Chapter 9
Non-viral Vector for Muscle-Mediated Gene Therapy

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Abstract Non-viral gene delivery to skeletal muscle was one of the first applications of gene therapy that went into the clinic, mainly because skeletal muscle is an easily accessible tissue for local gene transfer and non-viral vectors have a relatively safe and low immunogenic track record. However, plasmid DNA, naked or complexed to the various chemistries, turn out to be moderately efficient in humans when injected locally and very inefficient (and very toxic in some cases) when injected systemically. A number of clinical applications have been initiated however, based on transgenes that were adapted to good local impact and/or to a wide physiological outcome (i.e., strong humoral and cellular immune responses following the introduction of DNA vaccines). Neuromuscular diseases seem more challenging for non-viral vectors. Nevertheless, the local production of therapeutic proteins that may act distantly from the injected site and/or the hydrodynamic perfusion of safe plasmids remains a viable basis for the non-viral gene therapy of muscle disorders, cachexia, as well as peripheral neuropathies.

Keywords Naked · Complexes · Muscle · Vaccines · Hydrodynamic delivery

9.1 Introduction

Skeletal muscle can act as an effective platform for the long-term production (and secretion) of therapeutic proteins with systemic distribution and for the introduction of DNA vaccines eliciting strong humoral and cellular immune responses (for review see [1, 2]). Conversely, the treatment of hereditary neuromuscular diseases is particularly challenging for non-viral vectors. Among issues are as follows: (1) the size of the muscle tissue, which represents half of the total mass of the organism, (2) the poor accessibility of profound muscles or peripheral nerves, and (3) the progressive tissue remodeling along the natural history of some muscle diseases with active processes of necrosis/regeneration and fibrosis/lipidosis.

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On the other hand, non-viral vectors do bear interesting advantages over recombinant viruses. Non-viral vectors are made of plasmid DNA, naked or complexed to a variety of versatile molecules such as cationic lipids or polymers. They are (1) well characterized, and their structure can be fine-tuned [3], and (2) mostly non-immunogenic provided, they are not carrying protein motifs. This allows repeated administrations for chronic diseases, (3) comparatively easy to produce at a large scale [4], (4) less limited by size constraints, leaving the potential to deliver wide-type genetic material, as large as 100 kb [5] (this is far beyond the size of coding sequences such as the dystrophin cDNA for Duchenne muscular dystrophy), and non-viral vectors (5) can remain functional for a long period of time in skeletal muscles [6]. Episomal plasmid DNA can persist for life in rodents and for many years in larger animals if they are delivered into low turnover tissues, including the brain and spinal cord, heart, or muscle (for review see [7]).

Synthetic vectors have been constructed as substitutes to viral vectors for delivering therapeutic genes and many other drugs in humans [8]. The principle is based on the self-assembly of supramolecular complexes, often through electrostatic interactions between the positively charged vectors and the DNA negatively charged phosphate residues [9]. In these complexes, DNA is condensed and compacted and is less exposed to nuclease degradation. Among these, cationic lipid- and polymer-based systems have been the most extensively studied [10–12]. In early studies, DNA was encapsulated in neutral or anionic liposomes without changing the structures of the liposomes [9, 13]. The ratio between the cationic charge of the liposome and the negative charge of the DNA usually controls the size of complexes [14], typically in the range of 200 nm to 2 μm quasi-spherical particles with an ordered (often multilamellar) organization. Their positive total charge enables them of efficiently interacting with the negative residues of the cell membranes and internalizing into the cell, which occurs mainly through the endocytosis pathway [10, 15].

9.2 Systemic Delivery of Non-viral Vectors: An Update and Perspective

Systemic bio-distribution of non-viral vectors is dependent upon their capability of escaping from blood vessels in the target tissue. Vectors must be small enough (less than 500 nm) to cross through vascular endothelial cells and gain access to surrounding tissues [16]. Furthermore, they should also be designed so that they can be ignored by mononuclear phagocytes and have little interactions with plasma components to avoid aggregation [17, 18] and complement activation [19]. Another limitation with systemic gene delivery of complexes is their rapid clearance by the reticuloendothelial system or their entrapment within small capillaries leading to the accumulation within especially lung tissue [20]. This limitation can be improved by incorporating polyethylene glycol (*PEG*) lipids, leading to increased circulation time of the complex, and protein expression in distal tissues [21, 22]. The negatively charged components of the cell membrane (glycoproteins, proteoglycans, and

glycerophosphates) are able to interact with the positively charged systems triggering the non-specific endocytosis of cationic non-viral vectors. Increasing positive net charge, prolongation of the incubation time, or complex concentration can improve cell uptake by clathrin-mediated endocytosis of cationic lipids such as *DOTAP*/DNA or of cationic polymers such as *PEI*/DNA by clathrin-coated pits or potocytosis (through interaction with caveolae pits) [23, 24], receptor-mediated endocytosis, macropinocytosis, or lipid raft-mediated endocytosis [25, 26].

In contrast to viral vectors, non-viral gene transfer is not elicited to a large extent by active intake processes. Therefore, a sophisticated vector may be needed to facilitate the cellular uptake and appropriate intracellular processing of the transgene. Significant developments in artificial complexes combined different functions for improved gene transfer. Many cationic liposomes are normally accompanied by a neutral lipid such as dioleoylphosphatidylethanolamine (*DOPE*) or cholesterol. *DOPE* is frequently useful because it can fuse with other lipids when exposed to a low pH, as in endosomes, which triggers the release of the associated DNA into the cytosol [27]. Other popular modifications use ligand binding to *PEG*. Various targeting approaches have been investigated, including incorporation of peptides, antibodies, and sugar into the lipid vesicles to facilitate tissue targeting (for review see [28]). However, the association of all of these components results in complex structures that require thorough formulation and galenic studies.

After cell entry, intracellular barriers may impair successful gene delivery. Vectors need to escape from the endosomal or lysosomal membrane to avoid degradation of the plasmid DNA [29]. Endosomal release of DNA by cationic polyplex-based vectors may be based on the physical disruption of the negatively charged endosomal membrane after direct interaction with the cationic complex [30], or a "proton-sponge" phenomenon [11] resulting in osmotic swelling and endosomal membrane rupture, followed by the release of the polyplexes into the cytoplasm. Addition of a fusogenic helper lipid such as *DOPE* facilitates the formation of a destabilizing hexagonal phase with the endosome membrane and enhances gene expression by promoting the release of DNA from the endosomal compartment (Fig. 9.1 and [31]).

It should be mentioned the majority of cytoplasmic plasmids fail to reach the nucleus due to cytoplasmic nucleases. In contrast to short nucleic acids (such as oligonucleotides) which diffuse freely, the plasmid DNA imports to nucleus by an active transport process via the nuclear pore complex and receptor proteins that include importin α, β, and RAN [32]. Nuclear localization signals or designed peptides can be linked to the plasmid DNA to facilitate nuclear import (for review see [33, 34]).

A number of therapeutic concepts have been explored in humans using more or less refined non-viral gene delivery systems in the view of therapies for genetic disorders and of immunologic disorders. As of today, despite a number of very sophisticated chemistries, non-viral vectors were not completely satisfactory in transferring genes to muscle tissues following systemic administration. Many complexes show excellent transfection activity in cell culture, but most do not perform well in the presence of serum, and only a few are active in vivo [35]. They remain

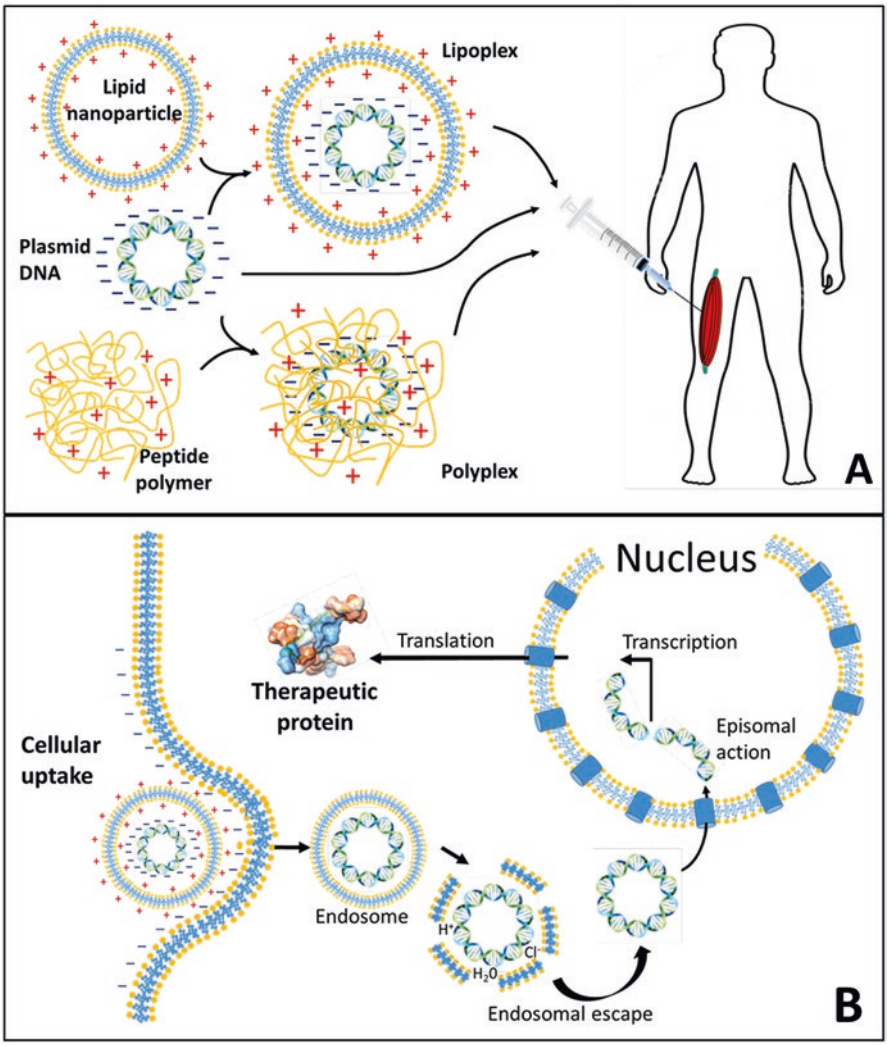

Fig. 9.1 Delivery options of non-viral vectors into skeletal muscles. (**a**) Examples of non-viral vectors, including negatively charged naked plasmid DNA (or polynucleotides) delivered either directly or combined with physical methods (ultrasound, electroporation) or complexed with various chemical entities such as cationic lipids or polymers. (**b**) Uptake pathways involve either fusion with the muscle cell membrane-, receptor-, clathrin-, caveolae-, or pinocytosis-dependent endocytosis. This is followed by endosome formation, escape from endosome, degradation, nuclear import of the plasmid DNA/polynucleotide, and transgene expression

at least 3 logs of magnitude less effective than viral vectors. Therapeutic doses require high concentrations of complexes. Besides the relatively large size of many synthetic vectors (often above 150 nm), the main obstacles in the use of synthetic complexes via systemic delivery are their aggregation, instability, toxicity, and

propensity to be captured by the mononuclear phagocyte system, leading to their rapid clearance by phagocytic cells in the liver, spleen, lungs, and bone marrow. These particles readily aggregate as their concentration increases. Toxicity is often linked to the colloidal instability of synthetic vectors resulting from interactions with molecules in biological fluids, leading to large aggregates. These aggregates, which are generally ineffective gene delivery agents, can be absorbed onto the surface of circulating red blood cells, or embolized in microvasculatures, preventing them from reaching the intended target cells. This opsonization process can also activate the complement system, one of the innate immune mechanisms against "foreign" particles within the bloodstream, which in turn activates the phagocytosis and initiates an inflammatory response [7, 19, 36]. Skeletal muscles possess poorly permeable, tight endothelial (maybe less in the case of chronically inflamed tissues) layers and a highly regulated microcirculation [37]. The implication is that one would not expect particulate systems to be distributed easily from the blood circulation to skeletal muscles. Thus, the prospects for non-viral particulate vector widespread distribution from the systemic circulation are limited at present. Only one systemic delivery attempt was initiated in a neuromuscular disease indication. This was in hereditary inclusion body myopathy in a single patient intravenously perfused with a lipoplex in a compassionate trial. The patient showed signs of increase of sialic acid-related proteins and stabilization in the decline of muscle strength [38].

The administration of vectors directly to the target tissue avoids most of the obstacles encountered by systemic delivery. However this approach remains hampered by the diffusion limitations and immune cell clearance in the interstitial region of the target organ. Indeed, transgene expression following direct intramuscular needle delivery of complexes is often localized in regions that are close to the injection site. This implies that the dispersion of colloidal particles within muscle is a critical issue, and there is a need for basic studies of the effect of formulation on dispersion within solid tissues such as skeletal muscle. Nevertheless this poor efficiency remains compatible with applications that require only low levels of the therapeutic proteins, such as genetic vaccines, cancer, or peripheral limb ischemia (Table 9.1).

Interestingly, retrograde transport seemed to be obtained as some gene expression was found in the peripheral and central nervous system following intramuscular administration [39]. Delivery of therapeutic genes to peripheral neurons upon a peripheral and minimally invasive intramuscular administration of polymeric nanoparticles was shown to be feasible in animal models [40].

9.3 "Naked" DNA

Naked DNA can be manufactured in a very cost-effective manner and is a very stable material that can be stored at room temperature for long periods of time following lyophilization. It is composed of a bacterial plasmid that contains the cDNA of the therapeutic gene under the transcriptional control of various eukaryotic

Table 9.1 Current status of non-viral vector clinical trials with skeletal muscle-based disease applications (update of Braun [7])

Condition	Transgenes/delivery procedure	Phase—status	Comments	References
Infectious disease vaccines				
HIV	18 plasmid products with various HIV-1 poly-epitopes (Gag, Pol, Vpr, Vpu, Nef, Rev, and Env) from subtypes A, B, and C, needle free injections or with ± elctroporation, adjuvant treatments (bupivacaïne), or immunostimulants (IL2/IL15), alone or in prime-boost regimens (with NYVAC-C, adenovirus 5, fowlpox, or MVA vectors. One trial with plasmid IL12 alone (completed Oct. 2016 and awaiting results)	I/II–C	Up to 8 mg plasmid, multiple (up to 3) injections. Not all trials show immune response. When observed, usually, the maximal CD4+ and CD8+ T cell responses occurred after 3 injections (transient or sustained). Always well tolerated.	[75–91]
HBV	Recombinant DNA yeast-derived hepatitis B vaccine (YDV), HBV epitope plasmid	I–C	Up to 1 mg 3 or 4 injections. Seroconversion in all patients. Increased immune response in 50% of the chronic carriers	[92, 93]
HCV	Plasmid: HCV nonstructural proteins NS3 4A, NS4B, and NS5A interleukin-12 (IL-12) + electroporation	I/II–O	Up to 9 mg	[94]
Ebola and Marburg	Biojector	I–O or C	Up to 8 mg, 3 injections. Immune responses in 20/20 vaccinees	[95, 96]
West Nile fever	WNV viral protein precursor transmembrane and envelope/ Biojector 2000	I–O	4 mg, 3 injections	
SARS	S protein of SARS-CoV/Biojector	I–O	4 mg/ 3 injections	[97]
Avian flu	Hemagglutinin 5 (H5)/Biojector	I–O	Up to 4 mg	[98]
HPV	Hpv-16 E6/E7, Hpv-18 E6/E7 followed by electroporatyion	III–O	6 mg. Efficacy in 165 CIN2/3 patients (phase II)	[99]
Cytomegalovirus	Plasmid ASP0113 or with phosphoprotein 65 and glycoprotein B epitopes in CMV-seropositive patients underegoing allogeneic hematopoietic cell transplant	II–C	Up to 5 mg. 25 or 68% immune response (seropositive vs seronegative patients). One trial with repeated 5 mg injections; safe, no CMV-viremia event in the treated patients	[100–102]

Malaria	PfCSP	I–C	Up to 2.5 mg, 3 injections. No immune response. Intramuscular jet injections better than needle intramuscular or intradermal jet injections	[103, 104]
Cancer				
Melanoma	4 trials: Mouse and human TYR DNA, gp100 ("self" nonmutated gp100 tumor antigen), or MART-1 antigens, AMEP + electroporation	I or II C	Up to 1.5 mg DNA. Safe and induced CD8+ T-cell responses in 7 of 18 patients. 2/5 trials failed to demonstrate significant clinical or immunologic responses	[105–109]
Breast cancer	2 tials: HER2/neu or mammaglobin-A	I–O		[110]
B-cell lymphoma	Tumor idiotype gp100 ± Biojector	I/II–C	Up to 1.8 mg, 3 injections	[111]
Cancers expressing HER-2 and-or CEA	HER-2, CEA prime plasmid, boost Ad5	I		[112]
Prostate	3 trials: PSMA27/pDom/with and without electroporation, or PSA + with GM-CSF (molgramostim) and IL-2 (aldesleukin)	I/II–O	Electroporation increases potency of the DNA vaccine. Anti-PSA immune response. A decrease in the slope of PSA in 2/8 patients	[113–115]
Breast, lung, or pancreatic cancer	hTERT immunotherapy alone or in combination with IL-12 DNA followed by electroporation	I/II–O	Up to 10 mg	
Allergies				
Adults allergic to peanut	Ara h1, h2, h3) lysosomal associated membrane protein plasmid	I–O		
Cardiovascular diseases				
Intermittent claudication/ arteriosclerosis	Plasmid-engineered zinc-finger transcription factor	I–O	For VEGF modulation; single injection	[116]

(continued)

Table 9.1 (continued)

Condition	Transgenes/delivery procedure	Phase—status	Comments	References
Severe peripheral artery occlusive disease (PAOD)	FGF-1	I–C	4 mg, 4 injections. No improvements in ulcer healing but 50% reduction in amputation risk (107 patients)	[117]
Critical limb ischemia	13 trials with naked plasmid-angiogenic factors: Stromal cell-derived Factor-1, HGF, pCK- VEGF165, bFGF	2 phase III–C	16 mg (in 16 injections). Trials showed significant reduction in pain and aggregate ulcer size, associated with an increased transcutaneous oxygen pressure. One phase II trial did not show difference between groups (104 patients) in secondary end points, including ankle-brachial index, toe-brachial index, pain relief, wound healing, or major amputation. One phase III trial failed (525 patients). One marketed product (Neovasculgen®: pCMV-vegf165 supercoiled) only in Russia	[118–128]
Peripheral artery disease	Plasmid Del-1 with poloxamer	II–C	A total of 84 mg of plasmid delivered as 42 intramuscular injections (2 ml per injection, 21 injections or 42 ml in in both lower extremities. Significant improvement in exercise capacity (105 patients)	[129, 130]
Thromboangiitis obliterans/ Buerger disease	HGF or VEGF165	I–C	4 mg/2 injections.Safety and improvement of ischemic symptoms at 12 weeks and 2 years after transfection. Improved perfusion to the distal ischemic limb and ulcers healing in 65–100% of the 22 patients	[131, 132]

Neurological and neuromuscular disorders				
Multiple sclerosis	Myelin basic protein	II–O	Phase I: Up to 3 mg plasmid (BHT-3009). Safe and antigen-specific immune tolerance with concordant reduction of inflammatory lesions on brain MRI	[133]
Duchenne muscular dystrophy	Full-length dystrophin	I–C	Up to 0.6 mg. Light dystrophin expression in the injected area. No immune rejection of the transgene	[66]
Diabetic peripheral neuropathy (also tested in ALS, criticla limb ischemia and foot ulcers)	HGF or VEGF-A transcription factor (zinc finger technology)	III–O	Up to 16 mg/injection. Phase II: Safe, pain score reduced in at least 50% patients	[134–136]
Cachexia	CpG-depleted plasmid GHRH + electroporation	I–O	Up to 3.5 mg	

O trial ongoing, *C* trial completed, *pCK* with Creatine Kinase promoter

regulatory elements and a bacterial origin of replication to allow production in bacteria. A strong promoter may be required for optimal expression in mammalian cells. For this, some promoters derived from viruses such as cytomegalovirus (CMV) or simian virus 40 (SV40) have been used. However, virally derived promoters, such as the CMV promoter, may not be suitable for applications to chronic diseases, as illustrated by the negative impact of inflammatory cytokines (interferon-γ or tumor necrosis factor-α) [41]. Thus, muscle-specific alternatives to the CMV promoter have been proposed, such as the desmin promoter/enhancer, which controls expression of the cytoskeletal protein desmin [42] or the creatine kinase promoter [43]. Even in vaccines, the vaccinating immune responses obtained were shown to be of a comparable magnitude to those in mice immunized with DNA vaccines containing nonspecific promoters.

For clinical efficacy and safety of chronic disease applications, it may be necessary to maintain appropriate levels of a gene product in order to prevent toxicity and to be able to modulate or resume transgene expression in response to disease evolution or immune problems. Artificial systems for the control of genes are based on two elements: a chimeric transcription factor responding to a small inducer or even electric field and an artificial promoter composed of multiple binding sites for the transcription factor followed by a minimal promoter. Inducible gene expression systems use endogenous elements that respond to exogenous signals or stress, such as cytokines, heat, metal ions, and hypoxia. However, neither muscle-specific nor inducible promoters in the absence of induction are devoid of leaky activity [44]. If hypomethylated bacterial CpG sequences are maintained on the plasmid DNA backbone or promoter elements, a T helper 1 (Th1) immune response (but only for a short period and with no induction of anti-DNA antibodies) can be generated which may however be advantageous in view of genetic vaccination, alone or in priming-boost regimens with viral vectors [45].

Following the serendipitous demonstration of transgene expression in skeletal muscle injected with naked DNA by Wolff [46], plasmid DNA has been used extensively in a variety of indications [7]. Uptake and expression of numerous transgenes have been demonstrated in various species following intramuscular administration of naked DNA. Expression peaks at around 7 days, followed by a slow decrease and a prolonged steady state (years), in case of non-immunogenic transgene. The very long-term expression is probably linked to the postmitotic state of skeletal muscles and the persistence of administered genetic material as an extrachromosomal episomal elements [47].

The efficiency of plasmid gene transfer into skeletal muscle (and other tissues) by direct injection is low (~1% of cell nuclei) and remains confined at the injection site (along the needle track) across species [48], and it further decreases with the plasmid size. Nevertheless, naked plasmid DNA administration was used in animal models to provide a systemic source of therapeutic protein, for genetic vaccination against pathogens and tumor cells or for therapeutic angiogenesis. In the later case, local gene delivery to focal lesions in the peripheral vasculature, for the production of highly active hormones, is ideally suited to the use of intramuscular or percutaneous vector delivery. In humans, intramuscular injections of naked plasmid encoding

angiogenic factors (such as VEGF165 or HGF) were used in small numbers of patients with critical limb ischemia and did demonstrate promising clinical efficacy for the treatment of peripheral arterial disease. Ischemic pain and ischemic ulcers in the affected limb were relieved or markedly improved in further trials ([49] and Table 9.1). Importantly, all those plasmid-based preclinical and clinical trials resulted in a very good safety record ([50] and Table 9.1). A meta-analysis of 12 clinical trials (1494 patients total) of local administration of pro-angiogenic growth factors (VEGF, FGF, HGF, Del-1, HIF-1alpha) using plasmid or viral gene transfer by intra-arterial or intramuscular injections showed that, despite promising results in single studies, no clear benefit could be identified in peripheral artery disease patients, irrespective of disease severity [51].

Locally injected naked DNA is being evaluated in muscle regeneration approaches such as myostatin propeptide gene gun delivery [52] and for genetic motoneuron disorders. In the later case, SMN induction in a mouse spinal muscular atrophy model was observed following intramuscular injection of a tetanus toxin C fragment plasmid [53].

Artificially or spontaneous regenerating muscle fibers display a higher, but still limited, efficiency of transfection [54]. Physical methods (electric or ultrasound pulses, ballistic gene gun), which either create transient pores in the cell membrane or increase passive diffusion, were shown to improve up to 100-fold gene transfer to skeletal muscles [55]. The pulse parameters and the type of material used (i.e., needle versus externally applied plate electrodes) are of critical importance [44]. Selective electro-sonoporation in a defined area using microbubble contrast agents showed increased plasmid-VEGF165 delivery in skeletal muscle allowing therapeutic angiogenesis in chronically ischemic skeletal muscles with undetectable tissue damage [56]. A slightly higher risk of random integration of plasmid DNA into genomic DNA may also be seen [57]. Still limited penetration of the genetic material in the tissue is obtained (in the range of ~1 cm). Widespread delivery to large or deep muscles remains challenging. Muscle damage and inflammation [58] are induced by these methods which peak at around 7 days and resolve at 3 weeks postinjection with both Th1 and Th2 immune responses potentially occurring [44]. Therefore, this strategy may not be suitable in already inflamed tissue such as DMD muscles.

9.4 Pressure-Mediated Gene Transfer

High levels of gene expression in the limb and diaphragm muscles have been achieved by the rapid injection of naked DNA in large volumes via locoregional hydrodynamic intravascular delivery with both blood inflow and outflow blocked surgically or using external tourniquets [59, 60]. The endothelium in muscle is continuous and non-fenestrated, showing low permeability to macromolecules, including plasmid DNA. The hydrodynamic pressure induces extravasation of the injected DNA, probably by expanding the endothelium and thereby making pores accessible

for DNA entry. The mechanism of plasmid DNA uptake by the muscle cells is still not clear and may involve both low-affinity receptor-mediated and nonspecific processes [1, 61]. The procedure safety is supported by a large body of data collected in mice, rats, dogs, and nonhuman primates. The edema caused by the injected fluid is resolved within 24 h and even the minimal signs of observed muscle toxicity clear within 2 weeks postinjection [62, 63]. The hind limb perfusion procedure is a rather quick and simple technique, which may be applied to chronic diseased muscles [64] or other chronic diseases such as anemia [65]. Based on successful preclinical studies using the *mdx* mouse and *golden retriever muscular dystrophy* (*GRMD*) dog models of Duchenne muscular dystrophy, and the positive (expression -though very low-, and safety) outcome of a phase I trial of intramuscular injection of MyoDys®, a full-length dystrophin plasmid, in Duchenne patients (the first completed gene transfer clinical trial in neuromuscular diseases) [66], the ground was set for a human clinical trial using MyoDys® into the forearm of Duchenne patients. A dose escalation study of single-limb perfusion with 0.9% saline was carried out in nine adults with muscular dystrophies under intravenous analgesia. The study led by Fan et al. demonstrated feasibility and safety up to 35% of limb volume in the upper extremities of the young adults with muscular dystrophy. Perfusion at 40% limb volume was associated with short-lived physiological changes in peripheral nerves without clinical correlates in one subject [67]. This study used lower cuff pressures than in our nonhuman primate studies (310–325 mm Hg vs. 450–700 mm Hg in nonhuman primates) [68, 69]. From our studies in the *mdx* mouse and *GRMD* dog models of Duchenne dystrophy, and in nonhuman primates, the minimal volume needed for efficient naked DNA limb perfusion is 40% of the limb volume [70]. Whereas arterial limb perfusion did not turn out to be safe in *GRMD* dogs (personal data not shown), up to ten consecutive naked DNA limb perfusions every other day appeared very safe in both dystrophic mice and dogs. Even though head-to-head comparison would be necessary, our studies suggested that gene transfer was higher in diseased muscles than in wild-type animals. We also noticed that the highest transfection efficiencies were found in nonhuman primates; up to 40% of limb muscles expressed reporter genes following a single-limb perfusion [68]. Therefore, limb perfusion of a naked DNA remains a valid approach to treat limb dystrophic muscles as an alternative to viral vectors in seropositive patients or in indications that require large transgenes with regional gene transfer [71].

Ex vivo approaches using gene-corrected stem cells with non-viral vectors are also being explored. Human artificial chromosome (HAC) vectors have the capacity to carry large genomic loci and to replicate and segregate autonomously without integration into the host genome. HAC vectors containing the entire human *dystrophin* gene (*DYS*-HAC) with its native regulatory elements allow dystrophin expression at levels similar to native dystrophin isoform expression levels. Since they can be stably maintained as episomal elements in host cells, the *DYS*-HAC could be introduced into several types of patient stem or progenitor cells for ex vivo therapy, e.g., induced pluripotent stem cells, mesoangioblasts, AC133, and mesenchymal stem cells [72]. One of the main issues, however, is the translatability of stem cell therapy in muscle disorders [73, 74].

9.5 Conclusion

The development of successful non-viral gene delivery systems to skeletal muscle is highly dependent on the proportion of muscle (or their innervating motoneuron) cells that need to be transfected. More than 25 years of research and testing in animal models and in human trials gear us toward two types of muscle-directed non-viral gene transfer applications:

1. Direct injection. This represents a far simpler but poorly efficient approach. Provided highly active gene products are used, non-viral gene therapy becomes increasingly amenable to infectious, cancerous, and peripheral ischemia diseases. Vectors could be both naked DNA and synthetic complexes.
2. Intravascular delivery. Simple intravenous perfusion of non-viral vectors is as of today far less practicable. Regional hydrodynamic delivery of naked DNA offers several advantages over viral vectors which hold potential for muscle diseases, including limb-girdle muscular dystrophies and peripheral neuropathies. Nevertheless, muscle gene therapy using systemic administration of non-viral vectors retains major hurdles that need to be overcome before any human applications.

Disclosure Author declares having no potential competing financial interests.

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