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Viral-mediated gene therapy for the muscular dystrophies: Successes, limitations and recent advances

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Abstract

Much progress has been made over the past decade elucidating the molecular basis for a variety of muscular dystrophies (MDs). Accordingly, there are examples of mouse models of MD whose disease progression has been halted in large part with the use of viral vector technology. Even so, we must acknowledge significant limitations of present vector systems that must be overcome prior to successful treatment of humans with such approaches. This review will present a variety of viral-mediated therapeutic strategies aimed at counteracting the muscle-wasting symptoms associated with muscular dystrophy. We include viral vector systems used for muscle gene transfer, with a particular emphasis on adeno-associated virus. Findings of several encouraging studies focusing on repair of the mutant dystrophin gene are also included. Lastly, we present a discussion of muscle compensatory therapeutics being considered that include pathways involved in the up-regulation of utrophin, promotion of cellular adhesion, enhancement of muscle mass, and antagonism of the inflammatory response. Considering the complexity of the muscular dystrophies, it appears likely that a multilayered approach tailored to a patient sub-group may be warranted in order to effectively contest the progression of this devastating disease.

Keywords: Dystrophin; Utrophin; Adeno-associated virus; Exon skipping; Antisense oligonucleotide

1. Introduction

The muscular dystrophies (MDs) are a heterogeneous group of inherited disorders characterized by progressive skeletal muscle degeneration. The clinical presentation of these disorders displays a broad spectrum of variability such as the degree and distribution of skeletal muscle involvement, cardiac/pulmonary involvement, age of onset, rate of progression, and mode of inheritance [1]. The diagnosis is often performed by examination of a muscle biopsy though in many cases genetic testing can be employed. Histologically, muscle biopsies may present with an increased number of myofibers with centrally located nuclei, an abnormally large myofiber size—both of which are characteristic of an enhanced degenerative and regenerative response, and in advanced cases replacement of myofibers with adipose, and connective tissue, in large part due to the loss of regenerative potential of dystrophic muscle fibers.

A variety of therapeutic options are being developed, particularly in small animal models, to counteract the muscle wasting associated with MD. These include autologous myoblast transplantation [2], stem cell transfer [3,4], pharmacological intervention [5–7], and gene repair/replacement strategies [8–13]. These therapies are not without significant hurdles. For example, cell-based approaches are hindered by a lack of efficient methods to introduce and engraft adequate numbers of muscle precursor cells into the bulk tissue. Gene transfer approaches are limited by difficulties associated with producing sufficient quantities of vector, cellular tropism of the vectors, ectopic gene expression, the need for methods to achieve whole-body delivery of the vector, and inherent immunological challenges. Recently, great strides have been

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made in overcoming these hurdles, and this latter topic will be an emphasis of this review. Ultimately, a combinatorial approach involving different therapeutic strategies may be necessary to combat the mechanical, signaling, and immune related mechanisms that lead to dystrophy.

The dystrophinopathies are X-linked recessive disorders caused by dystrophin deficiency. Since the discovery of the dystrophin gene and protein over 18 years ago [14,15], more than 30 different forms of muscular dystrophy have been molecularly identified [16,17]. The 427 kDa skeletal muscle specific isoform of dystrophin is a sarcolemmal protein that interacts with many other proteins to link the extracellular matrix to the cytoskeleton [18–20]. Dystrophin is composed of four major structural domains (Fig. 1) [21]. The N-terminal domain binds to F-actin filaments in the subsarcolemal cytoskeleton. The central rod domain includes 24 triple-helical domains, often referred to as spectrin-like repeats, and 4 strategically placed hinges that together provide flexible, spring-like properties that are likely critical during muscle contraction. The cysteine-rich (CR) domain along with the distal C-terminal (CT) domain, anchor to the plasma membrane through the dystrophin–glycoprotein complex (DGC) (Fig. 2). Thus, the structural components of dystrophin mediate a mechanical function that acts to stabilize and link the muscle cell membrane and cytoskeleton. The lack of a functional dystrophin protein results in the loss of the DGC and causes instability of sarcolemma. These deficiencies ultimately lead to chronic muscle damage and degenerative pathology.

Studies of components of the DGC in non-muscle tissues also strongly support a signaling role for some of these proteins [22–24]. The analogy of the DGC to integrin and caveolin-3 protein complexes is compelling: like the DGC, these are membrane proteins that, when mutated, cause muscular dystrophies [25–28]. In common with these multimeric complexes are shared signal transduction cascades that mediate cell survival/death pathways and cell defense mechanisms. The similarities suggest common pathogenetic mechanisms leading to cell death when the complexes and associated signaling functions are disrupted. For example, the DGC has been documented as interacting with signaling molecules such as calmodulin [29], Grb2 [30], extracellular signal-related kinase (ERK) [31,32], and neuronal nitric oxide synthase (nNOS) [33]. Membrane-associated integrin receptors exist as alpha- and beta-subunit heterodimers, which like the DGC mediate extracellular matrix

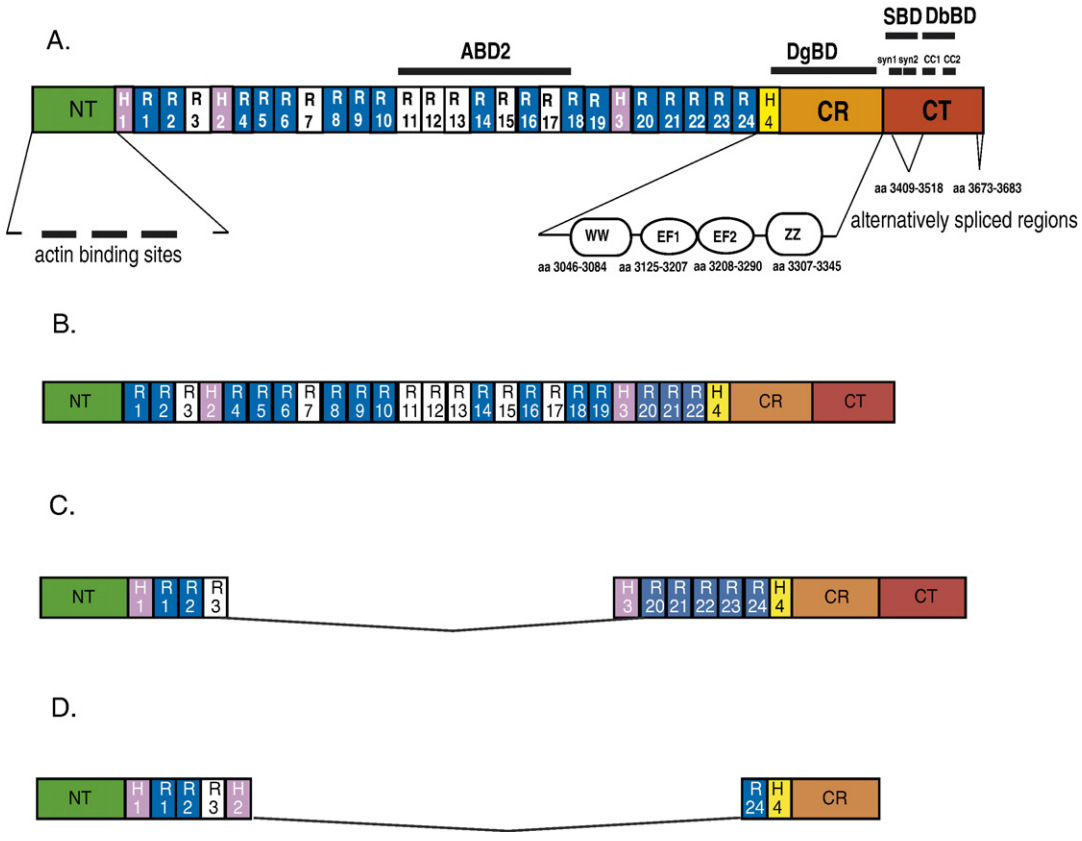

Fig. 1. Structural domains of the human dystrophin protein referred to in the text (scale is approximate). (A) Dystrophin (427 KDa); (B) Utrophin (395 KDa); (C) Mini-dystrophin ΔH2-R19Δ (227 KDa); (D) Micro-dystrophin ΔR4-R23 (132 KDa). ABD: actin-binding domains; H: hinge regions; R: spectrin-like repeats; CR: cysteine-rich domain; CT: C-terminal domain; CH: calponin homology motifs; WW: WW domain; EF: EF-hand motifs; ZZ: ZZ domain; DgBD: dystroglycan-binding domain; SBD: syntrophin-binding domain; DbBD: dystrobrevin-binding domain; syn: location of the syntrophin contact sites (syn1 spans amino acids 3427–3461; syn2 spans amino acids 3462–3483); CC: coiled coil motifs (CC1 spans amino acids 3506–3593; CC2 spans amino acids 3558–3593 as determined from the primary cDNA sequence). Hinge 4 spans amino acids 3041–3112.23 Basic repeats are shown in white, the others in blue. The location of the alternatively spliced exons is from Feener, CA Nature 1999.

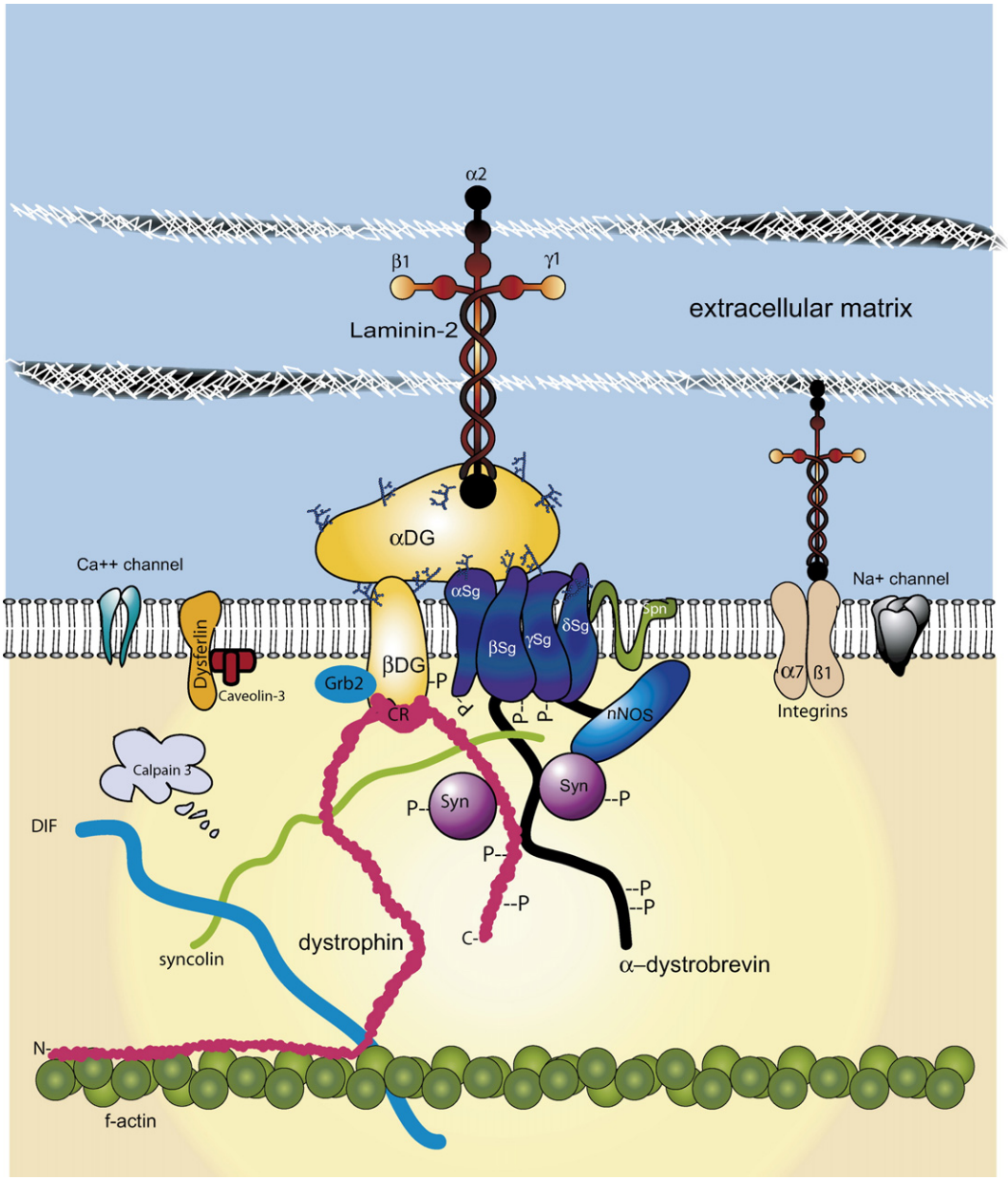

Fig. 2. Diagram of the dystrophin–glycoprotein (DGC) complex. This complex includes the integral components (dystrophin with the cysteine-rich (CR)), C-terminal (C) and N-terminal (N) regions, the sarcolemma associated α, β-dystroglycans, the α, β, γ, and δ-sarcoglycans (Sg), α-dystrobrevin, the syntrophins, sarcospan (Spn)), an extracellular ligand (laminin-2), intracellular binding partners (F-actin, desmin intermediate filament (DIF), and syncoilin (S)), non-associated caveolin-3 and dysferlin, and signaling molecules associated with the complex (Grb2, and nNOS). The dystroglycans and sarcoglycans are shown to be glycosylated, and phosphorylation sites are represented on dystrophin, α-dystrobrevin 1, β-dystroglycan, the syntrophins, and α-, β- and γ-sarcoglycans.

cell adhesion and focal adhesion complex formation. Focal adhesion kinase, paxillin and Rho A are examples of proteins involved in focal adhesion mechanotransduction. Commonality of signaling pathways to both the DGC and integrins includes the Ras/MAPK pathway by way of adaptor proteins such as Grb2. As with the DGC, caveolins serve as scaffolding proteins for signaling complexes at the membrane, and are known to compartmentalize multiple molecules involved in a variety of signaling pathways Caveolin is largely thought to inhibit cell growth and proliferation by modifying the function of a variety of molecules involved in signaling pathways such as nNOS [34], src tyrosine kinases [35], G-proteins [36], MAP kinase cascades [37], adenylyl cyclase [38], protein kinase A [39], protein kinase C (PKC) [40], epidermal or platelet derived growth factor receptor [41,42], or transforming growth factor receptor pathways [39]. Recently, caveolin-3, overexpression in cardiac myoblasts resulted in the inhibition of growth signal as demonstrated by calcium dependent attenuation of ERK signaling; suggested to be acting through PKC [43].

Perturbance of these and other signaling pathways could progressively add to the repetitive cycles of degeneration that over time leads to advanced muscle wasting.

The core skeletal muscle DGC is composed of dystrophin, the sarcoglycans (SGs) (α, β, γ, and δ-SG), dystroglycans

(DGs) (α and β-DG), sarcospan, and the syntrophins (Fig. 2). Additionally, a number of extra- and intracellular proteins are less tightly associated with the DGC, such as neuronal nitric oxide synthase (nNOS) [33], dystrobrevin [44,45], caveolin-3 [46] and laminin-2 [47]. The observation that expression of several DGC members was abnormal in a variety of different types of MD subsequently led to the realization that the genes for these DGC members were mutated in other forms of MD [21,48,49]. For the purpose of this review, Duchenne muscular dystrophy (DMD) will serve as the model for current viral-mediated gene transfer approaches being developed in animal models with the implicit assumption that these methods might also be applied to other forms of MD.

DMD is the most common inherited neuromuscular disorder, affecting 1 in 3500 newborn males worldwide [50]. DMD results from mutations within the dystrophin gene [51,52]. This gene is ~2.5 MB and consists of 79 exons, including 7 promoters with 3 full-length isoforms (expressed in muscle, brain, and cerebellar Purkinje neurons), and an additional 4 truncated isoforms (Dp260; Dp140; Dp116; and Dp71) produced from separate promoters within the gene. The gene is the largest identified to date, which may account for the high frequency of mutation [53]. Close to 30% of cases arise from spontaneous mutations [54]. Approximately 60% of mutations causing DMD are deletions of large segments of the gene usually including one or more exons [14,55,56], and a smaller ~5% of mutations are duplications of large segments of the gene [56]. Such mutations can result in mRNA instability, and/or bring about premature termination of translation leading to an unstable, incomplete protein that is nonfunctional and rapidly degraded. Some mutations in the dystrophin gene can lead to deletion of part of the coding sequence where the reading frame is maintained; such mutations result in a semifunctional, internally deleted dystrophin protein associated with a milder Becker muscular dystrophy (BMD) [14]. Lastly, former studies in transgenic mice have demonstrated not only the prevention of abnormal mechanical properties, but also non-toxic side effects of the dystrophin protein [57], further suggesting that DMD can be amendable to a gene replacement strategy.

2. Model systems

The development of potential treatments has been greatly assisted by the discovery of a natural genetic model of DMD: the *mdx* mouse [58]. The *mdx* mouse is dystrophin deficient owing to a point mutation that leads to a premature stop codon and the production of an unstable peptide [59]. Transgenic *mdx* mice expressing recombinant dystrophin cDNAs have revealed a wide therapeutic window, with prevention of pathology being achieved with dystrophin expression ranging from 0.2 times to 50 times normal endogenous levels [57,60,61]. Dystrophin expression can be restored in *mdx* mice by several routes, including drug administration, the use of anti-sense oligonucleotides targeted against the mutant exon, stem cell transplantation, and dystrophin gene transfer with viral or non-viral vectors. A related mouse model that more closely mimics the clinical picture in DMD patients is the dystrophin and utrophin double knock out (dko) mouse [62]. A somewhat more attractive model because of its larger size and severe phenotype may be that of the canine x-linked muscular dystrophy (*cxmd*) model [63,64].

3. Adenovirus

Gene transfer vectors derived from adenovirus (Ad) have provided a wealth of knowledge that includes but is not limited to various stages of the viral life cycle, viral tropism, transduction efficiency, and the interplay between viral infection and the host immune response. In brief, adenoviruses are medium-sized (90–100 nm), nonenveloped icosohedral viruses containing a double-stranded DNA genome. There are >50 immunologically distinct serotypes (6 subgenera: A through F) that can cause human infections, most of which are benign and are targeted to the respiratory tract. These subgenera are distinguished from one another based on differential hemagglutination patterns, oncogenic potential, and DNA G+C content [65]. Subgroup C serotypes 2 or 5 are the major types used as viral vectors. The wild type adenovirus genome is approximately 35 kb [66,67]. There are four early transcriptional units (E1, E2, E3 and E4), which have regulatory functions, and a late transcript, which codes for structural proteins. Progenitor or first generation vectors have the E1 and often the E3 gene deleted, with the missing E1 genes being supplied in *trans* from an E1 region integrated into a producer cell such as the human fetal kidney cell line 293 [68]. Second generation vectors additionally carry deletions of the E2b or E4 regions [69,70]. The most recent third generation or 'helper dependent' (hd) or gutted vectors contain only the inverted terminal repeats (ITRs) and a packaging sequence around the transgene with all of the necessary viral genes being provided in *trans* [71,72].

Attractive properties of Ad vectors included stable growth to very high titers ($>10^{11}$ particles /ml), a large transgene carrying capacity, very efficient transduction of target cells in vitro and in vivo, the ability to infect post-mitotic cells such as myotubes, the relative ease of genome manipulation and the ability to generate high levels of transgene expression. A first-generation adenovirus, with a limited insert capacity of ~8 kb, was the first viral vector to successfully deliver a human miniaturized dystrophin cDNA to *mdx* mice by intramuscular administration [73]. The cDNA used in this study was deleted for segments encoded on exons 17 to 48 producing a ~200 kd protein that lacks most of the central rod domain, and was previously observed in a BMD patient [74]. In this study correct localization of a minidystrophin protein within the injected muscle was found in ~50% of the myofibers and persisted for up to 3 months. As with any viral vector there are distinct advantages and disadvantages to its use (Table 1). Several of these limitations have been overcome with use of the helper-dependent or gutted adenoviral vectors (hdAd) that are deleted for all of the viral protein coding sequences. The use of these vectors has led to a reduced immune response, improved transgene expression, and larger transgene carrying capacity [75–77]. The use of hdAd to deliver dystrophin to neonatal or

Table 1
Properties of viral vector systems

Vector	(1st and 2nd gen.) *Adeno*	(Gutless) *Adeno*	*AAV*	*Lentivirus*
Genome	dsDNA	dsDNA	ssDNA	RNA
Capacity (kb)	~8.3 [a]	27–29	~4.8	~7.5–9[a]
Size (diameter)	~80 nm	~80 nm	~20 nm	~80 nm
Integration	No	No	No/Yes [b]	Yes
Non-dividing cell transduction	Yes	Yes	Yes	Yes
Duration of expression	Short	Long	Long	Long
CTL induction	Yes	Yes	No/Yes	No
Pre-existing immunity	Yes	Yes	No/Yes	No
Safety issues	Inflammation	Cytotoxicity	Insertional mutagenesis?	Insertional mutagenesis

[a] DNA payload size depends on the presence of accessory proteins.
[b] It is still unclear whether recombinant AAVs are capable of stable integration.

adult *mdx* mice has demonstrated long-term expression, restoration of the dystroglycan complex at the sarcolemma, and amelioration of the physiological and pathological indices of muscle disease [77–79]. Higher doses of vector were needed to treat adult *mdx* mice likely due to inefficient infection of mature muscle by Ad [77].

Even with improvements in Ad vectorology, problems remain. The episomal Ad genomes are gradually lost from the transduced myofibers. High-titer neutralizing antibodies arise against Ad after the first administration of vector, preventing repetitive administration of Ad vector of the same serotype [80]. The administration of high doses of either first-generation or hdAd results in the induction of innate and cellular immune responses, that at very high doses are associated with direct toxicity that can be lethal [81,82]. Further, systemic delivery of adenoviral vectors in mice leads to a significant drop in blood pressure likely due to the systemic activation of endothelial cells and increased production of nitric oxide, followed by destruction of Kupffer cells [83]. This acute toxicity is not prevented or reduced with hdAd. In the case of hdAd the capsid composition and structure are the same as in the first generation vectors and would not be expected to reduce the acute innate inflammatory response against the vector [84,85]. Several investigators are implementing methods to reduce the associated inflammation and toxicity. For example, coating the virus with polyethylene glycol can serve to mask Ad epitopes from preexisting immune responses by reducing protein–protein interactions [86]. The use of hdAd can limit the adaptive immune response against Ad proteins [81], but generation of immunity to the transgene dystrophin is somewhat controversial in the context of human gene therapy. Many DMD patients have large deletions within the dystrophin gene that prevent expression of epitopes encoded by deleted exons such that exogenously delivered dystrophin could be perceived as a neoantigen [87]. In this regard, co-delivery of immunomodulatory molecules such as CTL4AIg [88] is being re-examined in the context of hdAd dystrophin delivery. Investigators have shown prolonged dystrophin expression in immuno-competent mice with systemic gene transfer utilizing T-cell costimulatory blockade [89,90].

4. Lentivirus

An additional viral vector being tested for gene transfer to muscle is the HIV-derived lentiviral vector. This retroviral vector can be produced at moderate titers, can infect both dividing and non-dividing cells and be pseudotyped with a variety of different envelope glycoproteins [91]. In contrast to results with Ad vectors, these vectors do not induce a cytotoxic T-cell lymphocyte (CTL) response. To address safety concerns, genetic engineering has produced a lentiviral vector system that is self-inactivating (SIN) [92]. These vectors have a large deletion in the 3′ U3 region that contains the viral promoter and enhancer. This deletion is copied over to the 5′ region during reverse transcription which in turn abolishes LTR-driven transcription in transduced cells [91]. Furthermore, lentiviral vector systems can carry between 7.5 to 9 kb of cargo DNA, which is largely sufficient for cloning sizeable transgenes such as minidystrophin, selectable markers, and the promoters needed for expression. This feature is important, especially when using large tissue-specific promoters. Finally, lentiviral vectors have been shown to permanently transduce and stably express transgenes in muscle cells and their precursors, making them the delivery system of choice for muscle progenitor cell based gene transfer [93–95]. In adult skeletal muscle, satellite cells are the primary stem cells that are responsible for postnatal muscle growth, hypertrophy, and regeneration. These cells are normally quiescent but can activate to a proliferative state by extrinsic signaling mechanisms. Activation of the satellite cell population is dramatically enhanced in response to muscle damage or degenerative disease where myoblast generation is necessary for repair and growth. Upon activation, there is a proliferative phase where progeny proceed down the myogenic lineage pathway to become fusion competent myoblasts [96,97]. However, the transduction of satellite cells using lentiviral vectors would provide an attractive therapeutic option, particularly when robust isolation methodologies in the absence of activation and homing mechanisms become further identified. A large advantage of this therapy is the potential use of the patient's own cells and the inherent avoidance of the immune response. As with many ex vivo approaches there are challenges and this includes efficient isolation of satellite cells, development of optimal expansion conditions, enhancement of efficient muscle homing mechanisms, and minimization of insertional oncogenesis within active genes. Numerous other stem cells are also being studied for potential use in ex vivo gene therapy strategies for DMD, including mesangioblasts, SP cells and MAPC [3,98,99].

5. Adeno-associated virus

Among the viral vector systems being utilized today, one that has generated much optimism towards eventual treatment of muscular dystrophy is adeno-associated virus (AAV). AAV is classified as a Parvoviridae family member being of the genus

Dependovirus. Eleven strains of AAV (1–11) have been identified from primates thus far, and more than one hundred sequences representing novel clades of AAV have been reported [100–103]. AAV is a single stranded DNA virus (~4.7 kb) whose genome is flanked by inverted terminal repeats (ITRs) that are ~150 nucleotides in length. The ITRs are critical for stability and priming of genome replication through a "rolling hair pin" mechanism [104]. These elements contain all the cis-active elements necessary for replication and encapsidation of AAV genomes [105]. The recombinant-AAVs (rAAV) in use today are deleted of all viral genes, specifically the *rep* and *cap* genes that encode the nonstructural and structural proteins respectively. Viral packaging constraints limit the size of the transgene that may be included to the size of the wild type genome. rAAV for gene transfer has been developed using a pseudotyping approach that consists of a recombinant genome framed by the ITR of AAV serotype 2 and a capsid based on any one of the cloned AAV serotypes [106]. Recombinant vectors based on these variants demonstrate significantly different tropism for tissues such as cardiac muscle, skeletal muscle, liver, and lung as well as transduction differences in vitro versus in vivo [107–109].

For the expression of protein using rAAV there is the requirement of flanking ITRs, a promoter, transgene, and polyadenylation signal. The production of virions has vastly improved over the years. Refinement has allowed the use of a two plasmid system, where the Ad functions and Rep/Cap ORFs are supplied on a single plasmid [110,111]. The virions may be purified and concentrated from the cellular lysate by density gradient centrifugation, ion exchange or affinity chromatography [112–114]. In the case of large-scale production, column chromatography has a distinct advantage in terms of logistics. However, one must be aware that this procedure will not distinguish between virions that contain viral genomes from those that do not (empty capsids) as density gradient centrifugation allows. Several serotypes, for example, AAV2 and AAV6 have the capacity to bind heparin with a relatively high affinity thus allowing purification by heparin chromatography [112].

Many researchers have focused on the ability to systemically deliver rAAV carrying truncated versions of dystrophin to achieve body-wide transduction of the musculature. Though this is no small accomplishment, we must further address safety concerns of not only the viral vector, but also the transgene with more rigorous testing prior to proceeding to clinical trials. Irrespective to the transgene, the use of rAAV vectors is likely several years away from being a practical clinical therapy for DMD. However, there are several characteristics suggesting it as an attractive candidate for gene-replacement/corrective therapy. These include the nonpathogenic nature of AAV, the availability of alternative serotypes with muscle tropism, the ability to generate vectors at relatively high titer, a relatively low but not absent immunogenicity, and an ability to achieve long-term transgene expression. Further improvements in vector design with the goal of strictly limiting gene expression to muscle will undoubtedly add to the safety of AAV vectors on several levels. Optimization of methods to produce enough rAAV vector for routine use in patients, and the ability to develop techniques that allow transduction of enough of the musculature to provide a therapeutic benefit while minimizing toxicity will favorably influence the realization of therapeutic success in the clinic. In the future, with combined improvements in production and delivery approaches, these vectors likely will become more internally customized to a particular disease. Many of these promising technologies are new and likely to advance rapidly in the coming years. Analysis of specific and non-specific toxic effects will have to be a prominent aspect of future trials, and these will undoubtedly be balanced against the benefit of this type of treatment.

6. Gene targeting

Gene targeting is the replacement of an endogenous DNA segment with a homologous, exogenous segment of DNA by homologous recombination (HR). Homologous recombination technology has rapidly advanced the field of molecular genetics, but initially fell short of realization in terms of a gene repair strategy. This was primarily because vectors used for HR typically inserted into the host genome at random locations with a frequency greater than that of HR [115]. However, recently this area of research has been revitalized thanks to the efforts of several laboratories offering hope toward efficient chromosomal manipulation. A major advantage of the HR gene targeting approach is that the risk of random integration as is presented with retroviral mediated gene replacement [116] can be avoided or minimized. In one study HR was accomplished by using hdAd as a vector platform [117]. The frequency of HR using this system with an 18.6 kb region of homology reached $\sim 2\times 10^{13}$, while detection of random integration events was found to be ~5% per transduced mouse embryonic stem cell at a multiplicity of infection (moi) of 10. This is a vast improvement from the 10^{-5} to 10^{-7} per infected cell that was previously demonstrated using E1-deleted replication incompetent Ad to target the single-copy *Fgr* locus in mammalian cells [118]. It was also shown that HR could be accomplished on a relatively small number of cells, which would be of benefit in the stem cell field. Similarly, a recombinant adeno-associated virus (rAAV) vector has been used to correct dominant mutations in mesenchymal stem cells (MSCs) from patients with osteogenesis imperfecta [119], demonstrating that gene targeting by HR is possible for non-embryonic stem cells. In this study 6×10^5 to 2.3×10^4 MSCs underwent gene targeting. This strategy has an advantage of minimizing the possibility of insertional mutagenesis, and there is the further advantage that MSCs are typically non-immunogenic.

Recently, an exciting technical development has emerged with the use of chimeric zinc finger nucleases (ZFNs) where a FokI endonuclease domain is combined with a zinc finger protein. When designed properly, this system can exhibit exquisite sequence specificity and targeted DNA cleavage [120]. This study demonstrated a remarkable 18% level of gene-modified human cells without selection. DNA cleavage is followed mechanistically by stimulation of HR between the chromosome and the extrachromosomal DNA donor. By altering the specificity of the zinc finger domain one can alter

the target site specificity of the ZFN, making this a highly attractive methodology. The ability to engineer ZFNs to target essentially any sequence clearly establishes the utility of ZFN-driven genome editing. The same concerns of present-day gene therapy strategies exist, namely delivery method, associated toxicity, and a host immune response.

7. Muscle gene transfer

rAAV vectors support stable, long-term gene expression following infection of muscle cells, where they persist as an episome [121]. Serotypes that have demonstrated a particular tropism for the musculature include AAV1, 5, 6, and 8 [108,122–125]. These serotypes have demonstrated a transduction efficiency of up to 500 times greater than that of AAV2 [103,126–128]. Although wild type AAV is capable of integration into the host genome in the presence of Rep, integration of a rAAV genome into myonuclei has not been detected [129]. Long-term transgene expression of rAAV has been observed for over 5 years in dogs and rhesus macaques, and for 2 years in mice [130–134]. Vectors pseudotyped with capsids from AAV1, 6, and 8 display peak expression several weeks prior to that of AAV2 [126,135,136]. This delay in transduction is quite significant for a disease such as DMD where there is a rapid turnover of muscle fibers [137].

The route of administration of rAAV is a major consideration for the treatment of DMD, taking into account that body wide distribution would likely be necessary for an effective therapy. Previous efforts to target organs such as the diaphragm or heart, whose failure accounts for the majority of patient deaths, have utilized invasive surgical procedures for intravenous (IV) vector delivery using potentially toxic cofactors to induce vascular permeability [138]. Although such studies yielded beneficial results, they underscore the need to deliver a therapeutic vector in a noninvasive manner. In another approach, researchers administered viral vector to rodents intra-arterially using high pressure injections [139]. More recently, the ability to transduce the entire cardiac and skeletal musculature of a mouse has been accomplished with a single low pressure intravenous administration of rAAV6 [140]. The inclusion of vascular endothelial growth factor (VEGF) was shown to dramatically increase the efficiency of this method at lower vector doses [140]. This response to VEGF was not observed at higher vector doses ($>10^{12}$ vg). Importantly, IV administration of rAAV6 demonstrated efficient transduction of the diaphragm and intercostal muscles that are vital to respiration [140]. In a follow up study in which rAAV6 encoding human alkaline phosphatase was delivered by IV administration, persistence of transgene expression was demonstrated out to 20 months in mice (Fig. 3). Since this initial demonstration there have been

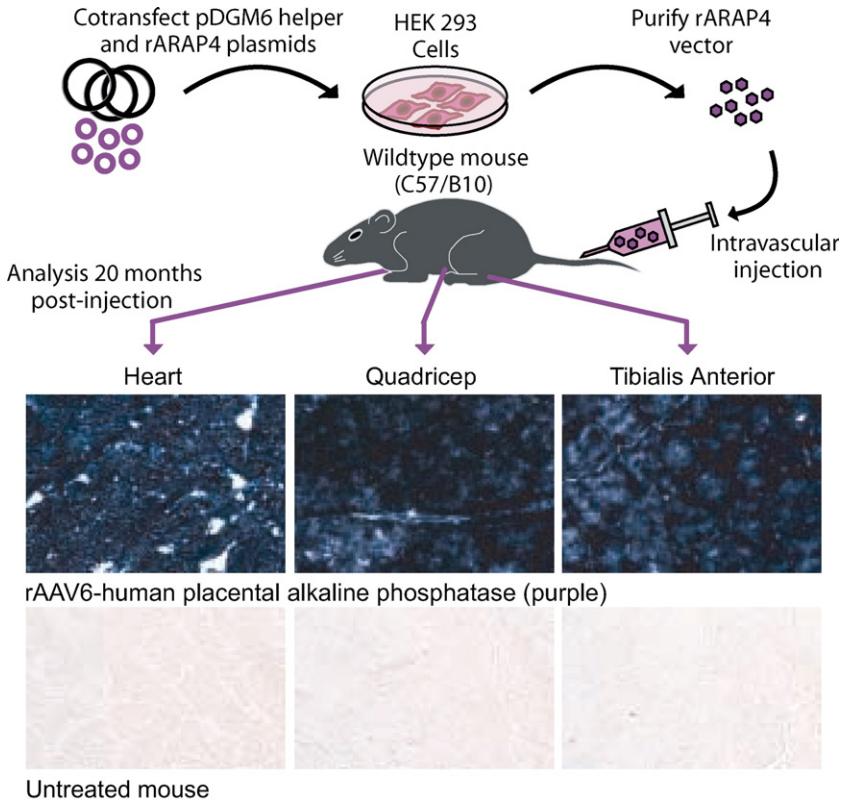

Fig. 3. Whole-body human placental alkaline phosphatase (hPLAP) gene transfer to the musculature using rAAV6. 1 × 1012 vg of rAAV6/RSV-hPLAP was injected into the tail vein of a 3-month-old wild-type mouse, and tissues were analyzed 20 months later. Vector was administered in a 300-μl bolus injection in physiological Ringer's solution containing 0.008% mouse serum albumin and 2 IU sodium heparin. VEGF was not used in this study. All striated muscles were found to express hPLAP as detected by NBT/BCIP (30 min following 90 min heat inactivation at 65 °C) in essentially every myofiber or myocyte. Shown are representative sections of the heart, quadricep, and tibialis anterior muscles. Top row, muscles from wild-type mice injected with rAAV/RSV-hPLAP; bottom row, muscles from wild-type mice injected with Ringers buffer solution.

numerous reports using various animal models that follow along a similar vein [125,141–148]. Additional optimization is warranted in order to allow for a lower effective dose in larger animal models, providing for a decreased potential for toxicity and a safer outcome. The low immune response elicited by rAAV vectors in animal studies suggests that this vector may have a distinct advantage over others such as Ad [134,149]. To date there is little evidence for rAAV vectors causing direct toxicity in either animal studies or human clinical trials. Doses up to 10^{12} vg/kg of rAAV have been well tolerated in human studies with no toxicity [134,150,151]. However, evaluation of clinical samples from a recent hemophilia trial revealed a cytotoxic T-cell (CTL) mediated immune response that was likely generated by memory $CD8^+$ T cells against the AAV capsid. This immune response resulted in a decline of transgene (Factor IX) expression and hepatic injury supported by an elevation of alanine and aspartate transaminases [152]. While these data are from a relatively small sample size, it should be considered carefully when proceeding to human clinical trials given the frailty of older DMD patients.

8. Gene repair strategies

It has recently been reported by the International Human Genome Sequencing Consortium (IHGSC) that the human genome contains ~25,000 gene loci covering ~1.7 Gb, with 231,667 protein-encoding exons. The tremendous size of the DMD gene represents an enormous target for genetic mutations. Approximately 20% of the mutations leading to DMD are point mutations or small insertions or deletions that effect one or a few exons [153]. With that in mind, nature has provided us with a clue for an alternative gene therapy approach to the gene replacement strategy. DMD patients occasionally have rare, dystrophin positive fibers (revertant fibers) that likely originate from exon skipping in the dystrophin gene and which generate a truncated transcript with a restored open reading frame [154]. Exon skipping therapy is based on the reading frame rule [155] which states that frame-shifting mutations in the dystrophin gene result in DMD, while frame-conserving ones primarily lead to a milder Becker muscular dystrophy (BMD). Thus, the ability to induce exon skipping has the potential to convert a DMD phenotype to a much less severe BMD phenotype. Several laboratories have demonstrated induction of exon skipping [12] using synthetic antisense oligonucleotides (AONs) [9,156,157]. These AONs bind to specific sequences in the pre-mRNA and thus disturb exon inclusion signals such as splice sites, intronic branch point sequences, or exonic splicing enhancer elements. In turn, this leads to removal of the targeted exon by inhibiting its inclusion within the processed mRNA.

One model to test the induction of exon skipping is through the targeted skipping of exon 23 in the *mdx* mouse. This exon contains a nonsense mutation that results in dystrophin synthesis being aborted prematurely. The researchers used an AON that targets the 5′splice-site of this exon, in combination with a drug carrier called F127 [156] (a block copolymer belonging to the group of amphiphillic pluronics used in the pharmaceutical industry). This drug carrier enhances the circulation time, metabolic stability, and transport of AONs across the cell membrane. It was demonstrated that AON-induced skipping of exon 23 could be therapeutic as up to ~20% of the muscle fibers in the *mdx* mouse were converted to a dystrophin positive state with positive functional consequences. The distribution of the dystrophin positive fibers was highly variable which was attributed to the regeneration process in individual *mdx* muscle fibers that led to differential uptake of the AONs. After repeated administration, dystrophin levels were shown to be 1–5% of normal. Absence of dystrophin expression in the heart and the increased dystrophin in older *mdx* mice compared to younger mice further indicate a role of the regenerative process on AON uptake. The lack of expression in cardiac tissue is a concern considering more than 30% of DMD-associated deaths occur as a result of cardiomyopathy [158].

In a more efficient and persistent strategy, rAAV was used to deliver AONs as part of either U1[157] or U7 small nuclear RNAs [9]. The latter study utilized a previously described antisense-plasmid system (U7mOPT) [159] that contains a modified U7snRNA gene (U7mOPT). This RNA, as a part of the U7 ribonucleoprotein particle, is involved in the processing of the 3′ end of histone pre-mRNAs within the nucleus through an antisense mechanism. Within the U7mOPT, the original U7 antisense sequence was replaced with two antisense sequences. These sequences target the branch point sequence in intron 22 and the 5′ splice site of exon 23. After intramuscular injection of the rAAV-U7-AON vector in *mdx* mice up to 77% of dystrophin positive fibers were observed, and 3 months following administration a 50% level was reported. Taking this approach one step further by using the systemic delivery capability of rAAV, it was demonstrated that AAV-mediated antisense-U1 small nuclear RNA expression resulted in effective dystrophin production through the hind limb of an *mdx* mouse, significant recovery of the functional properties in vivo, and lower serum creatine kinase levels, suggesting an overall decrease in muscle wasting [157].

In order to test the effectiveness of the AON strategy in the context of human dystrophin a *hDMD* transgenic mouse was developed with the full-length human DMD gene [160]. This model demonstrated that the targeting of human exons with AONs can induce their specific removal from the human DMD transcript. Further, showing exquisite specificity, no detectable effects in the homologous mouse dystrophin transcript were detected.

9. Immune evasion strategies

Any viral-mediated gene delivery method can potentially lead to a destructive host immune response against vector proteins. This result can occur by cell transduction and antigen presentation in the context of the major histocompatibility complex, resulting in elicitation of a CTL response that destroys transduced cells. It must also be considered that DMD patients, whose muscles do not express dystrophin, may be more likely to develop an immune response to vector-delivered dystrophin

than would patients who express low levels or truncated forms of dystrophin. Therefore, patients with different mutations may respond differently to gene transfer. Understanding the mechanisms by which vector-encoded proteins are recognized by the immune system in order to develop approaches to prevent induction of an immune response is of utmost importance. Various immunosuppressive protocols have been shown to potentially circumvent this dilemma. For example, transient depletion of CD4+ T cells during the initial exposure to rAAV allowed successful readministration of AAV vectors to skeletal muscle [161]. Similarly, the use of anti-CD40L antibodies, which block T cell activation of B cells, allowed prolonged gene expression in mouse lung [162]. These treatments would be expected to inhibit the initiation and expansion of a primary immune response and promote viral delivery of a transgene [161,163]. However, these approaches would be less effective in humans that possess an arsenal of pre-existing neutralizing antibodies that could block readministration of the viral vector.

Recently a large ($>10^6$) AAV-2 capsid library was generated with randomly dispersed capsid mutations in a process similar to DNA shuffling [164]. This approach was applied to a clinically relevant problem of AAV: preexisting immunity in the human population. In this case the authors applied repeated amplification of those AAV2 mutants that retained infectivity in the presence of antisera with increasing titer of neutralizing antibody [164]. The results were impressive: numerous antibody-evading mutants were obtained, one which showed the ability to moderately transduce cells while in the presence of a 1:2 antiserum dilution. When neutralizing antibodies to other AAV serotypes as well as crystal structures become available an AAV capsid regional immunogenic profile should become possible. These data have the potential to bring a wealth of knowledge to our current understanding of antigenicity of the AAV capsid and when combined with the knowledge of the altered tropism capacity of AAV serotypes [165] provides a powerful tool for therapeutic applications.

The promoter is an important consideration in determining both the level of expression and tissue specificity of rAAV-mediated gene transfer. Tissue specific gene expression within skeletal muscle has been achieved using the synthetic C5-12 promoter [166], the muscle creatine kinase (MCK) promoter [167] and the desmin promoter [168]. However, even when tissue-specific promoters are employed clearance of the vector from the bloodstream can occur from high levels of neutralizing antibody to the viral capsid or transgene product. It would be of great interest to specifically tailor transgene cassettes so that the gene expression could be prevented in undesirable cell types such as immune effector cells. Restricting expression with the use of tissue-specific promoters may also limit the potential efficacy of gene transfer by constricting the population of cells expressing the transgene.

Recently, micro RNA (miRNA) regulation was demonstrated within a lentiviral system to prevent transgene expression in hematopoietic lineages while permitting high expression in nonhematopoietic cells [169]. miRNAs are a family of small (~21mer) RNAs that, at least for those that have characterized targets, negatively-regulate gene expression at the post-transcriptional level [170–173]. The researchers incorporated four tandemly positioned copies of a 23-bp sequence (mirT) within the 3′ untranslated region of green fluorescent protein. This design provides complementary target sequence to multiple miRNAs with the intention of optimizing repression of the transgene. The selection of targets was based upon emerging constraints of miRNA mediated regulation [174,175]. This system was found to be an improved means for preventing expression in hematopoietic lineage cells when compared to a hepatocyte-specific albumin promoter which may have also better prevented cross-priming of the T-cell population to professional APCs. This improved ability to exclude expression was suggested to occur because post-transcriptional regulation can trump off-target promoter activity. The engineering of miRNA-mediated regulation into a viral vector could potentially allow for an exquisite layer of control over transgene expression at the level of transcription irregardless of the relative strength of the promoter, and as such potentially allowing avoidance of the host immune response.

10. Addressing the rAAV carrying capacity limitation

One of the most challenging aspects of developing rAAV for the treatment of DMD is the limited DNA packaging capacity of the capsid. The upper limit capacity of rAAV for foreign DNA is considered to be ~5 kb [176] while the coding region of dystrophin covers 11.2 kb of cDNA sequence. Three approaches have explored the ability to circumvent this size constraint, namely the minigene, trans-splicing, and recombination vector approaches. Patients with mild phenotypes of muscular dystrophy have been described that arise from large deletions within the dystrophin gene [74]. It was concluded that large portions of the gene are not vital for function (Fig. 1). Several transgenic *mdx* mice were engineered to carry different deletions throughout the four dystrophin domains in order to map the regions that are crucial for function. Mild phenotypes were associated with N-terminal domain deletions, indicating that this region is important but not essential for attachment to actin and the cytoskeleton [177]. The evaluation of a series of large deletions within the central rod domain indicated that as an entirety this structure is indispensable but the number of spectrin repeats can be markedly reduced [13,137,178]. Also the proper positioning of the repeats and the presence and configuration of the hinge regions is crucial. Deletions in the cysteine-rich domain cause severe dystrophy, also resulting in disrupted expression of the entire DGC [179]. The C-terminal domain was found not to be required for assembly of the full DGC [179,180]. Engineered to mimic and improve upon the exons 17–48 deletion in a BMD patient [74], the 6.2 kb minidystrophin construct (ΔH2-R19) contains 8 repeats and hinge regions 1, 3 and 4. This construct was found to be completely functional, in that *mdx* mice that expressed this mini-dystrophin demonstrated normal muscle morphology and force generation. Several microdystrophin constructs (3.6–4.2 kb) have also been developed that display a highly effective ability to support near normal muscle structure and function in

mice [137,178,181]. Truncated dystrophins carrying 4–7 spectrin-like repeats have been delivered to mouse muscle by rAAV. These constructs have been shown to ameliorate many of the dystrophic pathophysiological effects in the *mdx* mouse such as increased susceptibility to eccentric contraction-induced injury, high proportions of centrally-nucleated myofibers, and elevated levels of serum creatine kinase [13,137,140,167,182,183]. The micro-dystrophin found to have optimal characteristics of effectiveness and small size was the construct ΔR4-R23/ΔCT, which spans 3.6 kb of DNA and carried 4 spectrin-like repeats and hinges 1, 2 and 4 while lacking the C-terminal domain. Although most microdystrophins vastly improve the phenotype of *mdx* mice, restoration of the muscles to complete parity with those of wild type mice is not accomplished. There is not a full restoration of absolute or specific force generation, and the degree of correction is both age and muscle dependent [167,182]. Importantly, in young and old *mdx* mice, microdystrophin mediated correction of the abnormal properties of the diaphragm is significantly more effective than in limb muscles. This observation is noteworthy considering the phenotype in the diaphragm of the *mdx* mouse much more closely mimics the pathology found in human muscle and many patients succumb to respiratory failure. Even with these encouraging results it is possible that other more effective microdystrophins could be engineered.

Two such strategies have come to light that may allow larger minidystrophins to be expressed by rAAV delivery. The first is gene expression following trans-splicing. This approach utilizes two rAAV vectors with the transgene split between them. The 5′ gene portion is followed by a splice donor sequence from a natural dystrophin intron without a polyadenylation signal while the 3′ gene portion is preceded by a splice acceptor from the same intron, followed by a polyadenylation signal [182]. Expression of the large protein occurs in co-infected cells, where the viral genomes concatamerize, and cell mediated splicing generates an mRNA for a minidystrophin. RNA processing thus represents a crucial barrier to the efficiency of cellular transduction using this vector system. The sequence environment of these splice sites and their location within the split gene can have profound influences on transcription and splicing efficiencies [184,185].

Another system that utilizes a dual vector strategy is often referred to as recombination or overlapping rAAV vectors. In essence, two rAAV vectors containing overlapping fragments of a larger gene are delivered simultaneously to cells resulting in homologous recombination at the overlap such that the larger gene is reconstituted [186–188]. The efficiency with which transduction occurs using the recombination system can be influenced by the AAV serotype, desired tissue target, and the specific transgene overlap composition. In mouse lung it was demonstrated that rAAV6-Alkaline phosphatase (AP) was superior to that of rAAV2-AP, where the degree and distribution of transduction of the former recombinant vectors was similar to the parental intact vector. Similarly, rAAV6 recombination vectors have been found to be more efficient in skeletal muscle than rAAV2 counterparts [186]. Further, this study demonstrated that the transgene overlap can influence the recombination kinetics. This observation implies that sequence characteristics of the overlap region such as degree of secondary structure, and GC content should be taken into account when designing such a system.

11. Compensatory therapeutics

11.1. Utrophin

The spectrin superfamily of proteins includes dystrophin, utrophin (formerly called dystrophin-related protein, or DRP), DRP2, and dystrobrevin [189–191]. There is a high degree of sequence similarity between dystrophin and utrophin at both the DNA and protein levels [191,192]. Similar to dystrophin, utrophin is encoded by many small exons over a relatively large genomic region (~1 Mb) [193]. Additionally, transcription is carried out from several promoters [131], and several truncated isoforms have been identified [138,194,195]. In normal skeletal muscle utrophin is found concentrated at the myotendinous and neuromuscular junctions, whereas it is localized under the entire sarcolemma in regenerating fibers present in *mdx* skeletal muscle [196,197]. In skeletal muscle, utrophin is detectable in early fetal development over the sarcolemma where during development it is gradually replaced by dystrophin [198]. This difference in developmental expression has resulted in the proposal that utrophin may be the embryonic/neonatal form of dystrophin [199], implying that utrophin provides dystrophin-like function in embryonic and developing skeletal muscle. Recent evidence has come to light that may partially begin to explain some of these modest differences in terms of the structural/functional relationships of these two quite similar proteins. Alternative molecular mechanisms of beta-dystroglycan [200] and actin filament interactions [201–203] for utrophin when compared to dystrophin have been put forth. It may be that subtle differences or undisclosed areas within or adjacent to the N-terminal or cysteine-rich domains may impart a more significant contribution to binding affinity than previously considered. This would be of obvious importance when considering the development of micro or mini genes for therapeutic potential. In contrast to dystrophin, whose actin binding domain resides within the amino terminus and the central rod domain (Fig. 1); utrophin actin binding capacity resides in the continuous segment that includes the amino terminus and the first ten spectrin-like repeats of the rod domain [202]. Given this arrangement, and the degree of developmental expression, utrophin has been proposed to act as a stabilizer of newly formed actin filaments during costamere development [203]. Additionally, given the non-competitive nature of dystrophin/utrophin binding the overlapping expression of these two proteins may provide for an effective changing of the muscle fiber guard during the period shortly after birth of utrophin downregulation and dystrophin upregulation.

This expression pattern led to the hypothesis that utrophin may be able to functionally compensate for dystrophin in DMD patients [204]. This idea was supported by studies in *mdx* mice where it was found that elevation of utrophin levels in dystrophic muscle fibers restored sarcolemmal expression of

DGC members, and alleviated the dystrophic pathology. The concept of upregulating utrophin to allow for a replacement of dysfunctional or absent dystrophin provides promising therapeutic possibilities for DMD. For example, adenovirus delivery of utrophin in muscle has shown phenotypic improvement in *mdx* and double knock-out (*dko*) mice that are deficient for both utrophin and dystrophin [205,206]. In the *dko* mice administered the utrophin minigene neonatally, expression was detected 30 days post-injection in ~95% of myofibers within the limb muscle, providing protection from subsequent dystrophic damage in dystrophin deficient muscle. Further, transgenic *mdx* mice that expressed full-length utrophin mRNA had a more improved outcome in preventing dystrophy even with relatively low levels of expression [207]. Thus, experimental evidence suggests that a gene transfer approach focusing on the exogenous delivery of utrophin to DMD muscle fibers is a viable treatment option for this disease. As well, there is an added benefit to this approach; the lack of neoantigen production and subsequent immune response against the transgene that might result with the delivery of dystrophin [208].

Further evidence that utrophin can functionally compensate for the lack of dystrophin in muscle was provided by a transgenic *mdx* mouse line that expressed a mini-utrophin in muscle [209]. The results were striking: histochemical analysis demonstrated a dramatic decrease in fibrosis, necrosis, serum creatine kinase levels, and the frequency of centrally nucleated myotubes. It is noteworthy that no toxicity was associated with high-level expression of utrophin, even in non-muscle tissues [201,207,210]. Subtle structural differences between dystrophin and utrophin are known. For example, there is a short extension in the N-terminal domain that is absent from dystrophin that results in a greater binding affinity for actin. Also these two proteins do not appear to compete for binding sites on actin [203,211,212]. Furthermore, a difference can be found when comparing the rod domain of these two proteins though it is thought that this region has been under less selective pressure [74]. Obvious differences include a less defined first hinge following the N-terminal domain, two fewer spectrin-like repeats, and the lack of basically charged spectrin-like repeats that provide a second actin-binding domain in dystrophin [213]. These differences are in contrast to the cysteine-rich and C-terminal domains, which display ~80% amino acid sequence identity between dystrophin and utrophin [214].

12. Endogenous utrophin up-regulation

A number of labs have investigated the possibility of stimulating endogenous utrophin expression by pharmacological intervention. A challenge with this approach is achieving increased expression of utrophin along the entire length of myofibers to act as a dystrophin surrogate. In order to accomplish this one must begin to understand what motifs within the DNA are responsible for restricting utrophin expression at the neuromuscular junction and for allowing more widespread expression in developing and regenerating myofibers. Several labs are using such analyses to develop small-targeted molecules that might enable upregulation of the gene in all myonuclei. A more thorough understanding of trans-acting factors and their appropriate signaling pathways would be of great benefit towards the design and delivery of specific molecules to induce targeted transcriptional activation. Recently, the delivery of a small peptide region of the heregulin ectodomain to *mdx* mice increased utrophin expression and ameliorated the dystrophic phenotype [215]. The improvements appeared to be a result of utrophin up-regulation as the same treatment in the *dko* model failed to improve muscle pathology. However, the benefits obtained were not as dramatic (~2–3 fold) as those obtained previously by somatic gene transfer of mini-utrophin genes [205].

In addition to transcriptional regulatory mechanisms, utrophin appears to be regulated post-transcriptionally [216–219]. Regions within the 3′ untranslated region have been found to be responsible for targeting and stabilizing utrophin transcripts, allowing a potential means of sustaining increased levels in muscle. Studies in vivo have demonstrated that post-transcriptional processing plays a contributory role towards increased utrophin transcript stability in slow *versus* fast twitch muscle [219]. It has been proposed that this may be regulated by calcineurin levels [220]. This study indicated that transgenic mice overexpressing calcineurin showed increased utrophin mRNA levels, thought to be mediated through NFAT signaling, an effector of calcineurin. Inhibition of calcineurin resulted in an ~80% decrease in utrophin mRNA levels. An internal ribosomal entry site (IRES) has also been identified within the 5′ untranslated region of utrophin. This IRES has been shown to regulate the expression of utrophin during muscle regeneration [221]. Further, post-translational mechanisms have been implicated in utrophin expression such as the involvement of calpain-mediated proteolysis [222], and the GTPase RhoA was found to stabilize and aid in localization of utrophin protein in muscle cell cultures [223]. All together, each of these regulatory events represent potential targets for the development of strategies designed to increase endogenous utrophin in dystrophin deficient myofibers.

13. Muscle supportive therapy

In the absence of utrophin-based therapy, successful treatment of DMD will likely require gene replacement or correction of the genetic defect. However, given the complexity of the symptoms associated with this progressive disease it is to be expected that therapies may need to be tailored to a particular stage of the disease process. In addition to dystrophin family members, a variety of genes or compounds have been put forth as therapeutic candidates in an effort to address the varied arms of DMD pathogenesis. Most of these approaches are aimed at promoting cellular adhesion, reducing inflammation and eventual necrosis, and sustaining the regenerative capacity; all of which appear to aid in muscle stability. We will present a highlight of potential candidates that can be delivered to muscle using viral vectors many of which may even provide a synergistic effect in combination with dystrophin/utrophin delivery.

14. Promotion of cellular adhesion

Within muscle there are two major cell adhesion complexes that provide muscle fiber attachment to laminin in the basal lamina, the DGC and α7β1 integrin. In transgenic *mdx:utrn−/− dko* mice modest (2–3 fold) overexpression of integrin α7 was sufficient to significantly extend lifespan [224]. Further, the heterodimeric partner β1D was also restored, and the transgenic mice displayed reduced kyphosis and muscle disease pathology. Additionally, delivering α7 integrin with rAAV to muscle, as with utrophin, would have the advantage of not producing an exogenous protein that could be recognized as foreign by the immune system. An additional protein found to be involved in cellular adhesion is a component of the DGC, namely α-dystroglycan (α-Dgn). α-Dgn binds to several extracellular ligands, including laminin, agrin and perlecan in muscle (Fig. 2) [225–228]. Recent reports have shown that this interaction is strictly dependent on the glycosylation status of α-dystroglycan [229–231]. In one study a dramatic beneficial effect on degeneration and histopathology was observed in *mdx* mice that overexpressed N-acetyl galactosamine [232]. This result was suggested to strengthen the link to laminin in the extracellular matrix. Along that same line, overexpression of the glycoslytransferase LARGE was demonstrated to functionally bypass α-dystroglycan glycosylation abnormalities in a model of congenital muscular dystrophy [233]. It is possible that additional forms of post-translational modifications may serve as potential therapeutic targets to support muscle in multiple forms of muscular dystrophy.

15. Intracellular signaling and inflammation

ERKs act as an integrative point for multiple biochemical signals, and are involved in a wide variety of cellular processes. One such process is mechanical stress of muscle fibers where a strong correlation between peak tension and ERK phosphorylation in situ suggests these MAP kinases are particularly responsive to muscle damage [234]. Considering that myofibers in vivo are continually subjected to mechanical forces, disregulation of this pathway and others in response to mechanical stress could impart a considerable role on the progression of muscle pathogenesis. Another MAP kinase, JNK is also activated in mdx mice, and be involved in pathogenesis [235]. Nitric oxide, the product of nitric oxide synthase, functions as the major endothelial-derived relaxing factor. Skeletal-muscle-derived NO functions as an important vasodilator [236]. The activity and expression of nNOS are regulated by both myogenic and neurogenic factors while the association of nNOS with the sarcolemma is mediated by the DGC (Fig. 2). More specifically, nNOS is linked to the DGC through alpha-1-syntrophin PDZ–PDZ domain interactions where production is stimulated by muscle contraction. By a yet to be resolved mechanism, NO opposes alpha-adrenergic vasoconstriction. As a by-product of NO, increased production of cGMP may contribute to the inhibition of receptor-mediated Ca^{++} influx in vascular smooth muscle. In the case of DMD and the absence of dystrophin, nNOS is not properly localized to the sarcolemma [33]. In this circumstance nNOS protein is inappropriately localized within the cytosol, and the absence of nNOS from the sarcolemma can impede NO-mediated regulation of vasodilation [237,238]. DMD muscle was shown to have abnormal vasoconstriction during mild exercise, resulting in muscle ischemia induced by inadequate blood flow to actively contracting muscle [236].

The sarcoglycan (SG) complex is an integral subcomplex of the DGC, and in muscle consists of α, β, γ, and δ-SG (Fig. 2) all of which are glycosylated [49]. Evidence that the /δ-sarcoglycan core can associate with the C-terminus of dystrophin independent of dystroglycan or syntrophin was recently demonstrated [239]. It was further suggested that the interaction between the sarcoglycans and dystrophin is dependent upon the conformation of the sarcoglycan complex. This conformational change may also have an affect on membrane targeting or localization as well as post-translational modification events. The intracellular regions of α, β, and γ-SG have tyrosine phosphorylation sites and it has been shown in cell culture that adhesion leads to phosphorylation of each of these SGs [240]. In vitro, these SGs are phosphorylated by Src kinase [240], similar to the phosphorylation of focal adhesion proteins by integrin-activated kinases (FAK and Src). Recently it has been shown that the SG subcomplex plays a role in mechanical signal transduction in skeletal muscle, where changes in the phosphorylation status of ERK was demonstrated in muscles from γ-SG null ($gsg^{-/-}$) mice when compared to *mdx* muscles [31]. It would be interesting to determine if aberrant ERK phosphorylation that arises in these mice is associated with impaired nNOS signaling. Because the SG subcomplex has been suggested to interact with α-dystrobrevin [45], it may be that the absence of the SGs affects the manner in which nNOS is activated.

The use of antibodies to block the action of tumor necrosis factor alpha (TNF-α), a proinflammatory cytokine also involved in signaling, resulted in reduction in the breakdown of dystrophic muscle in *mdx* mice [241]. This result may be tied to NFκB activation [242,243] which appears to play an important role in muscle wasting [244]. The use of rAAV to deliver the TNF-α soluble receptor in conjunction with microdystrophin may be of benefit in more advanced cases of DMD.

The sarcolemma is also susceptible to microrupture in dystrophic muscles [245] which leads to increased levels of intracellular Ca^{2+} and by extension altered Ca^{2+} signaling, and calpain-mediated proteolysis resulting in muscle degeneration [246]. This idea is consistent with the original formulation of the calcium hypothesis of muscular dystrophy [247]. Interestingly, a recent subproteonomics analysis revealed that a major luminal Ca^{2+} binding protein, calsequestrin and the previously implicated Ca^{2+} shuttle element, sarcalumenin [248] are greatly reduced in *mdx* skeletal muscles [249]. By extension, Ca^{2+} exchangers, Ca^{2+} pumps, and Ca^{2+}-binding proteins may represent viable therapeutic targets for preventing muscle fiber degeneration. Alteration of Ca^{2+} cycling may prove useful in avoiding Ca^{2+}-related proteolytic processes, and future studies may address whether this can be combined with other muscle supportive therapies in order to improve long-term muscle mass and strength.

The identification of contributing factors to the dystrophic process, for example disregulation of signaling pathways, provides additional targets that may modify the disease process. Admittedly, the normal localization of signaling proteins to the sarcolemma likely will not be restored in the absence of an intact DGC, but promotion of muscle cell integrity and survival may be accomplished by exogenous regulation of their downstream effectors. It may be that a combination of agents to simultaneously provide mechanical muscle support, and to fortuitously regulate perturbed pathways could have a more significant benefit.

16. Myostatin and IGF-1

Two potential therapies have been considered for increasing muscle mass. The first is by delivery of insulin-like growth factor-1 (IGF-1). IGF-1 is a signaling peptide involved in muscle myogenesis, which works by enhancing myoblast proliferation, differentiation and survival. Moderate over-expression of IGF-1 in *mdx* muscles yielded increased muscle mass and strength with decreased necrosis and fibrosis [250,251]. Similarly, delivery of IGF-1 using rAAV has been shown to result in phenotypic improvement of dystrophic muscles [251,252]. One study suggested an increase in cellular survival was promoted in the absence of dystrophin by showing a dramatic increase in the phosphorylation status of the anti-apoptotic protein akt in *mdx* mice that received IGF-1 [253]. Another target candidate is the protein myostatin, a TGF-β family member which is a negative regulator of both muscle fiber number and size [254]. Although the precise mechanism by which myostatin inhibition results in increased muscle mass has not been fully elucidated, one report has proposed delayed differentiation coupled with enhanced activation, renewal, and proliferation of satellite cells as a likely mechanism [255]. Whatever the mechanism, it likely does not involve compensation by utrophin [256]. A number of studies have shown that there is indeed a benefit to dystrophic muscles from the blockade of myostatin. However, what the precise benefit is remains debatable. In one study, mice null for both dystrophin and myostatin were generated. As expected, there was increased muscle mass, though in contrast to a study that used neutralizing antibody to myostatin on young *mdx* mice [256], a significant reduction in necrosis and inflammation was not observed [257]. The *mdx* mouse may not be the best model considering that muscles in these mice do not have a robust regenerative capacity. This approach to therapy holds much promise particularly in combination with a dystrophin or utrophin replacement approach as there are several myostatin inhibitors that could be co-delivered systemically with rAAV, such as follistatin and the myostatin prodomain [254,258]. This approach would allow for an increase in the integrity of the sarcolemma membrane *via* restoration of the DGC, as well as added muscle mass and strength that may be necessary to provide a better clinical outcome. In addition, as we develop a better understanding of muscle biology and other biological processes such as aging and atrophy there may be signaling pathways that are revealed whose mediators can become candidates for additional vector-based modulation of muscle.

17. Conclusion

Conceptually the replacement of dystrophin by gene transfer represents an appealing and simplistic approach for the treatment of DMD. However, even though technological advances have allowed small successes in a variety of diseases and animal models, the ability to optimally integrate these advances into a therapy for a human disease that is as complicated as DMD likely requires a more complete understanding of the disease process. Understanding the underlying mechanisms of the degenerative process in the dystrophic muscle fiber is still preliminary and requires consideration of multiple mechanisms and approaches for analysis. Various processes that occur in normal and dystrophic muscle development should be considered in order to shed more light on how muscles degenerate. Finally, the effects on the entire musculature must also be considered because the challenge to retain functionality long-term in proper balance related to movement and posture of the patient will undoubtedly surface.

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