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Biomaterial-assisted gene therapy for translational approaches to treat musculoskeletal disorders

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ABSTRACT

Biomaterial-assisted gene therapy is a promising strategy for the treatment of various musculoskeletal disorders such as those concerning the articular cartilage, bones, tendons and ligaments, and meniscus as it can deliver candidate gene sequences in a spatially and temporally controlled manner in sites of tissue damage over prolonged periods of time that may be required to durably enhance the specific, natural repair mechanisms *in vivo* in direct, non-invasive procedures that avoid the arduous manipulation and implantation of patient-dependent cells genetically modified *in vitro*. In the present work, we provide an overview of the most up-to-date approaches and outcomes in experimental, relevant models of such disorders *in vivo* using biomaterial-guided gene transfer that may be employed in a near future to treat patients during a clinical intervention as a means to achieve an effective, safe, and persistent translational healing of musculoskeletal injuries.

1. Introduction

While various clinical options are available to address musculoskeletal disorders in millions of patients worldwide [1], none thus far has been capable of reliably regenerating the sites of tissue damage both in their native structure and mechanical functions.

The articular cartilage, the white gliding tissue that supports load transmission and joint mobility has a limited ability for self-repairing the absence of vascularization that may provide regenerative (progenitor) cells [2]. Articular cartilage lesions like those resulting from trauma (focal defects) or during progressive, degenerative osteoarthritis (OA) therefore do not heal by themselves or following classical reconstructive surgical interventions (marrow-stimulating techniques like microfracture, cell and tissue transplantation using autologous chondrocytes or mesenchymal stromal cells - MSCs, osteochondral cylinders) [3–5]. Despite such treatments, the repair tissue in sites of cartilage injury is generally of fibrocartilaginous nature (mainly type-I collagen) with poor mechanical properties and prone to progression into secondary OA [6,7], compared with the original hyaline cartilage composed of type-II collagen and proteoglycans, capable of withstanding mechanical stress [8].

Bone tissue with a hierarchical structure (type-I collagen fibers, nanohydroxyapatite - nHAp - matrix) to support the body weight and movements as points of attachment of muscles has an inherent ability for healing but not reliably for complex, large defects or fractures, resulting in non-unions [9]. Interventions based on autografts and devitalized cadaveric allografts are still problematic, with restricted graft availability and donor site morbidity (autografts) and inadequate tissue integration (allografts) [10].

Connective, poorly vascularized tendons and ligaments that store elastic energy and transmit forces for locomotion [11–15] are commonly predisposed to injuries that are critical issues in the clinics due to the modest healing capacities of these fibrous tissues and to their poor responses to the current therapies (sutures, auto-/allografts, prostheses) with the formation of tissues with reduced

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strength and mobility and the occurrence of adhesions, inflammatory responses, and fibrosis [16,17].

The fibrocartilaginous, connective meniscus that transmits weight-bearing forces promotes knee stability and proprioception, facilitates nutrition, and allows for cartilage lubrication, is highly susceptible to tears after trauma or related to age-associated degenerative OA [18–23], showing no full regenerative capabilities (particularly in the central avascular zone) even upon procedures of meniscal preservation, repair, and reconstruction (partial meniscectomy, sutures, autologous tissues and allografts, artificial substitutes) [24–28] that have several limitations (altered biomechanical properties and structure, instability, reduced vascularization, availability of and deleterious responses to the graft, extrusion, cartilage degeneration, subchondral bone edema) [23,29,30].

Gene therapy is a powerful technology to create tools for the prolonged delivery and production of therapeutic candidate gene sequences in musculoskeletal injuries over stable periods of time relative to the application of recombinant gene products displaying relatively short pharmacological half-lives (some hours) [27,31–34]. A novel concept to ameliorate human musculoskeletal gene therapy protocols *in vivo* is to provide gene-based treatments via biomaterial-assisted procedures, allowing for a spatiotemporal, controlled release of gene carriers and consequently of their products in sites of musculoskeletal damage [34,35]. Such systems may offer off-the-shelf compounds that support non-invasive, ubiquitous (patient-independent) direct treatments in clinical setups compared with a more complex, less convenient administration of genetically modified cells that requires repeated patient handling [27,31–34]. The following chapters discuss the principles of biomaterial-assisted gene therapy and provide an overview of translational applications for the treatment of musculoskeletal disorders in relevant models *in vivo*.

2. Biomaterial-assisted gene therapy: principles

Biomaterial-assisted gene therapy is based on the delivery of gene carriers via biocompatible, integrative, biodegradable, and biomimicking materials employed in tissue engineering procedures as a means to provide biological cues and support the endogenous reparative mechanisms in damaged musculoskeletal tissues by offering a cell-supporting scaffold in sites of injury [36–39].

2.1. Biomaterials for musculoskeletal applications

Various biomaterials have been manipulated in musculoskeletal research such as hydrogels and solid scaffolds derived from natural or synthetic materials (or from both) and displaying specific features to mimic the architecture and properties of the extracellular matrix (ECM) of the tissues, with both advantages and shortcomings for musculoskeletal repair [8,27,40–67].

Hydrogels are three-dimensional (3D) cross-linked polymer networks with a hydrophilic structure that makes them capable of holding large amounts of water for swelling (but without dissolution in water), being permeable to oxygen and nutrients [68]. They are biocompatible, easy to fabricate under mild conditions, and can be tailored and provided in a minimally invasive manner in tissue lesions of any size by arthroscopy as they can be provided as injectable formulations [69–71]. Natural hydrogels with a higher biocompatibility and biodegradability have been largely applied in translational tissue engineering strategies to treat injuries of the articular cartilage, bone, tendons/ligaments, and meniscus, based on fibrin, hyaluronic acid (HA), chitosan, collagen, peptides, gelatin, and alginate [72–86]. Synthetic hydrogels have more reproducible properties and they can be manipulated as “smart” systems to respond to a variety of environmental stimuli (pH, temperature, ionic strength, inflammation, etc) [87] and have been also employed in musculoskeletal tissue engineering using polyethylene glycol (PEG), polyacrylamide (PAM), polyvinyl alcohol (PVA), poly(lactic acid) (PLA), poly(lactide-*co*-glycolide) (PLGA), self-assembling peptides, poly(N-isopropylacrylamide) (pNIPAAm), polyethylene oxide- (PEO-) and polypropylene oxide- (PPO-) based copolymers (poloxamers or Pluronic®, poloxamines or Tetronic®), poly(ε-caprolactone) (PCL), HAp, and beta-tricalcium phosphate (β-TCP) [88–97] or mixed with natural compounds for the same purposes [98–104]. On the other side, hydrogels display lesser mechanical properties relative to solid scaffolds.

Mechanically stable solid scaffolds are well adapted to treat lesions affecting the musculoskeletal tissues as these are composed of a dense ECM exposed to external loads that lead to tissue deformation and/or strains. Solid scaffolds may be created with natural polymers such as HA, chitosan, collagen, gelatin, and silk [105–120] or using synthetic materials like PLA, poly-glycolic acid (PGA), PLGA, PEG, PCL, polyurethane (PU), HAp, β-TCP, poly(-propylene fumarate) (PPF), polyhydroxyalkanoates (PHA), bioactive glasses, titanium, and polyethylene terephthalate [121–142] that may be further mixed with natural compounds [143–148]. Solid scaffolds can be also combined with hydrogels to produce hybrid scaffolds (or composites) based on fibrin with either PU, PCL, β-TCP, or collagen, on alginate with PLA, PLGA, or β-TCP, on collagen with β-TCP, on agarose/PEG with PCL, on self-assembling peptides and PCL, and on pluronic F127 with β-TCP [149–161].

2.2. Gene therapy

Gene therapy consists in (i) the transfer of exogenous nucleic acid sequences (transgenes) in target cells or (ii) the endogenous editing of the genome of target cells for instance using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein-9 nuclease (Cas9) system, both using a gene carrier (vector) derived from nonviral systems or viruses (Table 1) [31–34,162–167].

Nonviral vectors avoid the risk to acquire replication competence specific of virus-derived vectors and do not activate immune responses in a recipient, making them safe gene carriers for *in vivo* applications. Yet, they are associated with low and short-term gene transfer efficiencies (some days) while necessitating cell division to permit gene nuclear translocation that occurs only in very few cell populations at the adult stage [168], making them more suitable for *ex vivo* (indirect) gene therapy procedures by transplantation of genetically modified cells. Viral vectors produced in musculoskeletal research from adenoviruses, retro-/lentiviruses, and adeno-associated virus (AAV) use natural entry pathways in target cells, with features inherent to each virus class. Adenoviral vectors permit high gene transfer efficiencies and therefore *in vivo* (direct) gene therapy approaches, but they are very immunogenic, relatively unsafe, and shortly effective (some days to a 1–2 weeks) [169]. Retro-/lentiviral vectors that have the ability to integrate in the genome of host cells may be durably functional. However, they have a relatively low gene transfer efficiency that makes them more adapted for *ex vivo* (indirect) gene therapy strategies while necessitating cell division (except for lentiviral vectors that originate from the pathogenic human immunodeficiency virus - HIV) and possibly activating tumor genes upon integration [170,171]. Recombinant, gutless AAV (rAAV) vectors are safe gene vehicles that are maintained under stable episomal forms in the targets and allow for high, prolonged gene transfer efficiencies (months to years) that support *in vivo* (direct) gene therapy settings [35,172].

Despite the value and versatility of gene transfer vectors, their optimal use for musculoskeletal gene therapy in the clinics

Table 1
Gene transfer vectors commonly used in musculoskeletal research.

Vectors	Advantages	Limitations	Maintenance
Nonviral vectors	. Not infectious . Not immunogenic	. Low, short-term efficiency . Cell division needed	episomal
Adenoviral vectors	. High efficiency . No need for cell division	. Short-term efficiency . Immunogenic	episomal
Retro-/lentiviral vectors	. Long-term efficiency	. Low efficiency . Cell division needed (not for lentiviral vectors) . Insertional mutagenesis	integration
rAAV vectors	. High, long-term efficiency . No need for cell division	. Humoral responses . Insertional mutagenesis?	mostly episomal

rAAV: recombinant adeno-associated virus.

[31–34,162,163,166] is still limited by a number of critical barriers that hinder their effective translational application. First, the vectors may disseminate to non-target organs and be cleared from the body [173,174]. Next, their access to the targets might be blocked by physical and biological barriers such as body fluids (synovial fluid), blood binding factors, adverse microenvironment (pH, hypoxia, enzymatic and inflammatory molecules), dense ECM components, and/or inhibitory clinical compounds (heparin) [175,176]. Finally, the vectors may raise toxic and (patient-specific) immune responses counteracting their efficacy (neutralizing antibodies and cellular immunity against the viral capsid proteins) [177–182] and have a low and short-term efficacy. Current strategies to address these issues include the transient immunosuppression of the host [183], a plasmapheresis and saline flushing [184], the use of alternative clinical compounds (hirudin) [175,176,185], and the genetic engineering of the viral vector capsid (chemical modification, epitope insertion, use of decoys, pseudotypes, hybrids, variants, mosaic/chimeric/mutant vectors) [186–193]. However, these strategies are either too complex and invasive, technologically arduous, or patient-specific, and have not met success in clinical setups yet, showing the critical need for new avenues of research that might more conveniently lead to systems applicable to all patients in clinical protocols.

2.3. Biomaterial-assisted gene therapy: controlled gene vector delivery

Combining gene therapy and tissue engineering procedures is a promising concept to overcome such hurdles by using a biomaterial as a cargo (or gene-activated matrix) to deliver a gene transfer vector in sites of musculoskeletal damage [34,35,194–200], based on the development of controlled drug delivery tools [201–203]. The controlled delivery and release of gene vectors from a biomaterial may: (1) enhance their spatial and temporal presentation and residence time in their targets; (2) minimize the doses of vector applied and prevent its dissemination; (3) provide stability against premature vector degradation; and (4) mask viral capsid epitopes to afford protection against immune responses. Gene vectors may be amenable to scaffold-mediated delivery upon encapsulation (loading during the preparation of the scaffold) or immobilization (adsorption on a scaffold formed beforehand) to allow for a gradient polymeric or substrate-mediated release, respectively, using hydrogels (vector diffusion process) or solid scaffolds (vector transfer from the substrate). Alternatively, the vector may be provided to the scaffold using bioprinting tools in defined 3D patterns [200,204].

3. Biomaterial-assisted gene therapy: applications for musculoskeletal disorders

Several experimental approaches using biomaterial-assisted gene therapy procedures have been reported in clinically relevant experimental models to develop treatments for disorders of the musculoskeletal system (Fig. 1 and Tables 2 and 3).

3.1. Articular cartilage

Micelles based on poloxamers and poloxamines have been employed to enhance the chondrogenesis of MSCs via delivery of rAAV vectors coding for the cartilage-specific sex-determining region Y-type high mobility group box 9 (SOX9) transcription factor *in vitro* with an increased deposition of ECM compounds (proteoglycans, type-II collagen), further affording protection against rAAV gene transfer inhibitors (viral uptake interfering agents such as heparin, anti-AAV capsid neutralizing antibodies) [205,206] and to heal experimental osteochondral defects by transfer of rAAV vectors carrying either SOX9 or the transforming growth factor beta (TGF-β) in explant cultures *in situ* by increasing the proliferative, pro-anabolic, and anti-hypertrophic activities in local chondrocytes (enhanced proteoglycan and type-II collagen deposition, reduced type-X collagen expression and mineralization) [207,208]. Carbon dot formulations have been also prepared to deliver rAAV vectors coding for SOX9 or TGF-β as a means to stimulate MSC chondrogenesis *in vitro* by enhancing cell proliferation and ECM deposition (glycosaminoglycans, type-II collagen) while containing undesirable osteogenesis and terminal differentiation (type-I and -X collagen deposition [209].

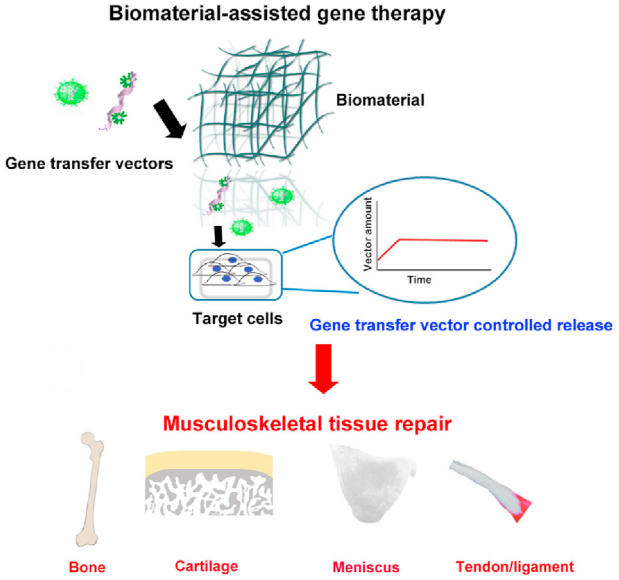

Fig. 1. Principle of biomaterial-assisted gene therapy for musculoskeletal regenerative medicine. Candidate non-viral and viral gene transfer vectors may be incorporated into biomaterial scaffolds that act as reservoirs to provide a spatially and temporally defined vector-controlled delivery and release in sites of musculoskeletal tissue damage (bone, cartilage, meniscus, tendon/ligament) over prolonged periods of time.

Table 2
Biomaterial-assisted gene therapy in musculoskeletal research: articular cartilage.

Systems	Vectors	Biomaterials	Genes	Models	Ref.
Micelles	rAAV	poloxamers,	SOX9	MSCs	[205]
		poloxamines	*lacZ*	eOCD	[206]
			TGF-β	eOCD	[207]
			SOX9	eOCD	[208]
Formulations	rAAV	carbon dots	SOX9, TGF-β	MSCs	[209]
Hydrogels	NV	HA	*luc*	MSCs	[210,212]
		PEG, OPF	*luc*	MSCs	[211]
		OPF	SOX trio, RUNX2	OCD	[214]
		pNIPAAm	*luc*	MSCs	[213]
		alginate/HAp	TGF-β, BMP-2	MSCs	[215]
	rAAV	fibrin	TGF-β	MSCs	[216]
		SALPs	*lacZ*, RFP	MSCs	[218]
		alginate, poloxamers	*lacZ*	MSCs	[217]
		poloxamers, poloxamines	SOX9	CD	[220]
		polypseudorotaxanes	*lacZ*	MSCs	[219]
Solid scaffolds	NV	chitosan, gelatin	TGF-β	ACs	[222]
		chitosan, gelatin, HAp	TGF-β, BMP-2	OCD	[226]
		collagen	IGF-I	ACs	[223]
			endostatin	MSCs	[225]
		collagen, GAG	*luc*	ACs	[221]
			IGF-I	ACs	[224]
		PLGA	SOX trio	OCD	[227]
	LV	PCL	TGF-β	MSCs	[228]
			IL-1Ra	MSCs	[229,230]
				iPSCs	[231]
	rAAV	PCL	*lacZ*, RFP	BMA	[232]
			SOX9	BMA	[233]
Hybrid scaffolds	NV	fibrin, PLGA	TGF-β	OCD	[234–236]

rAAV: recombinant adeno-associated virus; NV: nonviral vectors; LV: lentiviral vectors; HA: hyaluronic acid; PEG: polyethylene glycol; OPF: oligo(poly(ethylene glycol) fumarate); pNIPAAm: poly(N-isopropylacrylamide); HAp: hydroxyapatite; SALPs: self-assembling peptides; GAG: glycosaminoglycan; PLGA: poly(lactide-*co*-glycolide); PCL: poly(ε-caprolactone); SOX9: sex-determining region Y-type high mobility group box 9; *lacZ*: *E. coli* β-galactosidase; TGF-β: transforming growth factor beta; *luc*: Firefly luciferase; SOX trio: SOX5, SOX6, SOX9, RUNX2: runt-related transcription factor 2; BMP-2: bone morphogenetic protein 2; RFP: red fluorescent protein; insulin-like growth factor I; IL-1Ra: interleukin-1 receptor antagonist; MSCs: mesenchymal stromal cells; eOCD: experimental osteochondral defect; CD: chondral defect; ACs: articular chondrocytes; iPSCs: induced pluripotent stem cells; BMA: bone marrow aspirates.

Table 3
Biomaterial-assisted gene therapy in musculoskeletal research: bone.

Systems	Vectors	Biomaterials	Genes	Models	Ref.
Hydrogels	NV	alginate, collagen	SOX9	MSCs	[240]
		alginate, HAp	TGF-β, BMP-2	MSCs	[239]
	LV	gelatin	BMP-2	MSCs	[241]
Solid scaffolds	NV	chitosan	*lacZ*	MSCs	[243]
		collagen	BMP-4, PTH [1–34]	FD	[242]
			PTH [1–34]	TD, FD	[194]
			GFP	FD	[246]
			VEGF	RD	[244]
			luc	MSCs	[251]
			VEGF	FD	[252]
			BMP-2	TD	[247]
		collagen, HAp	*luc*	MSCs	[256]
			BMP-2	MSCs	[255,259]
			VEGF	MSCs	[259]
		collagen, PGA	BMP-2	MSCs	[248–250]
		gelatin, PGA	BMP-2	MSCs	[245]
		gelatin, collagen	BMP-2	MSCs	[245]
		gelatin, collagen, PGA	BMP-2	MSCs	[245]
		PCL	RUNX2	MSCs	[257]
		PLGA	TGF-β	MSCs	[258]
			TGF-β	FD	[258]
		PLLA, PLGA	GFP	MSCs	[253]
		Ti	BMP-2	MSCs	[260]
		Ti, PDLLA	BMP-2	MSCs	[254]
	AdV	β-TCP	RUNX2	FD	[261]
	rAAV	PCL	BMP-2	MSCs	[262]
			BMP-2	FD	[262]
		PLLA	BMP-2	MSCs	[263]

NV: nonviral vectors; LV: lentiviral vectors; AdV: adenoviral vectors; rAAV: recombinant adeno-associated viral vectors; HAp: hydroxyapatite; PGA: poly-glycolic acid; PCL: poly(ε-caprolactone); PLGA: poly(lactide-*co*-glycolide); PLLA: poly-L-lactide acid; Ti: titanium; PDLLA: poly(d,L-lactide); β-TCP: beta-tricalcium phosphate; SOX9: sex-determining region Y-type high mobility group box 9; TGF-β: transforming growth factor beta; BMP: bone morphogenetic protein; *lacZ*: *E. coli* β-galactosidase; PTH [1–34]: parathyroid hormone (amino acids 1–34); GFP: green fluorescent protein: VEGF: vascular endothelial growth factor; *luc*: Firefly luciferase; RUNX2: runt-related transcription factor 2; MSCs: mesenchymal stromal cells; FD: femoral defect; TD: tibial defect; RD: radial defect.

Hydrogels have been used to transfer non-viral [210–215] and rAAV vectors [216–220] using fibrin [216], HA [210,212], PEG and derivatives [211,214], pNIPAAm [213], self-assembling peptides [218], poloxamers and poloxamines [220], polypseudorotaxanes [219], and alginate with poloxamers [217] or with HAp [215] to transfer sequences for reporter genes [210–213,217–219] (Fig. 2) or for therapeutic SOX9 and the SOX trio (SOX5, SOX6, SOX9) [214,220], TGF-β [215,216], bone morphogenetic protein 2 (BMP-2) [215], and bone-specific runt-related transcription factor 2 (RUNX2) [214] as a means to activate MSC chondrogenesis *in vitro* [210–213,215–219] and to enhance the repair of focal cartilage defects *in vivo* [214,220]. For instance, moderate levels of transgene expression with release profiles of ~10 days were observed when incorporating plasmid DNA coding for luciferase (*luc*) gene/polyplexes into microporous hydrogel systems [210,212] and additional incorporation of micelles in hydrogel systems increased gene expression relative to hydrogel systems alone [211]. In addition, a bilayered porous oligo[PEG fumarate] hydrogel employed to co-deliver plasmid sequences coding for the chondrogenic (SOX trio) and osteogenic (RUNX2) transcription factors via poly(-ethylenimine)/HA complexes improved the healing of rat osteochondral defects relative to single gene delivery and to empty hydrogel systems [214]. Besides, release of an rAAV gene vehicle coding for TGF-β(1) from a fibrin glue scaffold had strong beneficial effects on the expression of cartilage-specific gene in MSCs [216]. Also, incorporation in and release of rAAV vectors from alginate/poloxamer (PF127) systems allowed to support elevated transduction efficiencies in MSCs without detrimental effects on the chondrogenic differentiation potential and viability of these cells *in vitro* [217]. Furthermore, controlled release of rAAV from self-assembling peptide hydrogels RAD16-I in a pure (RAD) form or combined with HA (RAD-HA) led to the successful genetic modification of MSCs without altering their viability nor chondrogenic commitment [218]. Next, delivery of rAAV vectors via supramolecular polypseudorotaxane gels was capable of promoting higher gene transfer efficiencies and cytocompatibility in MSCs compared with free vectors [219]. Most strikingly, overexpression of an rAAV SOX9 gene vehicle via *in situ* controlled release from a PEO-PPO-PEO hydrogel led to superior cartilage repair in full-thickness minipig chondral defects *in vivo* compared with hydrogel-free vector delivery [220].

Solid scaffolds have been also prepared to deliver non-viral [221–227], lentiviral [228–231], and rAAV vectors [232,233] using chitosan with gelatin [222] or with gelatin and HAp [226], collagen [223,225] with glycosaminoglycan (GAG) [221,224], PLGA [227], and PCL [228–233] to transfer sequences for reporter genes [221,232] or for therapeutic SOX9 and SOX trio [227,233], TGF-β [222,226,228], BMP-2 [226], insulin-like growth factor I (IGF-I) [223,224], interleukin-1 receptor antagonist (IL-1Ra) [229–231], and the anti-angiogenic endostatin factor [225] in order to target articular chondrocytes *in vitro* [221–224], stimulate chondrogenic and anti-inflammatory events in MSCs [225,228–230], in induced pluripotent stem cells (iPSCs) [231], and in bone marrow aspirates [232,233] *in vitro* (Fig. 3), and promote the repair of focal cartilage

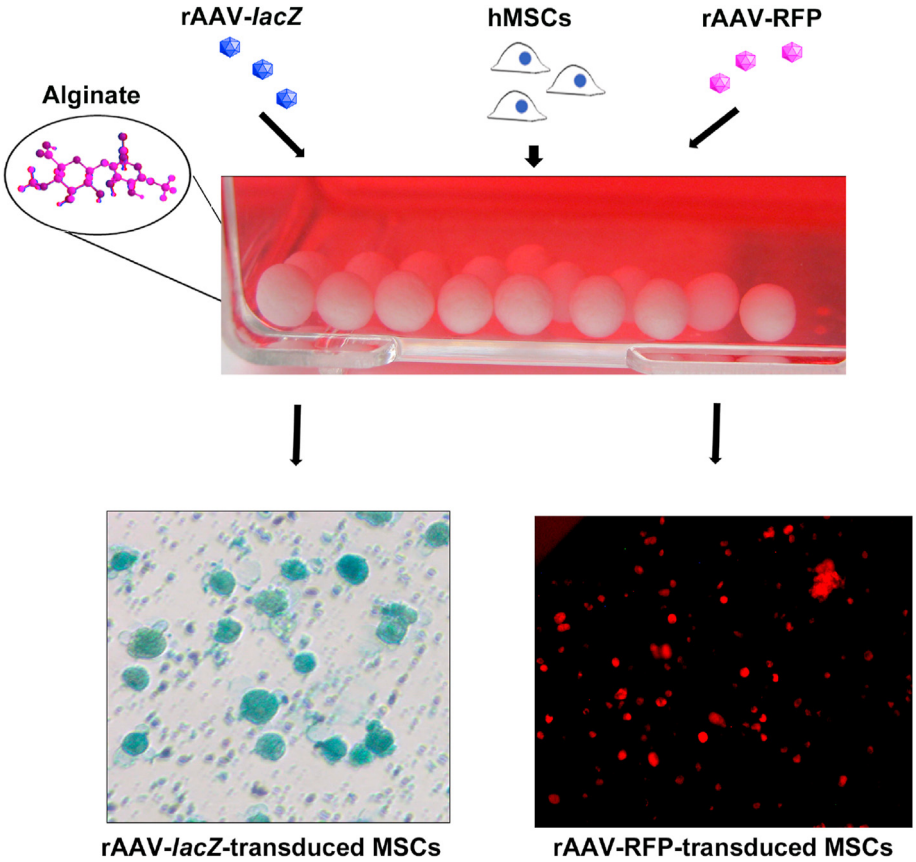

Fig. 2. Targeting of human mesenchymal stromal cells via alginate-assisted rAAV-mediated gene transfer. Human mesenchymal stromal cells (MSCs) were encapsulated in alginate spheres concomitantly with an rAAV vector carrying either the reporter gene coding for the *E. coli* β-galactosidase (*lacZ*), i.e. rAAV-*lacZ*, or the reporter gene coding for the red fluorescent protein (RFP), i.e. rAAV-RFP (top panel). Images (bottom panels) reveal the effective gene transfer and transgene expression in the treated MSCs after 10 days (left: *lacZ* expression detected by X-Gal staining, magnification x20; right: RFP expression visualized under fluorescent microscopy, magnification x10).

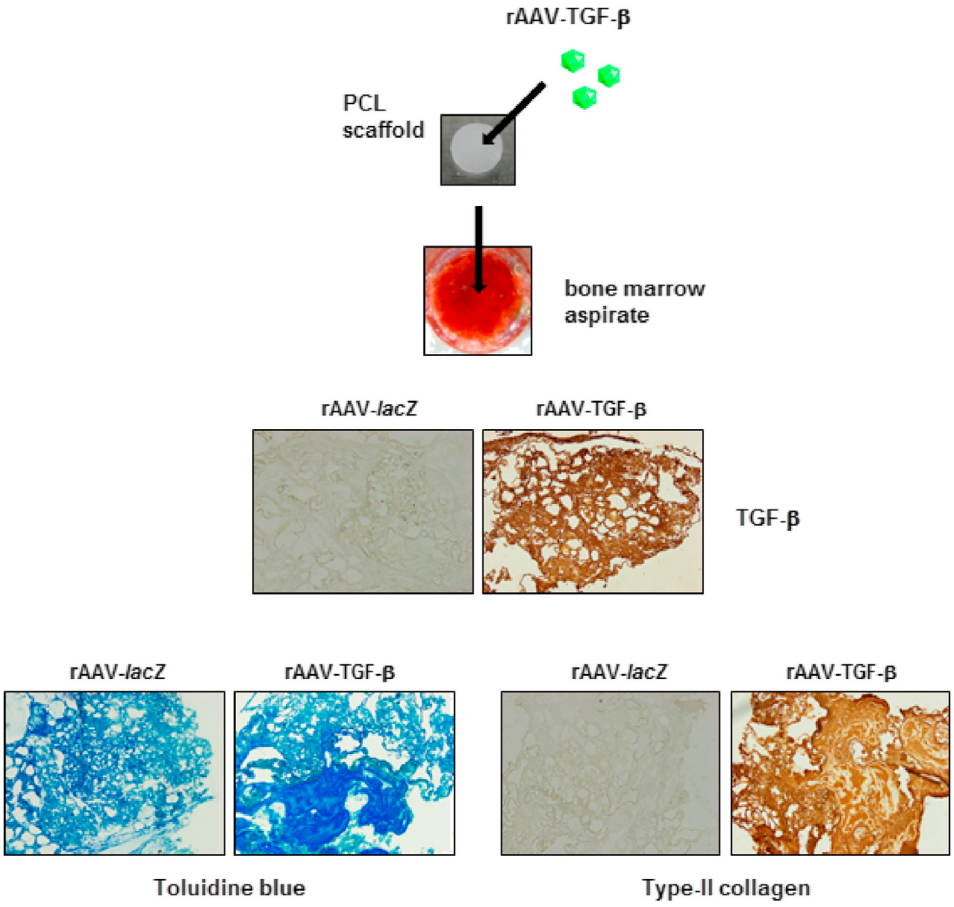

Fig. 3. Therapeutic PCL-assisted rAAV-mediated gene transfer in human bone marrow aspirates *in situ*. PCL films were coated with rAAV (therapeutic rAAV-TGF-β vector carrying the highly chondrogenic transforming growth factor beta - TGF-β - vs. reporter rAAV-*lacZ*) and next incubated with human bone marrow aspirates. Images reveal the effective gene transfer and transgene (TGF-β) expression in aspirates treated with PCL/rAAV-TGF-β relative to PCL/rAAV-*lacZ* after 21 days (magnification x10) (top panels) and the increased deposition of cartilage matrix components (proteoglycans by toluidine blue staining, left; type-II collagen expression by immunohistochemical detection, right) in response to PCL/rAAV-TGF-β application vs. PCL/rAAV-*lacZ* after 21 days (magnification x10) (bottom panels).

defects *in vivo* [226,227] (Fig. 4). Capito et al. [223] investigated the use of a type-II collagen-glycosaminoglycan (CG) scaffold as a non-viral system to deliver the gene for IGF-I to support cartilage repair. Sustained levels of IGF-I expression using such a setup significantly promoted cartilage tissue formation with chondrocyte-like cells, GAG accumulation, and type II-collagen deposition relative to control treatments lacking IGF-I. More recently, the same group monitored the effects of modifying the charge of a gelatin delivery system by cationization to deliver an IGF-I-coding plasmid as a means to genetically modify articular chondrocytes [224]. The result of the study revealed a 5-fold difference of IGF-I expression between groups treated with cationized particles compared with non-cationized ones. Transfection of cells seeded in type-II collagen-glycosaminoglycan (CG) scaffolds via cationized gelatin particles resulted in 60% higher GAG/DNA contents vs. untreated chondrocytes or chondrocytes transfected with gelatin alone. Also interestingly, implantation of a bilayered gene-activated osteochondral matrix consisting of both a plasmid TGF-β1-activated chitosan-gelatin scaffold (chondrogenic layer) and a plasmid BMP-2-activated HAp/chitosan-gelatin scaffold (osteogenic layer) in rabbit osteochondral defects led to concomitant cartilage and subchondral bone repair with good integration of the system with the native osteochondral tissues [226]. Besides, the group of Guilak et al. [228] demonstrated that delivery of a lentiviral vector coding for TGF-β3 via immobilization on PCL films led to the successful transduction of MSCs that produced a robust ECM in response to therapeutic (TGF-β3) transgene expression. The same group [229,230] further reported that administration of a lentiviral construct promoting the expression of IL-1Ra in a doxycycline (dox)-inducible manner via 3D woven PCL scaffold allowed for the production of a cartilage-specific ECM in MSCs in the presence of pathologic levels of IL-1 [229] and joint resurfacing in anatomically shaped cartilage constructs engineered with adipose-derived MSCs [230]. This group also showed that delivery of a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-inducible lentiviral vector coding for IL-1Ra inhibited pro-inflammatory pathways and ECM degradation in iPSC-based constructs treated with IL-1 [231]. Of further note, recent evidence showed the benefits of using PCL films grafted with poly(sodium styrene sulfonate) (pNaSS) as novel, highly effective systems for the controlled delivery of rAAV vectors to reparative bone marrow aspirates [232,233], allowing to enhance the biological activities and chondrogenic processes in the samples (ECM deposition) when delivering an rAAV SOX9 construct compared with the release of control (reporter) rAAV vectors or when using ungrafted films [233].

In addition, hybrid scaffolds composed of fibrin and PLGA were also used to carry nonviral vectors coding for TGF-β as systems to enhance the repair of focal cartilage defects *in vivo* [234–236]. For instance, Wang et al. [234] reported that a TGF-β-gene activated matrix based on fibrin/PLGA scaffolds seeded with MSCs enhanced

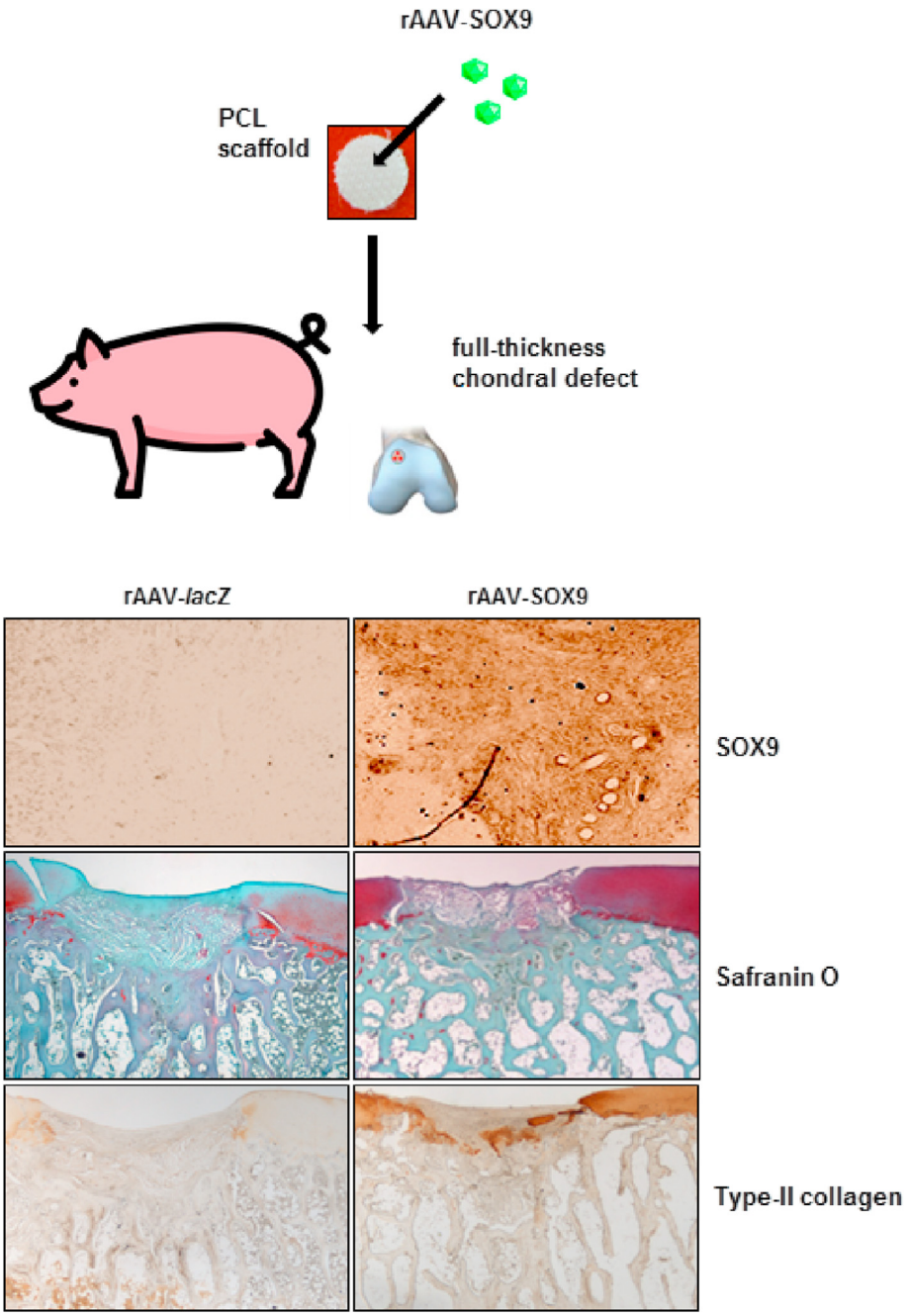

Fig. 4. Therapeutic PCL-assisted rAAV-mediated gene transfer in focal cartilage defects *in vivo*. PCL scaffolds were coated with rAAV (therapeutic rAAV-SOX9 vector carrying the highly chondrogenic cartilage-specific sex-determining region Y-type high mobility group box 9 transcription factor - SOX9 - vs. reporter rAAV-*lacZ*) and next applied to full-thickness chondral defects in minipigs after microfracture in the presence of bone marrow aspirate. Images reveal the effective gene transfer and intracellular transgene (SOX9) expression in the defects treated with PCL/rAAV-SOX9 relative to PCL/rAAV-*lacZ* after 4 weeks (magnification x20) and the increased deposition of cartilage matrix components (proteoglycans by safranin O staining, top; type-II collagen expression by immunohistochemical detection, bottom) in response to PCL/rAAV-SOX9 application vs. PCL/rAAV-*lacZ* after 4 weeks (magnification x4).

cartilage repair upon implantation in full-thickness cartilage defects in rabbits compared with the use of empty scaffolds. Of further note, complexation of a TGF-β-coding plasmid via PEO-b-poly (L-lysine) (PEO-bPLL) [235] or Lipofectamine [236] further increased the performance of these hybrid materials.

Of note, no studies are currently available on the potential of biomaterial-assisted gene therapy for the treatment of OA, although it has been evoked in recent reviews of the literature [200,237,238].

3.2. Bone

Hydrogels have been employed to deliver non-viral [239,240] and lentiviral vectors [241] using gelatin [241] and alginate with collagen [240] or with HAp [239] to transfer SOX9 [240], TGF-β [239], and BMP-2 [239,240] as a means to stimulate MSC osteogenesis *in vitro* [239–241]. Cunniffe et al. [239] developed a gene-activated bioink using RGD-γ-irradiated alginate and nHAp complexed to plasmid DNA and combined it with PCL and MSCs to

generate mechanically stable constructs. Delivery of a combination of genes coding for BMP and TGF-β from these constructs promoted a robust osteogenesis of encapsulated MSCs *in vitro*. When subcutaneously implanted in nude mice, these gene-activated matrices enhanced vascularization and mineralization compared with cell-free controls. In another attempt, collagen-I-alginate interpenetrating polymer network hydrogels were created to generate gene-activated matrices with nanocomplexed SOX9 polynucleotides and MSCs [240]. The results of the study demonstrated that SOX9 effectively induced MSC chondrogenesis and reduced the expression of hypertrophy markers compared with control matrices.

Solid scaffolds have been also prepared to deliver non-viral [194,242–260], adenoviral [261], and rAAV vectors [262,263] using chitosan [243], collagen [194,242,244,246,247,251,252] with HAp [255,256,259] or with PGA [248–250], gelatin with collagen, PGA, or both [245], β-TCP [261], PCL [257,262], poly-L-lactide acid (PLLA) [263] or PLGA [258] or both [253], or titanium and derivatives [254,260] as means to transfer sequences for reporter genes [243,246,251,253,256] or for therapeutic TGF-β [258], BMP-2 [245,247–250,254,255,259,260,262,263] and BMP-4 [242], RUNX2 [257,261], parathyroid hormone (amino acids 1–34) (PTH [1–34]) [194,242], and vascular endothelial growth factor (VEGF) [244,252,259] in order to activate MSC osteogenesis *in vitro* [243,245,248–251,253–260,262,263] and to heal bone defects *in vivo* [194,242,244,246,247,252,258,261,262]. Hosseinkhani et al. [245] impregnated a complex of cationized gelatin and plasmid DNA coding for BMP-2 in PGA fiber fabrics, collagen sponges, or collagen sponges reinforced by the incorporation of PGA fibers. Alongisde, the authors compared the levels of transgene expression obtained upon incorporation of MSCs in the different constructs under static, stirring, or perfusion culture conditions [249]. The results of the evaluation showed a more sustained release profile of plasmid DNA upon incorporation in PGA-reinforced sponges vs. other scaffolds and increased levels of B MP-2 expression via perfusion [245,249]. When the constructs were subcutaneously implanted in rats, higher levels of alkaline phosphatase (ALP) and osteocalcin expression were noted compared with groups impregnated or processed with the other methods [248]. Similar results were observed when incorporating plasmid BMP-2 DNA-nanoparticles using the same PGA-reinforced sponges [250]. In another study, a collagen-nHAp gene-activated matrix was developed by incorporating a plasmid BMP-2 DNA and MSCs [255]. In this work, enhanced osteogenesis in the cells was observed following nHAp-BMP-2 transfection both in 2D and 3D cultures at low levels of plasmid BMP-2 DNA. Dual delivery of plasmid BMP-2 DNA and plasmid VEGF DNA from the same constructs markedly promoted bone healing upon implantation in rat transosseous defects [259]. Other interesting work showed that release of an adenoviral vector carrying the coding sequence for the core-binding factor subunit alpha-1 (Cbfa1) from β-TCP/osteoprogenitor cells promoted bone formation upon subcutaneous implantation in rat bone defects [261]. Dupont et al. [262] also provided evidence that a self-complementary AAV (scAAV) vector coding for BMP-2 coated on 3D porous poly(ε-caprolactone) (PCL) scaffolds seeded with bone marrow- or amniotic fluid-derived mesenchymal stromal cells (MSCs) permitted increased mineral formation and bony bridging with stronger mechanical properties when implanted in critically sized immunocompromised rat femoral defects. Such findings corroborated work from Xue et al. [263] showing that an rAAV vector coding for BMP-2 encapsulated in a PLLA scaffold loaded with MSCs promoted bone formation when administered to an ectopic mouse model *in vivo* (muscle implantation).

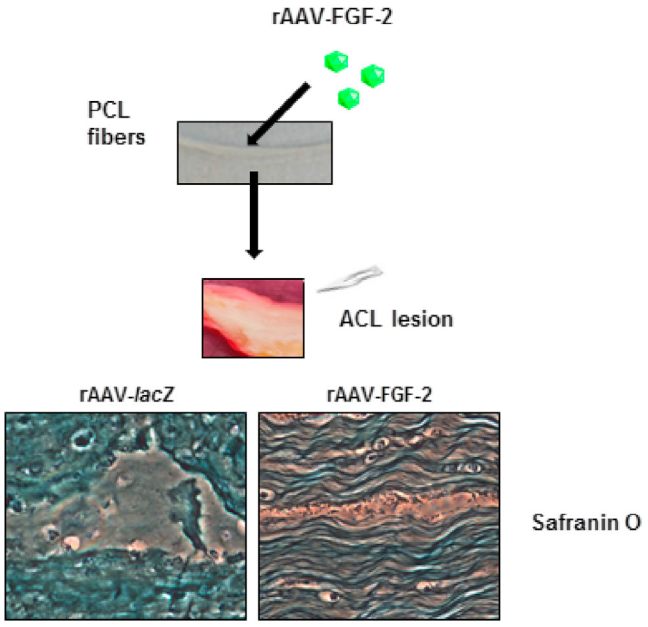

Fig. 5. Principle of therapeutic PCL-assisted rAAV-mediated gene transfer in injured ligaments. PCL fibers may be coated with rAAV (therapeutic rAAV-FGF-2 vector carrying the reparative basic fibroblast growth factor - FGF-2 - vs. reporter rAAV-*lacZ*) for application to ligament lesions like those affecting the anterior cruciate ligament (ACL). The figure shows the principle of the approach in a model of ACL lesion *in vitro* experimentally created with a scalpel. Healing may be monitored by evaluating the effects of the treatment on the extent (length, depth, 3D area) of the lesion.

3.3. Other musculoskeletal tissues

Interestingly, such approaches have not been reported yet to heal lesions affecting the tendons, ligaments, or meniscus, but they have been evoked in recent reviews of the literature [27,199] (Fig. 5).

4. Conclusions and perspectives

Several experimental studies in clinically relevant animal models *in vivo* currently validate the concept of biomaterial-assisted gene therapy to activate the repair processes in musculoskeletal tissues including the articular cartilage and bone and might further be envisaged to treat tendon, ligament, and meniscal lesions. Compared with the delivery of recombinant growth factors that exhibit very short half-lives and may be associated with adverse effects when used at high, supraphysiological doses [202], biomaterial-guided gene therapy may provide transgene expression profiles of therapeutic factors in a sustained, but controlled delivery manner by direct transfer of the candidate gene sequences in sites of tissue damage. Yet, even though numerous protocols have been conducted in patients using gene therapy [34,264,265] including for orthopedic applications [33,266–271], as well as tissue engineering approaches [25,47,50,57], there is only one clinical trial thus far reporting the feasibility of combining these two procedures by delivering a nonviral vector coding for VEGF via a collagen/HAp sponge for the treatment of mandibular bone defects in patients [272].

While gene-based translational applications are arduous, costly, and viewed as unsafe [265,273,274], the scarcity of biomaterial-assisted gene therapy trials may also reflect a need for more comprehensive experimental work to define optimal, i.e. effective/safe/non-immunogenic therapeutic compounds. This requires to

identify the most adapted vector system (vector class and dose), scaffold (hydrogels, solid scaffolds, hybrid materials), and gene (anti-inflammatory molecules, growth factors, transcription factors, signaling agents, etc.; individual vs. combined genes; genes that might be highly specific for the cartilage or for the bone to prevent unwanted effects in the adjacent/underlying tissue), the optimal level and duration of transgene expression and the most adequate control element/promoter (high level, tissue-specific, or disease-regulatable expression), as well as the route/occurrence of administration to avoid unwanted dissemination of the vectors to non-target locations (intra-articular injection, arthroscopy, arthrotomy; single vs. repeated administration). Other important points to consider include the manufacturing of vectors and of materials suitable for clinical applications (origin, clinical-grade nature, ease of fabrication, scalability, storage, pharmacokinetic release profiles, cost-effectiveness) that might be capable of penetrating dense tissues such as the articular cartilage and the bone prior to obtaining approval from regulatory agencies [275–278].

It remains to be seen how integrating scaffold/gene vector systems with the use of 3D bioprinting techniques that mimic structural features of the targeted injured tissue [204,239,279] or with that of orthobiologics (platelet-rich plasma - PRP, extracellular vesicles - EVs, etc.) as a personalized, biological augmentation/enrichment/adjuvant method [65,280–284] may be of added value for improved musculoskeletal therapy. Furthermore, the alternative translational use of innovative genome editing tools like those based on the Clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated protein-9 nuclease (Cas9) technology relative to classical (trans)gene therapy will require to expand our current knowledge on the benefits of such an approach in the musculoskeletal research area, as thus far only on study demonstrated the possibility to contain cartilage degeneration via rAAV-mediated editing of IL-1β and MMP-13 in experimental OA mice *in vivo* in a scaffold-free setup [285], aside from work showing the potential of CRISPR/Cas9 to preserve or rejuvenate MSCs and chondrocytes and to provide OA study models *in vitro* [286–294]. The effective and safe clinical use of CRISPR-Cas9 for genome manipulation will also necessitate a thorough benefit-risk assessment for musculoskeletal regenerative medicine compared with current clinical treatments for non-lethal orthopedic disorders and as this technology still faces a number of critical issues and challenges for adapted translation in patients (reproducibility, variability, off-target effects, immune responses against bacterial Cas9, existence of natural Cas9 inhibitors, efficiency *in vivo*, etc.) [295,296].

In conclusion, biomaterial-assisted gene therapy may provide adapted, novel tools as off-the-shelf systems that might be employed in conjunction with traditional clinical interventions for non-invasive treatments of musculoskeletal disorders in patients in near future by cooperation between scientists and clinicians and with the assistance and control of regulatory agencies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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