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
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
| 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 ± electroporation, adjuvant treatments (bupivacaine), 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 electroporation                                          | 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 undergoing 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]     |
| Disease                        | Vaccine/Immunotherapy | Phase | Dose/Details                                                                 | Source(s)       |
|-------------------------------|-----------------------|-------|-----------------------------------------------------------------------------|-----------------|
| **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                      | 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                 | 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                      | 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**                 | 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)
| 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, critical 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.

References

1. Wolff JA, Budker V (2005) The mechanism of naked DNA uptake and expression. Adv Genet 54:3–20. https://doi.org/10.1016/S0065-2660(05)54001-X

2. Lu QL, Bou-Gharios G, Partridge TA (2003) Non-viral gene delivery in skeletal muscle: a protein factory. Gene Ther 10(2):131–142. https://doi.org/10.1038/sj.gt.3301874

3. Belmadi N, Midoux P, Loyer P, Passirani C, Pichon C, Le Gall T, Jaffres PA, Lehn P, Montier T (2015) Synthetic vectors for gene delivery: an overview of their evolution depending on routes of administration. Biotechnol J 10(9):1370–1389. https://doi.org/10.1002/biot.201400841

4. Bondi ML, Craparo EF (2010) Solid lipid nanoparticles for applications in gene therapy: a review of the state of the art. Expert Opin Drug Deliv 7(1):7–18. https://doi.org/10.1517/17425240903362410

5. Magin-Lachmann C, Kotzamanis G, D’Aiuto L, Cooke H, Huxley C, Wagner E (2004) In vitro and in vivo delivery of intact BAC DNA—comparison of different methods. J Gene Med 6(2):195–209. https://doi.org/10.1002/jgm.481

6. Karmali PP, Chaudhuri A (2007) Cationic liposomes as non-viral carriers of gene medicines: resolved issues, open questions, and future promises. Med Res Rev 27(5):696–722. https://doi.org/10.1002/med.20090

7. Braun S (2008) Muscular gene transfer using nonviral vectors. Curr Gene Ther 8(5):391–405

8. Li S, Ma Z (2001) Nonviral gene therapy. Curr Gene Ther 1(2):201–226

9. Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci U S A 84(21):7413–7417
10. Behr JP (1994) Gene transfer with synthetic cationic amphiphiles: prospects for gene therapy. Bioconjug Chem 5(5):382–389

11. Boussif O, Lezoualc’h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci U S A 92(16):7297–7301

12. Wu GY, Wu CH (1987) Receptor-mediated in vivo gene transformation by a soluble DNA carrier system. J Biol Chem 262(10):4429–4432

13. Wu GY, Lee RJ (2000) Efficient gene delivery using anionic liposome-complexed polypelexes (LPDII). Biosci Rep 20(5):419–432

14. Almofti MR, Harashima H, Shinohara Y, Almofti A, Baba Y, Kiwada H (2003) Cationic liposome-mediated gene delivery: biophysical study and mechanism of internalization. Arch Biochem Biophys 410(2):246–253

15. Kostarelos K, Miller AD (2005) Synthetic, self-assembly ABCD nanoparticles; a structural paradigm for viable synthetic non-viral vectors. Chem Soc Rev 34(11):970–994. https://doi.org/10.1039/b307062j

16. Gomez JP, Pichon C, Midoux P (2013) Ability of plasmid DNA complexed with histidinylated IPEI and IPEI to cross in vitro lung and muscle vascular endothelial barriers. Gene 525(2):182–190. https://doi.org/10.1016/j.gene.2013.03.055

17. Li S, Tseng WC, Stolz DB, Wu SP, Watkins SC, Huang L (1999) Dynamic changes in the characteristics of cationic lipidic vectors after exposure to mouse serum: implications for intravenous lipofection. Gene Ther 6(4):585–594. https://doi.org/10.1038/sj.gt.3300865

18. Tseng WC, Jong CM (2003) Improved stability of polycationic vector by dextran-grafted branched polyethylenimine. Biomacromolecules 4(5):1277–1284. https://doi.org/10.1021/bm034083y

19. Plank C, Mechtler K, Szoka FC Jr, Wagner E (1996) Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. Hum Gene Ther 7(12):1437–1446. https://doi.org/10.1089/hum.1996.7.12-1437

20. Ishiwata H, Suzuki N, Ando S, Kikuchi H, Kitagawa T (2000) Characteristics and biodistribution of cationic liposomes and their DNA complexes. J Control Release 69(1):139–148

21. Lee H, Jeong JH, Park TG (2002) PEG grafted polylysine with fusogenic peptide for gene delivery: high transfection efficiency with low cytotoxicity. J Control Release 79(1-3):283–291

22. Ward CM, Pechar M, Oupicky D, Ulbrich K, Seymour LW (2002) Modification of pLL/DNA complexes with a multivalent hydrophilic polymer permits folate-mediated targeting in vitro and prolonged plasma circulation in vivo. J Gene Med 4(5):536–547. https://doi.org/10.1002/jgm.296

23. Labat-Moleur F, Steffan AM, Brisson C, Perron H, Feugeas O, Forstenberger P, Oberling F, Brambilla E, Behr JP (1996) An electron microscopy study into the mechanism of gene transfer with lipopolymamines. Gene Ther 3(11):1010–1017

24. Zuhorn IS, Kalicharan R, Hoekstra D (2002) Lipoplex-mediated transfection of mammalian cells occurs through the cholesterol-dependent clathrin-mediated pathway of endocytosis. J Biol Chem 277(20):18021–18028. https://doi.org/10.1074/jbc.M111257200

25. Conner SD, Schmid SL (2003) Regulated portals of entry into the cell. Nature 422(6927):37–44. https://doi.org/10.1038/nature01451

26. El-Sayed A, Harashima H (2013) Endocytosis of gene delivery vectors: from clathrin-dependent to lipid raft-mediated endocytosis. Mol Ther 21(6):1118–1130. https://doi.org/10.1038/mt.2013.54

27. Keswani RK, Lazebnik M, Pack DW (2015) Intracellular trafficking of hybrid gene delivery vectors. J Control Release 207:120–130. https://doi.org/10.1016/j.jconrel.2015.04.015

28. Bartsch M, Weeke-Klimp AH, Meijer DK, Scherphof GL, Kamps JA (2005) Cell-specific targeting of lipid-based carriers for ODN and DNA. J Liposome Res 15(1-2):59–92. https://doi.org/10.1081/LPR-64961

29. Lechardeur D, Sohn KJ, Haardt M, Joshi PB, Monck M, Graham RW, Beatty B, Squire J, O’Brodovich H, Lukacs GL (1999) Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer. Gene Ther 6(4):482–497. https://doi.org/10.1038/sj.gt.3300867
Non-viral Vector for Muscle-Mediated Gene Therapy

30. Zelphati O, Szoka FC Jr (1996) Mechanism of oligonucleotide release from cationic liposomes. Proc Natl Acad Sci U S A 93(21):11493–11498
31. Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ (1995) Cellular and molecular barriers to gene transfer by a cationic lipid. J Biol Chem 270(32):18997–19007
32. Wilson GL, Dean BS, Wang G, Dean DA (1999) Nuclear import of plasmid DNA in digitonin-permeabilized cells requires both cytoplasmic factors and specific DNA sequences. J Biol Chem 274(31):22025–22032
33. Hebert E (2003) Improvement of exogenous DNA nuclear importation by nuclear localization signal-bearing vectors: a promising way for non-viral gene therapy? Biol Cell 95(2):59–68
34. Simonson OE, Svahn MG, Tornquist E, Lundin KE, Smith CI (2005) Bioplex technology: novel synthetic gene delivery pharmaceutical based on peptides anchored to nucleic acids. Curr Pharm Des 11(28):3671–3680
35. Liu D, Ren T, Gao X (2003) Cationic transfection lipids. Curr Med Chem 10(14):1307–1315
36. Pouton CW, Seymour LW (2001) Key issues in non-viral gene delivery. Adv Drug Deliv Rev 46(1-3):187–203
37. Hudlicka O (2011) Microcirculation in skeletal muscle. Muscles Ligaments Tendons J 1(1):3–11
38. Nemunaitis G, Jay CM, Maples PB, Gahl WA, Huizing M, Yardeni T, Tong AW, Phadke AP, Pappen BO, Bedell C, Allen H, Hernandez C, Templeton NS, Kuhn J, Senzer N, Nemunaitis J (2011) Hereditary inclusion body myopathy: single patient response to intravenous dosing of GNE gene lipoplex. Hum Gene Ther 22(11):1331–1341. https://doi.org/10.1089/hum.2010.192
39. Kato N, Nakanishi K, Nemoto K, Morishita R, Kaneda Y, Uenoyma M, Ikeda T, Fujikawa K (2003) Efficient gene transfer from innervated muscle into rat peripheral and central nervous systems using a non-viral haemagglutinating virus of Japan (HVJ)-liposome method. J Neurochem 85(3):810–815
40. Lopes CD, Goncalves NP, Gomes CP, Saraiva MJ, Pego AP (2017) BDNF gene delivery mediated by neuron-targeted nanoparticles is neuroprotective in peripheral nerve injury. Biomaterials 121:83–96. https://doi.org/10.1016/j.biomaterials.2016.12.025
41. Qin L, Ding Y, Pahud DR, Chang E, Imperiale MJ, Bromberg JS (1997) Promoter attenuation in gene therapy; interferon-gamma and tumor necrosis factor-alpha inhibit transgene expression. Hum Gene Ther 8(17):2019–2029. https://doi.org/10.1089/hum.1997.8.17-2019
42. Kwissa M, von Kampen v K, Zurbriggen R, Gluck R, Reimann J, Schirmbeck R (2000) Efficient vaccination by intradermal or intramuscular inoculation of plasmid DNA expressing hepatitis B surface antigen under desmin promoter/enhancer control. Vaccine 18(22):2337–2344
43. Bartlett RJ, Secore SL, Singer JT, Bodo M, Sharma K, Ricordi C (1996) Long-term expression of a fluorescent reporter gene via direct injection of plasmid vector into mouse skeletal muscle: comparison of human creatine kinase and CMV promoter expression levels in vivo. Cell Transplant 5(3):411–419
44. Trollet C, Bloquel C, Scherman D, Bigey P (2006) Electrottransfer into skeletal muscle for protein expression. Curr Gene Ther 6(5):561–578
45. Verthelyi D (2006) Adjuvant properties of CpG oligonucleotides in primates. Methods Mol Med 127:139–158. https://doi.org/10.1385/1-59745-168-1:139
46. Wolff JA, Malone RW, Williams P, Chong W, Acscadi G, Jani A, Felgner PL (1990) Direct gene transfer into mouse muscle in vivo. Science 247(4949 Pt 1):1465–1468
47. Wolff JA, Ludtke JJ, Acscadi G, Williams P, Jani A (1992) Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. Hum Mol Genet 1(6):363–369
48. Jiao S, Williams P, Berg RK, Hodgeman BA, Liu L, Repetto G, Wolff JA (1992) Direct gene transfer into nonhuman primate myofibers in vivo. Hum Gene Ther 3(1):21–33. https://doi.org/10.1089/hum.1992.3.1.21
49. Trollet C, Scherman D, Bigey P (2008) Delivery of DNA into muscle for treating systemic diseases: advantages and challenges. Methods Mol Biol 423:199–214. https://doi.org/10.1007/978-1-59745-194-9_14
50. Gaffney MM, Hynes SO, Barry F, O’Brien T (2007) Cardiovascular gene therapy: current status and therapeutic potential. Br J Pharmacol 152(2):175–188. https://doi.org/10.1038/sj.bjp.0707315

51. Hammer A, Steiner S (2013) Gene therapy for therapeutic angiogenesis in peripheral arterial disease - a systematic review and meta-analysis of randomized, controlled trials. Vasa 42(5):331–339. https://doi.org/10.1024/0301-1526/a000298

52. Tsai SW, Tung YT, Chen HL, Yang SH, Liu CY, Lu M, Pai HJ, Lin CC, Chen CM (2016) Myostatin propeptide gene delivery by gene gun ameliorates muscle atrophy in a rat model of botulinum toxin-induced nerve denervation. Life Sci 146:15–23. https://doi.org/10.1016/j.lfs.2015.12.056

53. Olivan S, Calvo AC, Rando A, Herrando-Grabulosa M, Manzano R, Zaragoza P, Tizzano EF, Aquilera J, Osta R (2016) Neuroprotective effect of non-viral gene therapy treatment based on tetanus toxin C-fragment in a severe mouse model of spinal muscular atrophy. Front Mol Neurosci 9:76. https://doi.org/10.3389/fnmol.2016.00076

54. Vitadello M, Schiaffino MV, Picard A, Scarpa M, Schiaffino S (1994) Gene transfer in regenerating muscle. Hum Gene Ther 5(1):11–18. https://doi.org/10.1089/hum.1994.5.1-11

55. Favard C, Dean DS, Rols MP (2007) Electrotransfer as a non viral method of gene delivery. Curr Gene Ther 7(1):67–77

56. Yamashita Y, Shimada M, Tachibana K, Harimoto N, Tsujita E, Shirabe K, Miyazaki J, Sugimachi K (2002) In vivo gene transfer into muscle via electro-sonoporation. Hum Gene Ther 13(17):2079–2084. https://doi.org/10.1089/104304260395929

57. Wang Z, Troilo PJ, Wang X, Griffiths TG, Pacchione SJ, Barnum AB, Harper LB, Pauley CJ, Niu Z, Denisova L, Follmer TT, Rizzuto G, Ciliberto G, Fattori E, Monica NL, Manam S, Ledwith BJ (2004) Detection of integration of plasmid DNA into host genomic DNA following intramuscular injection and electroporation. Gene Ther 11(8):711–721. https://doi.org/10.1038/sj.gt.3302213

58. Wang S, Zhang C, Zhang L, Li J, Huang Z, Lu S (2008) The relative immunogenicity of DNA vaccines delivered by the intramuscular needle injection, electroporation and gene gun methods. Vaccine 26(17):2100–2110. https://doi.org/10.1016/j.vaccine.2008.02.033

59. Budker V, Zhang G, Danko I, Williams P, Wolff J (1998) The efficient expression of intravascularly delivered DNA in rat muscle. Gene Ther 5(2):272–276. https://doi.org/10.1038/sj.gt.3300572

60. Hagstrom JE (2003) Plasmid-based gene delivery to target tissues in vivo: the intravascular approach. Curr Opin Mol Ther 5(4):338–344

61. Budker V, Budker T, Zhang G, Subbotin V, Loomis A, Wolff JA (2000) Hypothesis: naked plasmid DNA is taken up by cells in vivo by a receptor-mediated process. J Gene Med 2(2):76–88. https://doi.org/10.1002/(SICI)1521-2254(200003/04):2<76::AID-JGM97>3.0.CO;2-4

62. Toumi H, Hegge J, Subbotin V, Noble M, Herweijer H, Best TM, Hagstrom JE (2006) Rapid intravascular injection into limb skeletal muscle: a damage assessment study. Mol Ther 13(1):229–236. https://doi.org/10.1016/j.ymthe.2005.07.699

63. Vigen KK, Hegge JO, Zhang G, Mukherjee R, Braun S, Grist TM, Wolff JA (2007) Magnetic resonance imaging-monitored plasmid DNA delivery in primate limb muscle. Hum Gene Ther 18(3):257–268. https://doi.org/10.1089/hum.2006.115

64. Duan D (2008) Myodys, a full-length dystrophin plasmid vector for Duchenne and Becker muscular dystrophy gene therapy. Curr Opin Mol Ther 10(1):86–94

65. Sebestyen MG, Hegge JO, Noble MA, Lewis DL, Herweijer H, Wolff JA (2007) Progress toward a nonviral gene therapy protocol for the treatment of anemia. Hum Gene Ther 18(3):269–285. https://doi.org/10.1089/hum.2006.186

66. Romero NB, Braun S, Benveniste O, Leturcq F, Hogrel JY, Morris GE, Barois A, Eymard B, Payan C, Ortega V, Boch AL, Lejean L, Thioloudelet C, Mourot B, Escot C, Chequel A, Recan D, Kaplan JC, Dickson G, Klatzmann D, Molinier-Frenckel V, Guillet JG, Squiban P, Herson S, Fardeau M (2004) Phase I study of dystrophin plasmid-based gene therapy in Duchenne/Becker muscular dystrophy. Hum Gene Ther 15(11):1065–1076. https://doi.org/10.1089/hum.2004.15.1065
67. Fan Z, Kocis K, Valley R, Howard JF Jr, Chopra M, Chen Y, An H, Lin W, Muenzer J, Powers W (2015) High-pressure transvenous perfusion of the upper extremity in human muscular dystrophy: a safety study with 0.9% saline. Hum Gene Ther 26(9):614–621. https://doi.org/10.1089/hum.2015.023

68. Hegge JO, Wooddell CI, Zhang G, Hagstrom JE, Braun S, Huss T, Sebestyen MG, Emborg ME, Wolff JA (2010) Evaluation of hydrodynamic limb vein injections in nonhuman primates. Hum Gene Ther 21(7):829–842. https://doi.org/10.1089/hum.2009.172

69. Wooddell CI, Hegge JO, Zhang G, Sebestyen MG, Noble M, Griffin JB, Pfannes LV, Herweijer H, Hagstrom JE, Braun S, Huss T, Wolff JA (2011) Dose response in rodents and nonhuman primates after hydrodynamic limb vein delivery of naked plasmid DNA. Hum Gene Ther 22(7):889–903. https://doi.org/10.1089/hum.2010.160

70. Zhang G, Wooddell CI, Hegge JO, Griffin JB, Huss T, Braun S, Wolff JA (2010) Functional efficacy of dystrophin expression from plasmids delivered to mdx mice by hydrodynamic limb vein injection. Hum Gene Ther 21(2):221–237. https://doi.org/10.1089/hum.2009.133

71. Braun S (2013) Gene-based therapies of neuromuscular disorders: an update and the pivotal role of patient organizations in their discovery and implementation. J Gene Med 15(11-12):397–413. https://doi.org/10.1002/jgm.2747

72. Stevenson FK, Ottensmeier CH, Johnson P, Zhu D, Buchan SL, McCann KJ, Roddick JS, King AT, McNicholl F, Savelyeva N, Rice J (2004) DNA vaccines to attack cancer. Proc Natl Acad Sci U S A 101(Suppl 2):14646–14652. https://doi.org/10.1073/pnas.0404896101

73. Berry SE (2015) Concise review: mesoangioblast and mesenchymal stem cell therapy for muscular dystrophy: progress, challenges, and future directions. Stem Cells Transl Med 4(1):91–98. https://doi.org/10.5966/sctm.2014-0060

74. Briggs D, Morgan JE (2013) Recent progress in satellite cell/myoblast engraftment—relevance for therapy. FEBS J 280(17):4281–4293. https://doi.org/10.1111/febs.12273

75. Catanzaro AT, Roederer M, Koup RA, Bailer RT, Enama ME, Nason MC, Martin JE, Rucker S, Andrews CA, Gomez PL, Mascola JR, Nabel GJ, Graham BS, Team VRC (2007) Phase I clinical evaluation of a six-plasmid multiclade HIV-1 DNA candidate vaccine. Vaccine 25(20):4085–4092. https://doi.org/10.1016/j.vaccine.2007.02.050

76. Cebere I, Dorrell L, McShane H, Simmons A, McCormack S, Schmidt C, Smith C, Brooks M, Roberts JE, Darwin SC, Fast PE, Conlon C, Rowland-Jones S, McMichael AJ, Hanke T (2006) Phase I clinical trial safety of DNA- and modified virus Ankara-vectored human immunodeficiency virus type 1 (HIV-1) vaccines administered alone and in a prime-boost regime to healthy HIV-1-uninfected volunteers. Vaccine 24(4):417–425. https://doi.org/10.1016/j.vaccine.2005.08.041

77. Chong SY, Egan MA, Kutzler MA, Megati S, Masood A, Roopchard V, Garcia-Hand D, Montefiori DC, Quiroz J, Rosati M, Schadeck EB, Boyer JD, Pavlakis GN, Weiner DB, Sidhu M, Eldridge JH, Israel ZR (2007) Comparative ability of plasmid IL-12 and IL-15 to enhance cellular and humoral immune responses elicited by a SIVgag plasmid DNA vaccine and alter disease progression following SHIV(89.6P) challenge in rhesus macaques. Vaccine 25(26):4967–4982. https://doi.org/10.1016/j.vaccine.2006.11.070

78. Cox KS, Clair JH, Prokop MT, Sykes KJ, Dubey SA, Shiver JW, Robertson MN, Casimiro DR (2008) DNA gag/adenovirus type 5 (Ad5) gag and Ad5 gag/Ad5 gag vaccines induce distinct T-cell response profiles. J Virol 82(16):8161–8171. https://doi.org/10.1128/JVI.00620-08

79. Eller MA, Eller LA, Opollo MS, Ouma BJ, Oballah PO, Galley L, Karnasuta C, Kim SR, Robb ML, Michael NL, Kibwika H, Wabwire-Mangen F, Graham BS, Birx DL, de Souza MS, Cox JH (2007) Induction of HIV-specific functional immune responses by a multiclade HIV-1 DNA vaccine candidate in healthy Ugandans. Vaccine 25(45):7737–7742. https://doi.org/10.1016/j.vaccine.2007.08.056

80. Goonetilleke N, Moore S, Dally L, Winstone N, Cebere I, Mahmoud A, Pinheiro S, Gillespie G, Brown D, Loach V, Roberts J, Guimarães-Walker A, Hayes P, Loughran K, Smith C, De Bont J, Verlinde C, Vooijs D, Schmidt C, Boaz M, Gilmour J, Fast P, Dorrell L, Hanke T, McMichael AJ (2006) Induction of multifunctional human immunodeficiency virus type 1
(HIV-1)-specific T cells capable of proliferation in healthy subjects by using a prime-boost regimen of DNA- and modified vaccinia virus Ankara-vectored vaccines expressing HIV-1 Gag coupled to CD8+ T-cell epitopes. J Virol 80(10):4717–4728. https://doi.org/10.1128/JVI.80.10.4717-4728.2006

81. Gorse GJ, Baden LR, Wecker M, Newman MJ, Ferrari G, Weinhold KJ, Livingston BD, Villafana TL, Li H, Noonan E, Russell ND, Network HIVVT (2008) Safety and immunogenicity of cytotoxic T-lymphocyte poly-epitope, DNA plasmid (EP HIV-1090) vaccine in healthy, human immunodeficiency virus type 1 (HIV-1)-uninfected adults. Vaccine 26(2):215–222. https://doi.org/10.1016/j.vaccine.2007.10.061

82. Graham BS, Koup RA, Roederer M, Bailer RT, Enama ME, Moodie Z, Martin JE, McCluskey MM, Chakrabarti BK, Lamoreaux L, Andrews CA, Gomez PL, Mascola JR, Nabel GJ, Vaccine Research Center 004 Study Team (2006) Phase 1 safety and immunogenicity evaluation of a multiclade HIV-1 DNA candidate vaccine. J Infect Dis 194(12):1650–1660. https://doi.org/10.1086/509259

83. Hanke T, Goonetilleke N, McMichael AJ, Dorrell L (2007) Clinical experience with plasmid DNA- and modified vaccinia virus Ankara-vectored human immunodeficiency virus type 1 clade A vaccine focusing on T-cell induction. J Gen Virol 88(Pt 1):1–12. https://doi.org/10.1099/vir.0.82493-0

84. Kelleher AD, Puls RL, Bebbington M, Boyle D, Ffrench R, Kent SJ, Kippax S, Purcell DF, Thomson S, Wand H, Cooper DA, Emery S (2006) A randomized, placebo-controlled phase I trial of DNA prime, recombinant fowlpox virus boost prophylactic vaccine for HIV-1. AIDS 20(2):294–297. https://doi.org/10.1097/01.aids.0000199819.40079.e9

85. MacGregor RR, Boyer JD, Ugen KE, Lacy KE, Gluckman SJ, Bagarazzi ML, Chattergoon MA, Baine Y, Higgins TJ, Ciccarelli RB, Coney LR, Ginsberg RS, Weiner DB (1998) First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. J Infect Dis 178(1):92–100

86. McCormack S, Stohr W, Barber T, Bart PA, Harari A, Moog C, Ciuffreda D, Cellera C, Cowen M, Gamboni R, Burnet S, Legg K, Brodnicki E, Wolf H, Wagner R, Heeney J, Frachette MJ, Tartaglia J, Babiker A, Pantaleo G, Weber J (2008) EV02: a phase I trial to compare the safety and immunogenicity of HIV DNA-C prime-NYVAC-C boost to NYVAC-C alone. Vaccine 26(25):3162–3174. https://doi.org/10.1016/j.vaccine.2008.02.072

87. Mulligan MJ, Russell ND, Celum C, Kahn J, Noonan E, Montefiori DC, Ferrari G, Weinhold KJ, Smith JM, Amara RR, Robinson HL, Network NNDHVT (2006) Excellent safety and tolerability of the human immunodeficiency virus type 1 pGAG/JS2 plasmid DNA priming vector vaccine in HIV type 1 uninfected adults. AIDS Res Hum Retrovir 22(7):678–683. https://doi.org/10.1089/aid.2006.22.678

88. Mwau M, Cebere I, Sutton J, Chikoti P, Winstone N, Wee EG, Beattie T, Chen YH, Dorrell L, McShane H, Schmidt C, Brooks M, Patel S, Roberts J, Conlon C, Rowland-Jones SL, Bwayo JJ, McMichael AJ, Hanke T (2004) A human immunodeficiency virus 1 (HIV-1) clade A vaccine in clinical trials: stimulation of HIV-specific T-cell responses by DNA and recombinant modified vaccinia virus Ankara (MVA) vaccines in humans. J Gen Virol 85(4):911–919. https://doi.org/10.1099/vir.0.19701-0

89. Tavel JA, Martin JE, Kelly GG, Enama ME, Shen JM, Gomez PL, Andrews CA, Koup RA, Bailer RT, Stein JA, Roederer M, Nabel GJ, Graham BS (2007) Safety and immunogenicity of a Gag-Pol candidate HIV-1 DNA vaccine administered by a needle-free device in HIV-1-seronegative subjects. J Acquir Immune Defic Syndr 44(5):601–605. https://doi.org/10.1097/QAI.0b013e3180417ecb6

90. Wang S, Kennedy JS, West K, Montefiori DC, Coley S, Lawrence J, Shen S, Green S, Rothman AL, Ennis FA, Arthos J, Pal R, Markham P, Lu S (2008) Cross-subtype antibody and cellular immune responses induced by a polyvalent DNA prime-protein boost HIV-1 vaccine in healthy human volunteers. Vaccine 26(8):1098–1110. https://doi.org/10.1016/j.vaccine.2007.12.024

91. Wilson CC, Newman MJ, Livingston BD, MaWhinney S, Forster JE, Scott J, Schooley RT, Benson CA (2008) Clinical phase 1 testing of the safety and immunogenicity of an epitope-
175
based DNA vaccine in human immunodeficiency virus type 1-infected subjects receiving
highly active antiretroviral therapy. Clin Vaccine Immunol 15(6):986–994. https://doi.org/10.1128/CVI.00492-07
92. Goilav C, Prinsen H, Piot P (1990) Protective efficacy of a recombinant DNA vaccine against
hepatitis B in male homosexuals: results at 36 months. Vaccine 8(Suppl):S50–S52; discussion
S60–S62
93. Mancini-Bourgine M, Fontaine H, Scott-Algara D, Pol S, Brechet C, Michel ML (2004)
Induction or expansion of T-cell responses by a hepatitis B DNA vaccine administered to
chronic HBV carriers. Hepatology 40(4):874–882. https://doi.org/10.1002/hep.20408
94. Latimer B, Toporovski R, Yan J, Pankhong P, Morrow MP, Khan AS, Sardesai NY, Welles
SL, Jacobson JM, Weiner DB, Kutzler MA (2014) Strong HCV NS3/4a, NS4b, NS5a, NS5b-
specific cellular immune responses induced in Rhesus macaques by a novel HCV genotype
1a/1b consensus DNA vaccine. Hum Vaccin Immunother 10(8):2357–2365. https://doi.org/10.4161/hv.29590
95. Martin JE, Sullivan NJ, Enama ME, Gordon JJ, Roederer M, Koup RA, Bailier RT, Chakrabarti
BK, Bailey MA, Gomez PL, Andrews CA, Moodie Z, Gu L, Stein JA, Nabel GJ, Graham BS
(2006) A DNA vaccine for Ebola virus is safe and immunogenic in a phase I clinical trial.
Clin Vaccine Immunol 13(11):1267–1277. https://doi.org/10.1128/CVI.00162-06
96. Sullivan NJ, Sanchez A, Rollin PE, Yang ZY, Nabel GJ (2000) Development of a prevent-
ive vaccine for Ebola virus infection in primates. Nature 408(6812):605–609. https://doi.org/10.1038/35046108
97. Sheets RL, Stein J, Manetz TS, Andrews C, Bailier R, Rathmann J, Gomez PL (2006)
Toxicological safety evaluation of DNA plasmid vaccines against HIV-1, Ebola, Severe Acute
Respiratory Syndrome, or West Nile virus is similar despite differing plasmid backbones or
gene-inserts. Toxicol Sci 91(2):620–630. https://doi.org/10.1093/toxsci/ kfj170
98. Cinatl J Jr, Michaelis M, Doerr HW (2007) The threat of avian influenza A (H5N1). Part IV:
development of vaccines. Med Microbiol Immunol 196(4):213–225. https://doi.org/10.1007/s00430-007-0052-3
99. Trimble CL, Morrow MP, Kraynyak KA, Shen X, Dallas M, Yan J, Edwards L, Parker RL,
Denny L, Giffear M, Brown AS, Marcozzi-Pierce K, Shah D, Slager AM, Sylvester AJ, Khan
A, Broderick KE, Juba RJ, Herring TA, Boyer J, Lee J, Sardesai NY, Weiner DB, Bagarazzi
ML (2015) Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA
vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepi-
thelial neoplasia 2/3: a randomised, double-blind, placebo-controlled phase 2b trial. Lancet
386(10008):2078–2088. https://doi.org/10.1016/S0140-6736(15)00239-1
100. Mori T, Kanda Y, Takenaka K, Okamoto S, Kato J, Kanda J, Yoshimoto G, Gondo H, Doi S,
Inaba M, Koidera Y (2017) Safety of ASP0113, a cytomegalovirus DNA vaccine, in recipi-
ents undergoing allogeneic hematopoietic cell transplantation: an open-label phase 2 trial. Int
J Hematol 105(2):206–212. https://doi.org/10.1007/s12185-016-2110-3
101. Smith LR, Wloch MK, Chaplin JA, Gerber M, Rolland AP (2013) Clinical development of
a cytomegalovirus DNA vaccine: from product concept to pivotal phase 3 trial. Vaccines
(Basel) 1(4):398–414. https://doi.org/10.3390/vaccines1040398
102. Wloch MK, Smith LR, Boutsaboualy S, Reyes L, Han C, Kehler J, Smith HD, Selk L,
Nakamura R, Brown JM, Marbury T, Wald A, Rolland A, Kaslow D, Evans T, Boeckh M
(2008) Safety and immunogenicity of a bivalent cytomegalovirus DNA vaccine in healthy
adult subjects. J Infect Dis 197(12):1634–1642. https://doi.org/10.1086/588385
103. Le TP, Coonan KM, Hedstrom RC, Charoenvit Y, Sedegah M, Epstein JE, Kumar S, Wang R,
Doolan DL, Maguire JD, Parker SE, Hobart P, Norman J, Hoffman SL (2000) Safety, toler-
ability and humoral immune responses after intramuscular administration of a malaria DNA
vaccine to healthy adult volunteers. Vaccine 18(18):1893–1901
104. Richie TL, Charoenvit Y, Wang R, Epstein JE, Hedstrom RC, Kumar S, Luke TC, Freilich
DA, Aguilar JC, Sacci JB Jr, Sedegah M, Nosek RA Jr, De La Vega P, Berzins MP, Majam
VF, Abot EN, Ganesan H, Richie NO, Banania JG, Baraceros MF, Geter TG, Mere R,
Bebris L, Limbach K, Hickey BW, Lanar DE, Ng J, Shi M, Hobart PM, Norman JA, Soisson LA, Hollingdale MR, Rogers WO, Doolan DL, Hoffman SL (2012) Clinical trial in healthy malaria-naive adults to evaluate the safety, tolerability, immunogenicity and efficacy of MuStDO5, a five-gene, sporozoite/hepatic stage Plasmodium falciparum DNA vaccine combined with escalating dose human GM-CSF DNA. Hum Vaccin Immunother 8(11):1564–1584. https://doi.org/10.4161/hv.22129

105. Hawkins WG, Gold JS, Dyall R, Wolchok JD, Hoos A, Bowne WB, Srinivasan R, Houghton AN, Lewis JJ (2000) Immunization with DNA coding for gp100 results in CD4 T-cell independent antitumor immunity. Surgery 128(2):273–280. https://doi.org/10.1067/msy.2000.107421

106. Rosenberg SA, Yang JC, Sherry RM, Hwu P, Topalian SL, Schwartzztuber DJ, Restifo NP, Haworth LR, Seipp CA, Freezer LJ, Morton KE, Mavroukakis SA, White DE (2003) Inability to immunize patients with metastatic melanoma using plasmid DNA encoding the gp100 melanoma-melanocyte antigen. Hum Gene Ther 14(8):709–714. https://doi.org/10.1089/humc.2012.240

107. Spanggaard I, Snoj M, Cavalcanti A, Bouquet C, Sersa G, Robert C, Cemazar M, Dam E, Vasquez B, Attali P, Mir LM, Gehl J (2013) Gene electrotransfer of plasmid antiangiogenic metargidin peptide (AMEP) in disseminated melanoma: safety and efficacy results of a phase I first-in-man study. Hum Gene Ther Clin Dev 24(3):99–107. https://doi.org/10.1089/humc.2012.240

108. Triozzi PL, Aldrich W, Allen KO, Carlisle RR, LoBuglio AF, Conry RM (2005) Phase I study of a plasmid DNA vaccine encoding MART-1 in patients with resected melanoma at risk for relapse. J Immunother 28(4):382–388

109. Wolchok JD, Yuan J, Houghton AN, Gallardo HF, Rasalan TS, Wang J, Zhang Y, Ranganathan R, Chapman PB, Krown SE, Livingston PO, Heywood M, Riviere I, Panageas KS, Terzulli SL, Perales MA (2007) Safety and immunogenicity of tyrosinase DNA vaccines in patients with melanoma. Mol Ther 15(11):2044–2050. https://doi.org/10.1038/sj.mt.6300290

110. Viehl CT, Frey DM, Phommaly C, Chen T, Fleming TP, Gillanders WE, Eberlein TJ, Goedegebuure PS (2008) Generation of mammaglobin-A-specific CD4 T cells and identification of candidate CD4 epitopes for breast cancer vaccine strategies. Breast Cancer Res Treat 109(2):305–314. https://doi.org/10.1007/s10549-007-9657-x

111. Timmerman JM, Singh G, Hermanson G, Hobart P, Czerwinski DK, Taidi B, Rajapaksra R, Caspar CB, Van Beckhoven A, Levy R (2002) Immunogenicity of a plasmid DNA vaccine encoding chimeric idioype in patients with B-cell lymphoma. Cancer Res 62(20):5845–5852

112. Smorlesi A, Papalini F, Pierpaoli S, Provinciali M (2008) HER2/neuDNA vaccination for breast tumors. Methods Mol Biol 423:473–485. https://doi.org/10.1007/978-1-59745-194-9_37

113. Pavlenko M, Roos AK, Nordquist LT, Slovin SF, Scher HI (2003) DNA vaccines: an active immunization strategy for prostate cancer. Semin Oncol 30(5):659–666

114. Rebar EJ (2004) Development of pro-angiogenic engineered transcription factors for the treatment of cardiovascular disease. Expert Opin Investig Drugs 13(7):829–839. https://doi.org/10.1083/sj.bjc.6602019

115. Nikol S, Baumgartner I, Van Belle E, Diehm C, Visona A, Capogrossi MC, Ferreira-Maldent N, Gallino A, Graham Wyatt M, Dinesh Wijeginghe L, Fusari M, Stephan D, Emmerich J, Pompilio G, Vermassen F, Pham E, Grek V, Coleman M, Meyer F (2008) Therapeutic angiogenesis with intramuscular NV1FGF improves amputation-free survival in patients with critical limb ischemia. Mol Ther 16(5):972–978. https://doi.org/10.1038/mt.2008.33
118. Baumgartner I, Pieczek A, Manor O, Blair R, Kearney M, Walsh K, Isner JM (1998) Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. Circulation 97(12):1114–1123
119. Belch J, Hiatt WR, Baumgartner I, Driver IV, Nikol S, Norgren L, Van Belle E, TAMARIS Committees and Investigators (2011) Effect of fibroblast growth factor NV1FGF on amputation and death: a randomised placebo-controlled trial of gene therapy in critical limb ischaemia. Lancet 377(9781):1929–1937. https://doi.org/10.1016/S0140-6736(11)60394-2
120. Comerota AJ, Throm RC, Miller KA, Henry T, Chronos N, Laird J, Sequeira R, Kent CK, Bacchetta M, Goldman C, Salenius JP, Schmiede FA, Pilsudski R (2002) Naked plasmid DNA encoding fibroblast growth factor type I for the treatment of end-stage unreconstructible lower extremity ischemia: preliminary results of a phase I trial. J Vasc Surg 35(5):930–936
121. Cui S, Guo L, Li X, Gu Y, Fu J, Dong L, Song H, Chen X, Lu Y, Hu C, Xiao F, Zhu D, Wu Z, Zhang Q (2015) Clinical safety and preliminary efficacy of plasmid pUDK-HGF expressing human hepatocyte growth factor (HGF) in patients with critical limb ischemia. Eur J Vasc Endovasc Surg 50(4):494–501. https://doi.org/10.1016/j.ejvs.2015.05.007
122. Freedman SB, Vale P, Kalka C, Kearney M, Pieczek A, Symes J, Losordo D, Isner JM (2002) Plasma vascular endothelial growth factor (VEGF) levels after intramuscular and intramyocardial gene transfer of VEGF-1 plasmid DNA. Hum Gene Ther 13(13):1595–1603. https://doi.org/10.1089/10430340260201680
123. Kim HJ, Jang SY, Park JI, Byun J, Kim DI, Do YS, Kim JM, Kim S, Kim BM, Kim WB, Kim DK (2004) Vascular endothelial growth factor-induced angiogenic gene therapy in patients with peripheral artery disease. Exp Mol Med 36(4):336–344. https://doi.org/10.1038/emmm.2004.44
124. Kusumanto YH, van Weel V, Mulder NH, Smit AJ, van den Dungen JJ, Hooymans JM, Sluiter WJ, Tio RA, Quax PH, Gans RO, Dullaart RP, Hospers GA (2006) Treatment with intramuscular vascular endothelial growth factor gene compared with placebo for patients with diabetes mellitus and critical limb ischemia: a double-blind randomized trial. Hum Gene Ther 17(6):683–691. https://doi.org/10.1089/hum.2006.17.683
125. Lazarous DF, Unger EF, Epstein SE, Airevalo JL, Chew EY, Quyyumi AA (2000) Basic fibroblast growth factor in patients with intermittent claudication: results of a phase I trial. J Am Coll Cardiol 36(4):1239–1244
126. Powell RJ, Simons M, Mendelsohn FO, Daniel G, Henry TD, Koga M, Morishita R, Annex BH (2008) Results of a double-blind, placebo-controlled study to assess the safety of intramuscular injection of hepatocyte growth factor plasmid to improve limb perfusion in patients with critical limb ischemia. Circulation 118(1):58–65. https://doi.org/10.1161/CIRCULATIONAHA.107.727347
127. Shah PB, Losordo DW (2005) Non-viral vectors for gene therapy: clinical trials in cardiovascular disease. Adv Genet 54:339–361. https://doi.org/10.1007/s00065-2660(05)54014-8
128. Shyu KG, Chang H, Wang BW, Kuan P (2003) Intramuscular vascular endothelial growth factor gene therapy in patients with chronic critical leg ischemia. Am J Med 114(2):85–92
129. Grossman PM, Mendelsohn F, Henry TD, Hermiller JB, Litt M, Saucedo JF, Weiss RJ, Kandzari DE, Kleiman N, Anderson RD, Gottlieb D, Karlsberg R, Smell J, Rocha-Singh K (2007) Results from a phase II multicenter, double-blind placebo-controlled study of Del-1 (VLTS-589) for intermittent claudication in subjects with peripheral arterial disease. Am Heart J 153(5):874–880. https://doi.org/10.1016/j.ahj.2007.01.038
130. Rajagopalan S, Olin JW, Young S, Erikson M, Grossman PM, Mendelsohn FO, Regensteiner JG, Hiatt WR, Annex BH (2004) Design of the Del-1 for therapeutic angiogenesis trial (DELTa-1), a phase II multicenter, double-blind, placebo-controlled trial of VLTS-589 in subjects with intermittent claudication secondary to peripheral arterial disease. Hum Gene Ther 15(6):619–624. https://doi.org/10.1089/10430340432314060
131. Isner JM, Baumgartner I, Rauh G, Schainfeld R, Blair R, Manor O, Razvi S, Symes JF (1998) Treatment of thromboangiitis obliterans (Buerger’s disease) by intramuscular gene transfer of vascular endothelial growth factor: preliminary clinical results. J Vasc Surg 28(6):964–973; discussion 973-965
132. Makino H, Aoki M, Hashiya N, Yamasaki K, Azuma J, Sawa Y, Kaneda Y, Ogihara T, Morishita R (2012) Long-term follow-up evaluation of results from clinical trial using hepatocyte growth factor gene to treat severe peripheral arterial disease. Arterioscler Thromb Vasc Biol 32(10):2503–2509. https://doi.org/10.1161/ATVBAHA.111.244632

133. Bar-Or A, Vollmer T, Antel J, Arnold DL, Bodner CA, Campagnolo D, Gianettoni J, Jalili F, Kachuck N, Lapierre Y, Niino M, Oger J, Price M, Rhodes S, Robinson WH, Shi FD, Utz PJ, Valone F, Weiner L, Steinman L, Garren H (2007) Induction of antigen-specific tolerance in multiple sclerosis after immunization with DNA encoding myelin basic protein in a randomized, placebo-controlled phase 1/2 trial. Arch Neurol 64(10):1407–1415. https://doi.org/10.1001/archneur.64.10.nct70002

134. Kessler JA, Smith AG, Cha BS, Choi SH, Wymer J, Shaibani A, Ajroud-Driss S, Vinik A, Group VD-IS (2015) Double-blind, placebo-controlled study of HGF gene therapy in diabetic neuropathy. Ann Clin Transl Neurol 2(5):465–478. https://doi.org/10.1002/acn3.186

135. Ropper AH, Gorson KC, Gooch CL, Weinberg DH, Pieczek A, Ware JH, Kershen J, Rogers A, Simovic D, Schratzberger P, Kirchmair R, Losordo D (2009) Vascular endothelial growth factor gene transfer for diabetic polyneuropathy: a randomized, double-blinded trial. Ann Neurol 65(4):386–393. https://doi.org/10.1002/ana.21675

136. Simovic D, Isner JM, Ropper AH, Pieczek A, Weinberg DH (2001) Improvement in chronic ischemic neuropathy after intramuscular phVEGF165 gene transfer in patients with critical limb ischemia. Arch Neurol 58(5):761–768