Gene Therapy in Large Animal Models of Muscular Dystrophy

Zejing Wang, Jeffrey S. Chamberlain, Stephen J. Tapscott, and Rainer Storb

Abstract

The muscular dystrophies are a group of genetically and phenotypically heterogeneously inherited diseases characterized by progressive muscle wasting, which can lead to premature death in severe forms such as Duchenne muscular dystrophy (DMD). In many cases they are caused by the absence of proteins that are critical components of the dystrophin-glycoprotein complex, which links the cytoskeleton and the basal lamina. There is no effective treatment for these disorders at present, but several novel strategies for replacing or repairing the defective gene are in development, with early encouraging results from animal models. We review these strategies, which include the use of stem cells of different tissue origins, gene replacement therapies mediated by various viral vectors, and transcript repair treatments using exon skipping strategies. We comment on their advantages and on limitations that must be overcome before successful application to human patients. Our focus is on studies in a clinically relevant large canine model of DMD. Recent advances in the field suggest that effective therapies for muscular dystrophies are on the horizon. Because of the complex nature of these diseases, it may be necessary to combine multiple approaches to achieve a successful treatment.

Key Words: antisense oligonucleotide; dog model; dystrophin; gene therapy; immunosuppression; muscular dystrophy; stem cell; viral vector

Introduction

Muscular Dystrophies

Muscular dystrophies are a group of heterogeneous diseases that primarily affect striated muscles throughout the body. Many of these myopathies are caused by mutations in genes that encode for structural proteins that link the cytoskeleton of muscle fibers to the extracellular matrix. The absence of functional proteins results in destabilization of the muscle membrane, increased muscle fragility and degeneration, and progressive muscle wasting, all of which compromise patients' mobility and, in the severe disease forms (Emery 2002) such as Duchenne muscular dystrophy (DMD[1]), lead to death.

DMD is a lethal X-linked recessive disease that affects 1 of 3,500 boys worldwide. It is caused by loss of the protein dystrophin, a critical component of the dystrophin-glycoprotein complex (DGC[1]) at the sarcolemma (Hoffman et al. 1987; Muntoni et al. 2003; Tyler 2003). The absence of dystrophin prevents assembly of the DGC, resulting in a functionally impaired sarcolemma; membranes then become highly susceptible to mechanical contraction-induced damage, which leads to cycles of myofiber necrosis and regeneration, and hence progressive loss of muscle mass. As muscle tissue is lost, it is gradually replaced by connective tissue and adipose cells (Foidart et al. 1981).

The clinical course of DMD is severe and progressive, although the disease phenotype and progression vary and may change over time. Affected individuals can be diagnosed at birth on the basis of elevated serum levels of muscle enzymes. They exhibit muscle weakness by age 5, lose independent ambulation, and succumb to respiratory failure or cardiomyopathy in their late teens or twenties (Muntoni et al. 2003; Tyler 2003). The disease differentially affects adjacent muscles and may even completely spare some muscles, such as the extraocular muscle (Khurana et al. 1995; Porter 1998). Increasing data suggest that secondary responses, such as inflammatory processes, may play major roles in promoting the pathology of dystrophin-deficient muscle through upregulation of major histocompatibility complex (MHC) molecules, various chemokines, and molecules necessary for the costimulation and eventual activation of T cells (Porter et al. 2003; Tidball and Wehling-Henricks 2005; Wiendl et al. 2005).

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[1]The definitions of this and other terms and abbreviations used in this article are in the Glossary on page 198.

Loss of calcium homeostasis may also be a cause of muscular dystrophy, but whether it is the primary cause of muscle fiber degradation or a secondary phenomenon resulting from fiber microlesions remains to be determined (Muntoni et al. 2003; Spencer and Mellgren 2002).

Despite the well-understood pathogenesis of DMD, the development of curative therapies targeting its primary causes remains a major challenge. However, several novel strategies have been the subject of preclinical studies and raise hope for the discovery of treatments for human patients. Here, we review results of cell- and viral vector–based gene replacement and repair as well as antisense oligonucleotide–mediated gene correction in a canine model of DMD.

Dystrophin and Dystrophin Deficiency

Dystrophin, the largest known gene, is located on the X chromosome, with 79 exons encoding a full-length 14,000 base pair (bp) mRNA distributed over more than 2 million bases of genomic sequences. Seven promoters linked to five different first exons give rise to various isoforms in a tissue-specific manner (Hoffman et al. 1987, 1988; Koenig et al. 1988; Muntoni et al. 2003). The full-length dystrophin protein is a large rod-shaped protein with a molecular weight of 427 kDa. It has four functional domains: the amino-terminus contains an actin-binding domain for anchoring dystrophin to the cytoskeleton; the central rod domain contains 24 spectrin-like repeats that constitute a flexible and elastic region with actin-binding properties; and the cysteine-rich and carboxyl-terminal domains interact with the DGC members (Abmayr and Chamberlain 2006; Hoffman et al. 1988; Koenig et al. 1988; Muntoni et al. 2003; Rando 2001). While the full-length dystrophin is normally expressed in striated muscle, smooth muscle, and neurons, multiple smaller dystrophin isoforms are exclusively or predominantly expressed in various nonmuscle tissues (e.g., the retina, glia, liver, and kidney) through the use of internal promoters and alternative splicing events (Byers et al. 1993; Lidov et al. 1995; Muntoni et al. 2003). Thus, although dystrophin deficiency is primarily manifested in muscle tissue, it can also lead to cognitive impairment (Muntoni et al. 2003). To date, there is no evidence that it leads to pathological abnormalities in other tissues.

Various mutations in the dystrophin gene can cause dystrophin deficiency, which presents as DMD or the milder Becker muscular dystrophy (BMD[1]). Intragenic deletions, the most common mutations, occur in 60-65% of DMD and BMD patients, duplication in 5-15% of the cases, and nonsense point mutations and other small mutations account for the remaining 20-35% (Muntoni et al. 2003). Disease severity is not simply related to the extent of a deletion or duplication but rather depends on whether it disrupts the normal open reading frame and allows expression of the dystroglycan-binding domain. Generally, in-frame mutations result in truncated yet partly functional dystrophin proteins and are associated with BMD, while frame shift mutations, which result in unstable RNA and complete absence of the dystrophin protein, are associated with DMD (Kerr et al. 2001; Muntoni et al. 2003). Information from studies of genotype-phenotype relationships in humans with partial deletion mutations and from *mdx* transgenic mice have shown that deletions in the N-terminal or the dystroglycan-binding domains of the dystrophin cause more severe clinical phenotypes (Harper et al. 2002b; Koenig et al. 1989; Muntoni et al. 2003).

Dystrophin interacts with integral and peripheral membrane proteins including dystroglycan, syntrophin, sarcoglycan, sarcospan, and dystrobrevin, which collectively constitute the dystrophin-glycoprotein complex. At the sarcolemma of striated and smooth muscles, the DGC spans the plasma membrane and provides a strong mechanical link connecting the intracellular γ-actin cytoskeleton to the extracellular matrix (Ervasti 2006). The absence of dystrophin prevents assembly of the DGC and reduces the levels of all DGC components (Lapidos et al. 2004; Muntoni et al. 2003). The resulting sarcolemma is mechanically fragile due to the inability to laterally transmit forces from within myofibers to the extracellular matrix, thereby rendering it highly susceptible to contraction-induced injuries that can trigger muscle necrosis (DelloRusso et al. 2001). A growing body of evidence has shown that the DGC is also a transmembrane signaling complex. Therefore, muscle cell death is likely related to disruption of cell survival pathways and cellular defense mechanisms that are regulated by signaling cascades (Lapidos et al. 2004; Muntoni et al. 2003; Thomas et al. 1998).

Animal Models of DMD

There are two naturally occurring animal models for DMD, X-linked *mdx* mice and X-linked muscular dystrophy dogs (*cxmd*[1]). The *mdx* mouse carries a single-point mutation in exon 23 of the dystrophin gene that results in a premature stop codon (Sicinski et al. 1989). Despite the absence of dystrophin expression in muscle, young *mdx* mice display a very mild phenotype, apart from the diaphragm, compared to DMD patients, especially with respect to cardiomyopathy. However, as the mice age the phenotype progressively worsens and they display a 20% reduction in lifespan (Chamberlain et al. 2007; Lynch et al. 2001). In addition to the original *mdx* mouse, researchers have characterized several additional strains that have different mutations and differential expression of dystrophin isoforms with a similar pathological phenotype (Cox et al. 1993b; Im et al. 1996). Canine *cxmd* results from a point mutation in a consensus splice acceptor site in intron 6 of the dystrophin gene, which leads to skipping of exon 7, a disruption in the open reading frame, and premature termination of translation (Howell et al. 1997; Sharp et al. 1992). In contrast to *mdx* mice, the clinical course of *cxmd* dogs is very similar to that of DMD humans, characterized by progressive muscle wasting, degeneration and fibrosis, and a shortened life span. But because of the *mdx* mouse's low cost and short gestation times, it remains the most widely used animal model (Allamand and Campbell 2000; Gregorevic et al. 2008; Sicinski et al. 1989; Stedman et al. 1991).

Investigators first identified and characterized canine X-linked muscular dystrophy (*cxmd*) in golden retrievers (Cooper et al. 1988; Kornegay et al. 1988, 1990; Valentine et al. 1988) and then in other breeds including rottweilers (Collins and Morgan 2003) and German shorthaired pointers (Schatzberg et al. 1999). One group produced a beagle model by crossing the golden retriever mutant with beagles (Shimatsu et al. 2003). Litters of golden retrievers crossed with beagles or mongrels have been raised at the Fred Hutchinson Cancer Research Center (Dell'Agnola et al. 2004).

Muscle lesions in *cxmd* start to develop in utero. Between 6 and 8 weeks of age affected pups begin to show clinical symptoms, which can be quite pronounced by 6 months. The dogs typically die from cardiac or respiratory malfunctions within days, months, or 2 to 4 years after birth (Howell et al. 1997; Valentine et al. 1986, 1988, 1992; Valentine and Cooper 1991). Because *cxmd* dogs require extra daily care for reasonable weight and health, the maintenance of a *cxmd* dog colony is challenging and expensive. However, their severe disease manifestations and progression make them the most useful preclinical animal model for testing therapeutic interventions that have promise for human DMD (Collins and Morgan 2003; Cooper et al. 1988; Howell et al. 1997).

Treatments

Conventional Treatment for DMD

In the absence of curative therapies for human DMD, therapeutic interventions control secondary symptoms with the aim of slowing progression of the disease and improving quality of life.

- The use of steroids such as oxandrolone and prednisone helps to increase protein synthesis and thus conserve muscle mass (Tidball and Wehling-Henricks 2004; Wagner et al. 2007; Zhao et al. 2004).
- Proteolytic systems are targeted for decreasing proteolysis in dystrophic muscle and slowing loss of muscle mass.
- Increased influx of calcium may be an option for increasing the activity of a calcium-dependent protease, calpain, in dystrophic muscle.
- Because overexpression of calpastatin, a natural inhibitor of calpain, reduces necrosis in *mdx* muscle (Spencer and Mellgren 2002; Tidball and Wehling-Henricks 2004), investigators have used β2-adrenergic agonists and pharmacophore to inhibit calpain activity (Burdi et al. 2006; Spencer and Mellgren 2002).
- The application of immunosuppressants such as glucocorticoids and anti-TNFα antibody reduces the inflammatory responses associated with the disease and delays pathology (Grounds and Torrisi 2004; Manzur et al. 2008; Wagner et al. 2007; Wehling-Henricks et al. 2004).

Although some of these treatments have resulted in improvements of muscle function and a slowing of disease progression in both the mouse model and human patients, adverse side effects have limited their usefulness. Hope for eventual correction of DMD disease symptoms rests with the stable, systemic introduction of a functional dystrophin gene into the muscles of DMD patients.

Developing New Strategies for Treating Dystrophin Deficiency

A number of studies have examined transplantations of normal stem cells such as hematopoietic stem cells (HSCs[1]), myoblasts, and stem cells derived from other tissue types as possible treatments for DMD and as a gene delivery system for therapeutic recombinant proteins (Camargo et al. 2003; Huard et al. 2003; Lee-Pullen et al. 2004; Parker et al. 2008; Partridge et al. 1998; Torrente et al. 2004). The disease phenotype of the *mdx* mouse model also shows improvement after the injection, either intramuscular or intravenous (Gregorevic and Chamberlain 2003; Gregorevic et al. 2004; Liu et al. 2005), of viral vectors encoding full-length or truncated but functional dystrophins (DelloRusso et al. 2002; Dudley et al. 2004; Harper et al. 2002b). Furthermore, it is possible to modify the mutant dystrophin mRNA by inducing skipping of the mutation-containing exons, through the use of specific antisense oligonucleotides, sometimes embedded in small nuclear ribonucleoproteins (snRNPs). This strategy can lead to restoration of an open reading frame, which has raised another possible approach for treatment (Alter et al. 2006; Bertoni 2008; Dunckley et al. 1998; McClorey et al. 2006).

However, these approaches are not without many significant hurdles, such as

- poor survival and limited dissemination of injected cells,
- immune responses to allogeneic cells, viral vectors, and neotransgene product (especially considering the preexisting inflammatory lesions in dystrophic muscle),
- unwanted ectopic gene expression, and
- inability to achieve bodywide muscle delivery (including cardiac tissue) of cells, vectors, and antisense oligonucleotides.

Stem Cells

Stem cells can serve as vehicles to deliver normal genes to mutant organisms. In the case of DMD, it is the hope that stem cells or their progeny carrying a wild-type dystrophin gene would home to the dystrophic muscle, proliferate, and differentiate to form new muscle fibers or fuse to existing myofibers, restoring the missing dystrophin, leading to assembly of the DGC, and eventually ameliorating muscle pathology and improving muscle function.

Hematopoietic stem cells. HSCs include multipotent cells that predominantly reside in the bone marrow and have the capacity for self-renewal and multilineage differentiation (Bellantuono 2004; Lakshmipathy and Verfaillie 2005).[2] Hematopoietic cell transplantation (HCT[1]) is a curative treatment for patients

[2]Multipotent cells also give rise to nonhematopoietic cells, as in bone and liver, vascular endothelial cells, and astroglia in the brain (Bhattacharya et al. 2000; Shi et al. 1998; Torrente et al. 2004), possibly through transdifferentiation or cell fusion (Bellantuono 2004) or recruitment of subpopulations of tissue-committed stem cells (Lakshmipathy and Verfaillie 2005; Torrente et al. 2004).

with hematological disorders (Storb 2003), but not, so far, for muscular dystrophy. Transplantation of wild-type bone marrow cells into mouse muscles with chemically or crush-induced injury or in the *mdx* mouse resulted in detectable donor-derived cells and dystrophin-positive fibers in skeletal and cardiac muscle in earlier studies (Bittner et al. 1999; Ferrari et al. 1998; Gussoni et al. 1999) but not in more recent ones (Kucia et al. 2005; LaBarge and Blau 2002; Torrente et al. 2004). Studies in seven *cxmd* dogs failed to demonstrate a contribution of HSC to skeletal muscle regeneration (Dell'Agnola et al. 2004), despite stable, complete, or near complete donor hematopoietic chimerism. There were no detectable contributions of bone marrow–derived cells to either skeletal muscle (assayed by reverse transcriptase polymerase chain reaction [RT-PCR] and immunoassays) or muscle satellite (cells assayed by clonal analyses). However, muscle cells from the HSC donors did survive (Dell'Agnola et al. 2004). Results from this clinically relevant dog model indicated that allogeneic HCT was of no therapeutic value. Moreover, injections of donor bone marrow cells in the muscles of *cxmd* dogs made tolerant by preceding DLA-identical HCT also failed to restore dystrophin expression or result in muscle regeneration (Kuhr et al. 2007a).

Muscle stem cells. Muscle-derived stem cells from healthy donors are of potential use for muscle regeneration in DMD patients. The ability of adult skeletal muscle to repair and regenerate itself after injury is largely attributable to muscle satellite cells, which represent a distinct population of myogenic precursors with self-renewal and muscle regeneration properties (Grounds and Davies 2007; Jankowski et al. 2002; Pirenne and Kawai 2004). In DMD, multiple cycles of muscle degeneration and regeneration are associated with a decreasing ability of the satellite cell to efficiently support muscle regeneration (Dhawan and Rando 2005); transplantation of normal myoblasts into dystrophin-deficient muscle may establish a normal myoblast reservoir for delivering dystrophin and regenerating muscle fibers. Fusion of mononucleated myoblasts either into multinucleated muscle fibers or with existing myofibers is a normal part of muscle repair. Normal myoblasts are able to fuse with dystrophic myoblasts to form myotubes that express dystrophin in vitro (Huard et al. 2003; Lee-Pullen et al. 2004). Similarly, intramuscular injection of myoblasts from normal donor mice reconstituted muscle fibers and led to dystrophin expression in *mdx* mice despite the rapid death of a high percentage of donor cells (Beauchamp et al. 1999; Jejurikar and Kuzon 2003; Lee-Pullen et al. 2004; Skuk et al. 2004). However, myoblast transplantation in DMD patients has been disappointing, with poor survival of injected cells, poor migration of newly introduced myoblasts to damaged areas, and immune responses to both the allogeneic donor cells and wild-type dystrophin (Gussoni et al. 1992; Huard et al. 1991, 1992; Mendell et al. 1995; Partridge 2000; Tremblay et al. 1993). Early studies of myoblast transplantation in dogs from the Tremblay group (Ito et al. 1998) suggested that the combination of three immunosuppressive drugs (FK506; cyclosporine, CSP; and RS-61443, now mycophenolate mofetil, or MMF) effectively controlled immune responses, compared to FK506 alone or CSP plus RS-61443, and allowed engraftment of allogeneic myoblasts for 4 weeks. However, FK506 and CSP-related toxicities were prominent in these dogs. An alternative way to induce tolerance to allogeneic donor myoblasts is to create mixed or complete donor hematopoietic chimerism in recipient dogs treated with DLA-identical HCT (Storb 2003; Storb et al. 1997). This strategy has successfully induced tolerance to donor-derived solid organ transplantation (e.g., kidney, liver, and pancreatic islet cells) in mice and dogs without the need for long-term conventional immunosuppression (Kuhr et al. 2007b). A recent study using *cxmd* dogs (Parker et al. 2008) demonstrated that DLA-identical HCT provided an immune-tolerant platform for subsequent transplantation and stable engraftment of HSC donor-derived myoblasts in the absence of pharmacological immunosuppression. Two chimeric dystrophic dogs received intramuscular injections of freshly isolated myoblasts. The level of wild-type dystrophin mRNA was increased to 6.5% of normal levels in one dog 10 weeks later, and to 1.3% in the other 24 weeks later, and correlated with increased dystrophin protein expression. It is thus possible to induce allogeneic tolerance to donor-specific myoblasts by allogeneic HCT, an option that raises hopes for future use of muscle stem cells as a means of delivering dystrophin to dystrophic muscle.

Mesoangioblasts. After researchers isolated mesoangioblasts from the dorsal aorta of mouse embryos, they found that these blood vessel–derived stem cells can differentiate into various tissue types, including skeletal and cardiac muscles (Cossu and Bianco 2003; De Angelis et al. 1999; Sampaolesi et al. 2003, 2006), and can cross the vascular barrier. Systemic delivery of donor mesoangioblasts via intra-arterial injection in dystrophic and *mdx*/utrophin null mice led to amelioration of muscle structure and function (Berry et al. 2007; Sampaolesi et al. 2003). Cossu and colleagues tested this approach in dystrophic dogs (Sampaolesi et al. 2006) by isolating wild-type or dystrophic mesoangioblasts from small blood vessels in the muscles of young dogs. They genetically modified dystrophic cells by transduction with a human microdystrophin using lentiviral vectors before injection into the femoral artery of the dystrophic dog of origin, and injected allogeneic cells under immunosuppression with CSP or rapamycin into the wild-type dog. All dogs also received steroids. Expression of dystrophin was detected in up to 70% of muscle fibers in the dogs that received wild-type mesoangioblasts, with improved muscle force generation and mobility. The results were encouraging, but critics emphasized the need to clarify several questions. For example, some studies in *mdx* mice and in DMD patients have suggested some benefit from CSP in ameliorating the dystrophic pathology and improving muscle strength (Davies and Grounds 2006; De Luca et al. 2005; Miller et al. 1997), but no dystrophic dogs that received the same drugs but without mesoangioblast transplantation were used in this study to exclude any effect of immunosuppression. Other questions concern the lack of a strong correlation between muscle function and the extent of expression of dystrophin, and the poor performance of genetically modified autologous cells compared to allogeneic cells despite production of human dystrophin in the canine muscle (Chamberlain 2006; Davies and Grounds 2006; Grounds and Davies 2007).

Viral Vector–Mediated Gene Therapy

The single gene mutation underlying DMD makes viral vector–mediated gene replacement an attractive strategy. Studies have focused on adenoviral (Ad), retroviral, and adeno-associated viral (AAV[1]) vectors and have shown that delivery via these vectors of full-length or truncated but functional dystrophin improves the disease phenotype of the *mdx* mouse model (Dudley et al. 2004; Gregorevic et al. 2004; Gregorevic and Chamberlain 2003; Liu et al. 2005). The level of dystrophin expression required for amelioration of skeletal muscle function in *mdx* mice may be above 20% of normal endogenous levels, with no toxicity seen from a fiftyfold overexpression (Cox et al. 1993a; Phelps et al. 1995; Wells et al. 1995), but the minimum and maximum levels of dystrophin expression in cardiac muscle remain to be determined (Duan 2006). Of the vectors used in these studies, Ad and AAV were tested in dog models.

Adenoviral vectors are among the most commonly used vectors for gene therapy (Ghosh et al. 2006) because of their large cloning capacity, nononcogenic nature, and ability to transduce both dividing and nondividing cells with high efficiency, including in heart and skeletal muscles. However, a major problem associated with early generations of adenoviral vectors has been robust immune reactions against both viral and transgene products in *mdx* mice and *cxmd* golden retrievers (Ghosh et al. 2006; Howell et al. 1998; Kay et al. 2001). "Gutted" adenoviral vectors lack all viral coding sequences and have a cloning capacity of about 35 kb, which is ideal for carrying large genes such as the full-length dystrophin gene (Hartigan-O'Connor et al. 2002). A gutted vector carrying full-length dystrophin can transduce muscle in *mdx* mice and *cxmd* golden retrievers, and result in expression of the dystrophin protein in muscle fibers despite humoral immune responses against dystrophin (DelloRusso et al. 2002; Dudley et al. 2004; Gilbert et al. 2001). However, gutted versions of Ad vectors are difficult to grow and scale up to pharmaceutical levels, humoral and cellular reactions in the hosts may result from the use of high vector doses, and intravenous administration of adenoviral vectors results in preferential localization to the liver (Chamberlain 2002; Ghosh et al. 2006; Schiedner et al. 1998). Moreover, Ad vectors do not integrate into the host genome for transgene expression; the relatively short-term expression of the transgene product requires repeated administration.

Retroviral vectors are attractive for treatment of genetic diseases when stable long-term integration in the genome is required. Retroviral vectors can hold up to 11 kb transgene cassettes and show a very low toxicity profile (Chamberlain 2002; Kay et al. 2001; Sinn et al. 2005). Oncoretroviral vectors require dividing cells for efficient cell entry, and transgene expression is dependent on the site of integration and cell division. Moreover, retroviral vectors integrate in somewhat random locations and thus can cause insertional mutagenesis and activation of nearby genes, including oncogenes (Baum et al. 2006). The development of leukemia in part due to ectopic activation of the Lmo2 oncogene in a clinical trial of gene therapy for severe combined immune deficiency disease has confirmed concerns about retroviral vector–based gene delivery (Hacein-Bey-Abina et al. 2002, 2003). Furthermore, the transduction efficiency of retroviral vectors carrying truncated dystrophin into the muscles of *mdx* mice has been poor and results in only minimal dystrophin expression (Chamberlain 2002; Kay et al. 2001).

Unlike the oncoretroviral vectors, lentiviral vectors based on the human immunodeficiency virus can carry 7.5 to 9 kb cargo DNA, transduce dividing and nondividing cells, and lead to stable expression of transgenes in muscle cells and muscle stem cells (Bachrach et al. 2004; Cudre-Mauroux et al. 2003; Kafri et al. 1997; MacKenzie et al. 2002).

The most promising vector for gene replacement has been AAV, a single-stranded DNA virus belonging to the Parvovirus family with more than 11 serotypes identified in both human and nonhuman primates (Gao et al. 2004, 2005; Rutledge et al. 1998). AAVs are advantageous for human muscle gene therapy because they transduce nonreplicating as well as replicating cells (myofiber and cardiomyocytes are postmitotic), and some serotypes (e.g., AAV1, 6, 8, and 9) exhibit high efficiency for the targeting of striated muscles (Athanasopoulos et al. 2004; Blankinship et al. 2006; Pacak et al. 2006; Wang et al. 2005; Warrington and Herzog 2006). There are no known pathogens associated with AAV in humans, and limited cellular immune responses have been reported after AAV-mediated gene delivery in mice. AAVs are small and can be produced at high titers for robust transduction of muscle through either direct injection or systemic delivery.

The use of AAV vectors as a gene delivery vehicle has shown promise both in preclinical studies and clinical trials for a number of acquired and inherited diseases (Athanasopoulos et al. 2004; Warrington and Herzog 2006). Stable local transgene expression has persisted for years in mice, large animals, and humans (Arruda et al. 2005; Jiang et al. 2006), but AAV vectors have a limited cloning capacity of less than 5 kb. In order to overcome the packaging limitation of AAV for treating DMD, a series of mini- and microdystrophin gene expression cassettes have been developed (Figure 1) based on information from studies of genotype-phenotype relationships in DMD and BMD patients and from transgenic studies in *mdx* mice (Harper et al. 2002a; Scott et al. 2002). While DMD is caused by frame shift mutations in the dystrophin gene, the milder BMD is typically caused by in-frame mutations in the dystrophin gene. The finding that some BMD patients with large deletions in the central rod domain display milder phenotypes than DMD patients suggested that this domain is of limited function and largely dispensable (Aartsma-Rus et al. 2006; Aartsma-Rus and van Ommen 2007; Abmayr and Chamberlain 2006; England et al. 1990). One of the smallest constructs, ΔR4-R23/ΔCT, is only 3.6 kb and contains the N-terminal domain, the first three and the last one of the 24 spectrin-like repeats, and the cysteine-rich domain (Figure 1) (Harper et al. 2002a).

Administration of AAV vectors containing these engineered constructs via either intramuscular or intravenous routes into *mdx* mice significantly improves muscle

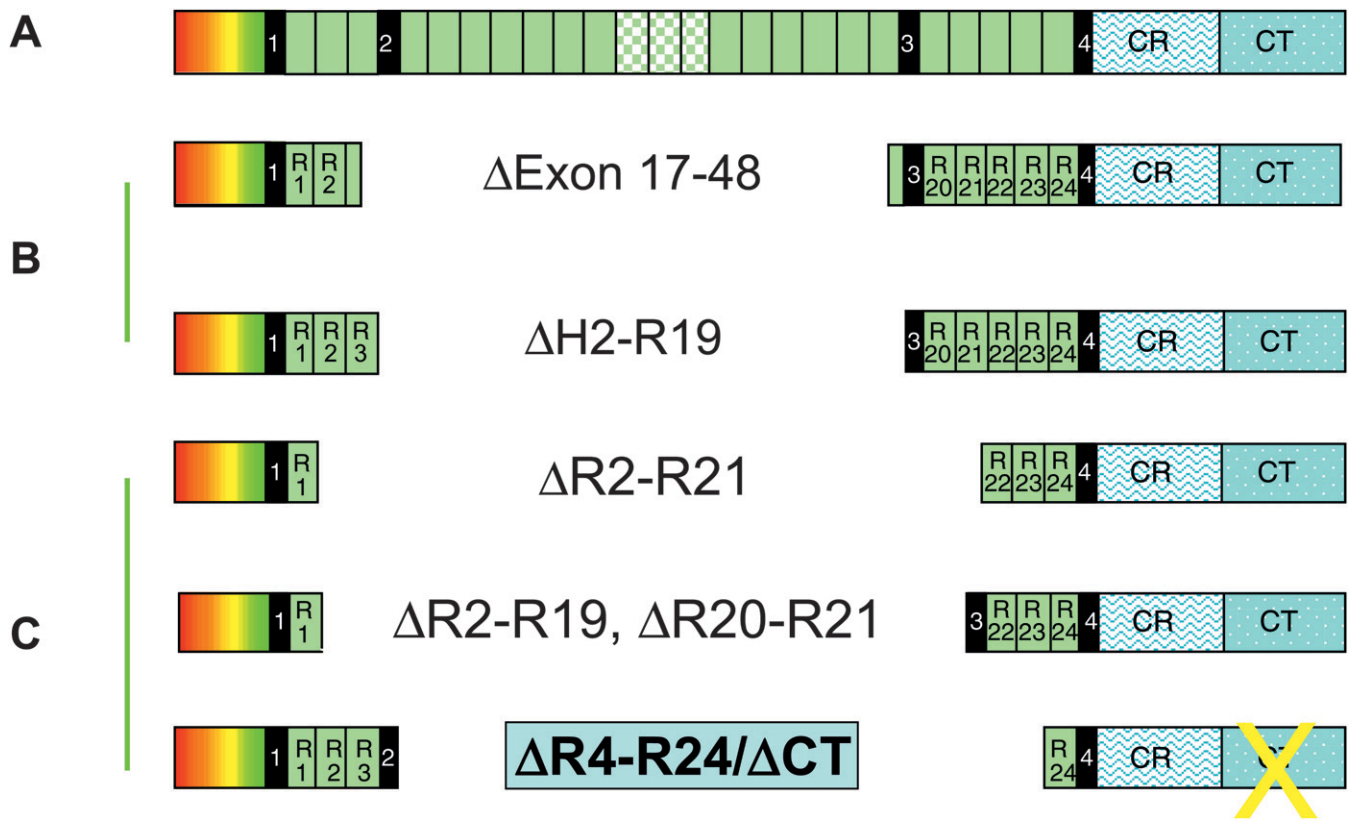

Figure 1 Full-length, mini-, and microdystrophin. A. Full-length dystrophin. B. Minidystrophins with the deletion of various numbers of spectrin repeats. C. Microdystrophins with the deletion of various numbers of spectrin repeats with or without C terminus. CR, cysteine-rich domain; CT, carboxyl terminus; R, spectrin repeat; Δ, deletion.

membrane integrity and muscle function (Gregorevic and Chamberlain 2003; Gregorevic et al. 2004; Harper et al. 2002a; Liu et al. 2005). The efficacy of this treatment was further evaluated in a clinically relevant *cxmd* model (Wang et al. 2007a). In sharp contrast to the murine studies, local intramuscular injection of AAV2 or AAV6 vectors into muscles of wild-type dogs induced robust T cell–mediated immune responses against AAV capsid proteins that peaked 4 weeks after vector injection and that limited long-term transgene expression. The immune responses are triggered regardless of the nature of transgene constructs, vector purification methods, or concentrations (from 5×10^9 to 5×10^{12} vector genomes/dog). A follow-up study (Wang et al. 2007b) addressed this issue in both wild-type and dystrophic dogs by evaluating immunosuppression regimens. The study demonstrated that a brief course of immunosuppression with a combination of CSP, MMF, and rabbit anticanine thymocyte globulin (ATG) was able to avert cellular immune responses against AAV6 vector carrying a canine microdystrophin, allowing sustained dystrophin expression in *cxmd* muscle, which was sufficient for restoring DGC assembly at the muscle membrane. This observation in a large, immunocompetent, random-bred dog model using a species-specific transgene has set the stage for further investigations of the specificity of immune responses to AAV vectors, the use of alternative, less toxic immunosuppressive regimens, and evaluation of the efficacy and efficiency of systemic ways of delivering transgenes to muscles (Arruda et al. 2005; Su et al. 2005) before embarking on human trials.

Another major challenge is how to achieve bodywide muscle transduction of therapeutic vectors. Systemic gene delivery via intravenous or intra-arterial injection of several AAV serotypes (AAV6, 8, and 9) in rodents demonstrated robust transduction throughout the body, including cardiac muscle (Gregorevic et al. 2004; Inagaki et al. 2006; Pacak et al. 2006; Wang et al. 2006). However, there is a significant difference in body size between rodents and large vertebrates, and the differences in anatomy and physiology, including vascular permeability, also pose great challenges to translation of this technique to humans. A recent study from Duan's group suggested that bodywide delivery of therapeutic genes may be an achievable goal in larger animals (Yue et al. 2008). In that study, four neonatal dogs (24 or 48 hours after birth) received single injections in the jugular vein of AAV9 vectors at doses ranging from 1×10^{11} to 2.5×10^{11} vg/g body weight. Robust whole-body skeletal muscle transduction occurred in all cases, and expression of the transgenes persisted for 6 months without detectable cellular infiltrations. One of the four pups contracted a deadly illness after vector administration and the authors suggested this

might have been due to a higher vector dose (2.5×10^{11} vg/g) given at an early age (24 hours after birth). Otherwise, the study results certainly raised hopes for potential application of systemic AAV delivery in human patients. However, one has to keep in mind when considering treatment for adult humans that the immune system in newborns is immature compared to that of adults, and vessel permeability may be different in young versus adult animals. Cardiomyopathy is a leading cause of death in DMD patients, and no cardiac muscle transduction was observed in this study, in contrast to murine data. The safety of widespread dissemination of virus throughout the body as a result of systemic delivery should also be taken into consideration when adapting these strategies to clinical trials.

Antisense Oligonucleotide–Mediated Gene Correction

Another strategy to convert a severe DMD phenotype to a milder BMD phenotype is to modulate splicing of the dystrophin mRNA and skip exons containing mutations that disrupt the open reading frame (Aartsma-Rus et al. 2003; Muntoni et al. 2003). Studies of targeted exon skipping have used several types of antisense oligonucleotides (AOs[1]), which include 2′-O-methyl phosphorothioate (2OMe) and modified alternatives, phosphorodiamidate morpholino oligomers (PMOs), and peptide nucleic acid (PNA) AOs. These studies are aimed at modulating gene expression rather than adding new genes (reviewed in Aartsma-Rus and van Ommen 2007; Foster et al. 2006). The mechanism of exon skipping is based on the binding of AOs to specific target sense sequences in the dystrophin pre-mRNA. This binding may either alter the local configuration of the pre-mRNA so the splicing machinery can no longer recognize it and therefore skips it, or interfere with components of the splicing machinery (Aartsma-Rus et al. 2003; van Deutekom and van Ommen 2003). The end result is to remove a portion of the mRNA and restore an open reading frame. Theoretical predictions have suggested that targeting one or more of only twelve exons in the dystrophin gene could lead to treatment options for approximately 75% of the mutations that cause human DMD (Aartsma-Rus et al. 2003; Aartsma-Rus and van Ommen 2007; Bertoni 2008; van Deutekom and van Ommen 2003). In vitro studies using human and mouse muscle cells (Aartsma-Rus et al. 2002, 2003) and in vivo studies using the *mdx* mouse (Alter et al. 2006; Dunckley et al. 1998; Vitiello et al. 2008) have shown promising results in restoring the open reading frame, thereby leading to expression of smaller, truncated, but functional dystrophins. A recent study from the Wilton group (McClorey et al. 2006) addressed the feasibility of this approach in the canine DMD model in vitro. The study evaluated the efficacy of 2OMe, PMOs, and peptide-linked PMOs to induce truncated in-frame dystrophin expression with the skipping of both exons 6 and 8 to restore the disrupted reading frame due to loss of exon 7 in cultured myoblasts isolated from golden retriever *cxmd* dogs. The group observed that 2OMe AOs were short-lived and caused moderate cell death at a low concentration of 600 nM, whereas PMO and peptide-linked PMO did not cause obvious toxicity to cells at a concentration of 20 μM. However, unconjugated PMO alone transduced cells poorly with limited expression of dystrophin induced at high concentration. In contrast, conjugated transport peptide facilitated transfection of PMOs and induced high levels of exon skipping for at least 10 days with high levels of dystrophin expression.

Whether or not antisense treatment can result in functional benefit remains to be tested in vivo in dogs with DMD. AOs are small, sequence-specific, and synthetic, and are considered relatively safe. They also have the advantage of being able to simultaneously correct all dystrophin isoforms (Aartsma-Rus et al. 2003; Wilton et al. 1999). But there remain several obstacles associated with the effectiveness of the treatment. AOs do not transduce cells efficiently, they can easily be degraded (which limits the duration of effectiveness), and they require repeated administration, probably weekly or monthly for the life of the patient. AOs linked to a modified U7 small nuclear RNA, normally involved in mRNA processing, achieved sustained dystrophin expression from skipped mRNA for more than 13 weeks in the limbs of the *mdx* mouse (Goyenvalle et al. 2004), thereby raising the hope for potential clinical application of this strategy. To date, AO treatment has not successfully induced dystrophin expression in cardiac muscle. In addition, the approach has the potential risk of causing nonspecific splicing aberrations at high concentration, and efficient systemic delivery methods will be required for muscle-specific targeting. Nevertheless, antisense therapy remains one of the more promising strategies for the treatment of most DMD patients.

Perspectives

The discovery and development of new therapy options for muscular dystrophies are ongoing. For stem cell–based approaches, investigators isolated a distinct subpopulation of muscle satellite cells in mice using a combination of markers including CXCR4 and β1-integrin (Cerletti et al. 2008). After intramuscular injection, these cells entered and renewed the endogenous satellite cell pool, participated in repairing damaged muscle by both fusing to existing muscle fibers and forming new fibers, and contributed to more than 90% of myofibers in injected muscle. There is growing interest in using nonviral transfer methods for delivering therapeutic genes, such as the use of naked plasmid DNA to deliver dystrophin cDNA constructs (reviewed in Rando 2007; Scime and Rudnicki 2008); intravascular injection of naked DNA into rodents and pigs showed encouraging results (Danialou et al. 2005; Wolff et al. 2005). PTC124, a newly identified chemical entity, promotes read-through of premature nonsense codon as well as the production of dystrophin protein in muscle cells derived from *mdx* mice and human patients with dystrophin genes containing nonsense mutations (Welch et al. 2007). Compensatory therapeutic strategies include the possibilities of promoting muscle regeneration and ameliorating the disease phenotype either (1) to increase the

expression of utrophin (a homologue of dystrophin) to compensate for impaired dystrophin function or (2) to inhibit myostatin (a negative regulator of muscle growth) and thus increase muscle mass (Bogdanovich et al. 2002; Fisher et al. 2001; Qiao et al. 2008, 2009; Wakefield et al. 2000).

The advances in the last two decades in the field of DMD therapeutics are remarkable and have energized the planning and implementation of phase I clinical trials. For example, high-density intramuscular injections of muscle stem cells in DMD patients are the subject of tests by the Tremblay group (Skuk et al. 2006), antisense oligonucleotides targeting exon 51 in DMD patients are being tested by van Deutekom and colleagues (2007), naked plasmids carrying the full-length dystrophin cDNA under the control of the cytomegalovirus (CMV) promoter have been injected into DMD and BMD boys (Romero et al. 2004), and the intramuscular injection of AAV vectors carrying functional human microdystrophin in DMD patients is under investigation by the Mendel group (Rodino-Klapac et al. 2007). The results from the studies that have been made public suggest the feasibility of these approaches in humans, but considerable further investigation is necessary to overcome hurdles such as immune responses, systemic delivery efficiencies, and long-term dystrophin expression. Ideally, such studies will be optimized in large animal models before a successful clinical translation. Ultimately, effective treatment may require combinations of several of these approaches.

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Glossary

AAV: adeno-associated virus, a single-stranded DNA virus belonging to the Parvovirus family; widely used as a gene transfer vehicle in gene therapy.

AOs: antisense oligonucleotides, used for targeted exon skipping; they can either bind to specific target sense sequences in pre-mRNA to alter its local configuration so the splicing machinery no longer recognize it and therefore skips it, or interfere with components of the splicing machinery.

BMD: Becker muscular dystrophy, a milder form of DMD associated with truncated yet partly functional dystrophin protein that results from in-frame mutations in the dystrophin gene.

cxmd: canine X-linked muscular dystrophy, caused by dystrophin deficiency due to frame shift point mutation in the canine dystrophin gene; its clinical and pathological courses are very similar to those of human DMD.

DGC: dystrophin-glycoprotein complex, which spans the plasma membrane and provides a strong mechanical link connecting the intracellular γ-actin cytoskeleton to the extracellular matrix at the sarcolemma of striated and smooth muscles.

DMD: Duchenne muscular dystrophy, a lethal X-linked form of muscular dystrophy caused by the loss of the protein dystrophin, a critical component of the DGC at the muscle membrane.

HCT: hematopoietic cell transplantation, a curative treatment for patients with many hematological disorders.

HSCs: hematopoietic stem cells, multipotent cells that are present predominantly in the bone marrow and have the capacity for self-renewal and multilineage differentiation.