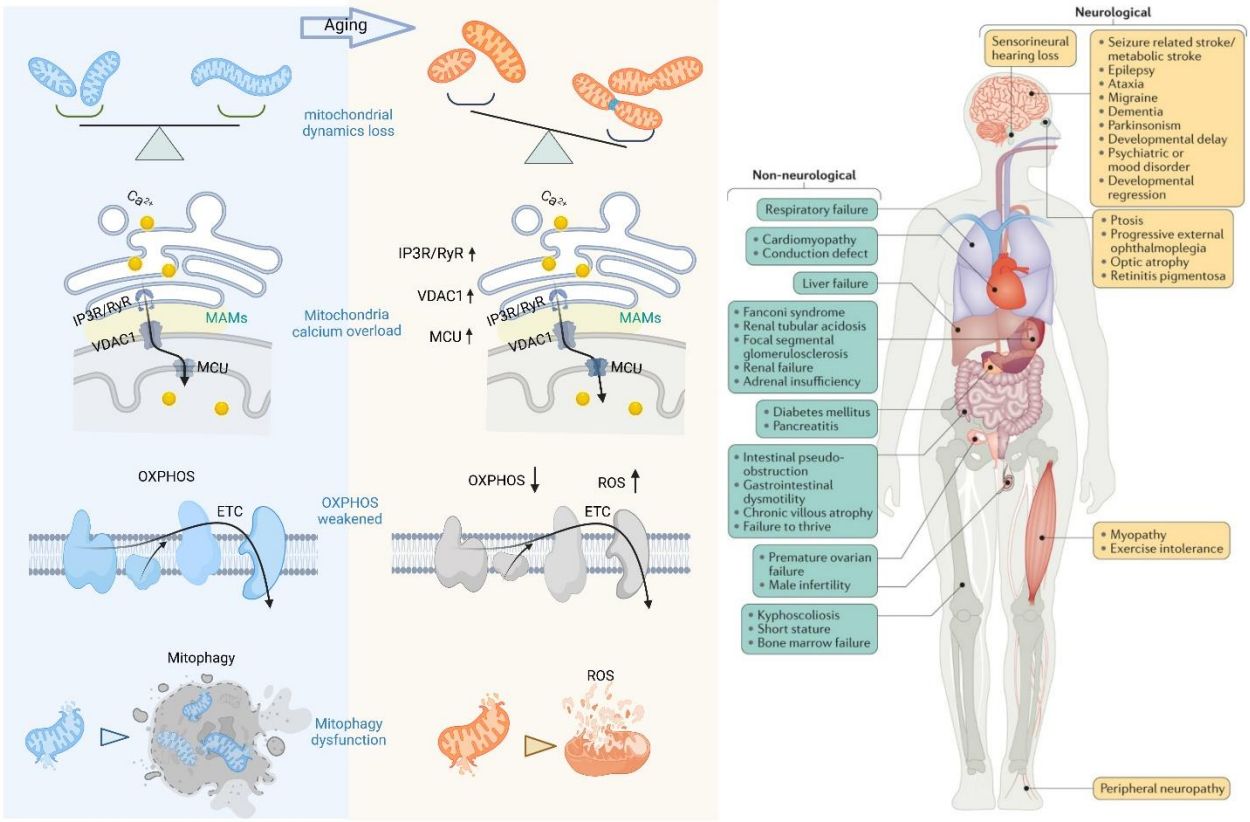


Figure 6. Mitochondrial biology in age-related diseases (created with BioRender).

Table 2. Therapeutic factors involved in major age-related mitochondrial diseases

Factors	**Models**	**Mechanisms**	**References**
c-myc	aging cardiomyocytes	mitochondrial metabolism	(Din et al., 2014)
PGC-1α	age-related pathologies (muscle, heart, liver, brain)	mitochondrial metabolism	(Nguyen et al., 2014; Wenz, 2011)
Pim-1	myocardial infarction	calcium homeostasis and mitochondrial function	(Din et al., 2014; Muraski et al., 2007)
PUM2	aged nematodes and aged mice muscle cells	mitochondrial dynamics and mitophagy	(D'Amico et al., 2019)
PFKFB3	cerebral ischemia-reperfusion injury	mitochondrial energy metabolism	(Burmistrova et al., 2019)
ATF3	idiopathic pulmonary fibrosis	mitochondrial homeostasis	(Bueno et al., 2018)

SIRT3	osteoporosis, cardiac hypertrophy	mitophagy and mitochondrial permeability	(Hafner et al., 2010; Ling et al., 2021)
SIRT4	ionizing radiation aging	mitochondrial dynamics and mitophagy	(Lang et al., 2017)
NEAT1	chronic obstructive pulmonary disease	mitophagy	(Q. Lin et al., 2022)
CoQ	heart and the liver of aged mice	ETC	(Genova et al., 1995)
MitoQ	cge-related endothelial dysfunction	oxidative damage to mitochondria	(Gioscia-Ryan et al., 2014)
BGP-15	type 2 diabetes	ETC	(Kozma et al., 2022)
GlcN	extends lifespan in nematodes and mice	mitochondrial metabolism	(S. Weimer et al., 2014)
IP3R2	age-related liver fibrosis	MAMs	(Ziegler et al., 2021)
CISD2	premature aging	autophagy	(Y. F. Chen, Kao, Chen, et al., 2009)
isradipine	Parkinson's disease	calcium uptake	(Guzman et al., 2018)
verapamil	age-related hematopoietic dysfunction	age-related hematopoietic stromal cell dysfunction	(Mansell et al., 2021)
α-Klotho	regeneration of aging muscles	maintenance of mtDNA integrity and mitochondrial function	(Sahu et al., 2018)
DRP-1	extending the lifespan of Drosophila melanogaster	mitochondrial fission	(Rana et al., 2017)
FIS1	skeletal muscle aging	mitochondrial morphology	(T. T. Lee et al., 2021)
Miro1	neuron disease	mitochondrial dynamics	(Nguyen et al., 2014)

Mitochondrial metabolic alterations from OXPHOS to glycolysis are frequently observed in senescent cells (Kang, Park, Choi, Choi, et al., 2017; Kang, Park, Choi, Kim, et al., 2017) whereas defending the efficiency of OXPHOS from being impeded was shown to be beneficial for the lifespan of living organisms (Barros, Bandy, Tahara, & Kowaltowski, 2004), suggesting that active repair and reversal of mitochondrial metabolism may be valuable to tackle aging. Specifically, Din *et al*. (Din et al., 2014) demonstrated that overexpressing regulators of mitochondrial biogenesis (cellular myelocytomatosis - c-myc, peroxisome proliferator activated receptor gamma coactivator

1 alpha - PGC-1α, proviral insertion in murine 1 - Pim-1) in a mouse model of cardiomyocyte aging was capable of reversing cardiac aging by maintaining mitochondrial function. Other gene regulation targets may include pumilio2 (PUM2), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), activating transcription factor 3 (ATF3), sirtuins 3 and 4 (SIRT3, SIRT4), and nuclear enriched abundant transcript 1 (NEAT1) (Bueno et al., 2018; Burmistrova et al., 2019; D'Amico et al., 2019; Hafner et al., 2010; Lang et al., 2017; Q. Lin et al., 2022; Ling et al., 2021). Besides, administering biomodulators may provide effective, alternative therapeutic approaches capable of modulating mitochondrial functions in response to aging such as coenzyme Q (CoQ), an important component in ETC, receiving electrons from complex I and complex III (Alcazar-Fabra, Navas, & Brea-Calvo, 2016). CoQ-deficient ETCs frequently appear in senescent cells and CoQ supplementation may optimize the mitochondrial functions by normalizing the $\Delta\Psi m$ and enhancing ATP synthesis in senescent cells (Tian et al., 2014). Mitoquinone (MitoQ) or O-(3-piperidino-2-hydroxy-1-propyl)nicotinic amidoxime (BGP-15) have been proven to modulate the $\Delta\Psi m$ and ROS contents to reverse aging-associated meiotic spindle defects in mice and humans (Al-Zubaidi et al., 2021). Weimer *et al*. (S. Weimer et al., 2014) also reported that D-glucosamine (GlcN) is able to increase the mitochondrial respiration by promoting the dependence of energy metabolism on OXPHOS while impairing glycolysis to contribute to an extension of the lifespan in many species (nematodes, aged mice).

The calcium overload in mitochondria is an important sign of aging (P. Gao et al., 2020) and the ER is an important source of mitoCa^{2+} through MAMs which achieve the spatial and functional coupling of the mitochondria with the ER via the inositol 1,4,5-trisphosphate receptor (IP3R/ITPR2)-glucose-regulated protein 75 (Grp75)-voltage-dependent anion channel (VDAC) (Rizzuto et al., 1998). In senescent cells, IP3R and VDAC are overactivated, resulting in unconstrained calcium flux into mitochondria from the ER (Decuypere et al., 2011; Rizzuto, De Stefani, Raffaello, & Mammucari, 2012) where calcium concentrations are 5- to 10-fold higher than in the mitochondria (Berridge, Lipp, & Bootman, 2000). The resulting calcium overload further triggers abnormal $\Delta\Psi m$ and elevated ROS (Peng & Jou, 2010), thus accelerating the aging process in a "vicious circle". Limiting calcium overload may therefore be another important approach to

suppress aging. For instance, Wiel *et al*. (Wiel et al., 2014) showed that inhibiting the IM mitoCa^{2+} uniporter holocomplex (MCU) membrane protein (the direct channel of calcium in the mitochondria) prevented a persistent accumulation of mitoCa^{2+} and that knocking down IP3R/ITPR2 and main channel of calcium exchange on MAMs can reduce ROS levels and inhibit senescence. Other significant factors to counterbalance aging by inhibiting mitoCa^{2+} include the tumor suppressor candidate 2 (Tusc2/Fus1), ATF3, CDGSH iron sulfur domain 2 (CISD2), isradipine, verapamil, and α-Klotho (Bueno et al., 2018; Y. F. Chen et al., 2020; Y. F. Chen, Kao, Kirby, & Tsai, 2009; Delcroix et al., 2018; Liu et al., 2020; Yu et al., 2013).

As dynamic cell organelles, the mitochondria perform a continuous cycle of fission and fusion called mitochondrial dynamics to ensure proper mitochondrial functions in response to nutrient demands, signal transduction, and external stress (Liesa & Shirihai, 2013). Abnormal mitochondrial dynamics is compromised in aging and related disorders, as evidenced by the occurrence of an aberrant (giant) mitochondrial morphology (Wilson & Franks, 1975) and maintaining or restoring the normal mitochondrial dynamics may allow to tackle the aging process in affected cells. For instance, up-regulating the cytosolic dynamin-related protein 1 (DRP-1 - a central protein that coordinates mitochondrial fission by mediating membrane contraction and fission) (Taguchi, Ishihara, Jofuku, Oka, & Mihara, 2007) can prolong the lifespan of Drosophila by facilitating mitophagy and improving the mitochondrial respiratory functions (J. E. Lee, Westrate, Wu, Page, & Voeltz, 2016; Rana et al., 2017). Other proteins that may be targeted include the mitochondrial fission factor (MFF), fission protein-1 (FIS1), mitochondrial Rho 1 (Miro1), mitochondrial dynamics protein-49 (MiD49), and mitochondrial dynamics protein-51 (MiD51), all involved in the mitochondrial dynamic networks and whose deviate from physiological expression during aging, by normalizing them in cells in order to restore the natural mitochondrial functions, morphology, and lifespan in living organisms (A. Sharma, Smith, Yao, & Mair, 2019).

3.4.2. Strategies targeting the mitochondria in oxidative disorders

Oxidative stress recapitulates a state of unbalanced antioxidant effects due to excessive levels of ROS leading to oxidative damage in the body that affects various intracellular biomolecules such as DNA, proteins, and lipids in the course of aging and of other human disorders (Schieber & Chandel, 2014). As the major ROS producer, the mitochondria that play crucial roles in the cellular emergency responses (oxidative stress, physical stimulation, calcium overload) are the first systems attacked (Ames, Shigenaga, & Hagen, 1993), resulting in the loss of elasticity of their membrane, in the disruption of the $\Delta\Psi m$, in mtDNA mutations, and in a reduction of ATP synthesis efficiency leading to mitochondrial dysfunction (Finkel, 2005). Mitochondria-targeted antioxidant therapy to reduce excessive ROS levels may therefore be an effective means to diminish or even prevent oxidative damage in affected cells and organs. Such a strategy by ROS scavenging and by the pharmacological manipulation of the mitochondrial biogenesis is based on the use of antioxidant moieties, of gene therapy, and of traditional Chinese medicine (TCM) monomers with antioxidant potency.

Since mitochondrial oxidative damage involves lipid peroxidation and as the binding of alkyl polypropylenes to the IMMs strongly exacerbates its damage (Su et al., 2019), initial research first focused on the use of antioxidants that are effective against lipid peroxidation. Ubiquitin (Kelso et al., 2001), tocopherol (K. Zhou, Yin, & Yu, 2005), lipoic acid (Brown et al., 2007), the peroxidase mimetic ebselen (MitoPrx) (Filipovska et al., 2005), and their derivatives have been extensively reported for their effectiveness against mitochondrial oxidative damage. MitoQ that is reduced by the complex II to an active ubiquitin antioxidant in the respiratory chain has been also particularly validated by driving the $\Delta\Psi m$ and lipid peroxidation in numerous clinical trials (James et al., 2007). Besides, the superoxide dismutase mimetic M40403 (MitoSOD) (resist $O_2^{\bullet-}$), MitoPrx (resist H_2O_2), TEMPOL (MitoTEMPOL) (resist OH•), vitamin E (MitoE) (resist OH•), and lipoic acid (MitoLip) (resist $O_2^{\bullet-}$, H_2O_2 and OH•) are additional antioxidants recognized for their ROS scavenging effects (Smith et al., 2008). To increase mitochondrial targeting, lipophilic cations such as triphenylphosphonium (TPP) that can bind to antioxidants, allowing them to pass the mitochondrial membrane (Liberman, Topaly, Tsofina, Jasaitis, & Skulachev, 1969; Murphy, 1997; Murphy & Smith, 2007), were introduced in a follow-up attempt,

greatly (100-fold) enhancing mitochondrial antioxidant uptake (Jauslin, Meier, Smith, & Murphy, 2003).

Regarding potential gene therapy interventions, several antioxidant genes were described to regulate the mitochondrial metabolism and to block oxidative stress by regulating OXPHOS metabolites. Among them, the mitochondrial signal transducer and activator of transcription 3 (STAT3) that manipulates the complex I dehydrogenase activity through a retrograde nicotinamide adenine dinucleotide (NAD^+) signal may improve cellular antioxidant activities (Lahiri et al., 2021). In addition to targeting OXPHOS metabolites, other gene regulation strategies may further suppress oxidative stress. For instance, the use of the hypoxia-inducible factor alpha (HIF-1α), a master regulator of tissue responses to oxidative pathological stimuli, may modulate damaging ROS levels that impair cardiac function in myocardial fibrosis after myocardial infarction (Janbandhu et al., 2022). Suppressive effects of HIF-α on mitochondrial oxidative stress were also documented for the treatment of liver fibrosis (H. S. Li et al., 2019), obesity (Gaspar & Velloso, 2018; K. Y. Lee, Gesta, Boucher, Wang, & Kahn, 2011), and insulin resistance (Y. S. Lee et al., 2014). Besides, overexpression of the neurofibromatosis-1 (NF1) was shown to advantageously increase the resistance to oxidative and mitochondrial respiration while reducing the levels of ROS production by 60% in Drosophila melanogaster through the adenylyl cyclase (AC)/cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling (Tong, Schriner, McCleary, Day, & Wallace, 2007).

The application of TCM has been performed for more than 2,000 years and in recent years, various TCM monomers have been reported in pharmacological research as effective antioxidants to treat a number of human diseases. The herbal salvia miltiorrhiza has been widely used for cardiovascular diseases as it can balance the production of ROS in cardiomyocytes (Fei et al., 2013; Ren, Fu, Nile, Zhang, & Kai, 2019) and reduce the oxidative damage of cardiac ischemia-reperfusion (Yin et al., 2013; Zhao, Jiang, Zhao, Hou, & Xin, 1996). The anti-inflammatory and anti-tumoral Atractylodes lactone extracted from the rhizome of Atractylodes macrocephala Koidz can counteract the oxidative stress associated with chronic kidney disease, reducing muscle wasting via inhibition of the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB or AKT)/mammalian target of rapamycin (mTOR) pathway (M. Wang et al., 2019). Other beneficial TCM compounds

may include curcumin, an inhibitor of oxidative stress and mitochondrial dysfunction in astrocytes (Daverey & Agrawal, 2016), the cuscuta pedicellata extract that improves oxidative stress caused by high-quality diet through gene protection (Mehanna, El-Sayed, Ibrahim, Ahmed, & Abo-Elmatty, 2018), and artemisinin protecting glutamate-mediated neuronal oxidative apoptosis by manipulating the $\Delta\Psi m$ and reducing ROS levels (S. P. Lin, Li, Winters, Liu, & Yang, 2018).

3.4.3. Strategies targeting the mitochondria in inflammatory diseases

Growing evidence supports the contribution of mitochondrial dysregulation to an inflammatory phenotype in numerous diseases such as rheumatoid arthritis (Fearon, Canavan, Biniecka, & Veale, 2016), multiple sclerosis (Voet, Prinz, & van Loo, 2019), thyroiditis (Burek & Rose, 2008), and type 1 diabetes (Vig, Lambooij, Zaldumbide, & Guigas, 2021). With aging or upon tissue damage, the mitochondria release undesired tricarboxylic acid metabolites and damaged mitochondrial components (mtDNA, cardiolipin, N-formyl peptides) recognized as damage-associated molecular patterns (DAMPs) that act as danger signals to trigger the immune system via pattern recognition receptors (PRRs) (Dela Cruz & Kang, 2018). For instance, circulating mtDNA gradually increases after 50 years of age (Pinti et al., 2014) correlating with an enhanced production of pro-inflammatory cytokines like in cultured monocytes (Pinti et al., 2014) and in elderly individuals (Pinti et al., 2014). Succinate, a tricarboxylic acid metabolite, induces HIF-1-mediated IL-1β production (Tannahill et al., 2013) and its accumulation promotes a reverse electron transport from complex II to complex I, a pattern resulting in a dramatic elevation of the ROS levels (Mills et al., 2016). Other mitochondrial metabolites with immunostimulatory effects include citrate, acetate, acetyl-CoA, and itaconate contributing to inflammation and immune responses (Mills, Kelly, & O'Neill, 2017). When stimulated by pathogen-associated molecular patterns (PAMPs) and DAMPs, MAMs are also involved in sensitive molecular signals by providing sites for the activation of the inflammasome as large protein complexes controlling the activation of the proteolytic caspase 1 enzyme that in turn regulates the maturation of IL-1β and IL-18 and cell death (pyroptosis via the formation of gasdermin D-mediated lytic pores in the plasma membrane), for instance the nucleotide oligomerization domain (NOD)-like receptor protein 3 (NLRP3) inflammasome (R. Zhou, Yazdi, Menu, & Tschopp, 2011). Owing to the importance of mitochondria in the

development of inflammation, several mitochondria-targeted strategies have been explored as anti-inflammatory therapeutic options focusing on mitophagy, ROS control, and on repressing the inflammasome.

The removal of dysfunctional and undesirable mitochondrial components such as mtDNA and ROS by mitophagy contributes to inflammation as DAMPs that are sensed by inflammatory signals (Dela Cruz & Kang, 2018). Interestingly, there is evidence that mitophagy combined with the mitochondrial unfolded protein response (UPRmt) can significantly mitigate LPS-mediated inflammatory myocardial injury (Y. Wang et al., 2021) and that the nuclear factor kappa B (NF-κB) and downstream p62 that recognize damaged mitochondria may activate mitophagy, contributing to homeostasis and tissue repair (Sliter et al., 2018). The protection of mitophagy against chronic inflammation has also been demonstrated in other disorders including colitis (Xu, Shen, & Ran, 2020), diabetes (de Maranon et al., 2022), and Alzheimer's disease (E. F. Fang et al., 2019).

Another promising strategy for inflammation is to control ROS production like by using the various approaches and compounds cited (MitoQ, BGP-15, MitoSOD, MitoPrx, MitoTEMPOL, MitoE, MitoLip, HIF-1α, NF1, TCM, etc.) (Al-Zubaidi et al., 2021; Fei et al., 2013; Janbandhu et al., 2022; S. P. Lin et al., 2018; Ren et al., 2019; Smith et al., 2008; To et al., 2020; Tong et al., 2007; Wiel et al., 2014; Yin et al., 2013; Zhao et al., 1996).

Restricting the inflammasome via mitochondrial regulation may further be beneficial to control inflammatory pathological phenotypes (D. Zheng, Liwinski, & Elinav, 2020) like during pathogen infection (human immunodeficiency virus - HIV) (Hernandez, Latz, & Urcuqui-Inchima, 2014), sepsis (Cecconi, Evans, Levy, & Rhodes, 2018), colitis (C. Chen et al., 2021), particulate matter (PM)2.5-mediated pulmonary pyroptosis (Huang et al., 2022), and more recently during the coronavirus disease 2019 (COVID-19) with its associated severe acute respiratory syndrome (SARS) due to the SARS coronavirus (SARS-CoV) caused by an excessive inflammatory storm led by NLRP3 (Freeman & Swartz, 2020) like using the MitoTEMPOL antioxidant that can regulate the formation of the NLRP3 inflammasome and reduce SARS-CoV viroporin 3a protein-induced IL-1β secretion for instance (I. Y. Chen, Moriyama, Chang, & Ichinohe, 2019).

3.4.4. Strategies targeting the mitochondria in mitochondrial diseases

Mitochondrial diseases represent a group of maternally inherited metabolic disorders caused by mutations in the mtDNA that lead to mitochondrial dysfunction, mostly affecting OXPHOS and the levels of ATP synthesis (Russell, Gorman, Lightowlers, & Turnbull, 2020) together with a loss of enzymatic intermediates, the accumulation of toxic metabolites, and the disruption of the Krebs and folate cycles (Gorman et al., 2016). Current therapeutic strategies for mitochondrial diseases are divided into small molecule therapy and mtDNA gene editing.

Supplementation with small molecules is currently the most commonly employed treatment modality for mitochondrial diseases as a means to remove toxic compounds, replenish defective enzymes or their analogs, or balance cofactors since toxic metabolites are often triggering the clinical phenotype of mitochondrial diseases. For instance, accumulation of hydrogen sulfide in patients with ethylmalonic encephalopathy (a disease caused by mutations in the ethylmalonic encephalopathy protein 1 (ETHE1) gene coding for a sulfur dioxygenase) may be reduced by a combined application of N-acetylcysteine and metronidazole (V. Tiranti et al., 2004; V. Tiranti & Zeviani, 2013; Viscomi et al., 2010). In addition, erythrocyte encapsulation of thymidine kinase phosphorylase was reported to improve the mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) syndrome in patients, a mitochondrial disease caused by thymidine phosphorylasethymine phosphorylase (TYMP) mutations leading to thymidine phosphorylase deficiency (Bax et al., 2013; Garone, Tadesse, & Hirano, 2011; Nishino, Spinazzola, & Hirano, 1999). A similar approach was applied to primary CoQ deficiency disorders such as encephalonephropathies with nephrotic syndrome, childhood-onset mitochondrial diseases, and isolated cerebellar ataxia using CoQ supplementation (Emmanuele et al., 2012; Quinzii, Emmanuele, & Hirano, 2014). Complementary therapy of enzyme cofactors (non-protein small molecules, metal hydrate ions) has also been successfully used like by supplementation of vitamin B12, a cofactor for flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), to treat mitochondrial diseases associated with FMN and FAD deficiencies (complex I deficiency due to acyl-CoA dehydrogenase protein 9 - ACAD9 - mutations, severe X-linked mitochondrial encephalomyopathy due to mutations in the apoptosis-inducing factor mitochondrion-associated 1 - AIFM1 - precursor with NAD-

dependent NADH oxidase activity) (Ghezzi et al., 2010; Giancaspero et al., 2015; Haack et al., 2010; Nouws et al., 2014).

The mtDNA gene editing technology may also offer powerful options to treat mitochondrial diseases (Zhong, Madry, et al., 2022) based on antigenomic mtDNA therapy or on the use of restriction endonucleases (Chinnery et al., 1999; Muratovska et al., 2001; Tonin et al., 2014), zinc-finger nucleases (ZFNs) (Gammage et al., 2016; Gammage, Viscomi, et al., 2018), transcription activator-like effectors nucleases (TALENs) (Bacman, Williams, Pinto, Peralta, & Moraes, 2013; Hashimoto et al., 2015; Y. Yang et al., 2018), and the clustered regularly interspaced short palindromic repeats/CRISPR-associated 9 (CRISPR/Cas9) (Gammage, Moraes, & Minczuk, 2018; Jo et al., 2015; Singh, Schoeb, Bajpai, Slominski, & Singh, 2018) to correct mtDNA variants (Cho et al., 2022; H. Lee et al., 2021; Mok et al., 2020; Nakazato et al., 2022; Silva-Pinheiro et al., 2022), especially with the emergence of double-stranded DNA deaminase (DddA)-derived cytosine base editors (DdCBEs) that can support the editing of C•G to T•A in mutated mtDNA sequences (Cho et al., 2022; H. Lee et al., 2021; Mok et al., 2020; Silva-Pinheiro et al., 2022). Still, single base editing is challenging due to the genome complexity and probably insufficient to fully reverse a diseased phenotype while it may lead to off-target modifications (Lei et al., 2022), all impacting the clinical translation potential of mtDNA gene editing.

3.4.5. Strategies targeting the mitochondria in cancer

Mitochondrial damage in tumor cells is associated with an abnormally weakened OXPHOS as such cells perform the glycolytic pathway to catabolize glucose in lactate to generate ATP even in the presence of sufficient oxygen(Warburg effect) (Warburg, 1956a, 1956b) while maintaining high ROS levels (Cross et al., 1987) that activate molecular signaling pathways promoting tumor cell proliferation (Schieber & Chandel, 2014). Regulating the mitochondrial energy metabolism may therefore be a powerful strategy for tumor therapy and several approaches have been attempted based on the use of specific pathway inhibitors or on the targeting of mitochondrial material metabolism and of mitophagy, including in conjunction with nanodelivery technologies to improve efficacy and reduce adverse reactions (**Figure 7**) (Fulda, Galluzzi, & Kroemer, 2010).

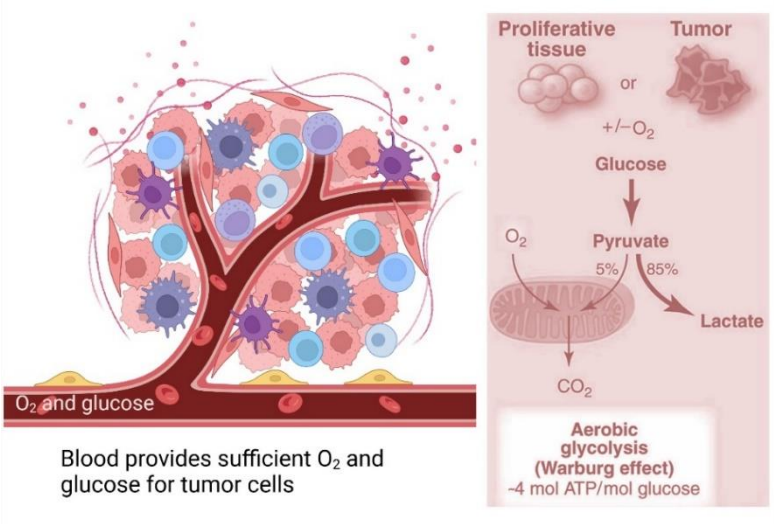


Figure 7. The Warburg effect in cancer (created with BioRender).

Different inhibitors of critical mitochondrial energy metabolic processes have been applied like for instance KUNB31 and geldanamycin, two inhibitors of the heat shock protein 90 (HSP90) that block ATP binding and hydrolysis in non-small cell lung cancer, multiple myeloma, ovarian cancer, melanoma, and renal cell carcinoma (Jurczyszyn et al., 2014; Mishra et al., 2021) or metformin, an ETC blocker of the complex I that can reduce the production of ATP in breast cancer, non-small cell lung cancer, renal cell carcinoma, melanoma, and colon cancer (El-Mir et al., 2000; Kasznicki, Sliwinska, & Drzewoski, 2014).

Besides, as tumor cells need a sufficient material basis to maintain their proliferation and metabolism, targeting mitochondrial metabolism is another attractive treatment against cancer. Tricarboxylic acid (TCA) cycle intermediates provide raw materials for the biosynthesis of macromolecules (Lukey, Wilson, & Cerione, 2013), with glutamine the major carbon source that replenishes TCA cycle intermediates and maintains their use in biosynthesis in tumor cells. Glutamine is converted to glutamate by the glutaminase (GLS) for further catabolism to produce beneficial α-ketoglutarate for the cellular material cycle (Weinberg & Chandel, 2015). Blocking this metabolic pathway is therefore an attractive approach in cancer therapy and indeed, multiple specific GLS inhibitors have been developed including the compound 968 and bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES), reducing glutamine catabolism and modulating the growth of glutamine-dependent tumors (Hensley, Wasti, & DeBerardinis, 2013; Shen et al., 2021).

Mitophagy is also involved in the rapid metabolism of tumor cells as another part of the mitochondrial "quality control" process by generating TCA cycle intermediates for tumor cell metabolism (Levy, Towers, & Thorburn, 2017). Interestingly, knocking-out the essential autophagy related 7 (ATG7) gene for mitophagy allows to increase the tolerance to starvation and to prolong the lifespan of mice with lung cancer (Strohecker et al., 2013). Chloroquine, another well-known inhibitor of autophagy, and its derivative hydroxychloroquine have been also reported for their excellent tumor suppressive effects (Briceno, Reyes, & Sotelo, 2003; Mahalingam et al., 2014; Rangwala et al., 2014). Besides, given that tumor cells are in a delicate oxidative balance adapted to different ROS levels than normal cells, disrupting this balance with high selectivity has been shown to be a potential therapeutic approach for tumors (Murphy & Smith, 2007; Pelicano et al., 2003; Trachootham et al., 2006).

3.4.6. Strategies targeting the mitochondria for regenerative medicine

The repair of damaged tissues is generally supported by the differentiation of progenitor (stem) cells, a critical process associated with pivotal biological activities (protein synthesis, genome replication, carbohydrate preparation) that necessitate large amounts of energy from the mitochondria. However, the contribution of mitochondria to stromal cell differentiation is not limited to providing energy but also probably associated with mitochondrial biogenesis and dynamics (Chakrabarty & Chandel, 2021; Q. Li, Gao, Chen, & Guan, 2017) in light of evidence showing that mitochondria are present at higher mass in differentiating cells, with a more elongated morphology, larger surface area, and lower membrane potential compared with their situation in pre-differentiated cells (Forni, Peloggia, Trudeau, Shirihai, & Kowaltowski, 2016; Hofmann et al., 2012; Quinn et al., 2013). Regulating the mitochondrial biological behavior may therefore be of strong value to promote stromal cell differentiation for tissue repair like by regulating mitochondrial dynamics (fission, fusion), the mitochondrial respiration, and mitoCa^{2+} uptake.

Changes in the mitochondrial morphology controlled by mitochondrial dynamics are closely related to the modulation of mitochondrial functions. Owing to the central role of the mitochondria in cellular processes, mitochondrial dynamics is extremely important in the cell cycle at the level of cell proliferation, differentiation, and aging and regulating

mitochondrial fission and fusion is thus an important way to influence the stromal cell fate (Wakabayashi et al., 2009). Several studies, for instance, reported the significant impact of the dynamin-related protein 1 (DRP-1), a molecule directing mitochondrial fission, on the differentiation of eukaryotic cells both *in vitro* and *in vivo*. Specifically, ablation of *Drp-1* in the mouse brain was shown to cause cerebellar hypoplasia in association with the presence of few giant mitochondria in Purkinje cells (instead of normally many short tubular mitochondria) (Wakabayashi et al., 2009) while neural cell-specific *Drp-1*(-/-) mice were reported to develop brain hypoplasia with reduced neurite numbers and abnormal synapse formation, shortly dying after birth (Ishihara et al., 2009). Accordingly, treatment with DRP-1 inhibitors such as the mitochondrial division inhibitor 1 (mdivi-1, an inhibitor of the guanosine triphosphate hydrolase (GTPase) activity of DRP-1) that prevents myotube formation and impairs the myogenic differentiation of myoblasts (B. Kim et al., 2013) or with the mitochondrial fission 1 protein (FIS1) involved in myeloid differentiation (Pei et al., 2018) may provide strong tools to address these critical problems. On the other side, there is evidence that mitofusin 2 (Mfn-2), a receptor located in OMMs and contributing to mitochondrial fusion, plays a key role in mammalian stromal cell differentiation and that overexpression of Mfn-2 in human induced pluripotent stem cells (hIPSCs) promotes their differentiation and maturation in neurons (D. Fang, Yan, Yu, Chen, & Yan, 2016).

The mitochondrial respiration, in particular the intermediate ROS product, is also important for stromal cell differentiation. Interestingly, while ROS is considered detrimental at unrestricted levels, closely regulated ROS levels may have a beneficial impact on cellular functions such as cell proliferation and differentiation (Atashi, Modarressi, & Pepper, 2015). For instance, appropriate ROS levels are capable of enhancing the adipogenic differentiation of MSCs by controlling specific signaling cascades (c-jun NH2-terminal kinase - JNK, p38 mitogen-activated protein kinase - p38 MAPK, extracellular regulated kinase - ERK, phosphatidylinositol 3-kinase/protein kinase B - PI3K/Akt - pathways) in these cells (Atashi et al., 2015). In contrast, inhibition of the mitochondrial respiration by hypoxia and mitochondrial ETC inhibitors was shown to significantly suppress the adipogenic differentiation of MSCs (Y. Zhang, Marsboom, Toth, & Rehman, 2013). Other studies demonstrated that overexpression of the superoxide dismutase 2 (SOD2), a mitochondrial antioxidant metalloenzyme, can significantly improve bone

differentiation and bone formation in mice with osteogenic differentiation defects by assisting SIRT3 and regulating the mitochondrial oxidative transition and respiratory activities (J. Gao et al., 2018). Finally, as an important intermediate of OXPHOS, providing the main carbon source for macromolecular synthesis and displaying antioxidant properties, glutathione was reported to increase the differentiation capacity of MSCs for bone regeneration (Shaban et al., 2017; T. Zhou, Yang, Chen, & Xie, 2019).

Apart from its effects on the mitochondrial metabolism, an imbalance in mitoCa^{2+} may also affect the regenerative capacity of cells and tissues (Duchen, 2000; Rizzuto et al., 2012; Szabadkai & Duchen, 2008). This concept is supported by the observation that increasing mitoCa^{2+} by inhibition of the mitochondrial calcium uptake 1 (MICU1, the "gatekeeper" of mitoCa^{2+} uptake in the MCU) attenuates the regenerative capacity of liver cells (Antony et al., 2016). In contrast, overexpression of MICU1 in a Streptococcus pneumoniae-mediated lung injury model can significantly reduce mitoCa^{2+} uptake and induce AT2 cells to alveolar cell differentiation as a beneficial process to promote alveolar repair (Ali et al., 2022) while MICU1 can increase α-ketoglutarate by reducing mitoCa^{2+} uptake and influence myofibroblast differentiation (Lombardi et al., 2019).

3.5. Implantation of regenerative mitochondria/rAAV platforms as an innovative osteoarthritis treatment

The emergence of the recombinant DNA technology has rapidly spawned a new therapeutic strategy - gene therapy - that aims at replacing defective genes or therapeutic protein products, leading to clinical benefit in patients with immunodeficiencies, hemoglobinopathies and metabolic and storage disorders (Dunbar et al., 2018). To date, multiple gene therapies received approval in the Western world such as Glybera® (approved by the European Medicines Agency EMA - in 2012 for the treatment of familial lipoprotein lipase - LPL - deficiency) (Watanabe, Yano, Tsuyuki, Okano, & Yamato, 2015), Luxturna® (approved by the U.S. Food and Drug Administration - FDA - for inherited retinal diseases in 2017) ("FDA approves hereditary blindness gene therapy," 2018), or Zolgensma® (approved by the FDA for spinal muscular atrophy - SMA - in 2019) (Ogbonmide et al., 2023). More than 200 phase II and III clinical trials are currently

underway, which could translate to as many as 40 new products being approved for clinical use within the next decade ("Gene therapies should be for all," 2021). Yet, serious treatment-related toxicities were evidenced in preclinical and evidence-based reports, including the neutralization of the viral particles by naturally existing anti-AAV capsid antibodies (Cucchiarini & Madry, 2019; Henning Madry et al., 2020; R. Yang, Chen, Guo, Zhou, & Luan, 2020) and malignancies caused by vector-mediated insertional activation of proto-oncogenes (High & Roncarolo, 2019; Kay, 2011), showing the critical need for alternative, safe platforms that can deliver therapeutic benefits without these accompanying risks.

In light of the critical roles of mitochondria in cellular biology and its dysfunction during the progression of osteoarthritis (Bock & Tait, 2020; Spinelli & Haigis, 2018; Zhong, Venkatesan, Madry, & Cucchiarini, 2022), an innovative approach was developed in this work based on the manipulation of human mitochondria as platforms to deliver rAAV vectors carrying the highly therapeutic IGF-I candidate gene via implantation (Zhong, Madry, et al., 2022) in autologous hOACs as a means to restore the otherwise altered functions of the mitochondria in these cells, with an extension using a biocompatible material (PF127) hydrogel to provide the mitochondria/rAAV platforms as future tools for the treatment of OA in human patients.

3.6. Controlled delivery of rAAV vectors with anti-inflammatory drugs as an innovative osteoarthritis treatment

As a potential alternative to the use of mitochondria in autologous implantation protocols for OA, rAAV vectors were applied to hOACs using a biocompatible material (type-I collagen) hydrogel with a recombinant anti-inflammatory drug, i.e. the rIL-1Ra in light of its protective effects in OA (Pelletier et al., 1997). The concept of controlled rAAV vector delivery via biocompatible materials has been described for its potential to allow for a more sustained and safer rAAV-based therapy that direct (biomaterial-free) gene transfer, avoiding potential viral vector neutralization by pre-existing anti-AAV capsid antibodies and extanding the potential therapeutic effects of the gene cargo (Cucchiarini & Madry, 2019; Henning Madry et al., 2020; R. Yang et al., 2020). The focus of such rAAV-based gene transfer in hOACs was placed on the cartilage-specific *sox9* gene due to the ability

of the SOX9 transcription factor to remodel the altered OA phenotype in particular via its protective effects against the loss of essential extracellular matrix components (proteoglycans, type-II collagen) and its beneficial effects on the restoration of their deposition (Bi, Deng, Zhang, Behringer, & de Crombrugghe, 1999; Lefebvre, Angelozzi, & Haseeb, 2019), especially when delivered via rAAV in direct (biomaterial-free) settings (Rey-Rico et al., 2018; J. K. Venkatesan et al., 2012; J. K. Venkatesan et al., 2017).

4. HYPOTHESES

The purpose of this project was to innovatively establish different, effective and safe therapies for human OA, based on (1) the autologous implantation of mitochondria as platforms of delivery of clinically adapted rAAV vectors in hOACs and (2) the hydrogel-based controlled delivery of rAAV vectors with anti-inflammatory drugs, in hOACs.

In the present study, the following two hypotheses were tested:

Hypothesis 1: The altered phenotype of hOACs can be managed by implantation of autologous mitochondria as platforms for therapeutic rAAV vector delivery.

Hypothesis 2 The altered phenotype of hOACs can be managed by hydrogel-based controlled delivery of therapeutic rAAV vectors with anti-inflammatory drugs.

To address these two hypotheses, the following approaches were developed:

To Hypothesis 1: Mitochondria were employed to generate mitochondria/rAAV platforms for the delivery of a therapeutic candidate rAAV vector carrying IGF-I (relative to control conditions) in autologous hOACs.

To Hypothesis 2: A type-I collagen hydrogel was employed to control the delivery of a therapeutic candidate rAAV vector carrying *sox9* with a recombinant anti-inflammatory IL-1Ra (relative to control conditions) in hOACs.

5. MATERIALS

5.1. Chemicals and reagents

Table 3. Chemicals and reagents used in the studies

Product	**Manufacturer**
1st Strand cDNA Synthesis Kit for RT-PCR (AMV)	Roche Applied Science, Mannheim, Germany
4-styrenesulfonic acid sodium salt hydrate (NaSS)	Sigma-Aldrich, Merck, Munich, Germany
AAVanced Concentration Reagent	System Bioscience, Heidelberg, Germany
AAV Titration ELISA	Progen, Heidelberg, Germany
ABC Reagent (Avidin-Biotin-Peroxidase-Reagent)	Vector, Burlingame, California, USA
anti-IGF-I (AF-291-NA)	R&D Systems, Minnesota, USA
anti-SOX9 (C-20)	Santa Cruz Biotechnology, Heidelberg, Germany
anti-type-I collagen (AF-5610) antibody	Acris, Hiddenhausen, Germany
anti-type-II collagen (II-II6B3) antibody	Ames, IA, USA
anti-type-X collagen (COL-10) antibody	Sigma-Aldrich, Merck, Munich, Germany
ATP Determination Kit	Thermo Fisher Scientific, Schwerte, Germany
BCA Protein Assay	Thermo Fisher Scientific, Schwerte, Germany
Brilliant SYBR Green QPCR Master Mix	Stratagene, Agilent Technologies, Waldbronn, Germany
BSA	Sigma-Aldrich, Merck, Munich, Germany
Cell Counting Kit-8	Sigma-Aldrich, Merck, Munich, Germany
Cell Proliferation Reagent WST-1	Roche Applied Science, Mannheim, Germany
collagenase	Worthington Biochemical, Lakewood, NJ, USA

DAB reagent	Vector, Burlingame, California, USA
DMEM	Sigma, Munich, Germany
DMMB dye	Sigma-Aldrich, Merck, Munich, Germany
eosin G	Roth, Karlsruhe, Germany
fast green	ICN Biomedicals, Eschwege, Germany
FBS	Sigma, Munich, Germany
fibrinogen/thrombin	Baxter, Volketswil, Switzerland
Fluorometric Intracellular ROS Kit	Sigma-Aldrich, Merck, Munich, Germany
formalin stock solution (37%)	Sigma-Aldrich, Merck, Munich, Germany
hematoxylin	Roth, Karlsruhe, Germany
HCl (1%)	Sigma-Aldrich, Merck, Munich, Germany
Hoechst 33358	Sigma-Aldrich, Merck, Munich, Germany
human type-I collagen hydrogel (VitroCol®)	Advanced BioMatrix, California, USA
hydrogen peroxide	Sigma-Aldrich, Merck, Munich, Germany
IGF-I Quantikine ELISA	R&D Systems, Wiesbaden, Germany
JC-1 fluorescent probe	MedChem Express, Mannheim, Germany
Mitochondria Isolation Kit	Thermo Fisher Scientific, Schwerte, Germany
papain	Sigma-Aldrich, Merck, Munich, Germany
penicillin	Sigma-Aldrich, Merck, Munich, Germany
Pierce Thermo Scientific Protein Assay	Thermo Fisher Scientific, Schwerte, Germany
pluronic® F127	BASF, Ludwigshafen, Germany

primers	Invitrogen, Sigma-Aldrich, Merck, Munich, Germany
rIL-1Ra	Sigma-Aldrich, Merck, Munich, Germany
RNeasy Protect Mini Kit	Qiagen, Hilden, Germany
RNase-Free DNase Set	Qiagen, Hilden, Germany
RNase-free water	Qiagen, Hilden, Germany
safranin O	Roth, Karlsruhe, Germany
sterile saline	Ecotainer, B. Braun Medical AG, Emmenbrucke, Germany
streptomycin	Sigma-Aldrich, Merck, Munich, Germany
TPCK treated-trypsin	Thermo Fisher Scientific, Schwerte, Germany
terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)	Thermo Fisher Scientific, Schwerte, Germany
xylene	Fischer, Saarbrücken, Germany

5.2. Solutions and buffers

Table 4. Solutions and buffers used in the studies

Solutions, buffers	**Ingredients**	**Volume, weight**
blocking buffer	BSA	6 ml
	PBS	200 ml
0.2% collagenase	collagenase	20mg
	PBS	10ml
DAB solution	H_2O	5 ml
	buffer (pH 7.5)	2 drops
	DAB reagent	4 drops
	H_2O_2	2 drops

fast green solution	fast green	200 mg
	H_2O	ad 1,000 ml
eosin solution	eosin G	10 g
	H_2O	ad 2,000 ml
	KH_2PO_4	9.07 g
	Na_2HPO_4	11.86 g
formalin solution (pH 7.4)	formalin stock solution	140 ml
	H_2O	ad 1,000 ml
hematoxylin solution	hematoxylin	10 g
	ethanol (100%)	120 ml
	sodium iodate	10 g
	$ALKSO_4$	200 g
	H_2O	ad 2,000 ml
HCl solution	HCl (40%)	5.4 ml
	H_2O	ad 200 ml
PBS	KCl (pH 7.2)	2.7 mM
	K_2HPO_4	1.7 mM
	NaCl	136 mM
	Na_2HPO_4-$7H_2O$	10 mM
safranin O solution	safranin O	1 g
	H_2O	ad 1,000 ml
trypsin solution (0.1%)	trypsin stock solution (25%)	800 µl
	PBS	ad 200 ml
trypsin stock solution	trypsin	25% (v/v)
	PBS	75% (v/v)

hydrogen peroxide (0.3%)	H_2O_2	0.6 ml
	H_2O	200 ml

5.3. Equipment

Table 5. Equipment used in the studies

Equipment	**Manufacturers**
autoclave AMA-240	Astell, Sidcup, England
Canon Powershot A480	Canon, Tokyo, Japan
digital camera CC-12 (on microscope BX45)	Soft Imaging System, Münster, Germany
refrigerator -20°C	Bosch, Gerlingen-Schillerhöhe, Germany
refrigerator -74°C Platinum 550	Angelantoni Industrie, Massa Martana PG, Italy
GENios microplate reader	TECAN, Crailsheim, Germany
incubator CB 150 (37°C)	Binder, Tuttlingen, Germany
magnetic stirrer RH basic 2	IKA, Staufen, Germany
microscopes BX45 and CK-2	Olympus, Hamburg, Germany
cover plate (plate sealer)	MD Bioproducts, Saint Paul, USA
Mx3000P QPCR operator system	Stratagene, Agilent Technologies, Waldbronn, Germany
heat plate HI 1220	Leica, Nussloch, Germany
Huawei 30 Pro	Huawei, Shenzhen, China
Transwell plates (pores: 3.0 μm)	Corning, New York, USA
water bath HI 1210	Leica, Nussloch, Germany

5.4. Software

Table 6. Software used in the studies

Software	**Company**
Adobe Illustrator	Adobe Systems, Mountain View, California, USA
Adobe Photoshop	Adobe Systems, Mountain View, California, USA
AnalySIS	Soft Imaging System GmbH, Münster, Germany
BioRender	BioRender, Toronto, Ontario, Canada
GraphPad Prism 8	GraphPad Software, California, USA
Image J.	National Institutes of Health, Bethesda, USA
Microsoft PowerPoint 2021	Microsoft, Redmond, USA
MxPro QPCR software	Stratagene, Agilent Technologies, Waldbronn, Germany

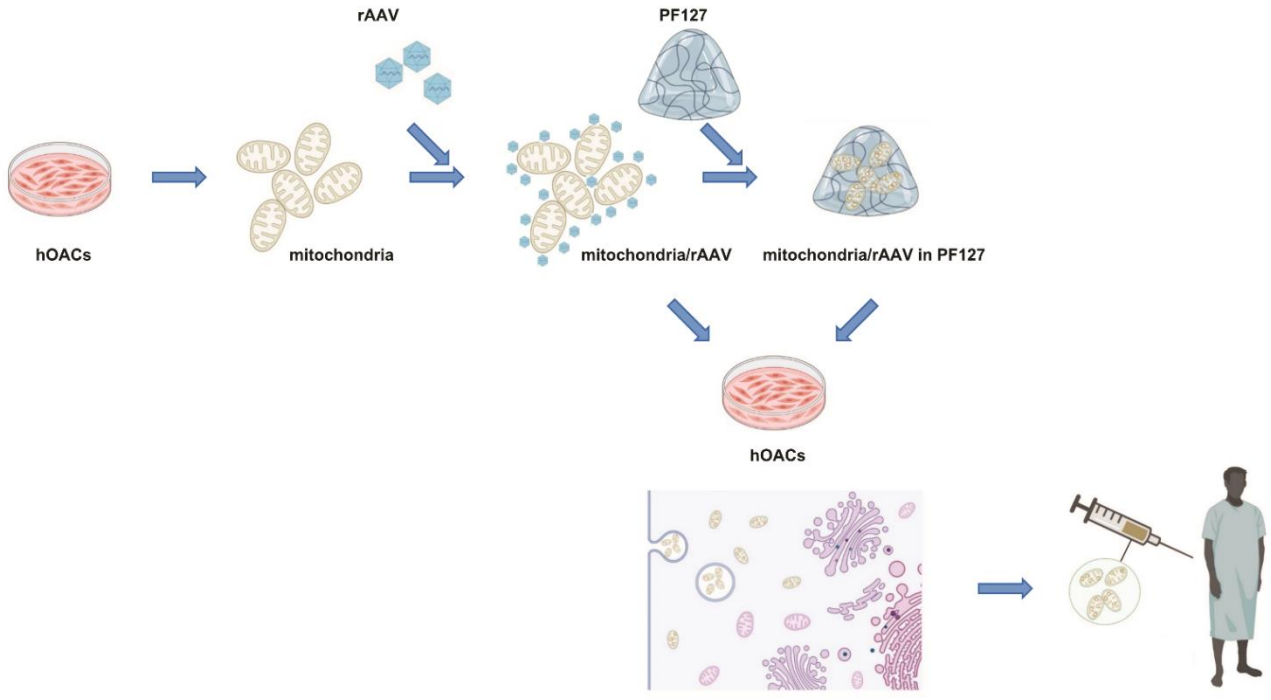
6. METHODS

6.1. Study design

6.1.1. Mitochondria/rAAV platforms

Mitochondria were isolated from hOACs, treated with rAAV vectors, and next implanted as mitochondria/rAAV platforms in autologous hOACs, using both reporter (*lacZ*, RFP) and candidate (IGF-I, *sox9*) rAAV vectors as proof-of-concept (*lacZ*, RFP) and to examine potential therapeutic effects (IGF-I, *sox9*) for future treatments of human OA (**Figure 8A**). In addition, the mitochondria/rAAV platforms were provided to hOACs using a biocompatible material (PF127) hydrogel (**Figure 8A**) using two approaches: formulation of the mitochondria/rAAV platforms in the PF127 hydrogel (plan I) and formulation of rAAV vectors in the PF127 hydrogel for mitochondria treatment (plan II) (**Figure 8B**).

A.



B.

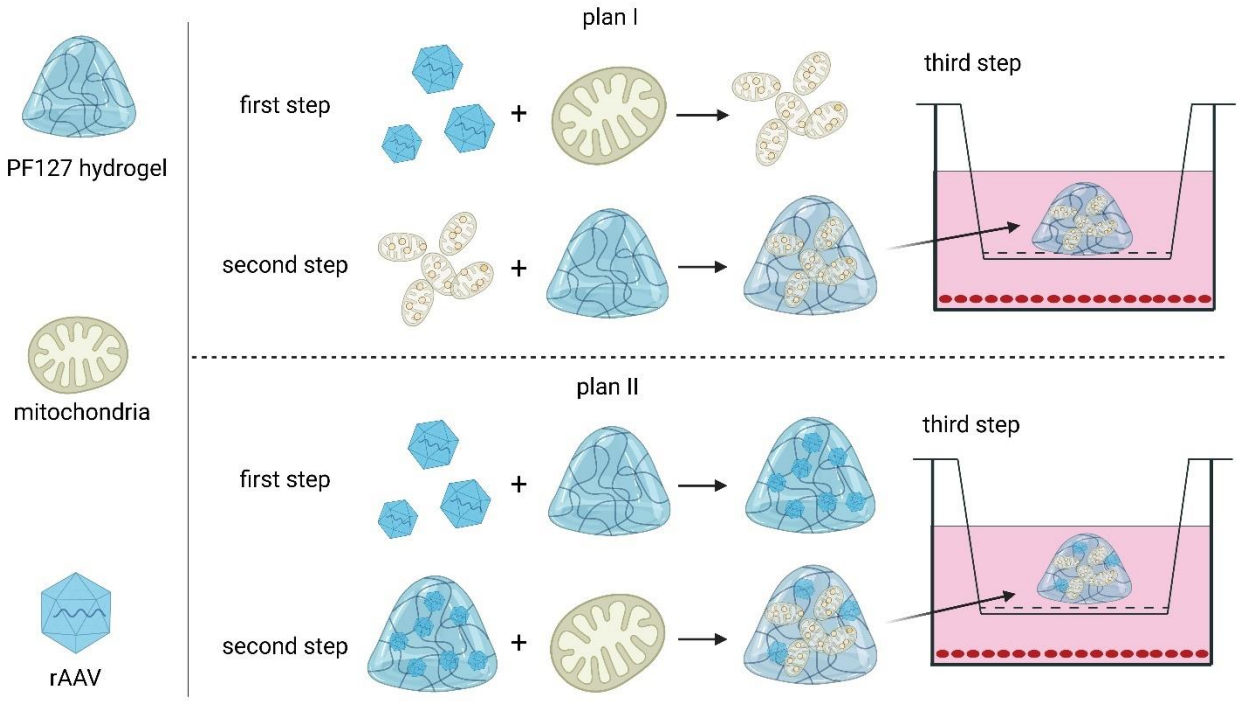


Figure 8. Design of the rAAV/mitochondria platforms for osteoarthritis. (**A**) Human mitochondria were isolated from hOACs treated with rAAV vectors, and the mitochondria/rAAV platforms were autologously implanted in the cells directly or via a biocompatible material (PF127) hydrogel for the future treatment of OA in patients. (**B**) For PF127 hydrogel-guided delivery, either the mitochondria were mixed with rAAV vectors and then the PF127 hydrogel was added to the mitochondrial/rAAV platform (plan I) or the PF127 hydrogel was mixed with rAAV vectors and then the mitochondria were added (plan II) prior to application to the hOACs (created with BioRender.com).

6.1.2. Controlled delivery of rAAV vectors with anti-inflammatory drugs

A type-I collagen hydrogel was employed to deliver candidate (*sox9*) rAAV vectors with the rIL-1Ra compound in hOACs relative to reporter (*lacZ*) rAAV vectors in order to examine potential therapeutic effects (*sox9*, rIL-1Ra) in these cells as a future treatment of human OA (**Figure 9**). Such a strategy may support direct intra-articular injections of the modified hydrogel in an *in vivo* scenario in OA patient.

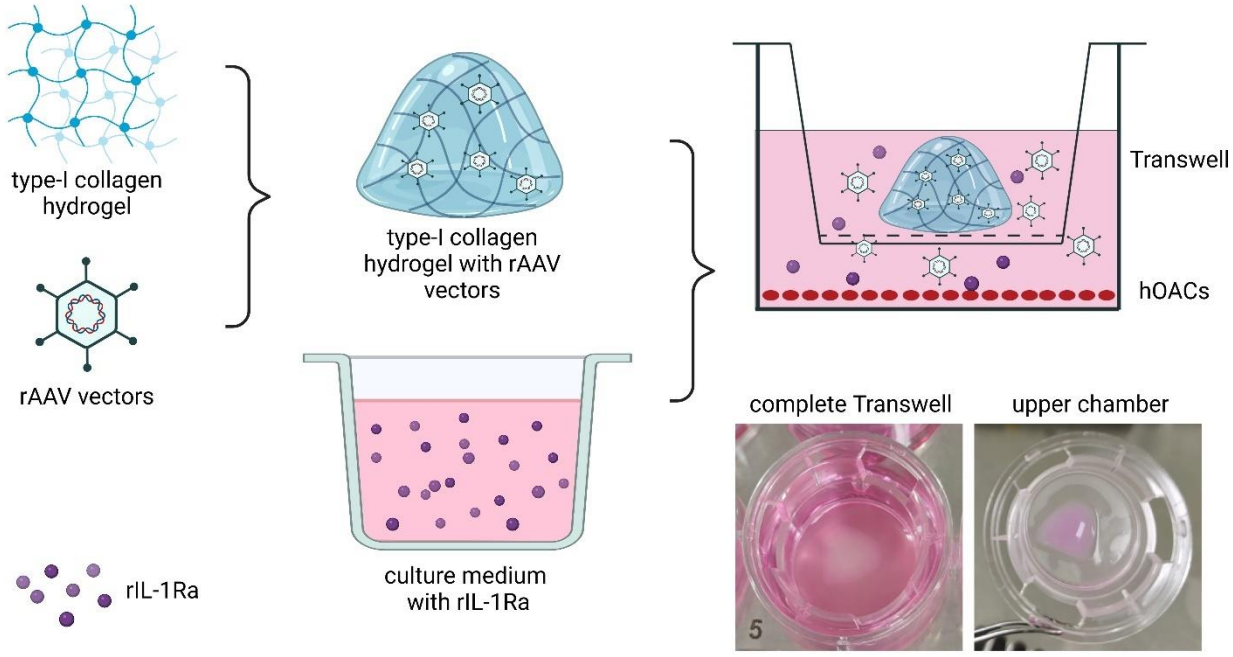


Figure 9. Design of the controlled delivery system of rAAV vectors with anti-inflammatory drugs. A type-I collagen hydrogel was formulated with rAAV vectors and the system was then placed in the upper chamber of a Transwell to ensure that the rAAV vectors can diffuse through the Transwell membrane to reach the lower chamber and potentially act on hOACs placed in the lower chamber in the presence of the rIL-1Ra (created with BioRender.com).

6.2. Human osteoarthritic chondrocyte cultures

Human OA cartilage was obtained from the knee 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 and all procedures were performed in accordance with the Helsinki Declaration. Informed consent was obtained from all participants. Human OA chondrocytes (hOACs) were isolated as previously described (J. K. Venkatesan et al., 2013). Briefly, the cartilage samples were washed in DMEM, diced in pieces (2 x 2 mm) and placed in DMEM, 100 U/ml penicillin G, and 100 μl/ml streptomycin (basal medium) with 0.2% collagenase (350-400 U/mg) for 16 h at 37ºC in a humidified atmosphere with 5% CO_2 (J. K. Venkatesan et al., 2013). The isolated cells were filtered using a 125-μm mesh to remove the undigested extracellular matrix (J. K. Venkatesan et al., 2013). The cells were incubated in DMEM, 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 μl/ml streptomycin (growth medium), plated in T75 flasks, and maintained at 37ºC in a humidified atmosphere with 5% CO_2 prior to the experiments with cells used at passage 2-3.

6.3. Plasmids and rAAV vectors

The constructs were derived from pSSV9, an AAV-2 genomic clone (Samulski, Chang, & Shenk, 1987, 1989). rAAV-*lacZ* carries the reporter *lacZ* gene for *Escherichia coli* β-gal, rAAV-RFP the reporter *Discosoma sp.* red fluorescent protein (RFP), rAAV-hIGF-I a 0.536 kb human IGF-I (hIGF-I) cDNA fragment, and rAAV-FLAG-h*sox9* a 1.7-kb FLAG-tagged human *sox9* (h*sox9*) cDNA fragment, all placed under the control of the cytomegalovirus immediate-early (CMV-IE) promoter (Rey-Rico et al., 2018; J. K. Venkatesan et al., 2012; J. K. Venkatesan et al., 2017). The vectors were packaged as conventional (not self-complementary) vectors using a helper-free, two-plasmid transfection system in the 293 packaging cell line (an adenovirus-transformed human embryonic kidney cell line) with the packaging plasmid pXX2 and the Adenovirus helper plasmid pXX6 as previously described (J. K. Venkatesan et al., 2013). The vector preparations were purified by dialysis and titered by real-time PCR, averaging 10^{10} transgene copies/ml (~1/500 functional recombinant viral particles) (Frisch et al., 2014; Ulrich-Vinther, Stengaard, Schwarz, Goldring, & Soballe, 2005).

6.4. Mitochondria isolation, modification, and implantation

Mitochondria were freshly extracted from hOACs (10^7 cells) using the Mitochondria Isolation Kit (Q. Zhang et al., 2010) to generate one unit of mitochondria isolate.

For direct rAAV-mediated mitochondria treatment, hOACs (5 x 10^4 cells) were seeded in growth medium for 24 h at 37ºC in a humidified atmosphere with 5% CO_2. Mitochondria isolate (one unit) was mixed with rAAV vectors (40 µl, 8 x 10^5 transgene copies) and DMEM (110 µl) for an incubation of 1.5 h at 37ºC and growth medium (150 µl) was next added to the rAAV-treated mitochondria for an incubation of either 24 h or 48 h at 37ºC. The rAAV-treated mitochondria were centrifuged, resuspended in growth medium, and implanted in the cultures of hOACs (multiplicity of infection = 16) for 24 h at 37ºC in a humidified atmosphere with 5% CO_2 prior to the experiments. Control conditions are indicated in the Results section for each specific experiment.

For PF127 hydrogel-guided rAAV-mediated mitochondria treatment, two approaches were developed. For plan I, DMEM (60 µl) was mixed with mitochondria isolate (one unit) and rAAV vectors (40 µl, 8 x 10^5 transgene copies) and incubated for 90 min at 37°C, then complete medium (100 µl) was added and incubated for 48 h at 37°C in a humidified atmosphere with 5% CO_2. PF127 hydrogels (24% w/v) were prepared in 10% sucrose at 4°C, then PF127 hydrogels (200 µl) were added to the mitochondrial solution to obtain PF127/mitochondria hydrogels (400 µl), incubated for 30 min on ice prior to application to hOACs. For plan II, the PF127 hydrogel (24% w/v) was prepared in 10% sucrose at 4°C. PF127 hydrogel (200 µl) was mixed with rAAV vectors (40 µl, 8 x 10^5 transgene copies). DMEM was mixed with mitochondria to prepare a total of 160 µl of solution, which was added to the rAAV/PF127 hybrid gel (400 µl), incubated for 30 min on ice and incubated for 48 h at 37°C in a humidified atmosphere with 5% CO_2 prior to application to hOACs. For both plan I and plan II, hOACs (5 x 10^4 in 6-well plate, multiplicity of infection = 16) were then cultured in the lower chamber of a Transwell, the medium was replaced after 24 h, and the abovementioned PF127/mitochondria hydrogel (400 µl) was placed in the upper chamber of the Transwell for 24 h at 37°C in a humidified atmosphere with 5% CO_2. Control conditions are indicated in the Results section for each specific experiment.

6.5. Type-I collagen hydrogel/rAAV system administration

hOACs (5 x 10^4 cells) were seeded in the lower chamber of a 6-well plate Transwell in growth medium for 24 h at 37ºC in a humidified atmosphere with 5% CO_2. Human type-I collagen hydrogel (200 µl) was mixed with rAAV vectors (60 µl, 1.2 x 10^6 transgene copies, multiplicity of infection = 24) and the type-I collagen hydrogel/rAAV system was immediately provided to the upper chamber of the Transwell. The cultures were maintained in growth medium in the presence (or absence) of rIL-1Ra (40 ng/ml) for 48 h at 37ºC in a humidified atmosphere with 5% CO_2 prior to the experiments. Control conditions are indicated in the Results section for each specific experiment.

6.6. Transgene expression

Transgene (RFP) expression was monitored under live fluorescence microscopy (Olympus CK-2) (Rey-Rico et al., 2015). Transgene (IGF-I, *sox9*) expression was monitored by immunostaining using specific antibodies, biotinylated secondary antibodies, and the ABC method using diaminobenzidine (DAB) as the chromogen (Cucchiarini, Terwilliger, Kohn, & Madry, 2009). The samples were examined under light microscopy (Olympus BX45) (Cucchiarini et al., 2009). To control for secondary immunoglobulins, samples were processed with omission of the primary antibody. Transgene (IGF-I) expression was also monitored by specific ELISA with measurements performed using a GENios microplate reader (Cucchiarini et al., 2009).

6.7. Biochemical analyses

Cell viability was monitored using the Cell Proliferation Reagent WST-1 with OD^{450nm} proportional to the cell numbers (A. Weimer et al., 2012) and Cell Counting Kit-8 (Zhong, Long, et al., 2022), and cell apoptosis using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) kit (A. Weimer et al., 2012). The DNA and proteoglycan contents were measured with Hoechst 33258 and by binding to the dimethylmethylene blue (DMMB) dye, respectively, with normalization to the total protein contents using the BCA Protein Assay (Cucchiarini et al., 2005; Y. J. Kim et al., 1988). The samples were examined under light microscopy (Olympus BX45) and measurements were performed using a GENios microplate reader (Cucchiarini et al., 2009).

6.8. Mitochondria functional tests

6.8.1. ATP contents

The supernatants of the hOAC cultures were collected by centrifugation (3 min, 1,500 g), frozen, and the ATP contents in the supernatants were detected using the ATP Determination Kit (Zhong, Long, et al., 2022) with normalization to the cell numbers. Measurements were performed using a GENios microplate reader (Cucchiarini et al., 2009).

6.8.2. Mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\Psi M$) the hOAC cultures was monitored using the JC-1 fluorescent probe (Perelman et al., 2012). The samples were examined under fluorescent microscopy (Olympus CK-2) (Rey-Rico et al., 2015).

6.8.3. ROS levels

The intracellular ROS levels the hOAC cultures were detected using the Fluorometric Intracellular ROS Kit (Yao, Long, Zhao, Zhong, & Li, 2020). Measurements were performed using a GENios microplate reader (Cucchiarini et al., 2009).

6.9. Histological and immunohistochemical analyses

The cultures were harvested and fixed in 4% formalin and stained with hematoxylin and eosin (H&E) (cellularity) and with safranin O (matrix proteoglycans) (Yao et al., 2020). Expression of IGF-I, type-II, -I, and -X collagen and of SOX9 was detected by immunocytochemistry using specific primary antibodies, biotinylated secondary antibodies, and the ABC method with DAB as the chromogen (Cucchiarini et al., 2009). The samples were examined under light microscopy (Olympus BX45). To control for secondary immunoglobulins, samples were processed with omission of the primary antibody.

6.10. Histomorphometry

The % of RFP-stained cells (number of RFP^+ cells relative to the total numbers of cells on immunocytochemically stained cultures), the indices of apoptosis (based on the results generated using the TUNEL kit), of safranin O staining intensity, and of IGF-I, type-I, -II, and -X collagen, and SOX9 immunostaining intensity (ratio of positively stained area to the total area evaluated on stained and immunocytochemically stained cultures), the $\Delta\Psi M$ (number of JC-1 aggregates to the number of monomers), the % of cell viability (based on the results generated using the Cell Counting Kit-8), the cell densities (cell number/mm^2 on H&E-stained sections) were measured at four randomized sites using Image J. (National Institutes of Health, USA) (Perelman et al., 2012; Rey-Rico et al., 2018; A. Weimer et al., 2012; Zhong, Long, et al., 2022).

6.11. 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 Set treatment (J. K. Venkatesan et al., 2018). RNA was eluted in 30 µl RNase-free water and reverse transcription was carried out with 8 µl eluate using the 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (J. K. Venkatesan et al., 2018). An aliquot of the cDNA product (2 µl) was amplified via real-time RT-PCR using the Brilliant SYBR Green QPCR Master Mix (J. K. Venkatesan et al., 2012) on an Mx3000P QPCR operator system as follows: initial incubation (95ºC, 10 min), amplification for 55 cycles (denaturation at 95ºC, 30 s; annealing at 55ºC, 1 min; extension at 72ºC, 30 s), denaturation (95ºC, 1 min), and final incubation (55ºC, 30 s) (J. K. Venkatesan et al., 2012). The primers used were dynamin-related protein 1 (*Drp-1*) (mitochondria fission) (forward 5'-TCAACCTCCGCGTCTACTC-3'; reverse 5'-GATCTGGAACTCGATGTCGGG-3'), mitofusin 1 (*Mfn-1*) (mitochondria fusion) (forward 5'-GAGGTGCTATCTCGGAGACAC-3'; reverse 5'-GCCAATCCCACTAGGGAGAAC-3'), tumor necrosis factor alpha (*TNF-*α) (inflammation) (forward 5'-AGAACCCCCTGGAGATAACC-3'; reverse 5'-AAGTGCAGCAGGCAGAAGAG-3'), *sox9* (cartilage-specific transcription factor) (forward 5'-ACACACAGCTCACTCGACCTTG-3'; reverse 5'-GGGAATTCTGGTTGGTCCTCT-3'), *COL2A1* (type-II collagen) (extracellular matrix marker) (forward 5'-GGACTTTTCTCCCCTCTCT-3'; reverse 5'-

GACCCGAAGGTCTTACAGGA-3'), interleukin 1 beta (*IL-1β*) (inflammation) (forward 5'-CCGTGCCTACGAACATGTC-3'; reverse 5'-CACACAGAAGCTCATCGGAG-3'), and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (forward 5'-GAAGGTGAAGGTCGGAGTC-3'; reverse 5'-GAAGATGGTGATGGGATTTC-3') (all 150 nM final concentration) (J. K. Venkatesan et al., 2012). Control conditions included reactions using water and nonreverse-transcribed mRNA. 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 and values were normalized to *GAPDH* expression using the $2^{-\Delta\Delta Ct}$ method (J. K. Venkatesan et al., 2012).

6.12. Statistical analysis

Each test and condition were performed in quintuplicate in four independent experiments using all the patients' samples. Data are expressed as mean ± standard deviation (SD). Statistical analysis and graphing were performed using GraphPad Prism 8. One-way analysis of variance with Tukey's LSD or Games-Howell *post hoc* tests was applied to evaluate differences between groups. *P* values ≤ 0.05 were considered statistically significant.

7. RESULTS

7.1. Implantation of regenerative mitochondria/rAAV platforms as an innovative osteoarthritis treatment

7.1.1. Efficacy of rAAV treatment in isolated human osteoarthritic chondrocyte mitochondria

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

Successful rAAV treatment in isolated hOAC mitochondria was achieved as noted by strong detection of a live fluorescent signal when applying a Cy3-labeled rAAV-*lacZ* vector ("Cy3-rAAV-*lacZ*") after 24 and 48 h relative to the control conditions (10% sucrose: "no vector"; unlabeled rAAV-*lacZ* vector: "rAAV-*lacZ*") where no such signal was detected (**Figure 10**).

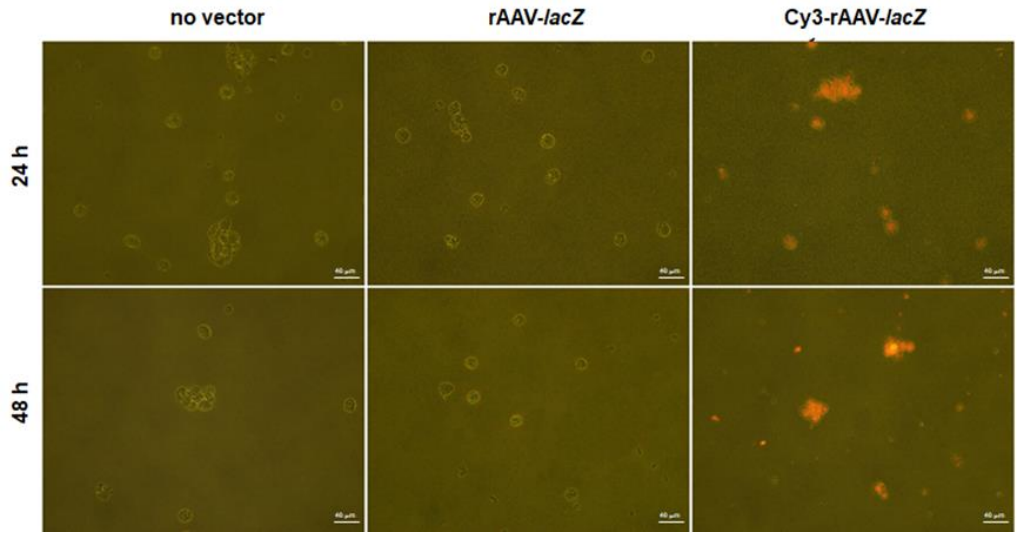


Figure 10. Efficacy of rAAV treatment in isolated human osteoarthritic chondrocyte mitochondria. The analyses were performed by detection of live fluorescence as described in the Methods (magnification x40; overlay; scale bars: 40 μm; all representative data).

7.1.2. Efficacy of mitochondria/rAAV platform implantation in human osteoarthritic chondrocytes

The feasibility of implanting mitochondria/rAAV platforms in hOACs was tested using reporter (rAAV-RFP, rAAV-*lacZ*) and candidate (rAAV-hIGF-I) vectors *versus* respective control conditions described below.

Successful implantation of reporter mitochondria/rAAV platforms in hOACs was achieved as noted by strong detection of a live fluorescent signal when applying rAAV-RFP-treated mitochondria ("mitochondria/rAAV-RFP") relative to the control condition (mitochondria without vector: "mitochondria/no vector") (**Figure 11A**), with significant 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. an ~ 38- and ~ 40-fold difference, respectively, $P \leq 0.0001$) (**Figure 11B**). Successful implantation of candidate mitochondria/rAAV platforms in hOACs was also achieved as noted by significant IGF-I expression levels monitored by immunodetection (**Figures 11C and 11D**) and ELISA (**Figure 11E**) when applying rAAV-hIGF-I-treated mitochondria ("mitochondria/rAAV-hIGF-I") relative to the control conditions (absence of mitochondria implantation: "no mitochondria"; mitochondria without vector: "mitochondria/no vector"; rAAV-*lacZ*-treated mitochondria: "mitochondria/rAAV-*lacZ*") (up to 2.2- and 8.4-fold difference after 24 h by immunocytochemical analysis and by ELISA, respectively, $P \leq 0.0001$) (**Figures 11C-11E**).

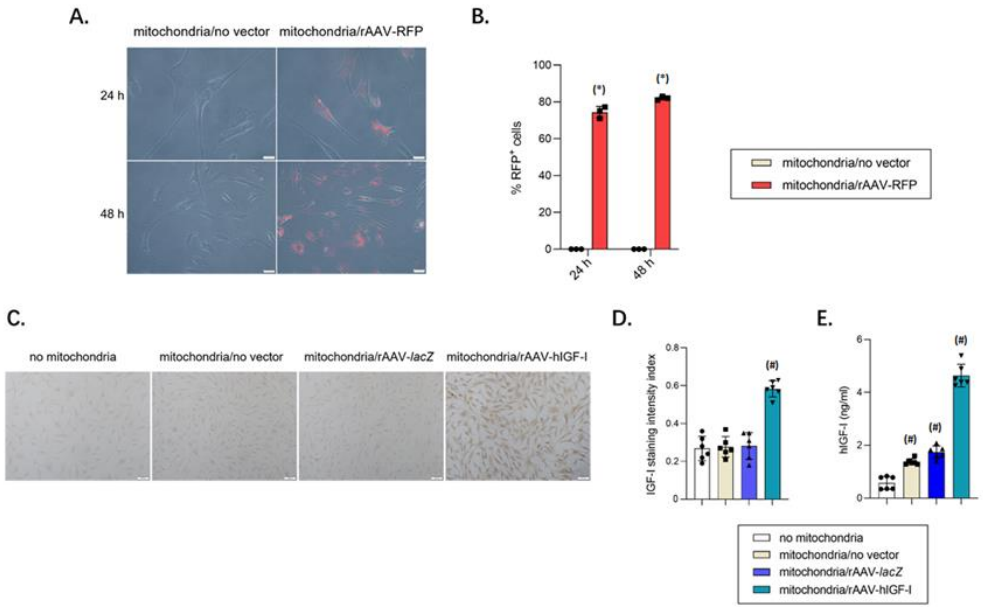


Figure 11. Efficacy of mitochondria/rAAV platform implantation in human osteoarthritic chondrocytes. The analyses were performed by (**A**) detection of live fluorescence (magnification x20 with overlay) with (**B**) histomorphometry, (**C**) IGF-I immunodetection (magnification x10) with (**D**) histomorphometry, and (**E**) IGF-I ELISA as described in the Methods (24 h after treatment; scale bars: 100 μm; all representative data). Data are given as means ± SD. Statistically significant relative to *the "mitochondria/no vector" treatment and to #the "no mitochondria" treatment.

7.1.3. Safety of mitochondria/rAAV platform implantation in human osteoarthritic chondrocytes

The safety of implanting mitochondria/rAAV platforms in hOACs was tested using a reporter (rAAV-RFP) vector *versus* control conditions (absence of mitochondria implantation, mitochondria without vector).

Safe, protective (proliferative) implantation of mitochondria/rAAV platforms in hOACs was achieved as noted by significantly increased levels of cell viability when applying rAAV-RFP-treated mitochondria ("mitochondria/rAAV-RFP") relative to the control condition in the absence of mitochondria implantation ("no mitochondria") (1.8- and 2-fold difference after 24 and 48 h, respectively, $P \leq 0.0001$) (**Figure 12A**). The presence of rAAV in the mitochondria ("mitochondria/rAAV-RFP") had no detrimental effects on the

cells relative to the control condition when mitochondria were provided without vector ("mitochondria/no vector") ($P \geq 0.986$) (**Figure 12A**). Safe, protective (anti-apoptotic) implantation of mitochondria/rAAV platforms in hOACs was also achieved as noted by significantly decreased levels of apoptosis when applying rAAV-RFP-treated mitochondria ("mitochondria/rAAV-RFP") relative to the control conditions (absence of mitochondria implantation: "no mitochondria"; mitochondria without vector: "mitochondria/no vector") (3- and 1.8-fold difference after 24 h, respectively, $P \leq 0.041$) (**Figures 12B and 12C**).

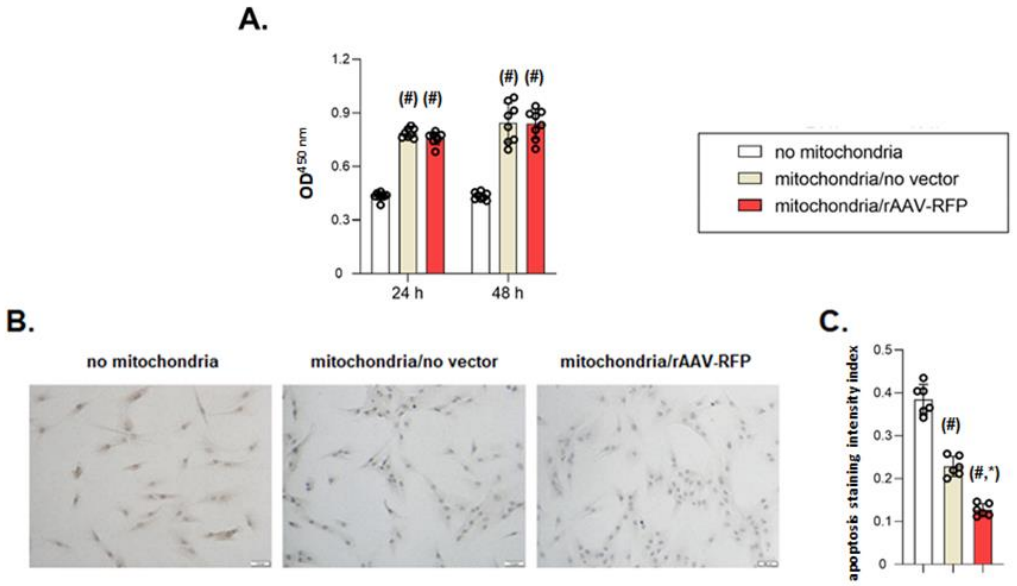


Figure 12. Safety of mitochondria/rAAV platform implantation in human osteoarthritic chondrocytes. The analyses were performed by (**A**) detection of cell viability ($OD^{450\ nm}$ using the WST-1 assay) and (**B**) detection of apoptosis (TUNEL assay; magnification x20; scale bars: 100 μm; all representative data) with (**C**) histomorphometry as described in the Methods (24 h after treatment). Data are given as means ± SD. Statistically significant relative to #the "no mitochondria" treatment and to *the "mitochondria/no vector" treatment.

7.1.4. Effects of mitochondria/rAAV-hIGF-I platform implantation on the biological activities of human osteoarthritic chondrocytes

The effects of implanting mitochondria/rAAV platforms on the biological activities of hOACs were tested using the candidate rAAV-hIGF-I vector *versus* control conditions (absence of mitochondria implantation, mitochondria without vector, rAAV-*lacZ*-treated mitochondria).

Implantation of rAAV-hIGF-I-treated mitochondria in hOACs ("mitochondria/rAAV-hIGF-I") significantly increased the levels of cell viability and the DNA contents relative to the control conditions (absence of mitochondria implantation: "no mitochondria"; mitochondria without vector: "mitochondria/no vector"; rAAV-*lacZ*-treated mitochondria: "mitochondria/rAAV-*lacZ*") (up to 1.9- and 1.8-fold difference after 24 h in cell viability and DNA contents, respectively, $P \leq 0.0001$) (**Figures 13A and 13B**) while significantly decreasing the levels of cell apoptosis (up to 4.2-fold difference after 24 h, $P \leq 0.0001$) (**Figures 13C and 13D**). It further significantly increased the deposition and contents of desirable matrix proteoglycans and type-II collagen relative to the control conditions ("no mitochondria", "mitochondria/no vector", "mitochondria/rAAV-*lacZ*") (up to 2-, 9.5-, and 1.7-fold difference after 24 h in the proteoglycan deposition and contents and in type-II collagen deposition, respectively, $P \leq 0.0001$) (**Figures 13E-13I**). It finally significantly decreased the undesirable deposition of osteogenic type-I collagen and hypertrophic type-X collagen relative to the control conditions ("no mitochondria", "mitochondria/no vector", "mitochondria/rAAV-*lacZ*") (up to 2.5-fold difference after 24 h in type-I and -X collagen deposition, $P \leq 0.0011$) (**Figures 13H, 13J, and 13K**). These effects were accompanied by significantly increased levels of cartilage-specific SOX9 expression relative to the control conditions ("no mitochondria", "mitochondria/no vector", "mitochondria/rAAV-*lacZ*") (up to 3.7-fold difference after 24 h, $P \leq 0.0001$) (**Figure 13H and 13L**).

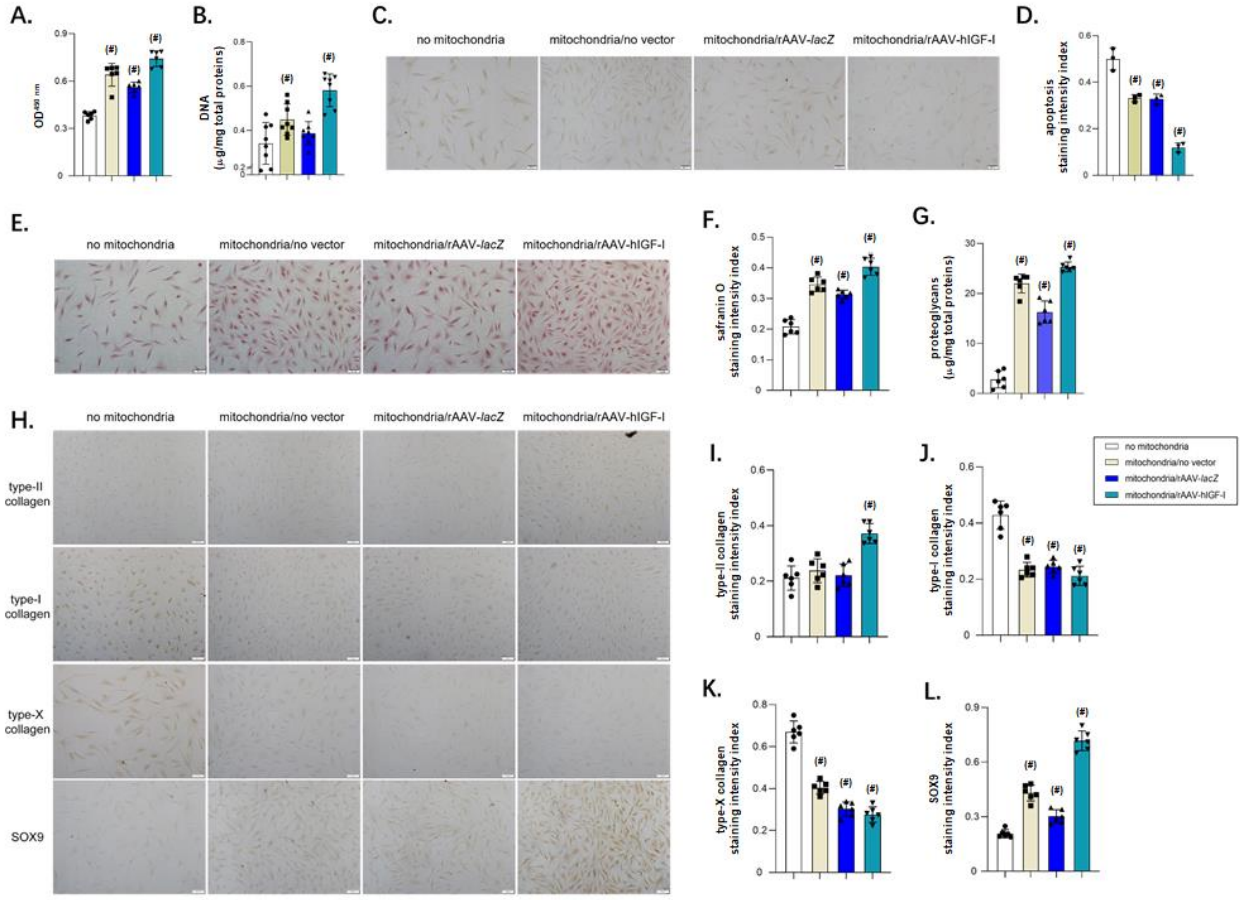


Figure 13. Effects of mitochondria/rAAV-hIGF-I platform implantation on the biological activities of human osteoarthritic chondrocytes. The analyses were performed by (**A**) detection of cell viability ($OD^{450\ nm}$ using the WST-1 assay), (**B**) detection of the DNA contents (Hoechst 33258 assay), (**C**) detection of apoptosis (TUNEL assay; magnification x20) with (**D**) histomorphometry, (**E-G**) detection of matrix proteoglycans (**E**: safranin O staining, magnification x20 with **F**: histomorphometry; **G**: DMMB assay), and (**H-L**) immunodetection (magnification x10;) with (**I-L**) histomorphometry as described in the Methods (24 h after treatment; scale bars: 100 μm; all representative data). Data are given as means ± SD. Statistically significant relative to #the "no mitochondria" treatment.

7.1.5. Effects of mitochondria/rAAV-hIGF-I platform implantation on the mitochondria functions of human osteoarthritic chondrocytes

The effects of implanting mitochondria/rAAV platforms on the mitochondria functions of hOACs were tested using the candidate rAAV-hIGF-I vector *versus* control conditions (absence of mitochondria implantation, mitochondria without vector, rAAV-*lacZ*-treated mitochondria).

Implantation of rAAV-hIGF-I-treated mitochondria in hOACs ("mitochondria/rAAV-hIGF-I") significantly increased the ATP contents and the mitochondrial membrane potential ($\Delta\Psi M$) relative to the control conditions (absence of mitochondria implantation: "no mitochondria"; mitochondria without vector: "mitochondria/no vector"; rAAV-*lacZ*-treated mitochondria: "mitochondria/rAAV-*lacZ*") (up to 1.6- and 5.7-fold difference after 24 h in the ATP contents and $\Delta\Psi M$, respectively, $P \leq 0.0006$) (**Figures 14A-14C**) while significantly decreasing the ROS levels (up to 3.2-fold difference after 24 h, $P \leq 0.0001$) (**Figure 14D**). These effects were accompanied by significantly decreased *Drp-1* expression levels (mitochondria fission), significantly increased *Mfn-1* expression levels (mitochondria fusion), and significantly decreased proinflammatory *TNF-*α expression levels relative to the control conditions ("no mitochondria", "mitochondria/no vector", "mitochondria/rAAV-*lacZ*") (up to 2.1-, 2-, and 4.5-fold difference after 24 h in *Drp-1*, *Mfn-1*, and *TNF-*α expression, respectively, $P \leq 0.0001$) (**Figures 14E and 14F**).

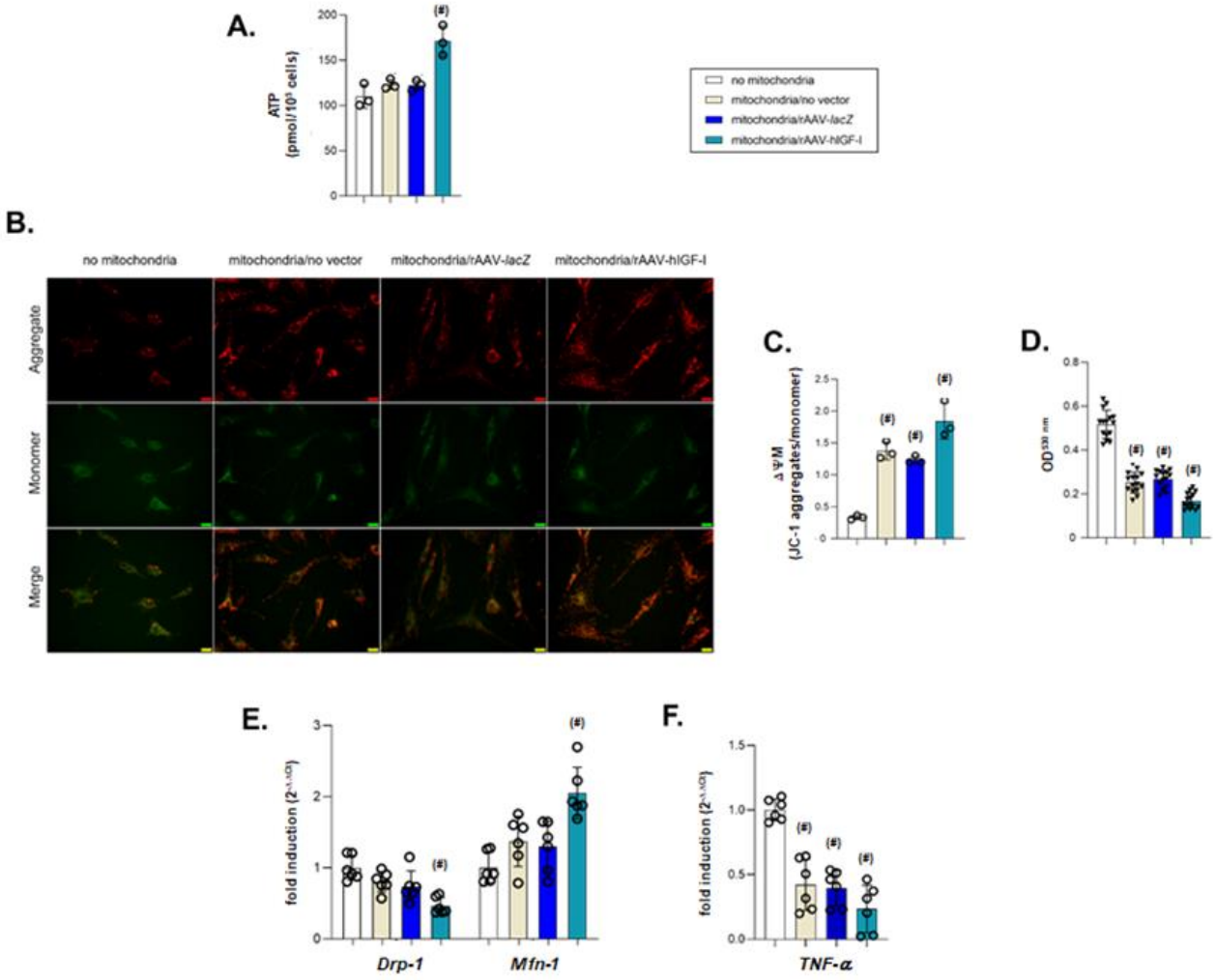


Figure 14. Effects of mitochondria/rAAV-hIGF-I platform implantation on the mitochondria functions of human osteoarthritic chondrocytes. The analyses were performed by (**A**) detection of the ATP contents (ATP Determination kit), (**B,C**) detection of the mitochondrial membrane potential ($\Delta\Psi M$) (**B**: JC-1 fluorescent probe, magnification x20; scale bars: 100 μm; all representative data with **C**: histomorphometry), (**D**) detection of the ROS levels ($OD^{530\ nm}$ using the Fluorometric Intracellular ROS kit), and (**E,F**) real-time RT-PCR (relative to the "no mitochondria" treatment) as described in the Methods (24 h after treatment). Data are given as means ± SD. Statistically significant relative to #the "no mitochondria" treatment.

7.1.6. Functional PF127 hydrogel-guided mitochondria/rAAV-hIGF-I platform implantation in human osteoarthritic chondrocytes

The feasibility of using functional PF127 hydrogels to guide the therapeutic implantation of mitochondria/rAAV platforms in hOACs was tested using the candidate rAAV-hIGF-I vector *versus* the absence of mitochondria implantation as the most significant control condition relative to other control treatments tested earlier (**Figures 11-14**).

Successful implantation of PF127 hydrogel-guided candidate mitochondria/rAAV platforms in hOACs was achieved as noted by significant IGF-I expression levels monitored by immunodetection (**Figures 15A and 15B**) and ELISA (**Figure 15C**) when applying PF127 hydrogel-guided rAAV-hIGF-I-treated mitochondria ("mitochondria/rAAV-hIGF-I in PF127") relative to the control condition (absence of mitochondria implantation: "no mitochondria") (4- and 4.9-fold difference after 24 h by immunocytochemical analysis and by ELISA, respectively, $P \leq 0.0001$) (**Figures 15A-15C**).

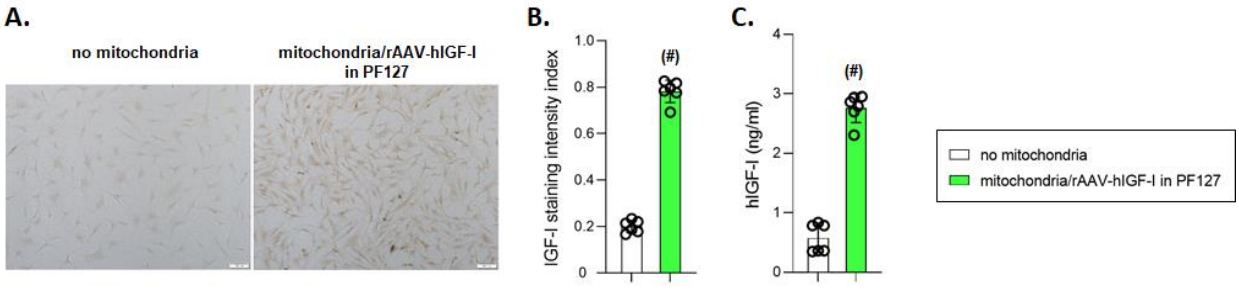


Figure 15. Effective PF127 hydrogel-guided mitochondria/rAAV-hIGF-I platform implantation in human osteoarthritic chondrocytes. The analyses were performed by (**A**) IGF-I immunodetection (magnification x10; scale bars: 100 μm; all representative data) with (**B**) histomorphometry and (**C**) IGF-I ELISA as described in the Methods (24 h after treatment). Data are given as means ± SD. Statistically significant relative to #the "no mitochondria" treatment.

Implantation of PF127 hydrogel-guided rAAV-hIGF-I-treated mitochondria in hOACs ("mitochondria/rAAV-hIGF-I in PF127") significantly increased the levels of cell viability relative to the control condition ("no mitochondria") (2.5-fold difference after 24 h, $P \leq 0.0001$) (**Figure 16A**) while significantly decreasing the levels of cell apoptosis (3.8-fold difference after 24 h, $P \leq 0.0002$) (**Figure 16B, 16C**). It further significantly increased the deposition and contents of desirable matrix proteoglycans and type-II collagen relative to the control condition ("no mitochondria") (1.9-, 3.9-, and 2.2-fold difference after 24 h in the proteoglycan deposition and contents and in type-II collagen deposition, respectively, $P \leq 0.0001$) (**Figure 7D-7H**). It finally significantly decreased the undesirable deposition of osteogenic type-I collagen and hypertrophic type-X collagen relative to the control condition ("no mitochondria") (up to 2.1-fold difference after 24 h in type-I and -X collagen deposition, respectively, $P \leq 0.0001$) (**Figure 16G, 16I, 16J**). These effects 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, $P \leq 0.0013$) (**Figure 16G, 16K**).

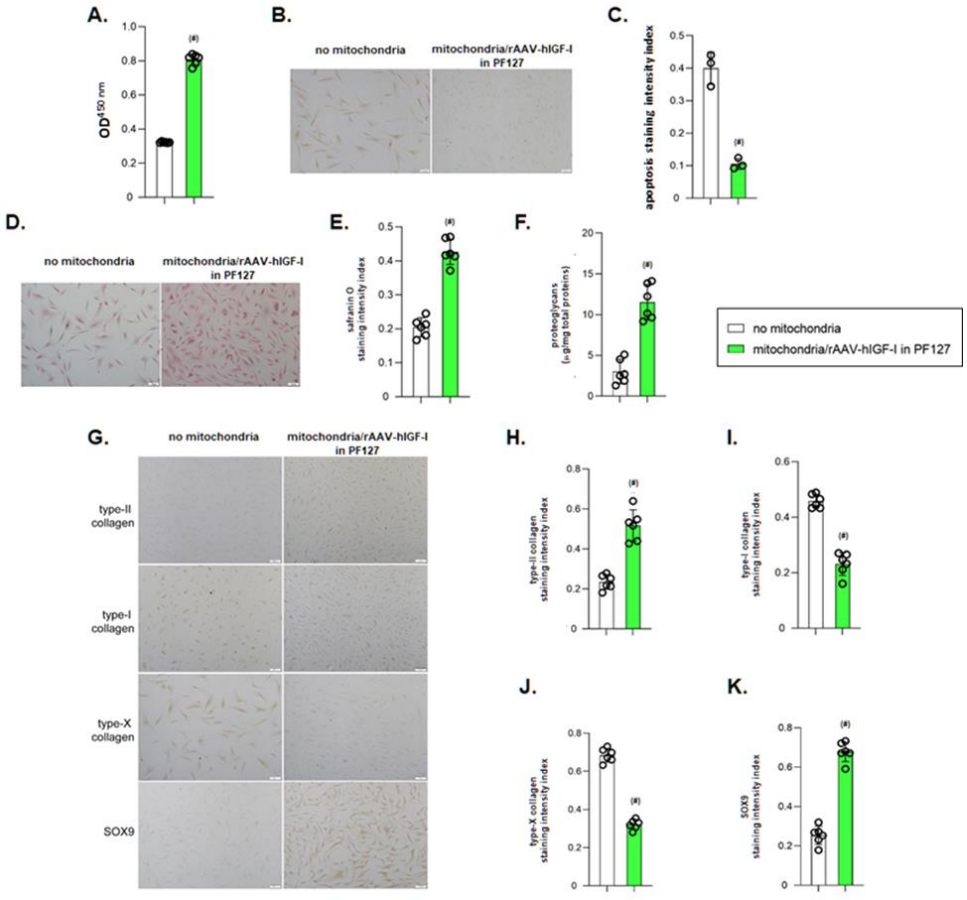


Figure 16. Effects of PF127 hydrogel-guided mitochondria/rAAV-hIGF-I platform implantation on the biological activities of human osteoarthritic chondrocytes. The analyses were performed by (**A**) detection of cell viability ($OD^{450\ nm}$ using the WST-1 assay), (**B**) detection of apoptosis (TUNEL assay; magnification x20) with (**C**) histomorphometry, (**D-F**) detection of matrix proteoglycans (**D**: safranin O staining; magnification x20; **E**: histomorphometry; **F**: DMMB assay), and (**G-K**) immunodetection (magnification x10) with (**H-K**) histomorphometry as described in the Methods (24 h after treatment; scale bars: 100 μm; all representative data). Data are given as means ± SD. Statistically significant relative to #the "no mitochondria" treatment.

Implantation of PF127 hydrogel-guided rAAV-hIGF-I-treated mitochondria in hOACs ("mitochondria/rAAV-hIGF-I in PF127") significantly increased the ATP contents and the $\Delta\Psi M$ relative to the control condition ("no mitochondria") (1.5- and 5.9-fold difference after 24 h in the ATP contents and $\Delta\Psi M$, respectively, $P \leq 0.050$) (**Figure 17A-17C**) while significantly decreasing the ROS levels (2.2-fold difference after 24 h, $\underline{P} \leq 0.0001$) (**Figure 17D**). These effects were accompanied by significantly decreased *Drp-1* expression levels, significantly increased *Mfn-1* expression levels, and significantly decreased *TNF-*α expression levels relative to the control condition ("no mitochondria") (2-, 1.1-, and 4.1-fold difference after 24 h in *Drp-1*, *Mfn-1*, and *TNF-*α expression, respectively, $P \leq 0.047$) (**Figure 17E, 17F**).

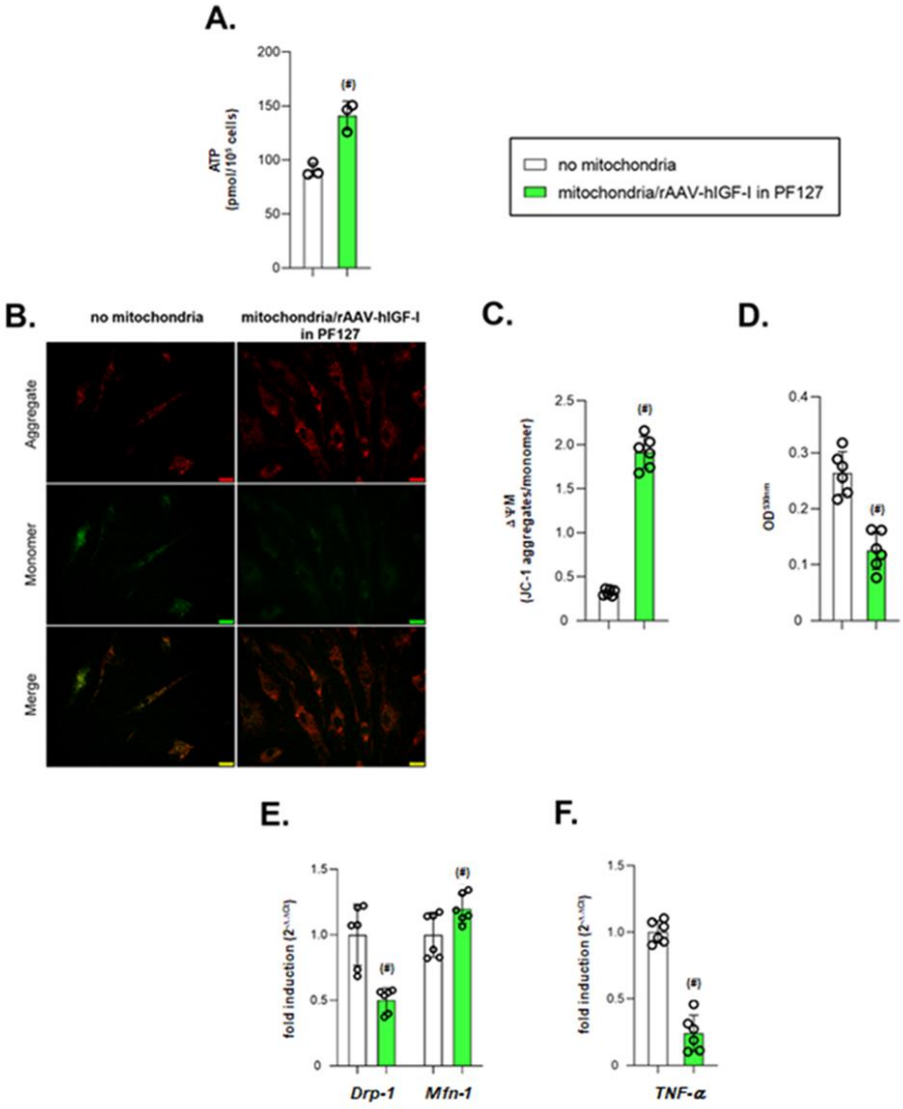


Figure 17. Effects of PF127 hydrogel-guided mitochondria/rAAV-hIGF-I platform implantation on the mitochondria functions of human osteoarthritic chondrocytes. The analyses were performed by (**A**) detection of the ATP contents (ATP Determination kit), (**B,C**) detection of the ΔΨM (B: JC-1 fluorescent probe, magnification x20; scale bars: 100 μm; all representative data with **C**: histomorphometry), (**D**) detection of the ROS levels ($OD^{530\ nm}$ using the Fluorometric Intracellular ROS kit), and (**E,F**) real-time RT-PCR (relative to the "no mitochondria" treatment) as described in the Methods (24 h after tratment). Data are given as means ± SD. Statistically significant relative to #the "no mitochondria" treatment.

7.2. Controlled delivery of rAAV vectors with anti-inflammatory drugs as an innovative osteoarthritis treatment

7.2.1. Efficacy of rAAV-mediated sox9 *overexpression in human osteoarthritic chondrocytes via type-I collagen hydrogel-guided rAAV vector delivery*

The type-I collagen hydrogel was first manipulated to test its ability to efficiently deliver the rAAV-FLAG-h*sox9* candidate vector in hOACs using a Transwell culture system relative to various control conditions.

Significantly increased SOX9 production was noted in the cells after 48 h via type-I collagen hydrogel-guided rAAV-FLAG-h*sox9* gene transfer in the absence or presence of rIL-1Ra ("coII/*sox9*" and "coII/*sox9*, rIL-1Ra" conditions, respectively) as noted by immunocytochemistry relative to the control conditions ("-/-", i.e. lack of vector and of hydrogel; "rIL-1Ra", i.e. sole presence of rIL-1Ra; "coII/*lacZ*", i.e. type-I collagen hydrogel-guided rAAV-*lacZ* without rIL-1Ra; "coII/*lacZ*, rIL-1Ra", i.e. type-I collagen hydrogel-guided rAAV-*lacZ* with rIL-1Ra) (up to 24.3-fold difference, $P \leq 0.05$), reaching levels similar to those observed upon direct, hydrogel-free rAAV-FLAG-h*sox9* gene transfer ("-/*sox9*" condition) (**Figure 18**).

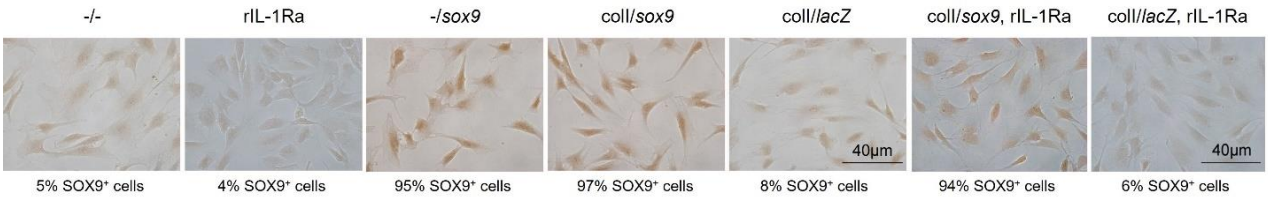


Figure 18. Detection of transgene (*sox9*) overexpression in human osteoarthritic chondrocytes via type-I collagen hydrogel-guided rAAV gene transfer. The analyses were performed by immunodetection with histomorphometry as described in the Methods (48 h after treatment; magnification x20; scale bars: 40 µm; all representative data).

7.2.2. Effects of rAAV-mediated sox9 *overexpression via type-I collagen hydrogel-guided rAAV vector delivery on the viability of human osteoarthritic chondrocytes*

The potential effects of type-I collagen hydrogel-guided delivery of the rAAV-FLAG-h*sox9* candidate vector on the viability of hOACs were next examined in the Transwell culture system relative to various control conditions. Significantly increased indices of hOAC viability were noted in the cells after 48 h in the "coII/*sox9*, rIL-1ra" condition as noted with the Cell Counting Kit-8 compared with all other conditions ("-/-", "rIL-1Ra", "-/*sox9*", "coII/*sox9*", and "coII/*lacZ*, rIL-1Ra") (up to 2.9-fold difference, $P \leq 0.05$) (**Figure 19A**). These results were corroborated by histological and histomorphometric findings via H&E staining (up to 1.9-fold difference, $P \leq 0.05$) (**Figure 19B**).

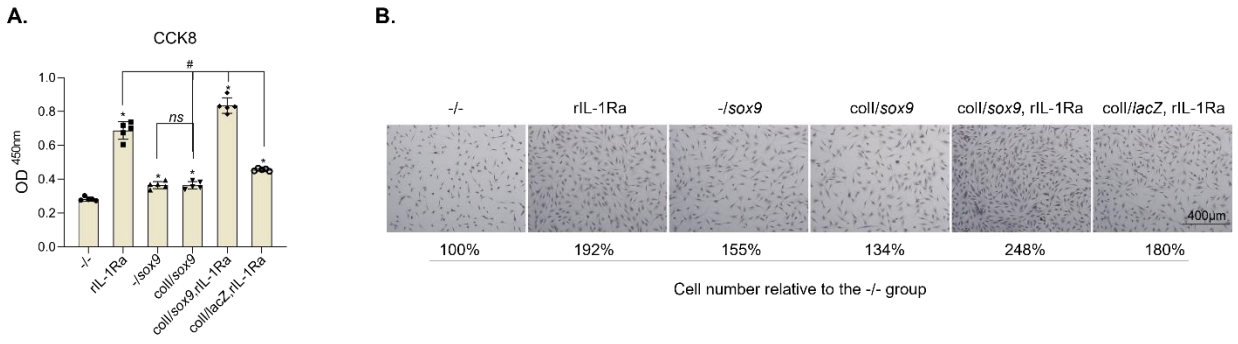


Figure 19. Effects of transgene (*sox9*) overexpression via type-I collagen hydrogel-guided rAAV gene transfer on the viability of human osteoarthritic chondrocytes. The analyses were performed by (**A**) detection of cell viability ($OD^{450\ nm}$ using the Cell Counting Kit-8) and (**B**) H&E staining with histomorphometry as described in the Methods (48 h after treatment; magnification x4; scale bars: 400 µm; all representative data). Data are given as means ± SD. Statistically significant relative to *the "-/-" treatment and to #the "coII/*sox9*, rIL-1Ra" treatment (*ns*: not significant).

7.2.3. Effects of rAAV-mediated sox9 *overexpression via type-I collagen hydrogel-guided rAAV vector delivery on the expression of cartilage-specific markers in human osteoarthritic chondrocytes*

The potential effects of type-I collagen hydrogel-guided delivery of the rAAV-FLAG-h*sox9* candidate vector on the expression of *sox9* and *COL2A1* and on the deposition of

proteoglycans in hOACs were next investigated in the Transwell culture system relative to various control conditions.

Significantly increased *sox9* and *COL2A1* expression was noted in the cells after 48 h in the "coll/*sox9*, rIL-1ra" condition as noted by real-time RT-PCR compared with all other conditions ("-/-", "rIL-1Ra", "-/*sox9*", "coll/*sox9*", and "coll/*lacZ*, rIL-1Ra") (except for *COL2A1* relative to "coll/*sox9*") (up to 4- and 2.8-fold difference, respectively, $P \leq 0.05$) (**Figure 20A**). Significant deposition of proteoglycans was noted in the cells after 48 h in the "coll/*sox9*, rIL-1ra" condition as noted by histological safranin O staining with histomorphometry compared with all other conditions ("-/-", "rIL-1Ra", "-/*sox9*", "coll/*sox9*", and "coll/*lacZ*, rIL-1Ra") (up to 2.2-fold difference, $P \leq 0.05$) (**Figure 20B**).

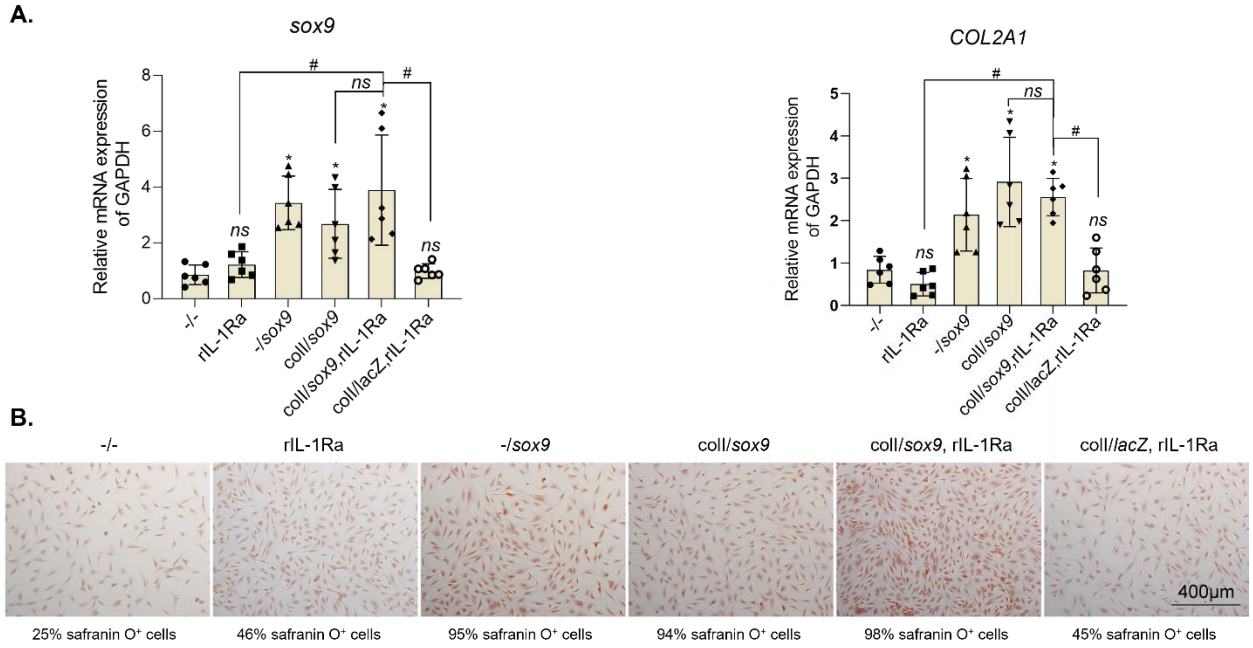


Figure 20. Effects of transgene (*sox9*) overexpression via type-I collagen hydrogel-guided rAAV gene transfer on the expression of cartilage-specific markers in human osteoarthritic chondrocytes. The analyses were performed by (**A**) real-time RT-PCR (relative to the "-/-" treatment) and (**B**) safranin O staining with histomorphometry as described in the Methods (48 h after treatment; magnification x10; scale bars: 400 µm; all representative data). Data are given as means ± SD. Statistically significant relative to *the "-/-" treatment and to #the "coll/*sox9*, rIL-1Ra" treatment (*ns*: not significant).

7.2.4. Effects of rAAV-mediated sox9 *overexpression via type-I collagen hydrogel-guided rAAV vector delivery on the expression of inflammatory mediators in human osteoarthritic chondrocytes*

The potential effects of type-I collagen hydrogel-guided delivery of the rAAV-FLAG-h*sox9* candidate vector on the expression of *IL-1β* and *TNF-α* in hOACs were finally investigated in the Transwell culture system relative to various control conditions.

Significantly reduced *IL-1β* and *TNF-α* expression was noted in the cells in the "coll/*sox9*, rIL-1ra" condition after 48 h as noted by real-time RT-PCR compared with all other conditions ("-/-", "rIL-1Ra", "-/*sox9*", "coll/*sox9*", and "coll/*lacZ*, rIL-1Ra") (except relative to "rIL-1Ra") (up to 2.7- and 2.3-fold difference, respectively, $P \leq 0.05$) (**Figure 21**).

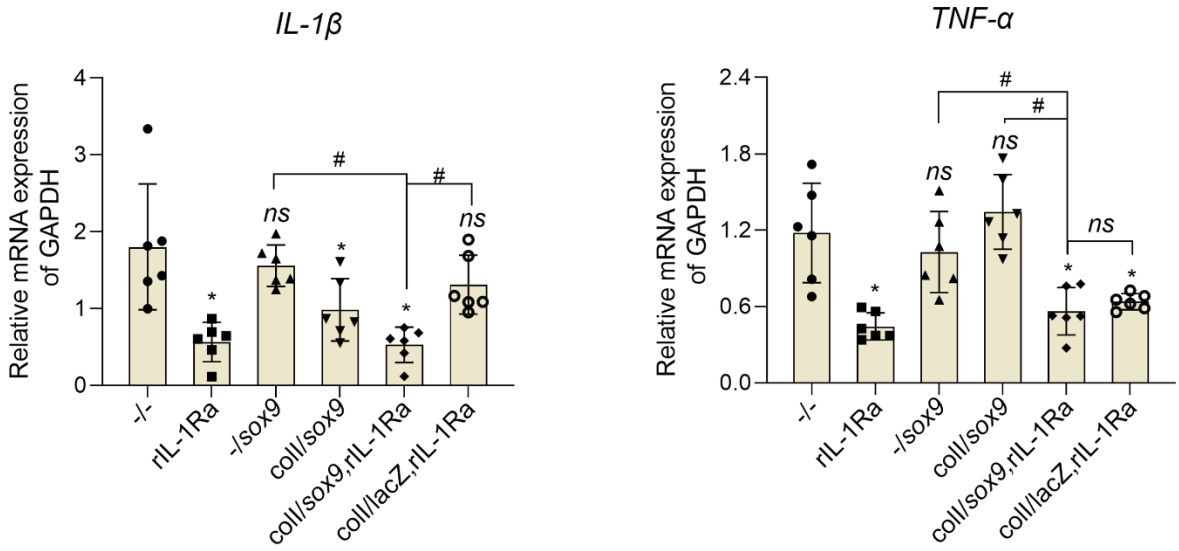


Figure 21. Effects of transgene (*sox9*) overexpression via type-I collagen hydrogel-guided rAAV gene transfer on the expression of inflammatory mediators in human osteoarthritic chondrocytes. The analyses were performed by real-time RT-PCR (relative to the "-/-" treatment) as described in the Methods (48 h after treatment). Data are given as means ± SD. Statistically significant relative to *the "-/-" treatment and to #the "coll/*sox9*, rIL-1Ra" treatment (*ns*: not significant).

8. DISCUSSION

The goal of the present study was to establish and test innovative treatments for human osteoarthritis based on (1) the autologous implantation of mitochondria as platforms of delivery of clinically adapted rAAV vectors and (2) the hydrogel-based controlled delivery of rAAV vectors with anti-inflammatory drugs *in vitro*. The analyses were performed in primary cultures of human osteoarthritic chondrocytes (hOACs) which represent the ultimate targets for therapy in this highly prevalent disease for which there is no causative cure to date.

8.1. Implantation of regenerative mitochondria/rAAV platforms as an innovative osteoarthritis treatment

The concept of employing mitochondria, the cellular powerhouses, as platforms for implantation in target cells to improve their functions and potency gained increasing interest, especially in the field of OA therapy (Zhong, Madry, et al., 2022), further taking advantage of the compatibility of these central organelles with the use of rAAV vectors (Zhong, Venkatesan, Madry, & Cucchiarini, 2022). The implantation of mitochondria/rAAV platforms may offer a protective environment for the gene vector, shielding it from degradation and immune responses. Such protection may be crucial for the maintenance of the vector integrity and bioactivity and to contain off-target effects, reducing the risk of potential (deleterious) side-effects associated with the treatment (Zhong, Madry, et al., 2022; Zhong, Venkatesan, Madry, & Cucchiarini, 2022).

OA is a complex disease where the articular chondrocytes undergo pathological changes in their mitochondrial functions (Blanco & Rego-Perez, 2018; Gavriilidis, Miwa, von Zglinicki, Taylor, & Young, 2013). To comprehensively address such issues in OA, it is critical to manage these alterations by restoring the mitochondrial functions at a cellular level. This was envisaged here by testing the autologous implantation of mitochondria as platforms to deliver reparative rAAV-based IGF-I gene vectors (A. Weimer et al., 2012).

In the present study, mitochondria freshly isolated from hOACs were employed to create platforms for the delivery of an rAAV-hIGF-I vector, further using a biocompatible PF127 hydrogel for transfer in hOACs as the ultimate cell targets for therapy in OA. This approach allowed for the successful overexpression of the candidate IGF-I gene compared with the control conditions tested here, leading to enhanced levels of cell viability, extracellular matrix deposition, and mitochondrial functions. Based on this proof-of-principle, work is now ongoing to test whether these platforms display further therapeutic benefits in hOACs over extended periods of time, also in the presence of anti-AAV capsid antibodies to verify protection of the platforms against neutralization. It will be also of critical importance to evaluate the approach in human OA articular cartilage explants (Rey-Rico et al., 2018; J. K. Venkatesan et al., 2012; J. K. Venkatesan et al., 2017) (also in the presence of anti-AAV capsid antibodies) and in clinically relevant *in vivo* models of OA (Maihöfer et al., 2021; Jagadeesh K Venkatesan et al., 2020) where neutralization and inflammation may naturally occur.

In conclusion, the implantation of mitochondria as platforms to deliver therapeutic rAAV vectors (IGF-I or other therapeutic candidates such as *sox9*, etc.) that may also be formulated in a biomaterial in the future (Gerwin et al., 2006) is a promising tool to tackle the pathological changes in the mitochondrial functions in OA and to offer hopes and improved quality of life for those affected by this serious, prevalent condition.

8.2. Controlled delivery of rAAV vectors with anti-inflammatory drugs as an innovative osteoarthritis treatment

The concept of slow, sustained release of gene vectors mediated by biomaterials also gained increasing interest in the field of OA therapy (Cucchiarini & Madry, 2019; Henning Madry et al., 2020; R. Yang et al., 2020), capitalizing on the controlled release of therapeutic agents to achieve higher efficiency and prolonged therapeutic effects (Gerwin, Hops, & Lucke, 2006). The slow release of gene vectors may ensure that the therapeutic gene remains present in the target tissue for a prolonged duration, increasing the likelihood of gene transfer and potentially leading to more sustained therapeutic effects of the candidate gene product (Maihöfer et al., 2021; Jagadeesh K Venkatesan, Falentin-Daudré, Leroux, Migonney, & Cucchiarini, 2020). In addition, biomaterials may also

provide a protective environment for the gene vector, shielding it from degradation and immune responses. Such protection may also be crucial for the maintenance of the vector integrity and bioactivity (Eoh & Gu, 2019). Finally, the controlled release of gene vectors may also contains off-target effects, reducing the risk of potential (deleterious) side-effects associated with the treatment (Dubey & Mostafavi, 2023).

OA is a complex disease where the joints undergo inflammation and cartilage degeneration (Goldring & Otero, 2011; Sokolove & Lepus, 2013). To comprehensively address such issues in OA, it is critical to manage both processes and consider a treatment approach that concurrently tackles them by reducing inflammation while promoting extracellular matrix repair. This was envisaged here by testing a combination of anti-inflammatory rIL-1Ra (Dinarello, Simon, & van der Meer, 2012; Jotanovic, Mihelic, Sestan, & Dembic, 2012) and reparative rAAV-based *sox9* gene transfer (Bi et al., 1999).

In the present study, a biocompatible type-I collagen hydrogel was employed as a biomaterial to guide rAAV-mediated *sox9* gene transfer with rIL1-Ra in hOACs as the ultimate cell targets for therapy in OA. This approach allowed for the successful overexpression of the candidate *sox9* gene compared with the control conditions tested here, leading to enhanced levels of cell viability and extracellular matrix deposition in the presence of the anti-inflammatoy rIL1-Ra that reduced the levels of inflammation in the cells, with an overall higher potency than when using direct (biomaterial-free) rAAV vector gene transfer. Based on this proof-of-principle, work is now ongoing to test whether this system displays further therapeutic benefits in hOACs over extended periods of time, also in the presence of anti-AAV capsid antibodies to verify protection of the hydrogel-guided rAAV vector gene transfer against neutralization. It will be also of critical importance to evaluate the approach in human OA articular cartilage explants (Rey-Rico et al., 2018; J. K. Venkatesan et al., 2012; J. K. Venkatesan et al., 2017) (also in the presence of anti-AAV capsid antibodies) and in clinically relevant *in vivo* models of OA (Maihöfer et al., 2021; Jagadeesh K Venkatesan et al., 2020) where neutralization and inflammation may naturally occur. In these future experiments, it will also be of the utmost importance to test the potential superiority of the hydrogel-guided rAAV vector gene transfer over the direct (biomaterial-free) rAAV vector gene transfer.

In conclusion, biomaterial-guided delivery of therapeutic rAAV vectors (*sox9* or other therapeutic candidates such as IGF-I, etc.) with anti-inflammatory drugs (rL1-Ra or other agents such as rTNF-α, etc.) that may also be formulated in a biomaterial in the future (Gerwin et al., 2006) is a promising tool to also tackle the multifaceted nature of OA and to also offer hopes and improved quality of life for those affected by this irreversible, debilitating condition.

8.3. Outlook

This work presented two advanced rAAV-based gene therapy systems for human OA: (1) a mitochondria-assisted rAAV platform therapy and (2) a hydrogel-guided rAAV therapy with an anti-inflammatory drug. The potential risks associated with rAAV, including the pathogenic proteins of the viral capsid, may be effectively addressed through these two strategies compared with platform- or hydrogel-free rAAV vector administration where the viral particles are in direct contact with the host organism. With pivotal roles in biological processes such as inflammation, oxidative damage, and regeneration, mitochondria (and their implantation) may avoid the use of additional drugs but they would likely need to be employed in a patient-matched manner, making their use (thus far) not universal. This is in contrast to the use of the hydrogel system that is devoid of cellular (patient-dependent) elements. One future strategy would be to develop a unique, universal source of mitochondria that could be used in a patient-independent manner. Besides, work based on hydrogels may be expanded to other drugs used already in patients, together with rAAV vectors (*sox9*, but also IGF-I, etc.), that all may be frozen to generate off-the-shelf therapeutics. Overall, the current work provides new avenues of research with high prospect in clinical applications for human OA based on the improvement of the mitochondrial membrane permeability using modified rAAV capsid proteins, on the optimization of mitochondrial membrane receptors to avoid mitochondrial immunogenicity, on the combination of new therapeutic substances to tackle autophagy deprivation, oxidative stress, and/or inflammation, and on the use of other biological materials to provide sustained release therapeutic models in OA chondrocytes (**Figure 22**).

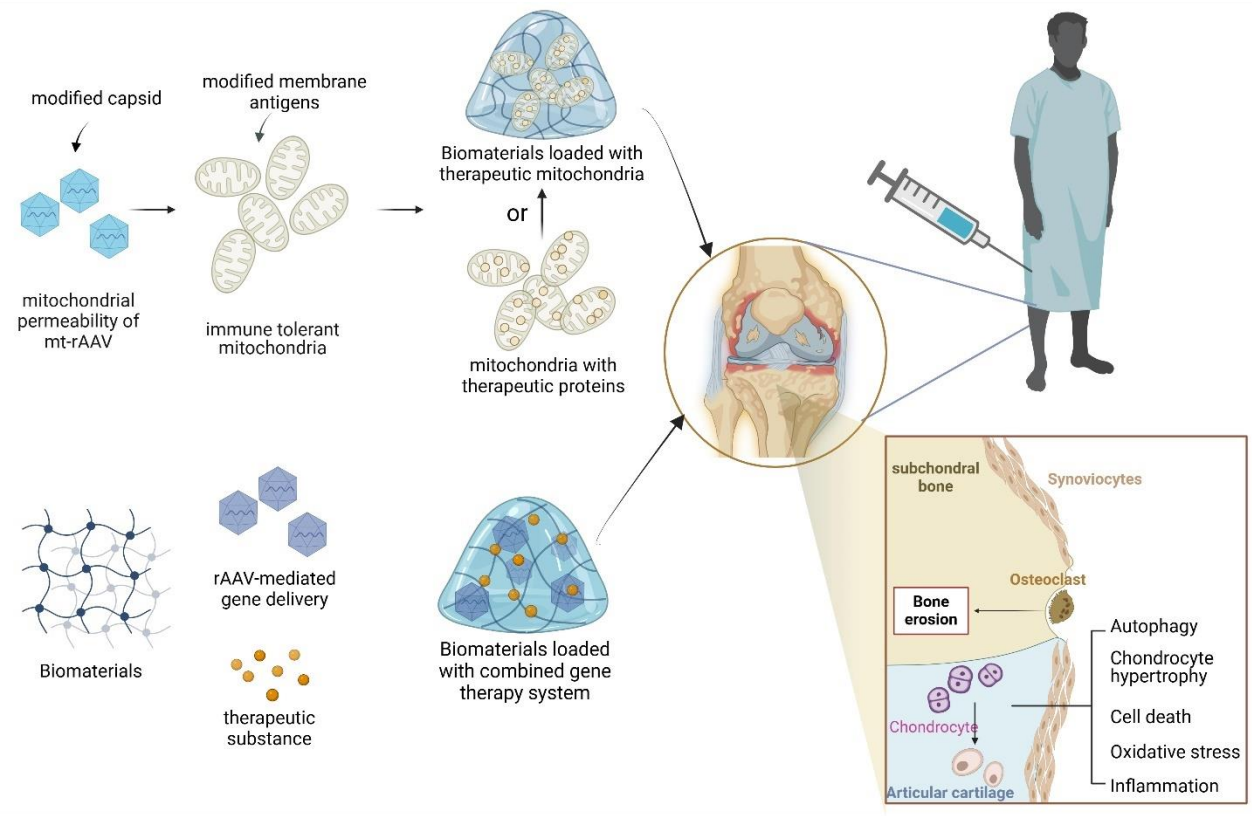


Figure 22. Perspectives of rAAV-mediated gene therapy as future human osteoarthritis treatments (created with BioRender.com).

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10. PUBLICATIONS AND PRESENTATIONS

10.1. Publications

1. **G. Zhong**, J.K. Venkatesan, H. Madry, M. Cucchiarini, Advances in human mitochondria-based therapies, Int J Mol Sci 2022, 24(1):608. Impact factor: 5.6 (2022)
2. **G. Zhong**, H. Madry, M. Cucchiarini, Mitochondrial genome editing to treat human osteoarthritis-A narrative review, Int J Mol Sci 2022, 23(3):1467. Impact factor: 5.6 (2022)
3. **G. Zhong**, W. Liu, J.K. Venkatesan, D. Wang, H. Madry, M. Cucchiarini, Autologous transplantation of mitochondria/rAAV IGF-I platforms in human osteoarthritic articular chondrocytes as a novel therapeutic concept for human osteoarthritis, Mol Ther *under review*.

10.2. Poster presentations

1. **G. Zhong**, J.K. Venkatesan, W. Liu, G. Schmitt G., H. Madry, M. Cucchiarini, Implantation of rAAV-treated mitochondria as an effective and safe protein replacement therapy to target human osteoarthritic articular chondrocytes. ORS 2024 Annual Meeting, February 2-6, 2024, Long Beach, CA, USA.
2. **G. Zhong**, J.K. Venkatesan, W. Liu, G. Schmitt, H. Madry, M. Cucchiarini, A novel mitochondrial-based rAAV-mediated IGF-I gene therapy to improve the human osteoarthritic phenotype. ORS 2024 Annual Meeting, February 2-6, 2024, Long Beach, CA, USA.
3. **G. Zhong**, J.K. Venkatesan, W. Liu, G. Schmitt G., H. Madry, M. Cucchiarini, Effects of combined type-I collagen hydrogel-guided rAAV-mediated *sox9* gene transfer and overexpression with an IL-1Ra on the phenotype of human osteoarthritic articular chondrocytes. 2024 OARSI World Congress on Osteoarthritis, April 18-21, 2024 Vienna, Austria.

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12. CURRICULUM VITAE

The curriculum vitae was removed from the electronic version of the doctoral thesis for reasons of data protection.