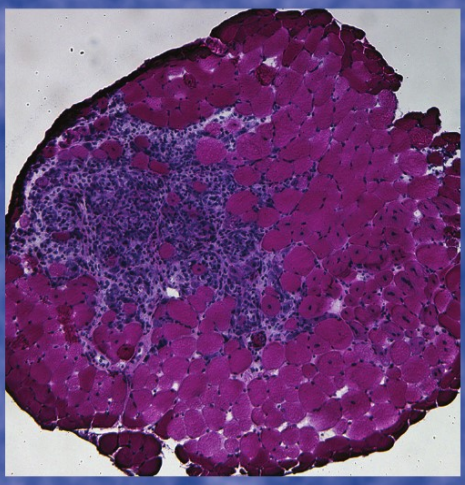
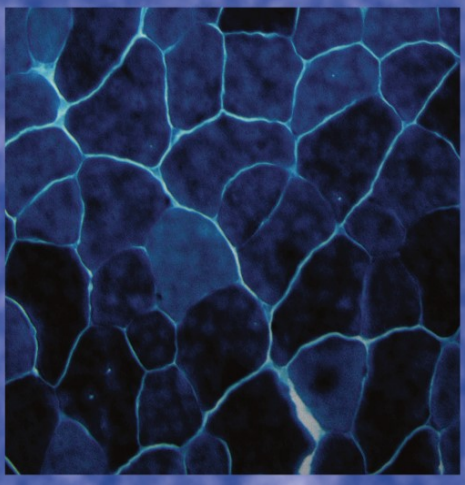
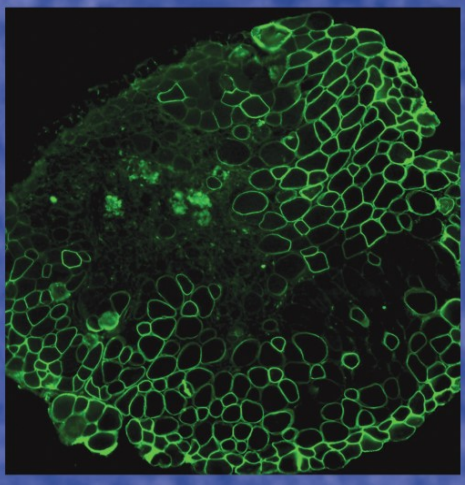
Dongsheng Duan

EDITOR

Muscle Gene Therapy







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Cover image: Background, transmission electron microscope image of purified recombinant adeno-associated virus (AAV) serotype-5 particles. Right top panel, an AAV serotype-9 virus carrying an alkaline phosphatase gene expression cassette was injected to the jugular vein of a newborn dog. Alkaline phosphatase expression was examined in skeletal muscle six months later on cryo-tissue section by histochemical staining. Right bottom panel, an AAV serotype-6 virus carrying a LacZ gene expression cassette was directly injected to the anterior tibialis muscle in a 2-month-old C57Bl/10 mouse. LacZ expression was examined one month later on cryo-tissue section by b-galactosidase staining. Left panels, an AAV serotype-6 virus carrying a micro-dystrophin gene expression cassette was directly injected to the extensor digitorium longus muscle in a 1-month-old utrophin/dystrophin double knockout mouse, a mouse model for Duchenne muscular dystrophy. Muscle histopathology and micro-dystrophin expression were examined at 1 month after gene therapy. Top panel, hematoxylin-eosin staining. Bottom panel, immunofluorescence staining for micro-dystrophin expression.

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To patients, their families and friends who have fought fiercely to defeat muscle diseases

To investigators who work diligently to find the cure for muscle diseases

Preface

Scene 1. *The year was 1890. A young couple brought their 5-year-old son to their physician. Unlike his peers, the boy had a hard time walking and his muscles were getting weaker. "What is the problem? Can you treat it?" the couple implored the physician. In the face of these serious questions from the apprehensive couple, the physician told them he had very little to offer. All he could tell them was that this was a muscle wasting disease commonly seen in young boys. A French physician named Duchenne de Boulogne described this disease in 1868 and it has been dubbed Duchenne muscular dystrophy. Six years later, the young boy succumbed to his disease.*

Scene 2. *A century later, it was 1990. A 2-year-old boy caught the attention of his physician. On physical examination, the physician noticed signs of delayed neuromuscular development. Laboratory testing revealed abnormally high blood creatine kinase levels. The clinical findings reminded the physician about a landmark study published in 1987. Louis M. Kunkel and his colleagues had cloned the first human disease gene called dystrophin. Mutations in the dystrophin gene have been identified as the culprit for Duchenne muscular dystrophy. A muscle biopsy and genetic testing are ordered immediately. It did not take long before the physician found that a small fragment of the dystrophin gene was indeed missing in the boy. "I am very sorry," the physician told the parents, "Your child suffers from Duchenne muscular dystrophy, the most common lethal muscle disease in boys. The genetic testing confirms the diagnosis." "Can you fix the mutated gene and cure the disease?" The worried parents asked. "I can put him on steroids and my colleagues can provide you with genetic counseling. But I am afraid that is all we can do." The boy died at age 20.*

Scene 3. *Another hundred years have passed and it is 2090. A young couple is anxiously waiting for the results of the whole genome sequencing for their newborn boy. The physician comes in, "I'm afraid there is bad news. We found a mutation in the dystrophin gene of your son. But don't worry, we will put him on gene therapy". The boy receives his treatment and lives a long active and healthy life thereafter.*

Muscle diseases, such as Duchenne muscular dystrophy (DMD), have afflicted humans for thousands of years as depicted in relief paintings of ancient Egyptian tombs. Quality of life is severely reduced and lifespan is shortened. Little was

known about the inner world of these diseases until the arrival of the molecular biology era. The discovery of the dystrophin gene in 1987 stands out as one of the great breakthroughs. Many more muscle disease genes have been cloned since then. Collectively, gene hunting has transformed the clinical practice by allowing accurate genetic diagnosis. Yet, a far more reaching implication of disease gene deciphering is gene therapy. Researchers now have the opportunity to tinker with the genetic tools to either repair or replace the defective gene.

The concept of therapeutic gene transfer was born within a decade of the discovery of the DNA double helix by Watson and Crick. The seminal studies of Kunkel and colleagues immediately generated great euphoria and hype among both researchers and the general public. It seems that a cure for muscular dystrophy by gene therapy is just around the corner. In reality, the development of muscle gene therapy has been far more challenging and frustrating.

DMD gene therapy started soon after the discovery of the gene, albeit with minimal knowledge of gene function, molecular pathogenesis or gene delivery vehicles. In the early 1990s, proof-of-principle successes were achieved by several groups using strategies from direct muscle injection of a plasmid carrying the full-length dystrophin expression cassette to retrovirus or adenovirus-mediated expression of a minimized dystrophin gene. Since then, enormous effort has been directed towards the development of an effective gene therapy for Duchenne muscular dystrophy and many other muscle diseases. We now know much more about the disease, the gene, the gene delivery vectors and the host immune response. Yet, the magic cure remains elusive.

Where are we? Is gene therapy ever going to work for muscle diseases? What is hindering muscle gene therapy? Can muscle gene delivery be used for treating other diseases? What is the clinical status of muscle gene therapy? With these questions in mind, this book was born. This is the first book entirely dedicated to muscle gene therapy. It encompasses a comprehensive and up-to-date evaluation of muscle gene therapy by leaders in the field.

Animal disease models have been extremely valuable in testing novel therapies. Preclinical studies in animals offers critical reassurance. For this reason, the book is started with a chapter on the animal models of muscle diseases. What follows are 15 chapters covering different aspects of muscle gene therapy.

Two chapters provide the current perspective on the design and application of antisense oligonucleotide-mediated exon skipping. This strategy aims at repairing the defective gene. It is not an imminent cure, but will significantly reduce the severity. Over the last decade, this technology has rapidly moved from encouraging findings in the rodent models to the large animal model and now human patients. The encouraging results from phase I human trials, as well as the exciting news on systemic exon skipping in dystrophic dogs, have greatly raised the hope that a clinical reality for muscle gene therapy is in sight.

Another major stride in the field is the development and application of adeno-associated viral vector (AAV) for muscle gene therapy. Approximately 40% of our body is made of muscle. Unlike many other tissues that have a restricted anatomic location, muscles are all over the place. For a long time, body wide gene delivery

has been a great challenge for muscle gene therapy. With the help of novel AAV serotypes, this obstacle has now at least been solved in rodents and dogs. For this and many other reasons, AAV has become the most favorite muscle gene delivery vehicle. It is thus not surprising that many chapters have elaborated on this highly promising technology.

One of the greatest lessons we have learned over the years is how our body's immune system responds to the gene delivery vehicle and the therapeutic protein produced by the vector. This is a hurdle we have to overcome in order to translate gene therapy efficacy from the bench to the bedside. This issue is addressed by a chapter on modulating immune response for muscle gene therapy.

Several chapters have also been included to address special issues in muscle gene therapy. These include gene therapy for respiratory and cardiac muscles, in utero gene therapy, delivering large therapeutic genes to muscle and the combinatorial approaches for muscular dystrophy gene therapy.

Gene therapy is a rapidly evolving field. It has benefited greatly from breakthroughs in basic biomedical research, such as RNA interference and stem cell research. Two chapters deal with these fascinating advances. These new tools expand the horizon of muscle gene therapy to diseases that were considered unapproachable previously. Also noteworthy are two chapters that describe the applications of muscle gene transfer to treat diseases that mainly affect other tissues or even using muscle as a target for genetic vaccine. Collectively, the possibilities of muscle gene therapy are endless.

The book concludes with a chapter on clinical trials of muscle gene therapy. When gene therapy was initially brought up as a concept, its simplicity and intuitive nature led many to believe that it would be a homerun. However, the road of dream chasing has been quite bumpy and full of unpleasant surprises. The death of a patient volunteer in a phase I clinical trial for a metabolic disease at the end of the 1990s made many people believe that gene therapy was merely a fancy toy for scientists which would never reach patients. As demonstrated by many encouraging examples in our final chapter, I have every reason to believe that muscle gene therapy has finally come of age. At the same time, I have no doubt that the road ahead of us is not straightforward. Painstaking effort will be needed to make the *Scene 3* described at the beginning of this preface a reality. Nevertheless, tomorrow will be better.

I am very grateful to chapter authors for their outstanding contributions. I would like to thank Springer editor Jeffrey Ciprioni for giving me the opportunity to edit this volume. Jeff and his colleague Elisa Weeks have been extremely helpful in guiding me through the jungle of book publishing. I would also like to thank Karen Ehlert for her administrative assistance in the preparation of this book. Special thanks are extended to my wife Yongping, my son Sean, and my parents and to Mr. and Mrs. Smaile and Drs. Markay Harlan and Howard K. Scott.

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Chapter 1
Animal Models for Inherited Muscle Diseases

Bruce F. Smith and Roberta Wrighten

Abstract Animal models of inherited muscle diseases are critical to our understanding of the pathophysiological mechanisms of these diseases and serve as surrogates in which approaches to therapy may be evaluated. Many models have been described, including those that have spontaneously occurred as well as models derived from mutagenesis and screening. This chapter focuses primarily on two species, the mouse and the dog, in which the majority of these models have been elucidated. However, inherited muscle disease is described in other species as well. For many of these models the nature of the mutation is known and the features of the disease are presented. Models of dystrophinopathy, limb-girdle muscular dystrophy, merosin deficiency, centronuclear myopathies as well as several other muscle diseases are discussed. In addition, information about husbandry and management of colonies of the animal models is presented.

1.1 Introduction

The identification of inherited muscle diseases in humans has been paralleled by the discovery of similar diseases in other animal species. These animals have been recognized as potential models of the corresponding human disease. Any discussion of human myopathies must therefore include the corresponding animal models. Such models may prove useful in the identification and testing of therapeutic approaches, as well as the understanding of pathogenic processes. With the discovery of the molecular basis of Duchenne Muscular Dystrophy (DMD), this disease has become the touchstone for animal models of inherited myopathies, and a significant number of models of DMD have been recognized in a variety of species. However, there are many additional diseases for which animal models have been identified. It is not the intent of this chapter to attempt to show the superiority of

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one model over another, but to provide an understanding of the strengths and weaknesses of each model in order to facilitate the use of these models.

The two most common species for models are the mouse and the dog. Each of these species can provide useful tools in the analysis of disease and this chapter will focus on these two species. The cat may provide some useful models and these will be discussed as well. In general, murine models allow relatively fast reproduction rates, and their small size makes rodents much less expensive to maintain. Dogs (and cats) represent an "intermediate animal model". The term intermediate is used intentionally here, as many researchers refer to dogs as "large animals" while the veterinary profession refers to them as "small animals". In reality, the dog is intermediate in size between the mouse and human patients, which allows therapies to be scaled up. In addition, canine models can be regarded as intermediate steps between fundamental experiments in murine models and the ultimate application of that knowledge in human patients.

Murine models can either be spontaneous, or induced. The latter approach involves both genetic approaches, such as knockout technology, as well as chemical and radiation induced mutagenesis. Since embryonic stem cell technology is not available for dogs or cats, all canine and feline models are spontaneous. Recognition of novel models in any species requires a surveillance program. In mice, this is often a component of the mutagenesis program itself. In dogs and cats, spontaneous neuromuscular disease is usually first recognized either by the veterinary pathologists examining specimens submitted for diagnosis or by the veterinary neurologists, attempting to make a diagnosis of neuromuscular disease. Once the inherited muscle disease is recognized, affected or carrier animals must be bred to create colonies that provide animals for further investigation. The critical issues affecting the expansion of animal models will be addressed later in this chapter.

1.2 Dystrophinopathies

In 1987, the protein responsible for DMD, and its milder relative Beckers muscular dystrophy (BMD) was identified in humans and named dystrophin (Hoffman et al. 1987). DMD is an X-linked disease that typically presents itself early in childhood as muscle weakness and progresses to muscle loss and fibrosis. Most boys with this disease die around the age of puberty or shortly thereafter from respiratory or cardiac failure. Dystrophin is a large cytoskeletal protein that is localized near the plasma membrane. The protein interacts with actin filaments, and members of the dystrophin-associated glycoprotein complex (DGC) that in turn spans the membrane, linking the cytoskeleton within the cell to the extracellular matrix. The majority of mutations in the human population are deletions, which are thought to be due to misalignment of dystrophin exons during meiosis, as these exons contain highly repetitive sequences. Mutations that maintain the dystrophin reading frame and allow the preservation of specific domains of the dystrophin protein often result in the milder form of the disease,

BMD, which may present as mild muscle weakness and may not be diagnosed until adulthood.

The classic murine model of DMD is the mdx mouse. This mutation was recognized in 1977 in the C57BL/10ScSn background. In 1989, the molecular basis of DMD in the mdx mouse was determined to be the substitution of T for C at position 3185, creating a nonsense codon (Sicinski et al. 1989). This premature stop occurs in exon 23 of the dystrophin protein. Mdx mice have a normal or nearly normal phenotype with a lifespan similar to that of the parental strain. Affected mice show central nucleation of many muscle fibers, indicating muscle degeneration and regeneration, but do not show weakness or fibrosis, with the exception of the diaphragm muscle, which is significantly affected (Stedman et al. 1991). If mdx mice are subject to eccentric contraction, such as walking on a downhill treadmill, they show significant muscle damage, leading the mice to become symptomatic (Sandri et al. 1997). Mdx mice show a significant number of the so called "revertant fibers", where fibers stain positive for dystrophin in affected animals. It is generally believed that few, if any of these fibers are true revertants of the point mutation, but rather, most are splicing variants that do not express the mutant exon 23. The elimination of exon 23 does not affect the reading frame, like a number of other exons of dystrophin, thus the remainder of the protein is produced in-frame. Because affected males achieve sexual maturity, it is possible to breed affected males to carrier and affected females thereby greatly increasing the relative number of affected mice per litter.

In an attempt to enhance the disease phenotype of the mdx mouse, it has been crossed with a model where the apparent paralog of dystrophin, utrophin, has been knocked out, generating the "double knockout" or dko model (Deconinck et al. 1997). Utrophin has been hypothesized to compensate for the lack of dystrophin in the mouse by a number of investigators, and utrophin knockout mice show no sign of the disease. However, dko mice show a phenotype that resembles that of human DMD, with a severe progressive muscular dystrophy and premature death.

Verne Chapman and colleagues created four additional models of DMD in mice in the late 1980s using using N-ethylynitrosourea (ENU) chemical mutagenesis (Chapman et al. 1989). These models were termed mdx 2–5CV. The mutation in the mdx 2CV strain has been determined to be a single base substitution in the splice acceptor sequence of intron 42. A series of different splicing variants therefore results, none of which maintain the normal open reading frame (Im et al. 1996). Similarly, mdx 3CV is due to a point mutation in intron 65 that creates a novel splice acceptor site, resulting in abnormally spliced RNA (Cox et al. 1993). The mdx 4CV model is due to a C to T transition in exon 53, resulting in a nonsense codon (CAA to TAA) (Im et al. 1996) and the mdx 5CV mutation is a 53 base pair deletion in the mRNA due to an A to T substitution in exon 10 that creates a novel splice donor site. This mutation results in the deletion of 53 bases and a frame-shift in the mRNA (Im et al. 1996). These models present unique opportunities to examine the contributions of dystrophin's multiple domains and promoters to contribute to disease presentation, progression, and pathogenesis. All four resemble mdx in the presentation of DMD. However, mdx 3CV mice show increased neonatal mortality (Cox et al. 1993).

In addition, this strain shows faint staining for dystrophin in muscles, similar to a subset of human patients. The mdx 4CV and 5CV strains show a tenfold reduction in revertant fibers when compared to the mdx model or mdx 2CV (Danko et al. 1992) (Table 1.1).

Spontaneously occurring DMD has been recognized in both dogs and cats. With the advent of molecular approaches and the identification of mutations in the dystrophin gene as the cause of DMD in humans, it has become possible to confirm, that the cause of the disease in these intermediate models are mutations in the dystrophin gene. Feline DMD is relatively rare, having been reported in three breeds of cat (Carpenter et al. 1989; Gaschen et al. 1992; Winand et al. 1994). The hallmark of this disease in cats is significant hypertrophy of muscle and the disease has been called hypertrophic feline muscular dystrophy (HFMD) due to this. Both the tongue and diaphragm can be severely affected resulting in the inability to drink due to swelling of the tongue and the inability to move food and water into the stomach when diaphragmatic hypertrophy constricts the esophagus. Some cats with DMD can lead nearly normal lives if they do not suffer these complications. Many do go on to develop sub-clinical cardiomyopathies and a few have been documented to develop acute, life-threatening rhabdomyolysis when given inhalational anesthesia. Interestingly, elevated serum creatine kinase (CK) levels cannot be used to identify affected or carrier animals at birth, but only begin to rise approximately two weeks postnatally.

Canine DMD has been diagnosed much more commonly than feline DMD, probably due to the severity of the disease in this species. Among the breeds in which DMD has been described are the Golden Retriever (Sharp et al. 1992), German Short-haired Pointer (Schatzberg et al. 1999), Rottweiler (Winand and Cooper 1994), Labrador Retriever (Bergman et al. 2002), Welsh Corgi (Woods et al. 1998), West Highland White (Smith, unpublished), English Springer Spaniel (Smith unpublished), Australian Labradoodle (Smith unpublished), Old English Sheepdog (Wieczorek et al. 2006) Grand Basset Griffon Vendeen (Klarenbeek et al. 2007) and Japanese Spitz (Jones et al. 2004). The Golden Retriever was the first

Table 1.1 Animal models of Duchenne muscular dystrophy

Species	Model	Comments
Mouse	Mdx	Mild disease
Mouse	Mdx 2CV	Mild disease
Mouse	Mdx 3CV	Mild disease, some faint staining
Mouse	Mdx 4CV	Mild disease, low number of revertant fibers
Mouse	Mdx 5CV	Mild disease, low number of revertant fibers
Mouse	Dko	Utrophin – dystrophin double knockout, severe disease
Cat	Multiple breeds	Hypertrophic changes predominate
Dog	Golden retriever	Original model, some revertant fibers, intron 6
Dog	German shorthaired pointer	Spontaneous knockout model
Dog	Welsh corgi	Intron 13 insertion
Dog	Labrador retriever	Intron 19 insertion

dog model of DMD identified and the first in which the mutation was determined. The mutation in this breed was found to be a point mutation in the splice acceptor site of intron 6 (Sharp et al. 1992). This results in exon 7 being spliced out of the mRNA, causing a frame-shift and subsequent termination. Some revertant fibers are noted in the Golden Retriever and these have been shown to be due to alternative splicing out of exons 3–9 or 5–12 (Schatzberg et al. 1998). The Golden Retriever mutation has also been bred onto the Beagle background. Recently, a male Golden Retriever was described who has the published mutation, but is very mildly affected. This dog has had multiple litters and some of his affected male offspring also show the extremely mild phenotype, indicating that the source of the effect may be autosomal in origin (Ambrósio et al. 2008).

Several other breeds have reached a similar level of molecular analysis and can be considered as alternative models to the Golden Retriever. The German Shorthaired Pointer was determined to be a spontaneous knockout mutation as these dogs are missing the entire dystrophin gene (Schatzberg et al. 1999). Consequently, there are no revertant fibers noted in the muscles of these dogs, making interpretation of results significantly easier. However, the lack of any dystrophin expression in these dogs also means that their immune systems are naïve and that even canine dystrophin may elicit an immune response. The mutation in the Japanese Spitz has not yet been published, however analysis of the protein using a panel of antibodies indicates that a severely truncated protein of 70–80 kdal (Jones et al. 2004). The mutations in the Welsh Corgi and the Labrador Retriever have been determined to be intronic insertions, in introns 13 and 19 respectively (Smith unpublished). In both cases, the insertions occur downstream of AG dinucleotides in the insertion which function as splice acceptor sites. Downstream, within the insertion, are putative splice donor sites, allowing the inserted material to act as novel exons. In-frame termination codons are present in these novel exons resulting in truncated dystrophin production. A small number of revertant fibers can be identified in both models with antibodies to epitopes beyond the mutation site, indicating that alternative splicing is most likely responsible for this low level expression.

Canine models exhibit many of the same clinical signs as boys with DMD. Affected puppies may be identified, often within hours after birth, by their elevated CK levels. These can be extremely high and while they often decrease during the first few weeks of life, CK levels remain elevated for the dog's life. Physical signs usually become apparent at 6–8 weeks of age when the puppies are noted to be smaller and to tire more readily than their normal siblings. Progression of the disease occurs over the ensuing 5–6 months, with loss of muscle mass, weakness and kyphosis as classical signs (Fig. 1.1). Microscopically, affected muscles show degeneration, regeneration, fatty infiltration, and fibrosis. Many dogs with DMD show similar cardiac disease to boys with this disease. Progression can vary between dogs with the same mutation, with some individuals requiring euthanasia within 6 months of birth, while other can survive into adulthood. However, with the exception of the Golden Retriever noted above, these "longer lived" affected dogs obviously show disease, need significant nursing care to be maintained and usually succumb around 2–3 years of age. In dogs with DMD, the typical reasons for euthanasia

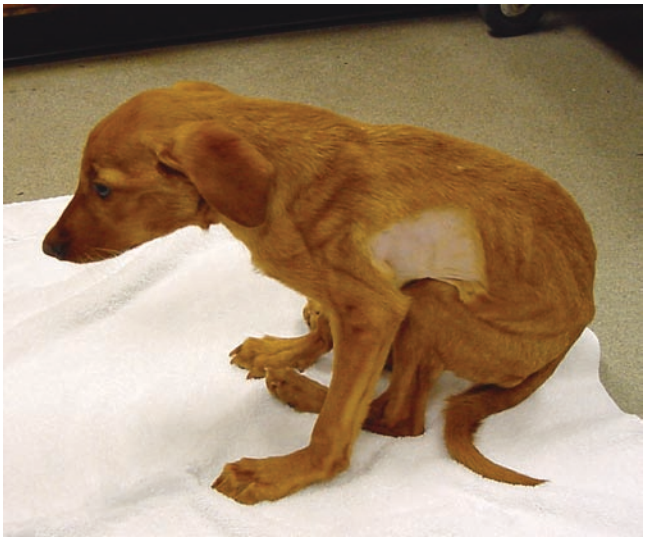

Fig. 1.1 DMD affected male yellow Labrador Retriever at 5 months of age. This dog shows severe muscle loss and fibrosis, kyphosis, weakness and hyperextension of the carpus and tarsus. This represents the extreme of this phenotype

are inability to eat, recumbency, respiratory disease secondary to compromised respiration and heart failure.

Animal husbandry in colonies of DMD dogs can be challenging. Dystrophic puppies have a higher neonatal mortality rate than normal puppies. Enhanced survival of the affected dogs requires precise timing of pregnancies, systematic surveillance of pregnant female dogs for signs of impending birth, and intensive observation perinatally. Dedicated facilities for whelping will help facilitate this process. Newborn puppies must be weighed multiple times each day to monitor weight gain and puppies need to be checked for dehydration and chilling frequently. Affected puppies can require bottle-feeding, either as a supplement to maternal feeding (preferred) or as their sole source of nutrition. The affected puppies become robust within a week or two of birth, and require little specialized care for the next month or two. However, once clinical signs begin to appear, the affected puppies may require significant additional nursing care. This includes the feeding of softer diets, regular cleaning and grooming, continued attention to weight gain, limited exercise, regular monitoring for respiratory obstruction and infections and regular assessment of disease progression. In older affected dogs, the extreme fibrosis associated with the disease may present appearance issues with animal care workers and regulatory personnel who are not familiar with the model as they may mistake the appearance of the dog for starvation. Some affected male dogs may live past puberty, and as a consequence can be used to breed female carriers and produce affected female dogs.

1.3 Limb-Girdle Muscular Dystrophies

Limb-girdle muscular dystrophy (LGMD) is a rare heterogeneous group of human diseases. Besides the characteristic presentation of weakness in the proximal limbs and shoulder or pelvic girdles, the subtypes of LGMD do not share many features in common. The group is classified, by mode of inheritance, into LGMD1 (autosomal dominant) and LGMD2 (autosomal recessive). The forms are further sub grouped based on the causative gene loci. Spontaneous animal models of LGMD are uncommon and the majority of models are in transgenic mice. Since mutations that result in LGMD sometimes result in other phenotypes, many of these animal models are useful beyond the scope of LGMD. In general the animal models of LGMD do not closely resemble the human phenotype, with a few exceptions. Although many of these models do not exactly replicate the human disease, they are still valuable tools in biomedical research, not only to further studies in understanding the mechanisms of the disease, but also to identify and test potential therapeutic targets (Table 1.2).

1.3.1 Myotilinopathy (LGMD1A)

Myotilin is a structural protein that plays a role in organizing and stabilizing the Z-disc in skeletal muscle. Human patients are affected with an adult onset of proximal limb and pelvic girdle weakness. A transgenic mouse model expressing the

Table 1.2 Animal models of limb-girdle muscular dystrophy

Type	Species	Protein	Comments
LGMD1A	Mouse	Myotilin	T57I transgenic mouse with human mutation
LGMD1B	Mouse	Lamin A	Both homozygotes and heterozygotes are symptomatic
LGMD1C	Mouse	Caveolin-3	Disease does not match human disease
LGMD2A	Mouse	Calpain 3	Mice are asymptomatic
LGMD2B	Mouse	Dysferlin	SJL/J mice have an inflammatory myopathy
LGMD2C	Mouse	γ-sarcoglycan	Knock out model, few signs of myopathy
LGMD2D	Mouse	α-sarcoglycan	Knock out model, few signs of myopathy
LGMD2E	Mouse	β-sarcoglycan	Knock out model, few signs of myopathy
LGMD2F	Mouse	δ-sarcoglycan	Knock out model, few signs of myopathy
LGMD2F	Hamster	δ-sarcoglycan	Bio14.6, predominantly cardiomyopathy
LGMD2C-F	Dog	unknown	Clinical myopathy, mutated sarcoglycan is unknown
LGMD2H	Mouse	Trim-32	Knockout model closely resembling human disease
LGMD2I	Mouse	Fukutin related protein	FKRP^{Tyr307Asn} point mutation is asymptomatic
LGMD2I	Mouse	Fukutin related protein	FKRP-NEO^{Tyr307Asn} null mutation is symptomatic

human myotilin cDNA with the T57I mutation, a mutation that affects humans with LGMD1A, was generated. These mice demonstrate the same muscle weakness in the upper forelimb and hindlimb muscles as human patients. On histopathologic examination, myofibrillar aggregates, fibrosis, and adipose infiltration are present in skeletal muscle (Garvey et al. 2006). The shared phenotype between human patients and the mouse line makes this model optimal for further investigation into therapeutic options.

1.3.2 Laminopathy (LGMD1B)

Lamin A/C are type V intermediate filament proteins of the nuclear lamina. They have a structural role in anchoring nuclear proteins and play a role in cell-signaling processes. Lmna –/– mice do not express lamin A or C proteins. Six or seven weeks after birth, affected mice die from muscular dystrophy and cardiomyopathy. The Lmna –/– mice exhibit abnormal gait with splayed hind limbs and reduced muscular strength. Histologic examination shows variable skeletal muscle degeneration and atrophy of cardiac myocytes in these mice (Sullivan et al. 1999). Heterozygous knockout mice (Lmna +/–) develop atrioventricular conduction defects as well as atrial and ventricular arrhythmias, symptoms present in human patients (Stewart et al. 2007). Both of these models resemble the human phenotype and are important to further investigate potential therapies.

1.3.3 Caveolinopathy (LGMD1C)

Caveolin-3 is a scaffolding protein at the plasma membrane of muscle and the principal protein in caveolae. Caveolae are small vesicular invaginations of the sarcolemma representing sub-compartments of the plasma membranes, which play an important role in signal transduction and vesicular transport. Cav-3 –/– mice express normal amounts of Cav-1 and Cav-2 in muscle and caveolin expression in other tissues types is normal. Clinical signs of muscular dystrophy are not present. However, histologic changes demonstrate muscular degeneration in the soleus and diaphragm muscles, and display t-tubule disorganization (Hagiwara et al. 2000, Galbiati et al. 2001). In addition, cardiomyopathy is present in Cav-3–/– mice, a feature not seen in human LGMD1C patients. These animals exhibit significant cardiac hypertrophy, dilatation, and reduced fractional shortening (Woodman et al. 2002) as well as cellular infiltration with accompanying perivascular fibrosis (Hnasko and Lisanti 2003). Despite the interaction between the DGC and caveolin, cav-3 –/– mice do not demonstrate any difference in DGC expression. Since the clinical signs in this model differ from human patients, it may be difficult to translate results; however, the model may be useful to investigate other potential functions of caveolin and potential phenotypes in humans.

1.3.4 Calpainopathy (LGMD2A)

Calpain 3 (CAPN 3) is an intracellular muscle specific nonlysosomal cysteine protease with a variety of cytoskeletal and myofibrillar substrates. CAPN 3 is vital for cytoskeletal rearrangements, sarcomere function, remodeling, and apoptosis (Kramerova et al. 2005). Knockout Capn3 –/– mice do not demonstrate any clinical myopathic signs throughout their lifespan. However on histologic examination progressive myopathy is evident. There are central nuclei present as well as areas of inflammatory infiltrates that increase as the animal ages (Fougerousse et al. 2003). This model was used to elucidate the pathophysiology of LGMD2A.

1.3.5 Dysferlinopathy (LGMD2B)

Dysferlin is involved in maintenance and repair of the sarcolemma. The spontaneous model for dysferlinopathy is the SJL/J mouse. This strain was initially used for research in autoimmune diseases and inflammatory myopathies before the mutation was identified. A 171 base pair deletion has been identified in the mRNA that results in the removal of 57 amino acids from the dysferlin protein (Bittner et al. 1999). The SJL/J model is characterized by a gradual loss of muscle mass and strength with evidence of increasing muscle pathology. Histological analysis shows fibers with central nuclei, indicating regeneration, size variability, fiber splitting, inflammatory infiltrates, necrosis and ultimately, replacement by adipose tissue (Vainzof et al. 2008). These changes primarily affect the proximal muscle groups (Bittner et al. 1999). SJL/J mice are similar to LGMD2B patients, including the inflammatory component.

1.3.6 Sarcoglycanopathies (LGMD2C–F)

Sarcoglycans are transmembrane glycoproteins that form a subcomplex within the DGC, creating a link between the cytoskeleton of the myocyte and the extracellular matrix. The Bio14.6 hamster was originally identified in 1962 as a spontaneous myopathy model having both skeletal and cardiac muscle involvement (Homburger et al. 1962). Prior to the identification of the causative gene locus, Bio14.6 was utilized as a cardiomyopathy model since the heart muscle is more affected than the skeletal muscle (Vainzof et al. 2008). Bio14.6 hamsters develop muscle fiber necrosis in the myocardium and the skeletal muscle. There is hypertrophy of myocardium ultimately leading to heart failure. Skeletal muscles also show significant degeneration. With the identification of a mutation consisting of the deletion of regulatory sequences and the first exon of δ-sarcoglycan, Bio14.6 was recognized as a model of LGMD2F (Nigro et al. 1997). However, unlike

Bio14.6, in the majority of human sarcoglycanopathy patients there is no evidence of cardiomyopathy (Nigro et al. 1997). In Bio14.6 animals there is a secondary reduction of α-, β-, and γ-sarcoglycan proteins. The variability in the reduction of expression of α-dystroglycan among Bio14.6 hamsters may account for the variable disease course, from the slowly progressive to rapid progression to death. Human sarcoglycanopathy patients share similar findings with respect to the progression of disease (Shelton and Engvall, 2005).

Knockout mice for each of the sarcoglycan genes (γ, α, β, δ-sarcoglycan), LGMD2C-F respectively, have been generated. Although each model exhibits progressive muscular dystrophy with varying severity, every model shares a secondary concomitant reduction of all other sarcoglycan proteins as well as other components of the DGC (Vainzof et al. 2008). The severity of dystrophic changes is comparable to those seen in mdx mice, except for the moderately affected sarcoglycan-α deficient mice.

Sarcoglycan-α null mice (sgca-null) do not show clinical signs of myopathy, despite the dystrophic evidence present on histopathological analysis (Duclos et al. 1998). Both sarcoglycan-β null (sgcb-null) and sarcoglycan-δ null (sgcd-null) mice exhibit more severe dystrophic changes on histopathological examination (Coral-Vasquez et al. 1999; Durbeej et al. 2000). These mice have larger and more pronounced areas of necrosis as well as central nuclei, calcification, fibrosis, and massive fatty infiltration. Cardiomyopathy is also present in both these models indicated by prominent areas of myocardial necrosis. In addition, these models exhibit disrupted expression of the DGC in smooth muscles due to the absence of β- and δ sarcoglycan, which are normally present in smooth muscles. This leads to vascular irregularities in the sgcb-null and sgcd-null models. Sgca, sgcb, and sgcd all demonstrate increased muscle mass and contraction-induced injury. The sarcoglycan-γ null (sgcg null) mice also demonstrate severe muscular dystrophy and cardiomyopathy. In contrast with the other sarcoglycan deficient models, sgcg mice do not show alteration in the expression of dystroglycans of the DGC, nor do they exhibit contraction-induced injury (Durbeej and Campbell, 2002). These murine models are valuable tools to evaluate therapeutic approaches as well as to further elucidate the role of each specific sarcoglycan.

Although specific mutations have not been identified, sarcoglycan deficiencies have been identified in a Boston Terrier, a Cocker Spaniel, and a Chihuahua. All three dogs exhibited failure to thrive, lethargy and exercise intolerance. Blood work results showed elevated creatine kinase (CK), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) indicating muscular damage. Histopathology showed varying fiber types, clusters of degeneration and regeneration with deposits of calcification. Immunohistochemistry demonstrated extremely reduced α, β, and γ-sarcoglycan expression (Schatzberg et al. 2003). These animals are the only report of sarcoglycanopathy in intermediate models and present the opportunity to study therapies in a model that closely resembles the human patient.

1.3.7 TRIM 32 mutations (LGMD2H)

Trim 32 is an E3 ubiquitin ligase involved in the maintenance and degradation of myofibrils during remodeling. TRIM 32 knockout (T32KO) mice were developed as a model to investigate LGMD2H and another myopathy caused by the same mutation, sarcotubular myopathy. Impaired muscle strength was evident in T32KO animals. On histology, muscle fibers have increased central nuclei as well as fiber splitting, ring fibers and angulated fibers. This model replicates human LGMD2H skeletal muscle pathology and offers a valid tool for future studies into the unclear mechanism of disease and eventually therapeutic options (Kudryashova et al. 2009).

1.3.8 Fukutin Related Protein Mutations (LGMD2I)

Fukutin related protein (FKRP) helps to glycosolate α-dystroglycan, a component of the DGC. Two murine lines were recently generated with knock-in mutations in FKRP. The first, $FKRP^{Tyr307Asn}$, has a point mutation common to human patients with LGMD2I and other FKRP mutation diseases. However, $FKRP^{Tyr307Asn}$ mice did not have any discernable abnormal phenotype. The second line, FKRP-$Neo^{Tyr307Asn}$, has a missense mutation with a retained neomycin selection cassette, which results in a null allele. Homozygous mice with this mutation showed reduced levels of FKRP transcripts and perinatal fatality. Histology of muscle demonstrates edema and hypoglycosylation of α-dystroglycan. Other phenotypic abnormalities found in this model affect the eye and brain in a manner similar to other diseases with alterations in FKRP (Ackroyd et al. 2009). These two lines will aid in the elucidation the function of FKRP in muscle cell biology and its role in eye and brain development.

1.4 Laminin α2 (Merosin) deficiency

In humans, mutations in the lama2 gene, which encodes laminin α2, cause merosin-deficient congenital muscular dystrophy 1A (MDC1A), one subset of a broad category of disease known as congenital muscular dystrophy (CMD). MDC1A is inherited in an autosomal recessive fashion. Laminin α2 is present in the basal lamina of skeletal muscle, as well as other cell types and combines with β and γ subunits to form laminin 2. A lack of laminin α2 results in a severe disease that includes hypotonia at birth, delayed motor development, an inability to walk, elevated CK levels and some structural and functional defects of the central white matter (Tomé et al 1994; Philpot et al. 1995).

Multiple murine models of laminin α2 deficiency have been identified. Michelson originally described the Dy mouse in 1955 as dystrophia muscularis (Michelson et al. 1955). The Dy2J mouse has a less severe phenotype, surviving

into adulthood. The mutation in the Dy2J model was determined to be a G to A substitution in a splice donor site (Xu et al. 1994). This mutation results in abnormal splicing products, one of which is translated into a protein that is missing 55 amino acids. While this deletion does not interfere with trimerization with β and γ subunits of laminin, it may prevent assembly into basement membranes. Dy3K mice were created by knockout technology and represent a severe phenotype, with death occurring around 5 weeks of age (Miyagoe 1997). Recently, a new lama2 mutation was described, nmf417, in which a point mutation substitutes Arg for Cys at position 79, disrupting a universally conserved CxxC motif in the N-terminal domain. These mice have similar muscle and nervous system signs to the other lama2 mutant mice but do not show disruption of the basal lamina (Patton et al. 2008).

Laminin α2 deficiency has been identified in both dogs and cats. In cats, affected breeds have been identified as Domestic Shorthair mix, Persian mix, Siamese, and Maine Coon Cat indicating the likelihood of at least 3–4 different mutations. The presence of the disease in mixed breed cats suggests that lama2 mutations might be present in multiple other breeds of cat as well. Affected cats present with a range of severity, including elevated CK levels, muscle atrophy, weakness, rigidity, especially of the pelvic limbs, tail and spine, and muscle contractures. Muscles of the affected cats show dystrophic changes and nerves show demyelination (O'Brien et al. 2001; Poncelet et al. 2003; Awamura et al. 2008).

Laminin α2 deficiency has been reported in a Brittany-Springer Spaniel mixed breed dog. This dog had a similar clinical presentation to humans and cats with this disease, including elevated serum CK, dystrophic changes in muscle biopsies, abnormal gait and muscle weakness (Shelton et al. 2001). As with the mixed breed cats, the presence of this disease in a mixed breed dog indicates that mutations of the canine lama2 gene may be present in at least 2 different breeds, although the ancestry of the affected dog is not discussed and inbreeding is also a potential reason for homozygosity.

Diagnosis of laminin α2 deficiency in both the dog and cats described above has been on the basis of antibody staining. As yet, no mutations have been published for these models. In addition, there is no indication that breeding colonies of dogs or cats with this disease have been established.

1.5 Centronuclear Myopathies

Centronuclear myopathies represent a broad category of muscle disease in which the myonuclei are located centrally within fibers. This is a hallmark of muscle regeneration and not pathognomonic for any one disease process. In humans, causes of centronuclear myopathies include myotubular myopathy (MTM), which can in turn, be broken down into three forms, X-linked MTM, due to defects in myotubularin (MTM1), autosomal recessive MTM due to defects in amphiphysin 2 (Bin1) and autosomal dominant MTM due to defects in dynamin (DNM2) (Laporte et al. 1996; Nicot et al. 2007; Bitoun et al. 2005).

In humans, X-linked MTM usually presents as severe myopathy with weakness and difficulty in eating and breathing. Infants often die from the disease by 1-year of age (Wallgren-Pettersson et al. 1995). A knockout mouse model has been created by homologous recombination in the myotubularin gene. These mice show a generalized and progressive myopathy beginning around 4 weeks of age, and progressing to death at 6–14 weeks. Muscles in these mice show an accumulation of central nuclei, as in the human disease (Buj-Bello et al. 2002).

The autosomal dominant form of MTM is usually later in onset and milder than the X-linked form. Weakness is more pronounced proximally, and affected patients may remain ambulatory into their 60's and beyond. Mutations in dynamin 2 cause this disease and there is some evidence that the location of the mutation in the gene may affect severity (Bitoun et al. 2005). No animal models of this disease have been described.

The autosomal recessive form of MTM is intermediate in phenotype between the X-linked and autosomal dominant forms. Facial weakness is a characteristic of this form and as with the dominant form, weakness is more pronounced proximally. Patients may have severe respiratory involvement, and some suffer cardiac involvement. In the absence of cardiorespiratory symptoms, the prognosis for these patients is better than for those with the X-linked form. Mutations in the amphiphysin 2 gene have been identified as being causative, at least in a sub-population of patients and interaction between dynamin 2 and amphiphysin have been demonstrated (Nicot et al. 2007). Amphiphysin has also been implicated as an anti-oncogene (Prendergast et al. 2009). A Bin1 knock mouse model has been developed, which shows a very high rate of perinatal mortality. Most Bin1 negative mice appear normal at birth, but fail to nurse and die within 24 hours. Bin1 negative embryos develop a ventricular cardiomyopathy, with the thickening of the ventricular wall, which results in occlusion of the chambers (Muller et al. 2003). This model represents a much worse scenario than that seen with human cases.

While no models of the three classical MTMs have been described in either the cat or the dog, a model of centronuclear myopathies in the dog has been identified that represents a disease that has yet to be recognized in humans or recreated in mice, autosomal recessive myopathy/muscular dystrophy (ARMD), in the Labrador Retriever (Fig. 1.2). Initially characterized in 1975, the disease results in the preferential loss of type 2 fibers, resulting in muscle weakness and atrophy (Kramer et al. 1976). As indicated by the name, the mode of inheritance is autosomal recessive. This disease has also been known as hereditary myopathy of Labrador Retrievers (HMLR), centronuclear-like myopathy, and Labrador Retriever myopathy. The appearance of affected dogs is characterized by muscle loss, which is especially evident in the pelvic and thoracic limb-girdles. This feature led to speculation that these dogs were a model of LGMD, however, this has not been ruled out. Affected puppies may show signs of the disease as early as 4–6 weeks of age. The disease progresses until approximately 1-year of age, when the dogs stabilize and may even slowly improve. Lifespan in affected dogs is normal or nearly normal. The incidence of heart failure in aged dogs may be increased. The disease phenotype is relatively mild; with no increase in CK.

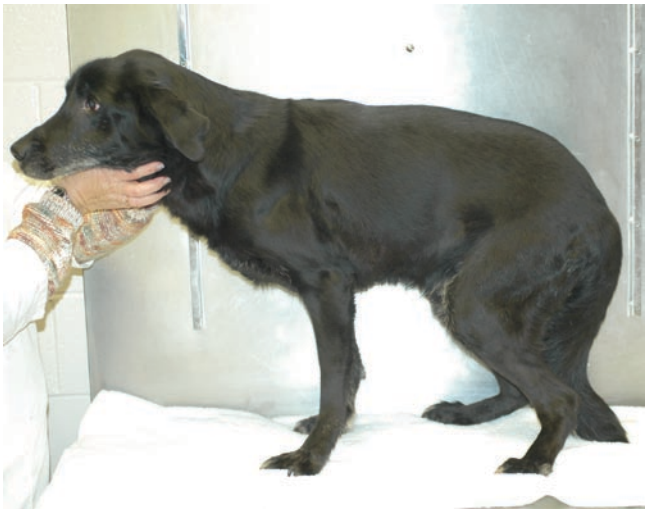

Fig. 1.2 ARMD affected female Labrador retriever at 7 years of age. This dog shows muscle atrophy, which is particularly evident in the temporal muscles and over the scapula. In addition, the dog shows mild kyphosis and laxity of the carpus and tarsus

Affected dogs are fertile and may be bred by artificial insemination. Litters are delivered by affected females, without assistance and these dogs can usually care for their own puppies, making it possible to produce litters with 100% of the puppies affected with the disease.

The genetic basis of ARMD has been determined to be a mutation in the protein tyrosine phosphatase-like member A (PTPLA) gene (Pele et al. 2005). The mutation consists of the insertion of a SINE element into exon 2 resulting in the production of multiple mRNA species. The pathophysiology of PTPLA deficiency is unclear, since the activity of the protein has yet to be determined. While the gene has sequence similarity to tyrosine phosphatases, this activity has not been confirmed. Preliminary analysis indicates that PTPLA is expressed ubiquitously in the tissues of juvenile and adult dogs (Smith unpublished).

1.6 Other Myopathies

1.6.1 Integrin α7 Deficiency

Integrin α7β1D is an important laminin receptor in skeletal muscle. The integrin β1D subunit is ubiquitously expressed, whereas the expression of integrin α7 is restricted to the skeletal muscle. Defects in the integrin α7 gene have been identified as a cause of congenital myopathy in humans (Hayashi et al. 1998). A knockout mouse model has been created with a null mutation in integrin α7. There is some

degree of embryonic lethality to the condition, with approximately 50% of homozygous null animals dying in utero between embryonic days 12 and 14. Null mice that are born are viable and can be bred to produce offsprings. These mice suffer from a myopathy and their muscles show classic dystrophic changes including central nucleation, split and necrotic fibers and variable fiber size. Integrin α7 deficient mice also show variable levels of pathology depending on the muscle examined, and there is significant disruption of the myotendinous junction, which is where the protein is highly concentrated in normal animals (Mayer et al 1997). No intermediate models of this disease have been identified, although decreased expression of integrin α7 has been noted in dogs with inflammatory myopathy (Ryckman et al. 2005).

1.6.2 Desmin related myopathy

Desmin related myopathies are a subtype of myofibrillar myopathy in human patients. With a heterogeneous presentation ranging from cardiomyopathy to myopathy and respiratory insufficiency, the diagnosis may be commonly missed in human patients. Inheritance is frequently autosomal dominant, but autosomal recessive inheritance has been seen. A large number of cases are sporadic, with no family history, indicating the potential for novel mutations. Some 20 mutations in desmin, and an additional mutation in αβ-crystallin, a desmin-associated protein, have been identified. Additional mutations in other desmin-associated genes probably remain to be identified, since chromosomal linkage studies provide strong evidence of their existence (Goldfarb et al. 2004).

A murine desmin knockout model has been developed. Not surprisingly, the trait is autosomal recessive in inheritance due to the complete loss of function from the mutation. Desmin null mice are viable and develop appropriately functioning cardiac, skeletal and smooth muscle, however all three types of muscle are defective. Muscle cells show degeneration, with a minority of cells showing severe disruption of their normal architecture. The most severely affected organ is the heart, which shows progressive degeneration, myocardial necrosis and calcification (Milner et al. 1996).

A canine model of desmin related myopathy has been identified in the Australian Shepherd dog. The affected dog showed increased CK levels, slowly progressive weakness, sparing of cranial muscles and cardiomyopathy. An accumulation of desmin was seen microscopically in affected muscles. No data was presented to determine if the disease was due to a mutation in the canine desmin gene or another desmin-associated protein gene (Shelton et al. 2004).

1.7 Additional Considerations

There are a number of general considerations involving the use of murine and intermediate species models that should be understood by the individuals interested in pursuing research in these models. These revolve around the expense, complexity and utility of these models.

Expense is a critical consideration when evaluating an animal model. Mice are typically group housed with per diem costs of $0.50–$1.00 per cage. Cats and dogs require significantly more space, food and care and consequently per diem costs for these species are usually $5.00–$10.00 per animal, depending on the particular facility. Thus, the cost of simply maintaining a single DMD carrier female dog may exceed $3,000 per year. These costs are magnified when one considers that female dogs are usually at least one year old before they can be bred for the first time. In addition, affected puppies or kittens born with an inherited muscle disease may require additional care beyond that offered by the standard per diem.

Some of the complexities surrounding these models relate to reproduction. Mice are generally bred by cohousing males and females and observing for successful breeding. Pregnancies can usually be timed by the appearing of a vaginal plug post coitus. If an opportunity to breed is missed, little delay is engendered as the mouse can be rebred within several weeks. Similar to mice, cats are generally bred by cohousing males and females when the females are in estrus. Since cats are induced ovulators, breeding usually results in the absence of estrus for approximately 60 days. However, if a cat is not bred, it will return to estrus within 3 weeks, providing multiple opportunities for breeding. Dogs are seasonally diestrus, meaning that they cycle approximately twice each year. If a breeding cycle is missed or is unsuccessful, the female dog cannot be rebred for an extended period of time. In addition, reproductive success in dog colonies is enhanced by rigorous monitoring and assisted reproductive technologies, such as artificial insemination. This becomes particularly critical when using affected animals as breeders or employing cooled or frozen semen. Whelping dates can be predicted in cats on the basis of ultrasound examination of the fetus and in dogs based on vaginal cytology and ultrasound examination.

The complexity and cost of intermediate animal models is balanced by their utility. This is particularly true when various sampling techniques are considered. For example, non-terminal blood collection from a mouse usually yields a volume of approximately 0.050 ml and terminal blood collection can yield as much as 1.0 ml. Individual cats and dogs can routinely provide sample volumes of 5 and 10 ml respectively on a weekly basis. A 25 kg dog can provide up to 500 ml of blood every 6 weeks, with the amount adjusted downward for smaller dogs and cats. These amounts allow routine biochemical and immunologic assessment of individual animals on a repeated basis over a period of time. Likewise, both dogs and cats are of sufficient size to allow multiple muscle biopsies in a single animal. This allows the progression of both the disease and potential therapeutic interventions to be assessed in a progressive manner in a single animal.

1.8 Summary and Future Directions

There have never been as many animal models of inherited muscle disease available as there are now, and it seems clear that the field will continue to expand over the coming years. With the availability of knockout technology and now RNAi, in

the mouse, it is possible to create murine models of nearly any known human gene mutation. The need for random mutagenesis and screening for muscle phenotypes has nearly disappeared and the focused nature of current technologies allows the rapid creation and identification of murine models. Many of these murine mutations are relatively close models of the human disease, however some display extreme phenotypes not seen in affected patients. While these latter models may not be as useful for the investigation of therapeutic interventions, they still educate us on the biology of the genes and proteins involved. Models of inherited muscle disease in the mouse have provided and will continue to provide the fundamental knowledge necessary to understand and perhaps treat these diseases. However, with the progress made on genetic therapies in mice, advances in the canine and feline genomes, and concerns about directly translating therapeutic results from mice to men, intermediate animal models have become increasingly useful and important. These models provide a stepping-stone between humans and laboratory mice. Intermediate animal models often show more similarities to the human disease than do their murine counterparts. In addition, the physical scale of the animals, their intact immune response, and their outbred nature, provide a realistic testing environment for therapies destined for human use.

Numerous human inherited muscle diseases exist for which neither murine nor intermediate species models have been created or discovered, and conversely, inherited muscle diseases exist in these species for which there is, as yet, no known human homolog. The intersection of these will result in still more animals models of these diseases. Ultimately, the rapid expansion in the number of models in the past decade predicts continued expansion in this area as additional knockout mice are created, and spontaneous disease models are identified in cats and dogs. Ultimately, it will be the combination of these models that allows us to understand the fundamental mechanisms of these diseases, and to offer patients novel and successful therapies.

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Chapter 2
In Utero Muscle Gene Transfer

Bhanu Munil Koppanati and Paula R. Clemens

Abstract Efficient, widespread transgene expression in the muscle is one of the major challenges of gene transfer for the treatment of genetic muscle disorders. A good example of this disease is the Duchenne muscular dystrophy (DMD), a progressive, degenerative disease whose clinical symptoms manifest after birth, but whose genetic defect causing the disease is present at conception. The availability of prenatal testing for genetic muscle diseases provides the basis for treatments in utero. In utero gene transfer has the potential to achieve widespread transgene expression in the muscle by accomplishing gene delivery when the tissue mass is small and the immune system is still immature. In this chapter, we present preclinical experience with gene delivery strategies to treat muscle disorders in utero. Important issues include experience with different gene delivery vectors in preclinical models, gene expression in muscle tissue, and effects on immunity.

2.1 Introduction

While some genetic disorders can be lethal to the embryo or fetus, many genetic disorders survive in utero, but subsequent to birth, lead to debilitating, lifespan-limiting conditions. Through early diagnosis and intervention during the fetal period in utero, gene delivery is uniquely positioned to intercede at the earliest possible stage of disease development. Duchenne muscular dystrophy (DMD) is an example of a disorder that can be identified by prenatal diagnosis, providing a window of opportunity to treat the disease before the signs and symptoms are clinically evident after birth. While in utero gene therapy is still in its infancy, numerous studies have been conducted in preclinical models of various genetic disorders to demonstrate the feasibility of the concept. Examples include cystic fibrosis (Larson

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et al. 1997; Rich et al. 1993), UDPglucuronyltransferase deficiency (Seppen et al. 2003), congenital blindness (Dejneka et al. 2004), α-thalassemia (Han et al. 2007), α-1-antitrypsin deficiency (Rosenfeld et al. 1991), and DMD (Reay et al. 2008).

The body mass of an organism increases dramatically from fetus to adult. Thus, gene delivery to the fetus provides an opportunity to target a higher percentage of cells than gene delivery later in life. In addition to the smaller target tissue of the fetus compared to the adult organism, the fetus also has less well-developed tissue barriers. Therefore, it is possible to accomplish gene delivery to the fetus with a lower dose and lower vector-to-tissue ratio, thereby lowering potential vector toxicities and decreasing vector production requirements. One study demonstrated that the degree of maturity of the muscle fiber basal lamina played an important role in the transduction efficiency of gene transfer by herpes simplex viral vectors when comparing neonatal and adult muscle target tissues (Huard et al. 1996). By extension, immaturity of the basal lamina in the fetus enhances widespread gene delivery to fetal muscle. A single intramuscular adenoviral vector injection in utero led to the transduction of multiple muscles, supporting the concept that muscle tissue barriers that impede vector spread in mature tissues are less of a barrier in the fetus (Reay et al. 2008).

While an immunological response that interferes with efficient gene delivery has been a significant concern in most postnatal gene therapy studies, immunity in the fetus is immature. Components of immunological immaturity during the fetal stage include a smaller number of immune cells, embryological immaturity of participating immune cells, variations in the early immune responses compared to the adult, and a lack of memory cells. Studies have shown that human fetal T lymphocytes secrete minimal levels of interleukin (IL)-2, IL-4, and interferon-gamma (IFNγ) when stimulated by phytohaemagglutinin or allogeneic stimulator cells (Zhao et al. 2002). Therefore, with gene delivery, the fetus likely cannot mount a significant immune response to the vector.

The development of immune tolerance either to the vector or the transgene has been addressed in several in utero gene therapy studies. In some preclinical experiments the fetal environment provided immune tolerance or ignorance to the transgene (Waddington et al. 2004b). Delivery of a lentiviral vector carrying the human factor IX (hFIX) cDNA and secreting hFIX protein into the fetal circulation elicited no humoral or celluar immune response against the protein (Waddington et al. 2004b). Similarly another prenatal study using systemic delivery of an adenoviral vector carrying hFIX showed evidence of immune tolerance to the transgene protein (Waddington et al. 2003a). Furthermore, this study demonstrated that five out of nine mice did not develop anti-hFIX antibodies in response to a postnatal administration of an hFIX adenoviral vector if the mice were given a prior prenatal administration of the vector. In contrast, mice treated solely with the postnatal administration of vector developed higher levels of anti-hFIX antibodies and experienced rapid loss of hFIX (Waddington et al. 2003a). Similarly Sabatino et al. demonstrated in utero delivery of adeno-associated viral (AAV) vector serotype 1 carrying hFIX achieved persistent expression of the transgene without humoral or cellular response to hFIX (Sabatino et al. 2007). These studies suggested that the

induction of postnatal tolerance to an exogenous transgene was a uniquely advantageous immunological aspect of fetal gene therapy as compared to postnatal gene therapy.

However, not all studies have suggested the development of immunological tolerance by in utero gene delivery. Although not to the degree observed with the humoral response seen in adult animals treated with AAV vectors (Brockstedt et al. 1999; Chao et al. 2000; Halbert et al. 2000), some fetal studies have shown a low level humoral immune response when treated with adenoviral or AAV delivery vectors (Lipshutz et al. 2001; Schneider et al. 2002; Vincent et al. 1995; Yang et al. 1999). Jerebtsova et al. demonstrated neutralizing antibody production in the setting of fetal gene delivery, but the antibody level was low and did not preclude readministration of the vector postnatal (Jerebtsova et al. 2002). Follow-up postnatal delivery of the vector not only triggered an adaptive immune response but also blocked transgene expression from a third vector administration indicating that the fetal administration did not result in immune tolerance to the vector or the transgene in this study (Jerebtsova et al. 2002). Taken together, these studies suggest that prenatal gene delivery offers immunological ignorance at a minimum, and may offer immunological tolerance in some settings.

One could postulate that the large number of proliferating progenitor and stem cells in embryonic and fetal tissue could provide an ideal environment for efficient gene transfer. If vectors that integrate in the genome transduce progenitor cells, then there is the potential of gene correction of all future progeny cells. Of the gene delivery vectors in common use, only moloney murine leukemia virus (MLV)-based retrovirus and lentiviral vectors integrate into the host cell genome (Fischer et al. 2004; Lewis et al. 1992; Naldini et al. 1996b). Very few muscle gene transfer studies demonstrate use of MLV-based retroviral vectors in utero (Tarantal et al. 2001). Lentiviral vectors were used for muscle gene transfer studies in utero, but ultimately resulted in tumor formation and studies were halted (Themis et al. 2005).

A unique aspect of differentiated muscle is that each muscle fiber postnatal and each myotube in the embryo and fetus is a syncytial cell containing multiple nuclei (Emerson and Hauschka 2004; Ontell and Kozeka 1984a). Therefore, even an episomal transgene or its expression product can diffuse a certain distance along the length of the fiber or myotube, an effect that could be further enhanced in utero due to tissue barriers that are not fully developed.

2.2 Fetal Muscle Development in Relation to In utero Gene Therapy

Muscle gene delivery in utero must consider the unique environment for developing the muscle in the embryo and fetus. The highly regulated processes of myogenic determination, differentiation, and development are precisely timed (Christ and Ordahl 1995; Rudnicki et al. 1993; Tajbakhsh et al. 1996, 1997); this developmental

program has important ramifications for muscle gene delivery in utero. Muscle tissue is derived from the paraxial mesoderm. Cells of the paraxial mesoderm form somites that give rise to the dermomyotome. The cells of myotome, derived from dermomyotome, further proliferate and migrate forming a mass of premuscle cells called myoblasts. Upon induction by extracellular signals, myogenic regulatory factors including myoD, myf5, myogenin and mrf4 are expressed in a regulated sequence. These signaling pathways ultimately result in the fusion of myoblasts to form myotubes (Christ and Ordahl 1995; Rudnicki et al. 1993; Tajbakhsh et al. 1996, 1997).

In humans, limb buds first appear at gestation day 28. By gestation day 38, the limb buds develop a central cartilaginous matrix segregating the dorsal and ventral premuscle masses. The myoblasts lining the future bone from the cartilaginous matrix arrange themselves in parallel arrays and by gestation day 45 myotubes begin to develop (Emerson and Hauschka 2004). A rapid increase in myotube formation is observed between gestation weeks 7 and 14. By the 20th week, muscle fibers are arranged in discrete bundles and cross striated with very few single cells persisting (Emerson and Hauschka 2004). Similarly in the mouse, embryonic myoblasts migrate from the dermatomytome to the future limb area and differentiate into multinucleated primary myotubes at approximately embryonic day 11 (E11) (Biressi et al. 2007; Buckingham et al. 2003). From E14 to E17, secondary myotubes begin to form on the scaffold of the primary myotubes (Biressi et al. 2007; Buckingham et al. 2003; Duxson and Usson 1989; Ontell et al. 1988; Ontell and Kozeka 1984a, b; Ontell et al. 1993). Therefore, most preclinical murine in utero muscle gene delivery studies have been performed at E15 or E16 when secondary myotubes are forming and while many tissue barriers such as basal lamina and immunity are still quite rudimentary.

2.3 Candidate Diseases for In utero Muscle Gene Therapy

Although, ideally all genetic diseases are targets for in utero gene therapy, those that manifest signs and symptoms shortly after birth and are associated with early mortality have the greatest potential to benefit from fetal gene transfer. Treatment of DMD is the principal in utero muscle gene transfer application in preclinical disease models (Reay et al. 2008). DMD is an example of a lethal, degenerative genetic muscle disease where patients generally succumb to respiratory or cardiac complications in the second or third decade of life. The dystrophin gene, whose mutation causes DMD, encodes the protein dystrophin. The dystrophin protein is expressed in all striated muscles and plays an important role in muscle structure and integrity. Although a proportion of DMD cases are caused by new mutations and would not be suspected during the fetal stage without specific screening, the majority of cases are inherited in an X-linked recessive pattern (Blake et al. 2002; Emery 1993). Therefore, future pregnancies carrying male fetuses are tested because of prior affected family members. Dystrophin gene mutations can be determined

prenatally from chorionic villus or amniotic fluid samples (Prior and Bridgeman 2005). Similar to DMD, the allelic disorder Becker muscular dystrophy could also be treated in utero.

Autosomal dominant muscular dystrophies could be considered for in utero gene transfer as DNA diagnostics and gene transfer vectors for these rare disorders are developed. Autosomal recessive muscular dystrophies and metabolic muscle diseases are also candidates for in utero muscle gene transfer although the absence of extensive family pedigrees for autosomal recessive disorders makes detection of these disorders in utero more challenging. In utero diagnostic screening studies must be developed in parallel with in utero treatment strategies. Among the metabolic muscle diseases, Pompe disease is particularly attractive as a disease target because DNA and protein diagnostic studies are readily available, and a lethal, infantile onset of the disease heightens the need for in utero treatment. Gene replacement studies have been extensively pursued in animal models of Pompe disease, but not for in utero treatment in preclinical models (Sun et al. 2005; Ziegler et al. 2008).

2.4 Vectors for In utero Muscle Gene Therapy

Preclinical studies of in utero vector-mediated gene transfer have been pursued utilizing numerous animal models, including mice, rats, rabbits, guinea pigs, sheep, and nonhuman primates (Baldwin et al. 1997; Bilbao et al. 2005b; Cohen et al. 1998; Hatzoglou et al. 1990; Holzinger et al. 1995; Larson et al. 1997; Lipshutz et al. 1999a, b, c; McCray et al. 1995; Mitchell et al. 2000; Schachtner et al. 1999; Sekhon and Larson 1995; Senoo et al. 2000; Tarantal et al. 2001; Vincent et al. 1995; Wang et al. 1998; Yang et al. 1999). Numerous routes of vector delivery have been explored, as will be discussed below.

The success of gene therapy depends on the ability of vectors to deliver genes without being pathogenic. Viral vectors have been an integral part of the development of gene therapy approaches. Technological developments in viral vectors have led to larger scale production, improved purification, and decreased pathogenicity. Ever growing efforts have successfully improved the performance of gene transfer in utero. For preclinical in utero gene therapy studies, the vectors employed have included the retroviridae family of viral vectors (example: lentivirus, MLV-based retrovirus), adenovirus, AAV, and several others. Each vector has specific advantages and disadvantages for the application of muscle gene transfer in utero.

2.4.1 Lentiviral Vector

Lentiviruses belong to the genera retroviridae which also includes MLV-based retrovirus. Lentivirus has a double-stranded RNA genome and a viral envelope. Lentiviral vectors carry up to 8 kb of transgene and randomly integrate into the

host genome. The commonly employed lentiviral vectors in gene therapy are immunodeficiency viruses such as human immunodeficiency virus -1 (HIV-1) and feline immunodeficiency virus (FIV) and equine infectious anemia virus (EIAV). Hybrid vectors are generated with heterologous envelope glycoproteins such as G glycoprotein of the vesicular stomatitis virus (VSV-G) which provides broad tropism (Johnston and Power 1999; Johnston et al. 1999; Kim et al. 1998; Naldini et al. 1996a, b; Poeschla et al. 1998; Srinivasakumar et al. 1997; Zufferey et al. 1997).

The capability of lentiviral vectors to integrate into quiescent cells suggested their potential to target cells for gene replacement (Lewis et al. 1992; Naldini et al. 1996b). Long term extensive expression in the heart, muscle, and diaphragm for up to 1 year was observed after the EIAV vector was injected into the fetal circulation (Waddington et al. 2003b). Furthermore, muscle gene delivery of lentiviral vector was achieved through intraperitoneal, intrathoracic, intramuscular, and systemic administration routes in utero. Systemic delivery of an EIAV-based lentiviral vector maintained transgene expression up to 15 months post-injection in diaphragm, intercostal, and limb muscles (Gregory et al. 2004). MacKenzie et al. demonstrated that lentiviral vectors transduce myogenic stem cells. They demonstrated that the vector can transduce stem cells by introducing a postnatal notexin-induced injury, and showing that in utero lentiviral vector-transduced myogenic precursor cells participated in the formation of regenerated muscle fibers (MacKenzie et al. 2005).

Lentiviral vectors can be pseudotyped with different envelope proteins. Studies have also shown that HIV vector-pseudotyped with Mokola or Ebola envelope proteins efficiently transduced muscle cells in utero (MacKenzie et al. 2002). Studies from Tarantal et al. demonstrated that a VSV-G pseudotyped HIV-1-based vector can transduce muscle tissues in rhesus monkey fetuses (Tarantal et al. 2001). However, it is unlikely that gene delivery using HIV-based vectors would be used for in utero human clinical trials since even a very low possibility of conversion to active HIV infection would be unacceptable. Furthermore, due to improvements in the FIV-based vector system, FIV-based vectors offered advantages similar to HIV-based vectors, while avoiding many of the disadvantages. While lentiviral vectors have demonstrated long-term expression, their major drawback is the high incidence of oncogenesis following in utero gene delivery using these vectors, which drastically curtails their clinical application (Themis et al. 2005).

2.4.2 Moloney Murine Leukemia Virus-Based Retroviral Vector

Another member of the genera retroviridae commonly studied in gene therapy is the amphotropic MLV-based retrovirus (Romano et al. 1999; Shinnick et al. 1981; Weiss 1998; Weiss and Wrangham 1999). MLV-based retroviral vectors contain a single-stranded, linear, positive-sense RNA molecule of approximately 8000 nucleotides. In some studies in utero, MLV-based retroviral vectors have been pseudotyped with VSV-G envelope proteins (Tarantal et al. 2001). MLV-based retroviral

vectors infect dividing cells and integrate into the host cell genome providing the rationale that MLV-based retroviral vectors could offer permanent gene replacement in utero (Miller et al. 1990). While studies have shown short term expression in canine mucopolysaccharidosis Type I animal models, they have not demonstrated long term gene expression and transgene integration into the germ line (Meertens et al. 2002).

Relatively few retroviral in utero studies transducing muscle tissues have been performed. One study showed prenatal systemic delivery of an MLV-based retroviral vector that exhibited expression in various tissues with minimal expression in the muscles (Tarantal et al. 2001). The low transduction efficiency, the requirement of dividing cells and instability in the presence of body fluids are significant limitations of the MLV-based retroviral vector for in vivo muscle gene transfer. Furthermore, in recent years a study demonstrated premalignant proliferation and lymphoproliferative disease in an X-SCID trial in which study participants received transplantation of retroviral vector-transduced, autologous bone marrow-derived $CD34^+$ cells (Hacein-Bey-Abina et al. 2003). The potential for malignant transformation is a significant concern that is heightened for in utero application.

2.4.3 Adenoviral Vector

Adenoviruses have a nonenveloped capsid and a 36 kb double-stranded DNA genome that remains as an episome in an infected cell. Adenoviral vectors for gene replacement therapy have been developed from adenovirus through genetic modification to make them replication-deficient (Haecker et al. 1996; Kochanek et al. 1996; Lai et al. 2002). First- and second-generation adenoviral vectors have deletions of one or more adenoviral early genes. Helper-dependent, high-capacity adenoviral vectors have been developed to circumvent the unfavorable immune response associated with earlier generation vectors. The reduced immunogenicity of the high-capacity adenoviral vector and its large insert size capacity for carrying the 14 kb dystrophin cDNA were significant advances for muscle-directed, adenoviral vector-mediated gene transfer (Clemens et al. 1996; Kochanek et al. 1996).

The feasibility of in utero gene delivery utilizing adenoviral vectors has been tested by various groups (Bilbao et al. 2003, 2005b; Bouchard et al. 2003; Christensen et al. 2000; Holzinger et al. 1995; Iwamoto et al. 1999; Larson et al. 2000, 1997; Lipshutz et al. 1999a, b, c; McCray et al. 1995; Reay et al. 2008; Sylvester et al. 1997; Turkay et al. 1999; Vincent et al. 1995; Yang et al. 1999). Adenoviral vectors can infect both dividing and nondividing cells and can achieve high levels of gene transduction. However, adenoviral vectors induce an immune response to the viral capsid proteins and this has been a major concern in adenoviral vector-mediated gene delivery in postnatal settings where host immune systems are fully developed (Acsadi et al. 1996; Gilgenkrantz et al. 1995; Tripathy et al. 1996; Yang et al. 1994). The development of antibody-mediated immune responses to capsid and transgene proteins after in utero muscle gene transfer has been variable

between studies (Jerebtsova et al. 2002; McCray et al. 1995; Schneider et al. 2002). Schneider et al. showed absence of immune responses against adenoviral capsid proteins and the transgene product (Schneider et al. 2002). However, even in one study in which neutralizing antibodies against capsid proteins were detected, a second postnatal vector administration was successful (Jerebtsova et al. 2002).

An in utero muscle gene transfer study demonstrated the vector delivery efficiency of first-generation adenoviral vectors as compared to high-capacity adenoviral vectors and demonstrated transgene expression persistence for at least 5 months (Bilbao et al. 2005b). This study observed a mosaic pattern of transduced muscle fibers expressing vector transgene similar to two previous studies utilizing first-generation adenoviral vectors for in utero muscle gene transfer (Mitchell et al. 2000; Yang et al. 1999). One of the latter studies demonstrated persistence of transgene expression up to 16 weeks postnatal after an in utero intramuscular injection of first-generation adenoviral vectors with transgenes driven by the human cytomegalovirus (HCMV) promoter (Mitchell et al. 2000). Furthermore, this study demonstrated a confined focus of transgene expression in the liver in 8 weeks and only minimal evidence of inflammation in the muscle or liver. No neutralizing antibodies were observed in this study.

In addition, fetal sheep gene transfer into thoracic muscles and pleural cavity using adenoviral vectors also demonstrated efficient transduction into respiratory muscles (Weisz et al. 2005). Furthermore, the feasibility of less invasive, ultrasound guided intramuscular gene delivery into specific muscles of the sheep fetus was demonstrated using adenoviral vectors (David et al. 2003).

Relatively few studies utilizing adenoviral vector gene delivery in utero have been performed in disease models. Toward the treatment of hfIX deficiency, an adenoviral vector carrying hfIX was injected intramuscularly in C57BL/6J or MF1 mice in utero. This approach resulted in therapeutic serum levels of hfIX that persisted up to 6 months of the study (Schneider et al. 2002).

Recently Reay et al. performed muscle gene transfer experiments in utero in the mouse model of DMD, the mdx mouse. A high-capacity adenoviral vector carrying the full-length dystrophin cDNA was injected intramuscularly into the mdx mouse in utero resulting in properly localized recombinant dystrophin expression, restoration of the dystrophin associated glycoprotein complex, and suggestion of functional benefit (Reay et al. 2008).

The potential of targeting adenoviral vectors to alternate receptors to enhance muscle gene delivery has been tested primarily in postnatal studies. However, limited vector targeting studies have been pursued in an attempt to enhance in utero muscle gene delivery. Native adenoviral infection is initiated by binding of the adenoviral fiber protein to the coxsackievirus and adenovirus receptor (CAR) followed by internalization mediated by viral penton base and integrin αv proteins (Bergelson et al. 1997, 1998; Wickham et al. 1994, 1993). It was postulated that high expression of integrin αv proteins on fetal muscle could provide a ligand for vector targeting. To target αv integrin on fetal muscle cells for in utero muscle gene transfer, RGD-modified high-capacity adenoviral vectors were generated and tested (Bilbao et al. 2003). The study showed that unmodified vector transduction was

more efficient than RGD-modified high-capacity adenoviral vector transduction, demonstrating that the loss of CAR-mediated transduction was not sufficiently compensated by any gains provided by the abundant αv integrin expression on fetal muscle (Bilbao et al. 2003).

2.4.4 Adeno-Associated Viral Vector

Recently, AAV vectors have been utilized most extensively in preclinical and clinical muscle gene therapy studies. AAV is a nonenveloped, single-stranded DNA virus with a gene insert size capacity of about 4.7 kb for AAV serotype 2 (Srivastava et al. 1983). The discovery of different AAV serotypes has led to AAV vectors that have differing tissue tropisms and transduction efficiencies. AAV infect dividing and non-dividing cells and appear to have lower tendency to induce immunity compared to many other vectors. AAV vectors have been studied in utero in various animal models such as mice (Bilbao et al. 2005a), rats (Garrett et al. 2003), rabbits (Boyle et al. 2001), and primates (Garrett et al. 2003; Larson et al. 2000; Nathwani et al. 2007).

The first AAV vector muscle gene transfer studies in utero were done with direct intramuscular injections into the fetus. Mitchell et al. showed that the intramuscular injection of AAV2 vector with a transgene driven by the HCMV promoter produced persistent expression in muscles up to 3 months after birth (Mitchell et al. 2000). Similarly, Schneider et al. showed β-galactosidase expression in muscle tissues up to 6 weeks after in utero intramuscular injections of AAV2 vector carrying a CMV-driven *lac*Z transgene (Schneider et al. 2002). In addition, another study by Schneider et al. using AAV2 and AAV5 vectors by intramuscular injection demonstrated muscle transgene expression up to 18 weeks (Schneider et al. 2002). Furthermore, intramuscular injection studies of AAV1 (Bilbao et al. 2005a), and AAV2 vectors into fetal muscle demonstrated good transgene expression (Mitchell et al. 2000; Yang et al. 1999). Moreover Bilbao et al. showed that intramuscular injection of AAV1 vector transduction to be 20 times higher compared to AAV2 vector transduction of skeletal muscles 4 weeks after in utero treatment (Bilbao et al. 2005a). This high transgene expression from an intramuscular administration of AAV1 vector in utero compared to AAV2 vector was further corroborated by Sabatino et al (Sabatino et al. 2007).

There is limited experience with systemic AAV vector gene delivery to muscle in utero. Intraperitoneal injection of AAV2 vector carrying an EF1α promoter-driven luciferase transgene demonstrated transgene expression in various tissues including heart (Lipshutz et al. 2001). This study demonstrated the absence of germ line integration, hepatotoxicity, and antibodies against the vector or the transgene (Lipshutz et al. 2001). Bilbao et al. demonstrated that systemic delivery of AAV1 vector in utero transduced diaphragm significantly better than the limb muscle tissue (Bilbao et al. 2005a). The recently utilized serotypes of AAV vector, such as AAV8 vector carrying a *lac*Z reporter gene, showed a high level of transduction in the diaphragm and a more moderate and mosaic pattern of transduction of multiple

limb muscles and heart (BMK and PRC, in press). Furthermore, recently in utero gene delivery of an AAV8 vector carrying a truncated dystrophin cDNA not only restored the dystrophin associated glycoprotein complex proteins, α-sarcoglycan and β-dystroglycan, but also provided a functional benefit in *mdx* diaphragm (BMK and PRC, unpublished data).

2.4.5 Other Vectors

Studies have also shown that less known vectors such as Sendai virus vectors also have provided muscle transgene expression up to 48 hours after intramuscular injection in utero (Waddington et al. 2004a). While several groups have performed experiments in utero with nonviral vector gene transfer (Gharwan et al. 2003; Luton et al. 2004; Mason et al. 1999; Sato et al. 2004), further studies will be required to enhance the efficacy of such vectors for in utero muscle gene transfer. Yoshizawa et al. have successfully used gene gun to transfer plasmids in mid-gestational mouse fetuses and suggested the possibility of using gene gun techniques to deliver vectors to muscle tissues in utero (Yoshizawa et al. 2004). Another interesting approach tested in a few studies was to genetically target stem cells with gene delivery vectors *ex vivo* and transplant them into the fetus (Casal and Wolfe 1997; Kantoff et al. 1989; Lutzko et al. 1999). The therapeutic and proliferative advantage, if any, of such modified cells over the native cells in fetal muscle gene therapy needs to be explored.

2.5 Summary and Future Directions

Preclinical studies have demonstrated the feasibility of introducing an exogenous gene into the muscle of the developing fetus, although the optimal timing and best mode of delivery have yet to be defined. In utero gene therapy is still in its infancy for clinical application. While there are many potential advantages, the various safety and ethical implications must also be considered. With advanced techniques such as fetoscopy, ultrasound, and fetal surgical methods, it is possible to achieve in utero gene delivery while limiting the invasiveness of the procedure to both the fetus and the mother. While the aim of in utero gene therapy is to provide an optimal therapeutic benefit, every attempt must be made to minimize potential complications. Therefore, careful monitoring for birth defects and any long term side effects of in utero gene transfer will be required.

Important perinatal considerations of in utero gene transfer to the muscle include minimizing any increased risk of preterm labor, infection, and fetal loss. Human clinical approaches to fetal muscle have included fetal muscle biopsy (Heckel et al. 1999; Kuller et al. 1992) and ultrasound guided fetal gluteal muscle injection of corticosteroid, employed to improve fetal lung maturation (Ljubic et al. 1999). Therefore, the surgical approaches that would be required for human in utero gene transfer have precedence.

Some of the many factors concerning optimal timing of injecting the viral vector include prevention of insertional mutagenesis, avoidance of integration into the germ line and avoidance of deleterious immunity. Insertional mutagenesis, which could possibly lead to oncogenesis, can be avoided by using vectors that do not integrate into the host cell genome. Germ line integration can be minimized by injecting the vector into fetal tissue after the process of gonadal compartmentalization is completed in the fetus. In mouse, primordial germ cells complete colonization into gonadal primordium by E13 (Hogan et al. 1994). Similarly in humans the primordial germ cells are completely compartmentalized by the seventh week of gestation (Gillman.J 1948). By choosing the appropriate fetal developmental stage for gene delivery, germ line integration can be minimized. It is clear from the variable results to date that further studies of immunity and the development of tolerance in the setting of muscle gene transfer in utero are required.

One of the drawbacks of postnatal gene therapy is hepatic toxicity. Although some in utero studies in guinea pigs have shown liver transduction (Senoo et al. 2000), which could be due to the choice of the promoter, most evidence suggests low levels of liver transduction following in utero muscle or systemic gene delivery (Bilbao et al. 2005a, b; Boyle et al. 2001). Similar to AAV vector-mediated gene transduction in utero, AAV vector-mediated gene delivery in neonatal mice yielded significantly lower levels of liver transduction compared to gene delivery in adult mice (Wang et al. 2008, 2005). While additional studies are needed to understand the mechanisms of low liver transduction in most in utero studies, the high proliferative state and unique properties of the fetal liver during development could play a role (Lansdorp et al. 1993; O'Donoghue and Fisk 2004; Taylor et al. 2002). Another consideration that impacts the ultimate safety of in utero gene transfer is the transplacental spread of vector from the fetus to tissues of the pregnant female. Studies have shown the presence of antiadenovirus and antitransgene antibodies in maternal serum (Bilbao et al. 2005b). Although these antibodies were not neutralizing (Bilbao et al. 2005b), immune tolerance, liver toxicity and other consequences need to be considered.

In this chapter, we have given the results of the studies on the unique potential, the different vectors used to date in experimental studies, and the challenges of in utero muscle gene transfer. Future advances will depend on continued vector development, a better understanding of effects on target and nontarget tissues and the induction of immunity and strategies to safely enhance efficient gene transduction of the muscle tissue for long-lasting therapeutic benefit.

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Chapter 3
Gene Therapy for the Respiratory Muscles

Gawiyou Danialou and Basil J. Petrof

Abstract The diaphragm and other ventilatory muscles constitute a vital pump for respiration. Death is an inevitable consequence when this pump fails, unless artificial ventilatory support is provided. In many neuromuscular disorders for which gene therapy is being considered (e.g., Duchenne muscular dystrophy), muscle weakness involves the diaphragm and other respiratory muscles, thereby leading to the development of ventilatory failure. Therefore, it is critically important that effective methods be developed for targeting the respiratory muscles by gene therapy approaches. In this chapter, we review the normal physiology of the respiratory muscles and the particular challenges associated with evaluating the efficacy of any future applications of gene therapy to the respiratory muscles in humans. We also review the current state of affairs with respect to preclinical animal models of candidate diseases for respiratory muscle gene therapy, which have pointed to several challenges as well as promising areas for future progress in this area.

3.1 Introduction

The diaphragm and other respiratory muscles comprise a ventilatory pump, analogous to the heart, upon which the act of breathing depends. In many neuromuscular diseases for which gene therapy is being considered, muscle weakness involves the respiratory muscles to an equal or even greater extent than other skeletal muscles. In several diseases (e.g., Duchenne muscular dystrophy, amyotrophic lateral sclerosis), there is a relentless progression to respiratory failure, leading inevitably to death unless mechanical ventilation is provided. In addition to the muscles of inspiration such as the diaphragm, expiratory muscles are often weak as well, resulting in impaired cough and clearance of secretions, which predisposes to respiratory complications. Some patients with neuromuscular diseases also have weakness of

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the upper airway muscles, which can lead to repeated episodes of aspiration pneumonia or to the development of obstructive sleep apnea syndrome. Finally, although not considered to be respiratory muscles per se, weakness of the musculature that supports the spinal column leads to chest wall deformities such as kyphoscoliosis, which can in turn produce a major restrictive impairment of the lungs. In view of all of the above, it is clear that for many neuromuscular disorders, any application of gene therapy that hopes to prolong survival will need to effectively target the respiratory muscles (Petrof 1998).

The diaphragm is the primary muscle of inspiration and is normally responsible for generating about two-thirds of the resting tidal volume. It is comprised of costal and crural muscular portions having distinct attachments and mechanical actions upon the chest wall, which both undergo shortening during inspiration. The diaphragm is innervated by the phrenic nerves bilaterally, which receive their neural input from the third to fifth cervical roots emerging from the spinal cord. There is also a rich vascular supply to the diaphragm, originating from the phrenic, intercostal, and internal mammary arteries. The diaphragm is normally recruited with each inspiration during resting and breathing, along with the parasternal intercostals. Under conditions of high ventilatory demand, with underlying lung pathology present, or when the diaphragm is weakened by disease, the so-called "accessory" muscles of inspiration are also activated to aid in the inspiratory effort. These consist of neck muscles (e.g., scalene, sternocleidomastoid), muscles of the upper thorax (e.g., pectoralis, trapezius), and the nonparasternal external intercostals. Important expiratory muscles include the abdominal muscles as well as the internal intercostals. The respiratory muscles present unique challenges for human gene therapy, both because of their relative inaccessibility anatomically in some instances (e.g., for the diaphragm) and the fact that the clinical evaluation of respiratory muscle function is less straightforward than for the limb musculature.

3.2 Respiratory muscle functional assessment: implications for human gene therapy

In patients with neuromuscular disorders, respiratory symptoms are often minimal until relatively late in the course of the disease, due to the inherently large reserve of the respiratory system. Respiratory muscle involvement may also be masked because patients with weak limb muscles have limitations in their overall activity levels (e.g., if wheelchair-bound), thereby reducing the magnitude of the physiologic challenge faced by the respiratory system. Furthermore, changes in lung spirometry and the development of hypercapnia (carbon dioxide retention in the blood) are usually late phenomena. Although measurement of the forced vital capacity (FVC) is the most useful test for determining overall respiratory prognosis and the risk of hypercapnia in most neuromuscular disorders (Fallat et al. 1979; Rideau et al. 1981; Phillips et al. 2001), it is an insensitive measure of the respiratory muscle strength. In this regard, it is not unusual for respiratory muscle strength

to be reduced by over 50% before the appearance of a reduction in vital capacity (Braun et al. 1983). Therefore, more sensitive and direct tests of respiratory muscle function would almost certainly be required in order to ascertain any early or modest benefits of gene therapy.

In clinical practice, respiratory muscle force-generating capacity is determined indirectly from the measurement of pressures rather than muscle tension per se. The maximum inspiratory pressure (Pi_{max} or MIP) and expiratory pressure (Pe_{max} or MEP) generated against an occluded airway and measured at the mouth, are most often used. Normal predicted values have been established in both adults and children, and these tests can be performed during the routine follow-up of neuromuscular patients. In adults, hypercapnia is generally not observed until the MIP falls below 30 cm H_2O (Braun et al. 1983). The lowest value of MEP found to be consistent with production of a satisfactory cough was on the order of 50–60 cm H_2O (Szeinberg et al. 1988). Maximal sniff nasal pressure (SNIP, or sniff Pi_{max}) is a relatively new test of inspiratory muscle strength, which involves measurement of the pressure generated within an occluded nostril during a maximal sniff maneuver. It has been reported that sniff Pi_{max} is easier to perform and more reliable in some neuromuscular patients (Stefanutti et al. 2000). The tests of respiratory muscle function discussed above, all have the advantage of being noninvasive and relatively easy to perform serially over time, which are major advantages for any future clinical trials.

Although the MIP and MEP are more sensitive for detecting respiratory muscle weakness than the vital capacity, they are nonetheless tests of global inspiratory or expiratory muscle function, and do not allow one to determine the effects of a gene therapy intervention on a specific respiratory muscle. To more specifically evaluate the diaphragm, thin balloon-tipped catheters can be swallowed by the patient under topical anesthesia, and placement of these catheters in the esophagus and stomach allows one to measure intrapleural and intraabdominal pressure swings during breathing. The pressure difference across the diaphragm (transdiaphragmatic pressure, or Pdi) can thus be measured, and Pdi measurements performed during a maximal inspiratory effort will specifically reflect the force-generating capacity of the diaphragm (Laporta and Grassino 1985). However, it is important to recognize that Pdi can also be altered by changes in chest wall configuration (e.g., with kyphoscoliosis) and lung volume, and these factors must be controlled for as much as possible when making the measurements (Bellemare and Grassino 1982).

Since all of the above are volitional tests, submaximal or variable patient efforts can (and often do) result in falsely low or inconsistent values, which will further complicate the assessment of any potential benefits of gene therapy on respiratory muscle function. To deal with this problem, several groups of investigators have employed direct stimulation of the phrenic nerves (Similowski et al. 1989; Laghi et al. 1996). Cervical magnetic stimulation is a technique which allows both phrenic nerves to be supramaximally stimulated using a magnetic coil placed over the cervical vertebrae, thereby eliminating any volitional component (Similowski et al. 1989). Importantly, the procedure is painless (as opposed to bilateral electrical stimulation of the phrenic nerves), and can be used in combination with either:

(1) muscle-specific albeit invasive measurements of Pdi, or (2) noninvasive measurements of global inspiratory muscle pressure generation (i.e., at the mouth during airway occlusion) (Hamnegaard et al. 1995).

Another functional outcome measure of potential use for evaluating the effectiveness of future gene therapy interventions is respiratory muscle endurance. This has been evaluated by a number of different methods in patients with various neuromuscular disorders. Although not a pure test of endurance, the classical maximal voluntary ventilation (MVV) maneuver can be reduced in patients with neuromuscular diseases even when the vital capacity is normal. Others have measured the maximal sustainable ventilation or pressure produced by the respiratory muscles over predetermined periods of time, or the maximal time for which a subject is able to sustain a predetermined percentage of the MVV or maximal Pdi (Matecki et al. 2004; Smith et al. 1988; Vilozni et al. 1994; Wanke et al. 1994).

Finally, there are newly emerging areas of respiratory muscle evaluation which are conceptually attractive due to their noninvasive nature and the ability to track changes over time. One such approach is the ultrasound, which can be used to image the diaphragm provided there is little or no air intervening between the probe and the muscle. The normal diaphragm is poorly echogenic, whereas the diseased diaphragm muscle in Duchenne muscular dystrophy was found to be associated with increased echogenicity (De Bruin et al. 1997). The thickness of the diaphragm during relaxation at different lung volumes, tidal breathing, and static inspiratory efforts can also be determined (Ueki et al. 1995; Cohn et al. 1997). When skeletal muscles contract, they also produce vibrations and low frequency sounds which can be detected by microphones placed on the skin surface (Oster and Jaffe 1980). This signal, called a phonomyogram, can be recorded during respiratory maneuvers and has been correlated with the muscle tension developed during contractions (Maton et al. 1990). Phonomyograms of the diaphragm during phrenic nerve stimulation were shown to be modified by the onset of muscle fatigue (Petitjean and Bellemare 1994). Whether such methods could be used to noninvasively monitor the efficacy of therapeutic gene transfer to the human diaphragm remains to be determined.

3.3 Challenges for gene transfer to the respiratory muscles: lessons from animal models

Table 3.1 shows a list of some of the more common or prototypical neuromuscular disorders which can affect the diaphragm and other respiratory muscles. As can be seen, respiratory muscle weakness can be caused by a large and diverse number of diseases acting at different levels of the nervous system, including the central nervous system, the spinal cord, the nerves, the neuromuscular junction, and the muscle. In principal, gene therapy could be used to treat several of these conditions, either by correcting an underlying genetic defect or by supplying some other therapeutic gene product. For the purposes of this chapter, we will limit our discussion to disorders which are potentially treatable by gene transfer to the muscle itself,

Table 3.1 Neuromuscular Disorders Affecting Respiratory Muscle Function

Muscular Dystrophies
Duchenne Muscular Dystrophy
Becker Muscular Dystrophy
Limb-Girdle Muscular Dystrophy
Myotonic Dystrophy
Facioscapulohumeral Muscular Dystrophy
Congenital Muscular Dystrophy
Metabolic Diseases of Muscle
Acid Maltase Deficiency
Mitochondrial Myopathies
Carnitine Palmityl Transferase Deficiency
Other Myopathies
Polymyositis or Dermatomyositis
Corticosteroid-Induced
Diseases of the Neuromuscular Junction
Myasthenia Gravis
Eaton-Lambert Syndrome
Diseases of Peripheral Nerve
Charcot-Marie-Tooth Disease
Friedreich's Ataxia
Chronic Inflammatory Demyelinating Polyneuropathy
Guillain-Barre Syndrome
Diseases of the Motor Neuron
Amyotrophic Lateral Sclerosis
Postpolio Syndrome
Spinal Muscular Atrophy
Other Diseases Involving the Spinal Cord
Traumatic Injury
Multiple Sclerosis

with a particular focus on the diaphragm. In animal models, gene transfer to the diaphragm has been accomplished through one of 3 major routes of vector administration: (1) direct intramuscular diaphragmatic injection; (2) intracavitary (pleural, peritoneal or in utero) injection; and (3) intravascular administration.

3.3.1 Direct Intramuscular Diaphragmatic Injection

The diaphragm can be accessed for direct intramuscular injection of gene transfer vectors into its abdominal surface via a surgical laparotomy. Using such an approach, Davis and Jasmin (1993) and Petrof et al. (1996) first reported successful results of plasmid DNA-mediated reporter gene transfer to the diaphragms of normal mice and rats, respectively. Petrof et al (1995) also evaluated the physiological consequences of adenovirus (first generation)-mediated reporter gene transfer to the diaphragm in

both normal and dystrophin-deficient mdx (murine model of Duchenne muscular dystrophy) mice. At 1 week after intradiaphragmatic adenovirus vector administration, a substantial level of transgene expression within the injected area of the diaphragm could be demonstrated. One month later, however, elimination of transgene expression was observed, along with a significant decrease in force production by both normal and mdx diaphragms. This was attributed to an adverse host immunological response against the vector and/or reporter transgene, since immunosuppression with cyclosporine was able to alleviate the adverse effects of adenovirus vector administration upon diaphragmatic force-generating capacity (Petrof et al. 1995).

Subsequent studies examined the effects of delivering therapeutic genes by intradiaphragmatic injection in mice. Decrouy et al. (1997) reported that intradiaphragmatic injection of plasmid DNA containing the 6.3 kb Becker-like dystrophin minigene had beneficial effects upon sarcolemmal membrane stability in the diaphragmatic fibers of mdx mice. Yang et al (1998) also found that adenovirus-mediated transfer of the dystrophin minigene was able to achieve successful dystrophin gene transfer and alleviate histopathological evidence of disease progression in the mdx mouse diaphragm. However, as with the previous study of diaphragm muscle treated by first-generation adenoviral vectors (Petrof et al. 1995), vector-related toxicity and adverse host immune responses were a limiting factor, and immunosuppression was required in order to maintain transgene expression over several weeks in the diaphragm. This problem was partially addressed in a later study which used a third-generation or "gutted" adenoviral vector deleted of all viral genes and containing two full-length dystrophin transgenes in the mdx mouse diaphragm (Matecki et al. 2004). Under these conditions, full-length dystrophin expression remained stable for 30 days without the need for continuous immunosuppression, and treated diaphragms showed a significantly improved resistance to the force deficits induced by high-stress eccentric muscle contractions.

Taken together, the above studies demonstrate that intradiaphragmatic injections of gene transfer vectors can produce the expected physiological effects, which are similar to the findings observed after administering these vectors by intramuscular injection in other skeletal muscle groups. Moreover, in human patients it should be feasible to perform direct intramuscular injections into the diaphragm via modern laparascopic techniques, including in patients with neuromuscular disease and severe diaphragmatic weakness (DiMarco et al. 2005; Onders et al. 2008). However, as with intramuscular injections in other muscle groups, a major problem is the restricted area of vector diffusion within the muscle tissue which is largely limited to the immediate vicinity of the injection site. Therefore, in human patients it is likely that a very large number of injections would be required to cover a substantial surface area of the diaphragm.

3.3.2 Intracavitary Injection

Several studies have demonstrated that the diaphragm can be successfully targeted for gene transfer during the fetal period, by injecting gene transfer vectors directly into the uterine cavity. In a murine model of Pompe disease (Glycogen storage

disease type II) which is defined by a lack of the enzyme acid α-glucosidase (GAA), Rucker et al (2004) injected adeno-associated virus vectors (AAV1 and AAV2) into the fetal peritoneal cavity on day 15 of gestation. These authors found that several weeks after birth, GAA levels in the diaphragm were significantly increased, and this was associated with improvements in histopathology and the contractile function of the diaphragm up to 6 months of age. Using fetal mice of the same gestational age, Gregory et al (2004) showed high-level LacZ gene transfer to the diaphragm by equine infectious anemia virus (EIAV) lentivirus vectors pseudotyped with VSV-G. By combining intrapleural and intraperitoneal injections of EIAV in the fetus, not only the diaphragm but also the intercostal muscles and abdominal musculature were effectively transduced over a large proportion of their surface area. It should be noted that in the above studies there was little or no transgene expression in the distant muscles or organs, suggesting that the vectors were accessing the tissues locally rather than through their absorption into the systemic circulation (Rucker et al. 2004; Gregory et al. 2004). Moreover, an added advantage of performing gene transfer during the fetal period was that host immune responses against the involved vectors or transgenes were either mitigated or completely eliminated in mice (Rucker et al. 2004; Gregory et al. 2004). Jimenez et al (2005) administered HIV-1 derived lentiviral vectors into the peritoneum of early gestation macaque monkeys under ultrasound guidance, and also found consistent expression within the diaphragm. Remarkably, transgene expression in the diaphragm was reportedly sustained for up to 3 years (Tarantal et al. 2006). Finally, it has been reported that somite-derived cells expressing LacZ and injected into the uterine circulation of pregnant mdx mice can lead to significant transgene expression within the diaphragm at 2 months after birth, although the level of engraftment is low (Torrente et al. 2000).

During the postnatal period, the abilities of intraperitoneal or intrapleural administration of different vectors to transduce the diaphragm have also been evaluated. Howell et al. (1997) reported that after intraperitoneal injection of plasmid DNA containing a LacZ transgene together with lipofectin in Golden Retriever Muscular Dystrophy (GRMD) dog pups, positive staining could be found in the diaphragm, intercostal and abdominal muscles, albeit at extremely low levels. Huard et al (1995) performed intraperitoneal injection of first-generation adenoviral vectors in 2-day old rats, and showed significant transgene expression after 5 days in the diaphragm. In this study, a substantial adenovirus level in the blood was also demonstrated in the first few hours after intraperitoneal injection of the vector, raising a question as to whether transduction of the diaphragm may have been mediated through the systemic circulation. In adult mice, Mah et al (2004) compared the efficiencies of gene transfer to the diaphragm after direct local application ("painting") of AAV serotypes 1, 2 or 5 onto the peritoneal surface of the muscle. Interestingly, they also compared transduction efficiencies when the vectors were applied as free virus or mixed with a water-soluble, glycerine-based gel. These investigators found that at 6 weeks after vector delivery, the highest transduction of the diaphragm was achieved with gel-based application of vectors (AAV1 > AAV2 > AAV5), and diaphragmatic transduction was demonstrated not only for a reporter gene but also for the GAA transgene in the murine model of

Pompe disease. On the other hand, another group reported that in adult mice which received intrapleural instillation of AAV vectors containing a luciferase transgene, higher levels of diaphragmatic transduction were attained with AAV5 as compared to AAV2 (De et al. 2004). Blankinship et al (2004) showed high-level transduction of the diaphragm and intercostal muscles of adult mice after a single injection of AAV6 into the thoracic cavity. In addition, in the same study intraperitoneal injection of AAV6 in newborn mice achieved widespread transduction throughout the musculature, including the diaphragm.

Overall, the literature to date indicates that intracavitary injection of certain gene transfer vectors can achieve substantial local transduction of the respiratory muscles in animal models. These approaches are generally more effective in fetal or newborn animals, presumably due to incomplete formation of physical barriers to vector dispersion in the diaphragm and other respiratory muscles at this stage of development, as well as potentially higher levels of viral receptors and coreceptors within immature skeletal muscle tissues.

3.3.3 Intravascular Injection

Plasmid DNA can transduce hindlimb muscles with high efficiency, including in large animal models (Danialou et al. 2004; Hagstrom et al. 2004; Zhang et al. 2001) and, if delivered intraarterially or intravenously under conditions of high injectate volume and pressure together with a temporary obstruction of venous outflow from the targeted limb. Liu et al (2001) have presented evidence that a similar strategy can be applied to the diaphragm of mdx mice, by clamping the vena cava immediately following injection of plasmid DNA into the tail vein.

Huard et al (1995) reported that after intracardiac injection of first-generation adenoviral vectors in 2-day old rats, there was significant transduction of the diaphragm. However, adenovirus vectors generally perform poorly for this purpose in adult skeletal muscles due to difficulties in breaching the endothelial barrier and the relatively low levels of its receptors in mature muscles (Cho et al. 2000). AAV6 and AAV8 are superior to adenoviral vectors for intraarterial delivery to mouse hindlimb muscles, and AAV8 was also found to be effective for this purpose in macaques (Rodino-Klapac et al. 2007), although the diaphragm was not evaluated.

The most dramatic examples of successful intravascular delivery of gene transfer vectors to the diaphragm and other respiratory muscles have been achieved with AAV vectors. Gregorevic et al (2004) initially reported that by combining intravenous AAV6 administration and treatment with vascular endothelial growth factor (VEGF) to increase vascular permeability, it was possible to transduce most major skeletal muscle groups of the body in adult mice, including the intercostal muscles. However, a notable exception was the diaphragm, which demonstrated poor transduction. This latter finding was likely related to the version of creatine kinase promoter (CK6) used in the vector, which has been shown to have poor activity in the murine diaphragm (Salva et al. 2007). Indeed, when using a constitutively

active viral (CMV) promoter within AAV6 injected by the tail vein, Gregorevic et al (2008) more recently achieved high-level microdystrophin expression in the diaphragms of older (20 months) mdx mice, and also demonstrated an improved resistance of the diaphragm to contraction-induced injury under these conditions. In keeping with these results, Salva et al (2007) showed robust microdystrophin expression in the mdx mouse diaphragm after intravenous injection of AAV6 containing a muscle-specific promoter (MHCK7) that demonstrates superior activity to the CK promoter in oxidative muscle fibers. Furthermore, the latter results were achieved in adult mdx mice without the need for concomitant vascular permeabilization treatment with VEGF.

Several studies have examined the ability of intravenously administered AAV to correct diaphragmatic pathology in the murine model of Pompe disease. In this model, the diaphragm and other skeletal muscles exhibit an abnormal accumulation of glycogen within muscle fibers, resulting in a loss of force-generating capacity. As mentioned earlier, the disease is caused by a lack of the glycogen-metabolizing enzyme GAA in lysosomes. After intravenous delivery of AAV vectors encoding GAA, substantial correction of the diaphragmatic phenotype has been demonstrated (Cresawn et al. 2005; Mah et al. 2005; Sun et al. 2005). Interestingly, transduction of the diaphragm itself is not absolutely required for amelioration of the diaphragmatic pathology, as GAA secreted from other transduced organs (eg. liver) can be taken up by distant muscles (Ding et al. 2001). However, the development of neutralizing antibodies against GAA is a frequent problem which reduces this "distant cross-correction" effect over time (Cresawn et al. 2005; Sun et al. 2005), suggesting that direct transduction of the diaphragm may be a better long-term strategy. Studies in the GAA-deficient mouse model have also indicated that in addition to AAV6, other pseudo types such as AAV1 in newborns (Mah et al. 2005) and AAV7, AAV8 or AAV9 in adults (Sun et al. 2008), are able to transduce the diaphragm with reasonably high efficiency following intravenous administration.

Finally, there is currently considerable interest in the potential of antisense oligonucleotides to restore dystrophin expression by inducing "exon skipping" in the Duchenne muscular dystrophy. In the mdx mouse, intravenous delivery of specific 2-*O*-methyl phosphorothioate antisense oligoribonucleotides mixed together with the triblock copolymer F127 induced dystrophin expression in the diaphragm, intercostals, and abdominal muscles at low levels (Lu et al. 2005). The same group later showed that morpholino phosphorodiamidate antisense oligonucleotides can be delivered repeatedly by the intravenous route in adult mdx mice, resulting in a cumulative increase in dystrophin expression in intercostal and abdominal muscles, and to a lesser extent within the diaphragm (Alter et al. 2006). More recently, cell-penetrating peptides containing arginine, 6-aminohexanoic acid, and/or β-alanine conjugated to morpholino phosphorodiamidate antisense oligonucleotides have been delivered intraperitoneally or intravenously to mice, and demonstrated a high level of efficacy in the diaphragm (Jearawiriyapaisarn et al. 2008). In mdx mice injected intravenously with these peptide-conjugated morpholinos on a daily basis for 4 days, dystrophin expression was reportedly restored to 40–50% wild-type levels for up to 17 weeks after the last treatment. This is a level of dystrophin

restoration which would be expected to have major functional benefits and could even completely prevent abnormal contractile function if instituted early enough in the disease process.

3.4 Summary and Future Directions

Despite major technical and biological obstacles, several promising methods for achieving effective respiratory muscle gene transfer have emerged over the past few years. These developments are exciting and raise a real prospect for gene therapy of the respiratory muscles in the not too distant future. A major challenge is to now refine and adapt these methods to large animal models which are more relevant and analogous to the physiological scale encountered in human patients. Further advances in minimally invasive surgical techniques for targeting the diaphragm and its circulatory system, as well as ongoing developments in vector biology and improvements in our understanding of the human immune response to gene therapy interventions, will also be essential in this effort.

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Chapter 4
Muscular Dystrophy Gene Therapy in Small Animal Models

Chunping Qiao and Xiao Xiao

Abstract Muscular dystrophies are inherited neuromuscular disorders characterized by progressive muscle loss and weakness. The morbidity and fatality associated with the diseases and a lack of effective treatment have prompted urgent search for novel therapeutics. Gene therapy is one of the frontiers. Currently, adeno-associated viral (AAV) vector-mediated gene transfer offers a powerful tool for muscular dystrophy gene therapy for both skeletal as well as cardiac muscles, by means of local, regional, and systemic deliveries. However, AAV has a packaging limit smaller than 5,000 nucleotides. Larger genes such as dystrophin will need to be truncated to functional miniature versions to be packaged in AAV particles. In this chapter, we will illustrate how gene therapy with AAV vectors is applied to small rodent muscular dystrophy models including those that mimic Duchenne muscular dystrophy (the dystrophin-deficient mdx mice), congenital muscular dystrophy (the laminin $\alpha 2$ knockout dy/dy mice), and limb-girdle muscular dystrophy (the delta-sarcoglycan deficient TO-2 hamsters). Challenges in larger animal studies and prospects for clinical trials in muscular dystrophies will be briefly discussed.

4.1 Introduction

Muscular dystrophies are hereditary, degenerative disorders of muscles, primarily the skeletal and cardiac muscles, resulting from defects in genes that encode a diverse group of proteins (Emery 2002). In the severe forms of the disease, the patients gradually lose ambulation and are confined to wheelchairs and eventually to bed. Currently, there is no efficacious treatment for any form of muscular dystrophies. Clinical management involves respiratory care and treatment of cardiological complications. Thus, there is an urgent need for novel therapeutics.

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Gene therapy, especially gene replacement therapy, offers a straightforward and attractive strategy. Conceptually, the most direct and effective treatment of these diseases would be via gene transfer to restore the missing component. In practice, we have to identify a gene transfer vehicle that can safely, efficiently, and stably deliver the therapeutic genes to the affected muscles before clinical gene therapy for muscular dystrophy becomes a reality.

Since the first examples of long-term gene transfer and expression in skeletal muscles of immunocompetent mice (Kessler et al. 1996; Xiao et al. 1996), adeno-associated virus (AAV) (Berns and Giraud 1995; Flotte 2005; Grieger and Samulski 2005) has proven to be the most successful in vivo gene transfer system currently available for muscle-directed gene therapy. There are a number of reasons for that. Firstly, AAV is capable of infecting a broad range of cell types and tissues (Muzyczka 1992), including skeletal and cardiac muscle cells (Li et al. 2003). Secondly, AAV's small particle size facilitates its diffusion in muscle tissue and the penetration of extracellular matrix barriers. It is the smallest virus with a diameter of ~20 nm. The physical particle of AAV is even smaller than the naked plasmid DNA (Hammermann et al. 1998; Yoshida et al. 1998). Thirdly, AAV vectors are generally considered as a safe vector system for gene therapy. Wild-type AAV does not appear to cause any disease in humans. Recombinant AAV vectors have eliminated most of the viral genes except two inverted terminal repeats (ITRs). This adds another safety feature that not only prevents the generation of helper virus via homologous recombination, but also mitigates the possibility of immune reactions caused by undesired viral gene expression. Lastly and more importantly, AAV can render long-term gene transfer mainly through episomal persistence (McCarty et al. 2004). Over the past decade, AAV vectors have become a promising vector for gene therapy of many genetic diseases, including hereditary muscular dystrophies.

In this chapter, we will describe how gene therapy is practically applied in small animal models as a treatment for several forms of muscular dystrophies, specifically, Duchenne muscular dystrophy (DMD), congenital muscular dystrophy, as well as limb-girdle muscular dystrophy.

4.2 Gene Therapy for Duchenne Muscular Dystrophy

DMD is a very severe X-linked inherited disorder and is one of the most common recessive disorders in the human population. It is characterized by rapid loss of muscle fibers and ensuing weakness resulting in loss of mobility and eventual premature death (Hoffman et al. 1987; Koenig et al. 1987; Kaemmerer et al. 2000). DMD is caused by recessive mutations in the dystrophin gene which is the largest gene known to date. The dystrophin gene spans nearly 3 million bp on the X-chromosome (Koenig et al. 1987) with a high rate of de novo mutations. Despite our understanding on the molecular mechanism of DMD, there is still no efficacious treatment available. Since DMD is recessively inherited, delivering a functional version of the dystrophin cDNA by gene replacement therapy could be an

effective treatment that would not require correction of the mutant allele on the X-chromosome.

A major hurdle in AAV-mediated gene replacement therapy for DMD is AAV's packaging limit, which allows for genes smaller than 5 kb. With a size of 14 kb, full-length dystrophin cDNA precludes being packaged into AAV vectors. There are two existing strategies to solve this problem. One strategy is named "split AAV vectors" (or trans-splicing AAV vectors) through which the large cDNA was split into two pieces and separately packaged into two individual viral particles (Duan et al. 2000; Sun et al. 2000; Chao et al. 2002). The other strategy is to create miniature versions of dystrophin genes ideal for AAV packaging while maintaining functional domains (Wang et al. 2000). In this chapter, we will only describe the latter strategy which is being investigated in a phase I gene therapy clinical trial.

4.2.1 Creation of Minidystrophin Genes

Dystrophin is an enormous rod-like protein (427 kDa) localized beneath the inner surface of myofiber plasma membrane in both skeletal and cardiac muscles (Hoffman et al. 1987). Dystrophin functions through four major structural domains. The N-terminal domain binds to the F-actin of cytoskeletal structures, whereas the C-terminal cysteine-rich (CR) along with the distal C terminus (CT), anchors to the plasma membrane via dystrophin-associated protein (DAP) complexes. The central rod domain contains 24 triple-helix rod repeats and four hinges (Fig. 4.1) (Koenig and Kunkel 1990). Thus, dystrophin cross-links and stabilizes the muscle cell membrane and cytoskeleton.

The muscle isoform of dystrophin is encoded on a 14 kb mRNA. Transgenic animal studies have shown that this cDNA can fully prevent dystrophy in mdx muscles (Cox et al. 1993). The idea that truncated dystrophin could be functional came from observations that some mildly affected BMD patients have deletion mutations that remove large portions of the gene (McCabe et al. 1989). The deletion mutations are positioned in the central rod domain of dystrophin, which suggests that a major portion of the rod domain is dispensable. In addition, transgenic studies in mdx mice indicate that CT domain may be dispensable (Rafael et al. 1996) as well.

Bearing this in mind, our group has for the first time created mini-dystrophin genes, which are small enough to be packaged into AAV vectors and yet contain essential functions in protecting muscles from dystrophic phenotypes (Wang et al. 2000). The mini-dystrophin genes, as small as only one-third of the 11-kb full-length dystrophin coding sequence, are significantly smaller than the 6.3-kb Becker-form mini-dystrophin gene (England et al. 1990) that was widely used in transgenic and gene therapy studies in mdx mice (Ragot et al. 1993). To ensure sufficient physical flexibility of the mini-dystrophin protein, all of our constructs such as Δ3849 still retain at least five rod repeats (R1, R2, R22, R23, and R24) and two hinges (H1 and H4) in the central rod domain (Fig. 4.1). Construct Δ3990 has

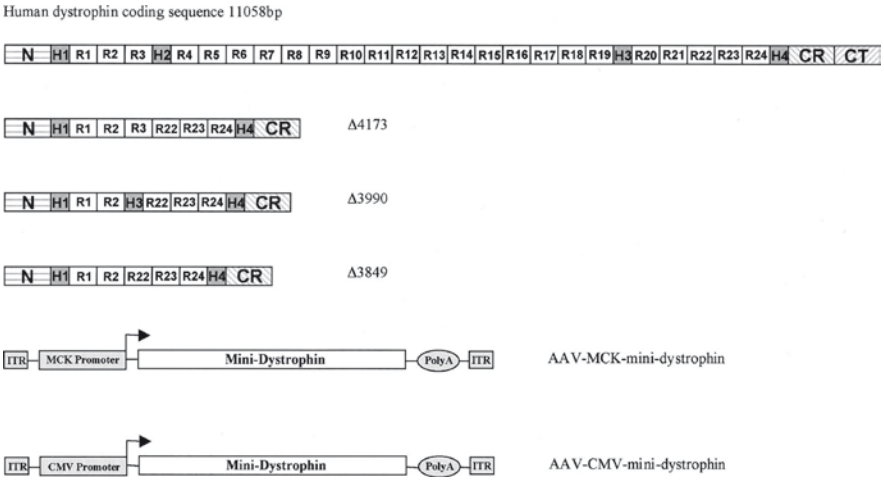

Fig. 4.1 Construction of highly truncated mini-dystrophin genes. Dystrophin has four major domains: the N-terminal domain (N), the CR domain, the CT domain, and the central rod domain, which contains 24 rod repeats (R) and four hinges (H). The mini-dystrophin genes were constructed by deleting a large portion of the central rods and hinges and nearly the entire CT domain (except the last 3 aa). This figure was previously published in Proc Natl Acad Sci U S A 97(25): 13714–13719 by Wang et al. (2000)

an additional hinge (H3), whereas construct Δ4173 contains an additional rod (R3) (Fig. 4.1). The rationale of deleting the central portion of the rod domain while preserving both distal rod repeats in our minigenes is based on the fact that those distal repeats were naturally retained in the mild Becker muscular dystrophy patients, who had large in-frame deletions in the rod domain (England et al. 1990). Later on, Dr. Chamberlain's group (Harper et al. 2002) and other groups (Fabb et al. 2002; Sakamoto et al. 2002) have also generated the similar micro-dystrophins, and further proved that those miniature versions of dystrophin genes are fully functional to protect muscle pathology in mdx mice. Those studies are very important fundamental contributions to the development of gene therapy regime for DMD (also see review articles (Chamberlain 2002; Athanasopoulos et al. 2004; Blankinship et al. 2006)).

4.2.2 Rescue of Muscular Dystrophic Pathology by Delivery of AAV-Minidystrophin

When the AAV vector containing mini-dystrophin vectors were delivered into mdx mice, a DMD mouse model, they showed efficient mini-dystrophin expression. The most proficient expression was mediated by Δ3849 and Δ3940, followed by Δ4173. Our later experiments were all based upon Δ3849 or Δ3940 constructs. Additionally,

the expression of mini-dystrophin protein also restored the DAP complex (data not shown), which was missing in mdx mice.

The expression of mini-dystrophin was also effective in protecting the plasma membrane from mechanical damage; a myofiber integrity test was performed by i.v. injection of Evans Blue dye. Evans Blue is a widely used red fluorescent dye that is excluded by the healthy myofibers, but is taken up by the dystrophic myofibers containing leaky cell membranes caused by contractile damage. As shown in Fig. 4.2, muscle from healthy mice revealed uniform dystrophin staining across the muscle sections and no uptake of the dye by the myofibers (Fig. 4.2, top row). The AAV vector-treated mdx muscle showed results consistent with the healthy muscle, thus the mutual exclusivity of dystrophin expression and dye uptake (Fig. 4.2, three middle rows). Dye uptake (red fluorescence) was found only in myofibers that lacked mini-dystrophin expression in the areas not transduced by AAV vectors (Fig. 4.2, three middle rows). By contrast, the untreated mdx muscle revealed absence of dystrophin and extensive dye uptake (Fig. 4.2, bottom row). These results unequivocally demonstrated the physiological functionality of mini-dystrophins in maintaining membrane integrity and protecting myofibers from mechanical damages. Recent studies in our laboratory have further demonstrated that the mini-dystropin genes delivered by AAV1 vectors were able to prolong the general health, muscle forces and life-span of utrophin/dystrophin ($utrn^{-/-}$ mdx) double knockout mice (Lu et al. 2008). When systemically delivered with AAV9 vectors, mini-dystrophin genes displayed much improved therapeutic effects, which resulted in near normal lifespan and restoration of fertility of the double knockout mice (Xiao unpublished results).

4.3 Gene Therapy for Laminin α2-Deficient Congenital Muscular Dystrophy

Laminin α2-deficient congenital muscular dystrophy (MDC1A) is a very severe, autosomal recessive muscle wasting disease that often leads to death in early childhood (Allamand and Guicheney 2002; Emery 2002). It is caused by mutations in LAMA2, the gene encoding laminin α2, which assembles with the β1 and the γ1 chain to laminin-2, the main laminin isoform present in the basement membrane of muscle fibers and peripheral nerves (Meinen and Ruegg 2006). Basement membranes are highly structured sheets of extracellular matrix molecules that "laminate" a variety of cells including myofibers. The major role of laminin-2 in the muscle is to interconnect the myofiber extracellular basal lamina with the plasma membrane. In laminin-α2 deficient individuals, the basal lamina is corrupted and the trans-membrane cytoskeletal structure is lost. As a consequence, muscle fibers lose their stability and degenerate.

Similar to other forms of muscular dystrophies, there is no efficacious treatment available for MDC1A. Overexpression of laminin α2 chain via AAV vector will be extremely difficult, because the size of laminin α2 cDNA (over 9 kb) is far beyond

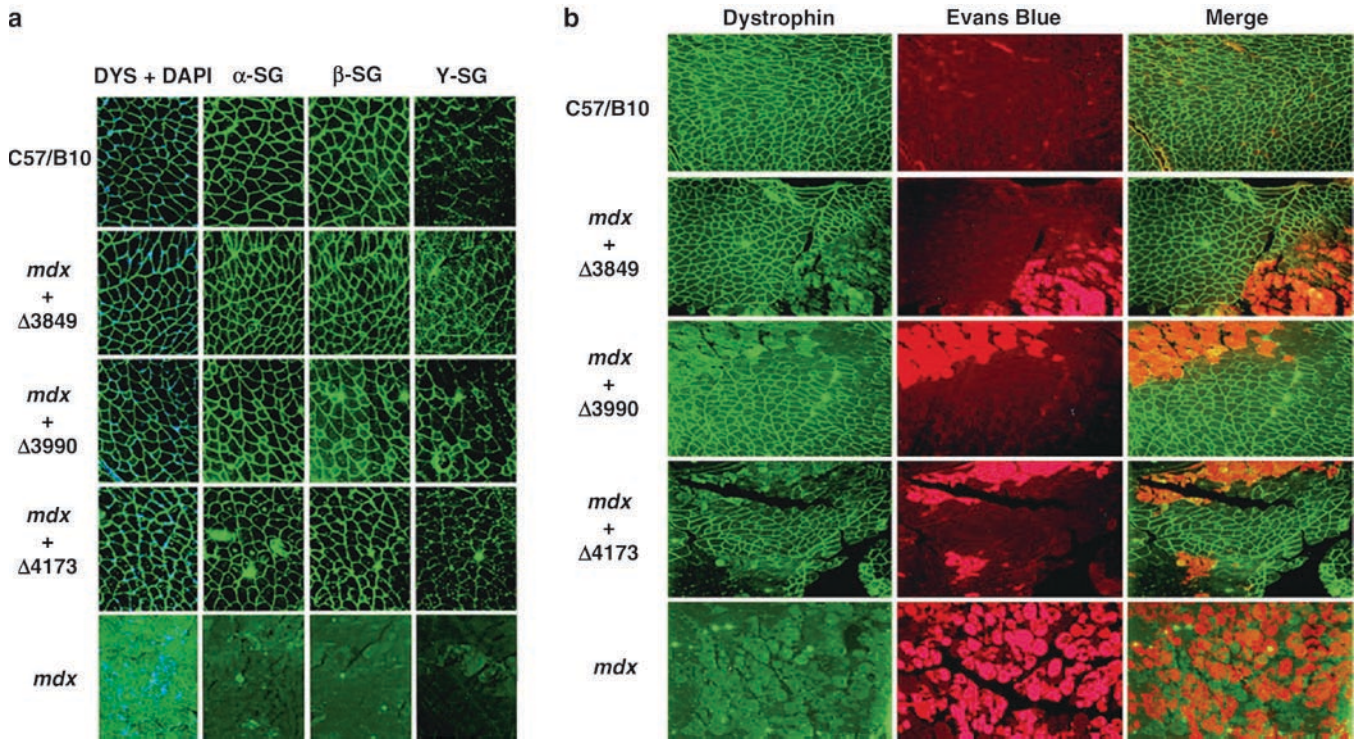

Fig. 4.2 Protection of myofiber membrane integrity by overexpression of mini-dystrophin in mdx mice. Normal dystrophin and mini-dystrophin expression was visualized by IF staining (*Left*, green). The leaky myofibers were visualized by the uptake of Evans Blue dye (*Center*, red fluorescence). Note the mutual exclusivity between dystrophin expression and Evans Blue dye uptake as shown by the merged images (*Right*). Photographs were taken with a ×10 lens. This figure was previously published in Proc Natl Acad Sci U S A 97(25): 13714–13719 by Wang et al. (2000)

AAV's packaging limit (5 kb). Moreover, laminin α2 must become incorporated into laminin heterotrimer to be functional. As several domains of laminin α2 contribute to its functionality, it is also highly challenging to generate a miniaturized version without losing its function (Meinen and Ruegg 2006). On the other hand, delivering a surrogate gene, which harbors similar functions as laminin α2 to interconnect basal lamina with plasma membrane, can also render therapeutic effect on MDC1A deficiencies.

A miniature version of the agrin gene (mini-agrin) is one of such good alternatives (Moll et al. 2001; Qiao et al. 2005). Agrin, well known for its role in the organization of the nerve–muscle synapse (Bezakova and Ruegg 2003), shares with laminin α2 the ability to bind to α-dystroglycan, a protein that is involved in the linkage of basement membranes to the muscle sarcolemma. Moreover, an amino-terminal domain of agrin confers binding to all laminins. Transgenic overexpression of a mini-agrin consisting of the laminin-binding and the α-dystroglycan binding domain markedly improved the stability, function, and regenerative capacity of muscle in dy^w/dy^w mice (Moll et al. 2001). Our group has tested AAV-mini-agrin-mediated gene therapy in the same animal model, and has achieved profound therapeutic effect (Qiao et al. 2005)

By a single dose intraperitoneal injection of AAV1-mini-agrin vector to the neonatal MDC1A disease mice, we have achieved body-wide over expression of mini-agrin protein in striated muscles (Qiao et al. 2005). The over expression of mini-agrin protein in the skeletal muscle restored the structural integrity of myofiber basal lamina, inhibited fibrosis, and ameliorated dystrophic pathology (Fig. 4.3a). Furthermore, the treatment also led to improved whole body growth (Fig. 4.3b), increased motility, and extended the life span of the diseased mice. A review paper regarding the importance of our work was written by Meinen and Ruegg (2006). Although significant efficacy has been achieved in our study, AAV-mini-agrin mediated gene therapy for MDC1A deficiency is still far from ideal. The growth curve of the treated mice still lagged behind the wild-type littermate, and their nerve defect was not overtly improved. Nevertheless, our work points to a new direction and offers a practical approach to gene therapy for patients suffering from MDC1A.

4.4 Gene Therapy for Limb-Girdle Muscular Dystrophies

Limb-girdle muscular dystrophies are a group of heterogeneous inherited neuromuscular diseases caused by mutations in a number of different genes including genes encoding sarcoglycans (SG) α (LGMD 2D), β (LGMD 2E), γ (LGMD2C), and δ (LGMD 2F) (Lim and Campbell 1998; Hoffman 1999). Sarcoglycans are small transmembrane proteins associated in equal stoichiometry on the muscle cell membrane to form a heterotetramer, termed the SG complex. Primary deficiency of any single SG protein generally results in partial or complete disappearance of the entire SG complex on the sarcolemma, leading to muscular dystrophies now

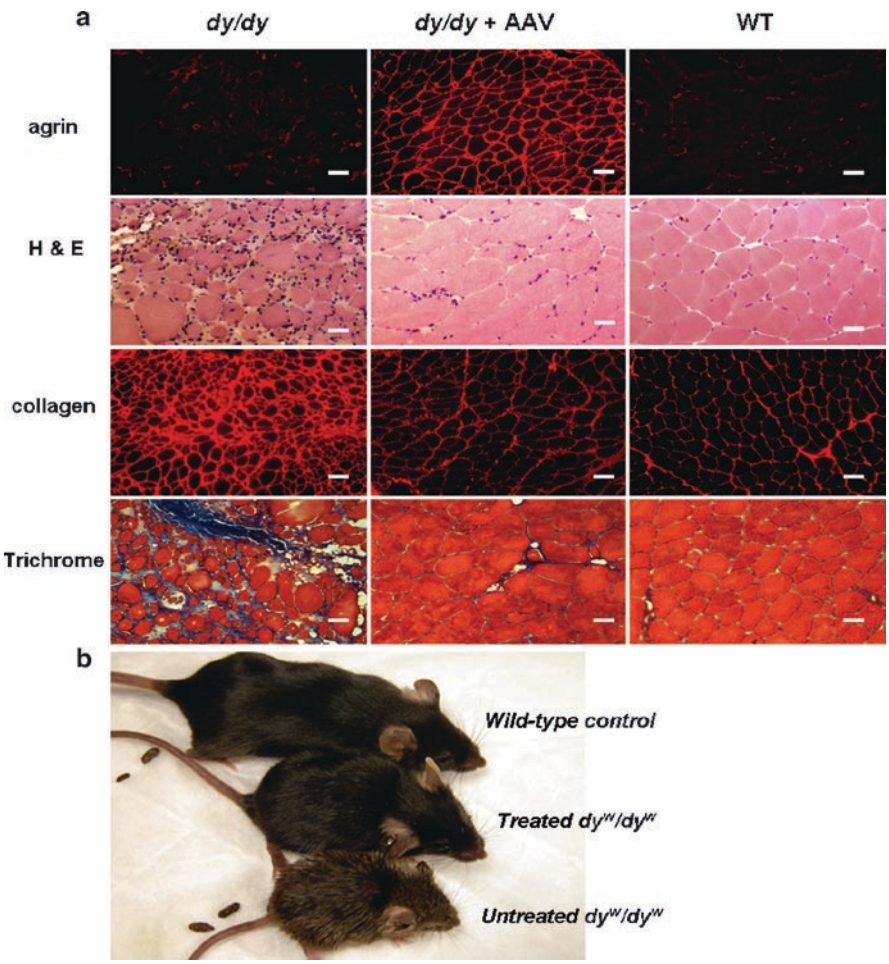

Fig. 4.3 Improvement of the disease phenotype of congenital muscular dystrophic mice by overexpression of mini-agrin. (**a**) Local overexpression of mini-agrin gene and amelioration of histopathology in the dystrophic muscle of *dy/dy* mice. (**b**) Whole-body photograph of one-and-a-half-month-old wild-type control, the AAV-treated and untreated dy^{w}/dy^{w} mice. This figure was previously published in Proc Natl Acad Sci U S A 102(34): 11999–12004 by Qiao et al. (2005)

collectively called sarcoglycanopathies (Piccolo et al. 1995; Jung et al. 1996). The lack of effective treatment necessitates the search for innovative therapeutic strategies, such as gene therapy.

Unlike the dystrophin gene, the full-length cDNA for each of the sarcoglycan subunits is within the cloning capacity of AAV. Thus, it is relatively easier to perform AAV-based gene therapy for the sarcoglycan-derived cases of LGMD. Toward this

goal, several groups have initiated AAV-mediated gene therapy for LGMD based upon δ-sarcoglycan deficient hamster model (Li et al. 1999; Xiao et al. 2000; Kawada et al. 2002; Li et al. 2003), γ-sarcoglycan-deficient mouse model (Cordier et al. 2000), and α- and β-sarcoglycan-deficient mouse models (Dressman et al. 2002; Pacak et al. 2007). The above studies demonstrate efficient and long-term transgene of β- (Dressman et al. 2002), γ- (Cordier et al. 2000), and δ-sarcoglycan (Li et al. 1999; Xiao et al. 2000) expression, as well as recovery of physiological function deficits (Xiao et al. 2000) in treated skeletal muscle after intramuscular injection of AAV vector. AAV1-mediated α-sarcolgycan gene therapy has entered a phase I clinical trial led by Dr. Jerry Mendell of Nationwide Children's Hospital in Ohio.

Since most LGMD disease models also develop cardiomyopathy, seeking an efficient route to deliver AAV vector to the heart is important for LGMD gene therapy. In an early proof-of-principle study, we have delivered AAV2 vectors into the coronary artery ex vivo in heterotopically transplanted hearts of the δ-SG deficient hamsters, and obtained widespread and long-term therapeutic gene transfer in cardiomyocytes. In addition, Kawada et al. have achieved significant therapeutic effect in rescuing cardiomyopathy by direct myocardium injection under open chest surgery (Kawada et al. 2002).

Although the above studies demonstrate significant efficacy of AAV-mediated gene therapy for LGMD, they are far from ideal. The delivery routes used by those studies are either through intramuscular injection, or via intramyocardium injection under complicated surgery. Similar to DMD, LGMD is a body-wide muscle disease. Finding a convenient method to deliver AAV vector to the muscles throughout the whole body, including the heart, is highly desirable. Fortunately, with the discovery of new AAV serotypes (Gao et al. 2002), efficient systemic delivery of AAV vector to the whole body is now attainable. Since sarcoglycan genes are small (~1 kb), they can be readily packaged into the newly developed AAV vector cassette, named self-complementary or double-stranded AAV (McCarty et al. 2001; Wang et al. 2003), which mediates more robust and faster transgene expression than traditional single-stranded AAV vectors.

Taking advantage of both double-stranded AAV vector and new AAV serotypes, we have tested the feasibility of systemic delivery of AAV-δ-SG into the TO-2 hamster model. A single dose intravenous injection of double-stranded AAV serotype 8 vector carrying human δ-SG gene, without any physical or pharmaceutical interventions, achieved nearly complete gene transfer and tissue-specific expression in the heart and skeletal muscles of the diseased hamsters (Wang et al. 2005; Zhu et al. 2005). Broad and sustained restoration of the missing δ-SG gene in the TO-2 hamsters corrected muscle cell membrane leakiness throughout the body and normalized serum creatine kinase levels (Fig. 4.4a, b). Furthermore, the restoration of δ-SG gene, in both the skeletal and the cardiac muscles, has greatly improved histology, cardiac functions and the endurance of treadmill running (Fig. 4.4c). The lifespan of the animals was dramatically extended as well (Zhu et al. 2005) with a group of treated TO-2 hamsters having survived for more than 2 years (data not shown). The above results showed another example of successful body-wide gene therapy for muscular dystrophies after AAV-mediated systemic therapeutic gene delivery in animal models.

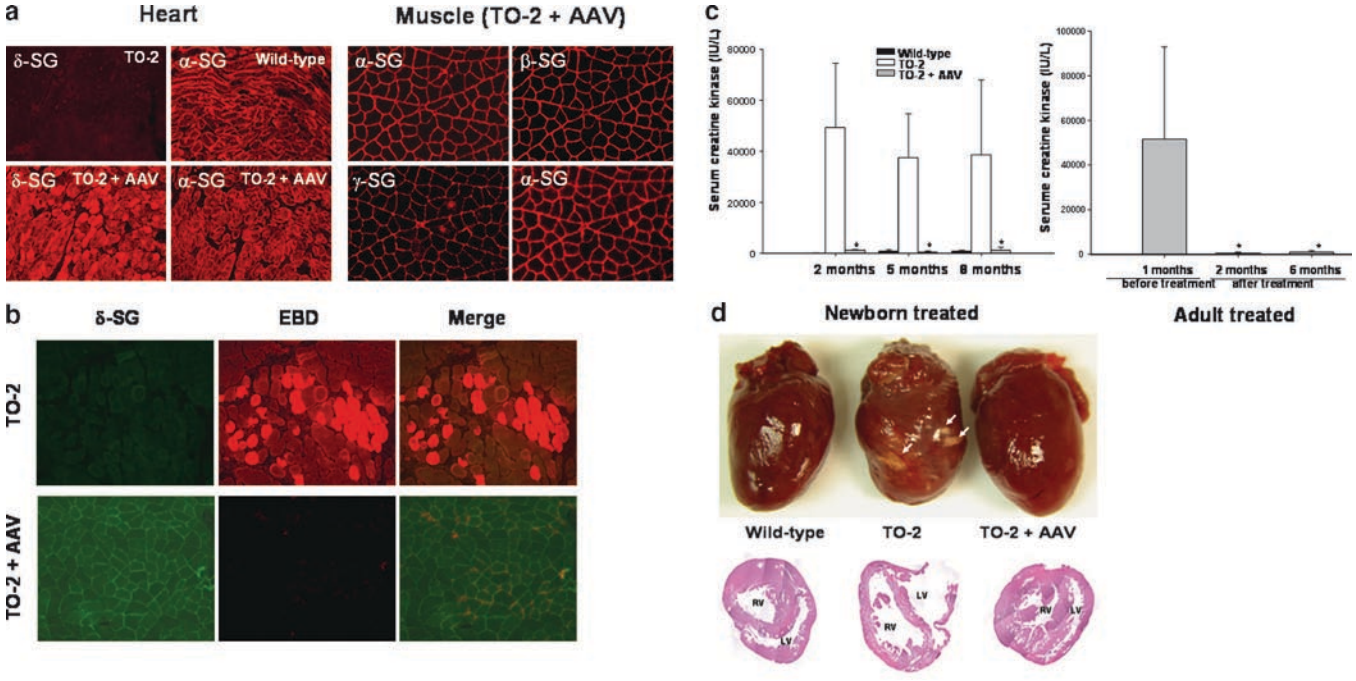

Fig. 4.4 The efficacy of overexpression of delta-sarcoglycan in TO-2 hamsters. (**a**) Recovery of SGs (α, β, γ, and δ) onto cell membrane of heart and TA muscles at 2.5 months after AAV8 intraperitoneal injection in neonates. **(b)** Evans blue dye uptake assay results. (**c**) Serum creatine kinase (CK) levels of wild-type F1B, untreated TO-2, and AAV8-treated TO-2 hamsters measured at different time courses. *Statistically significant ($n=3$–5 per group; $p<0.01$). (**d**) Gross appearance of the hearts (*top*) and hematoxylin and eosin staining of the transverse sections (*bottom*) of hearts from 9-month-old wild-type F1B, TO-2 control, or TO-2 treated with AAV at 10-day age. Arrows indicate visible calcification. This figure was previously published in Circulation 112, 2650–2659 by Zhu et al. (2005)

4.5 Summary and Future Direction

In summary, AAV-mediated gene therapy for muscular dystrophies on small animal models by systemic therapeutic gene delivery has been very successful. It can fundamentally restore the defective gene leading to functional recovery of the disease phenotype. In this chapter, we described how gene therapy was applied to multiple forms of muscular dystrophies, particularly, DMD, congenital muscular dystrophy, as well as limb-girdle muscular dystrophy. These studies, along with the work of many other groups, have laid a solid foundation for the development of gene therapy regime for muscular dystrophy patients. In recent years, tremendous efforts have been made to translate the encouraging results from small animal models to large animal models, and eventually to muscular dystrophy patients. One of the examples is the translation from mdx mice to the golden retriever muscular dystrophy (GRMD) dogs and to DMD patients. A number of new challenges absent in the mouse studies are met in the studies involving the GRMD dogs, which are a few hundred times larger than mice, manifest more severe and diverse disease phenotypes, and more importantly, come with more complex immune systems. Cellular immune responses against AAV vectors that were not seen in mice have been observed in the GRMD dogs (Wang et al. 2007a, b). These new challenges call for efforts on improvement of targeted gene delivery, tissue-specific therapeutic gene expression, immune modulation and new vector development and large-scale production technologies. Given the concerted efforts and exponential advances in muscular dystrophy gene therapy research and development over the past 10 years, we are confident that the challenges will be conquered one by one, and gene therapy for muscular dystrophies will eventually succeed.

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Chapter 5
Antisense-Mediated Exon Skipping for Duchenne Muscular Dystrophy

Annemieke Aartsma-Rus and Gert-Jan B van Ommen

Abstract Duchenne muscular dystrophy (DMD) is a severe, progressive neuromuscular disease for which there is currently no cure available. The disease is caused by mutations in the dystrophin-encoding *DMD* gene that disrupt the open reading frame and completely abolish dystrophin function. Antisense-mediated exon skipping is a promising approach that is close to clinical application. It aims to modulate pre-mRNA splicing such that the reading frame is restored, allowing for the generation of partly functional dystrophin proteins, as found in less severe Becker muscular dystrophy. Proof-of-principle for this approach has been obtained in patient-derived cell cultures and animal models carrying different types of mutations (deletions, duplications, small mutations and splicing mutations). In theory this approach should be applicable to almost 80% of all patients, but as different exons need to be skipped to restore various mutations, skipping a single exon is beneficial to a subset of patients. All DMD exons can currently be skipped and antisense oligonucleotides to induce exon 51 skipping (applicable to the largest group (13%) of patients) are in the early phase of clinical trials.

5.1 Introduction

Deletions of one or more exons in the dystrophin-encoding *DMD* gene are associated with a very severe and progressive neuromuscular phenotype (Duchenne muscular dystrophy (DMD)), a milder phenotype (Becker muscular dystrophy (BMD)), but have also been found in asymptomatic individuals (Ferreiro et al. 2008; den Dunnen et al. 1989; Muntoni et al. 2003; Angelini et al. 1994; Aartsma-Rus et al. 2006b). This apparent discrepancy can be explained by the effect mutations have on dystrophin functionality (Monaco et al. 1988). In muscle fibers, dystrophin connects the actin cytoskeleton with its N-terminal domain to the

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extracellular matrix via a transmembranal protein complex with its more C-terminal cysteine-rich domain (Fig. 5.1) (Koenig et al. 1988; Koenig and Kunkel 1990). This connection protects muscle fibers from damage during muscle contraction. In DMD patients, mutations generally disrupt the open reading frame and protein translation is truncated prematurely (Aartsma-Rus et al. 2006b; Hoffman et al. 1988). The resulting dystrophin cannot fulfill its linker function and consequently muscle fibers are very sensitive to damage induced by muscle fiber contraction. During several rounds of regeneration, the body tries to compensate for the loss of damaged muscle fibers, but eventually fibers are permanently lost and replaced by adipose and connective tissue, a process that is accompanied by loss of muscle function.

By contrast, deletions that maintain the open reading frame and affect the part of the transcript encoding the domain that intersperses the actin-binding and cysteine-rich domains (the so-called central rod domain) result in a phenotype that can vary from severe BMD to (close to) asymptomatic (Aartsma-Rus et al. 2006b; Angelini et al. 1994; Hoffman et al. 1988). The amount of internally deleted protein produced, inversely correlates with the severity of the disease (Hoffman et al. 1988). In addition, the functionality of the resulting dystrophins is determined in part by the location of the deletion: deletions in the proximal part of the central rod domain generally lead to very mild or absent phenotypes, whereas deletions in the distal part result in typical BMD and deletions affecting one of the two N-terminal actin-binding domains are found in more severely affected BMD patients (Aartsma-Rus et al. 2006b; Beggs et al. 1991). Interestingly, huge in-frame deletions affecting over two-thirds of the central rod domain have been described in mildly affected BMD patients (Dastur et al. 2008; England et al. 1990; Mirabella et al. 1998). However, larger in-frame deletions result in DMD (Fanin et al. 1996). This implies that a small part of the central rod domain is essential, but a complete domain is not a prerequisite for (partial) dystrophin functionality.

This phenotypic difference forms the basis for the therapeutic antisense-mediated exon skipping approach, which aims to reframe DMD transcripts to allow synthesis of internally deleted, partly functional BMD-like proteins (van Deutekom and van Ommen 2003). Reframing is achieved through small synthetic RNA or DNA oligomers complementary to a target exon (so-called antisense oligonucleotides (AONs)) that hide the exon from the splicing machinery. Consequently, the exon is skipped, the open reading frame restored, resulting in the synthesis of BMD-like dystrophins. As treatment for DMD patients is currently only symptomatic, conversion to a BMD phenotype, with a (much) slower disease progression, better preserved muscle function and (near) normal life expectancy would be a huge step forward.

In this chapter we will explain how this approach can be employed for different mutation types, we will describe in vitro and in vivo proof-of-concept studies, and will discuss AON design and future directions. We will only briefly go into the different AON backbone chemistries and optimization of systemic delivery, as these topics are discussed in Chap. 6.

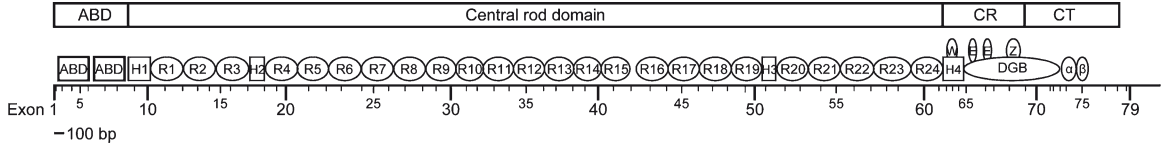

Fig. 5.1 The location of the different dystrophin domains aligned to the cDNA. The N-terminal region of dystrophin contains two actin-binding domains (ABD). Reputedly, a third actin-binding domain is encoded by exons 35–44. The central rod domain consists of 4 hinge regions (H) and 24 spectrin-like repeat domains (R1–R24). At the C-terminus the essential cysteine-rich domain (CR) contains the dystroglycan-binding domain (DGC), two EF-hand motifs (E), a WW1 domain (W) and a ZZ domain (Z). The final C-terminal domain (CT) contains binding sites for α- and β-syntrophin (α and β, respectively)

5.2 Exon Skipping for Different DMD Mutations

While the aim of antisense-mediated exon skipping is the same for each mutation type (i.e., reframing the transcript) a slightly different approach is required for different mutation types (Fig. 5.2) (Aartsma-Rus et al. 2009a). For the most common mutation type, a deletion of one or more exons, generally skipping one additional exon will reframe the mutated transcript (Fig. 5.2a). Proof-of-concept for this approach was first obtained in patient-derived myogenic cultures with a deletion of exon 45 (van Deutekom et al. 2001). Treatment with AONs targeting exon 46

Fig. 5.2 Exon skipping strategies for different DMD mutations. (**a** and **b**) Exon skipping for deletions. (**a**) For most deletions skipping a single exon will restore the open reading frame. In this example a deletion of exon 45 disrupts the open reading frame (indicated by a conversion from dark to light-gray exons). Through blocking of exon 46 by an antisense oligonucleotide (AON) this exon is skipped by the splicing machinery and exon 44 is joined to exon 47, which is in-frame (all exons are dark-gray). (**b**) For some deletions (e.g., deletion exon 46–50) the reading frame can only be restored through the skipping of two exons simultaneously (double exon skipping). This is achieved using a combination of AONs targeting the individual exons (exon 45 and 51 in this example). While single exon skipping leads to only two types of transcripts (with and without skipping), double exon skipping leads to four different transcripts, i.e., no skipping, skipping of the first exon only, skipping of the last exon only and skipping of both exons. Only the last type will have a restored reading frame and will allow the generation of Becker-like dystrophins. (**c**–**e**) Exon skipping for point mutations. (**c**) When a mutation disrupts a splice site, this exon is no longer recognized by the splicing machinery (the acceptor splice site of exon 7 is disrupted in this example). This generally leads to exon skipping and the result on RNA level is thus similar to that of a deletion event. In this example skipping of both exons 6 and 8 restore the open reading frame. It has been observed that exon 8 skipping is always accompanied by exon 9 skipping. However, as exon 9 is in-frame, exon 6, exon 8 and exon 9 triple exon skipping is also frame restoring. (**d**) Small mutations within an exon can disrupt the open reading frame or introduce a premature stop codon, as is the case in this example for exon 23. Since this exon is in-frame, skipping it will maintain the open reading frame, while bypassing the mutation. (**e**) Small mutations in out-of-frame exons are somewhat more challenging as here skipping the mutated exon (exon 43 in this example) will disrupt the open reading frame and skipping of an additional exon (exon 44) is needed to restore the reading frame. (**f–h**) Exon skipping for duplications. (**f**) Single exon duplications are ideal targets for exon skipping, as skipping either the original or the duplicated exon (exon 45 in this example) will restore the wild type transcript. (**g**) For most single exon duplications skipping is too efficient and both exons are skipped (exon 44 in this example), which will result in another out-of-frame transcript. Through triple exon skipping of both exons 44 and an additional exon (exon 43 in this example) the reading frame can be restored. (**h**) Exon skipping is challenging for multiple exon duplications (exon 43–45 in this example). Only skipping the duplicated exon (exon 43 in this example) will restore the open reading frame. However, the AON can not discriminate between the original and the duplicated exon, leading to various products, only one of which is in-frame. For some multiple exon duplications, skipping of two exons is required, which increases the number of possible outcomes even more and complicates matters further. (**i**, **j**) Exon skipping for rare mutations. (**i**) Intronic mutations can create new splice sites and cause the insertion of aberrant pseudoexons (pe) between the original exons (between exon 11 and 12 in this example). This insertion generally disrupts the open reading frame and/or contains premature stop codons. Targeting the aberrant exon with an AON will restore the normal transcript.

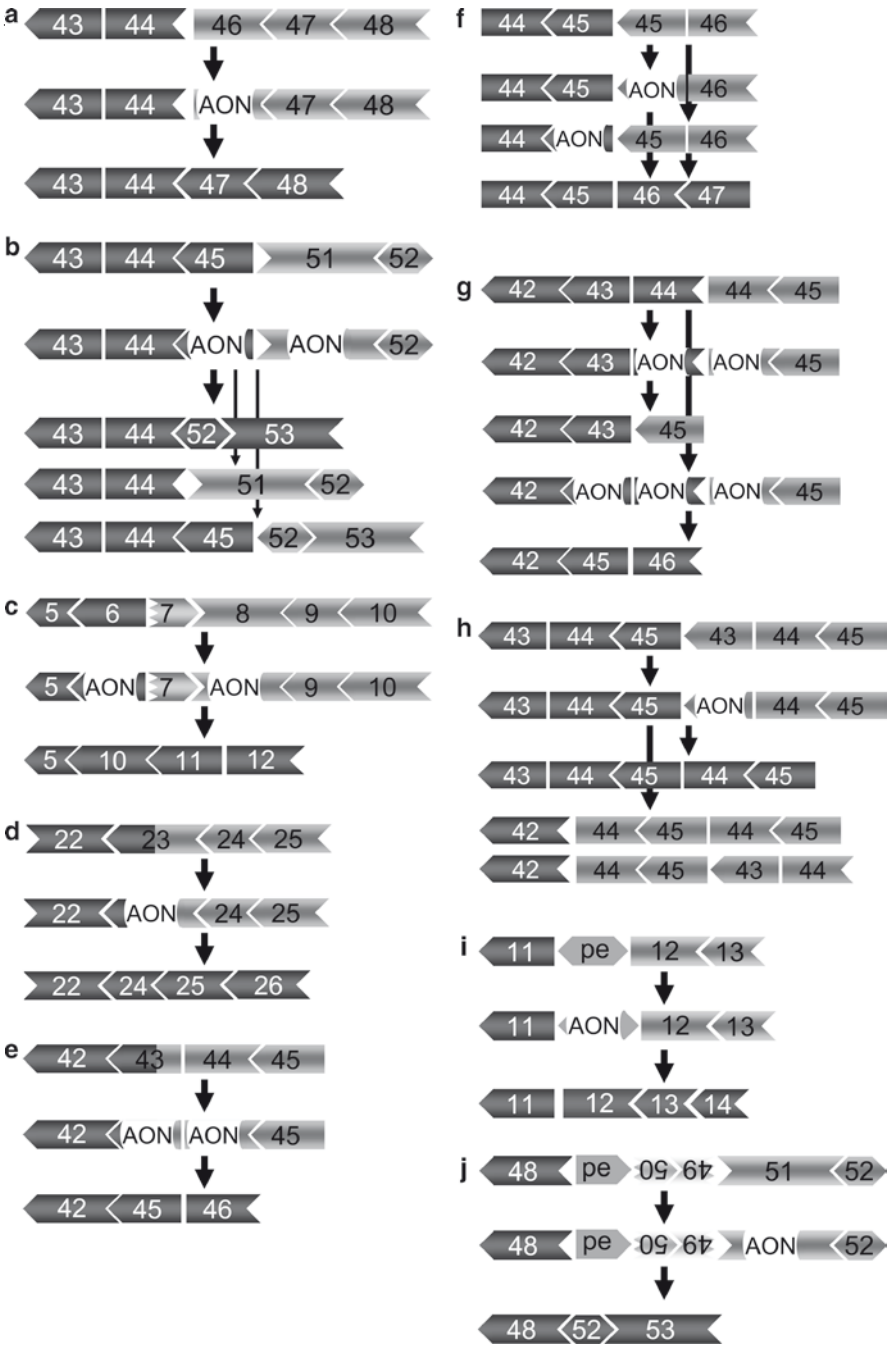

Fig. 5.2 Continued (**j**) Inversions are rarely found in DMD patients. In this example exon 49 and 50 have been inverted and are no longer recognized as exons. The inversion event also resulted in the partial activation of a pseudoexon in intron 50 between exon 48 and 51. Skipping of the pseudoexon and exon 51 would restore the open reading frame. Notably, using AONs targeting only exon 51 resulted in the skipping of both the pseudoexon and exon 51, thus restoring the reading frame

indeed resulted in exon skipping on RNA level and dystrophin restoration in over 75% of treated myotubes. The broad applicability of exon skipping has since then been confirmed in numerous patient-derived cells carrying deletions (Aartsma-Rus et al. 2003; Arechavala-Gomeza et al. 2007; Takeshima et al. 2001). In each case the reading frame and dystrophin synthesis was restored. For a small subset of deletion patients, skipping one exon is not sufficient to restore the reading frame, but skipping of two additional exons is required (Fig. 5.2b). Treatment with a combination of AONs directed against the two target exons is expected to result in four different transcripts: the wild type, transcripts where either the first or the second exon is skipped and transcripts where both exons are spliced out. This was indeed observed on RNA levels after treating deletion exon 46–50 cells with a combination of exon 45 and exon 51 AONs (Aartsma-Rus et al. 2004). Fortunately, the major transcript (75%) consisted of the reframed transcript where both exons were skipped simultaneously. This was accompanied by dystrophin restoration in over 70% of treated myogenic cells, which is only slightly lower than the 75% restoration rate generally observed for single exon skipping in patient-derived cells.

Small mutations in exons can also disrupt the reading frame, e.g., through disruption of a splice site, a small deletion or insertion or through a point mutation that creates a premature truncation codon. Splice site mutations generally lead to exon skipping; e.g., the golden retriever muscular dystrophy (GRMD) model has a mutation in the splice acceptor site of exon 7 (Sharp et al. 1992). This results in several out-of-frame transcripts, but only exon 7 is skipped in the major transcript. Here, the reading frame can be restored through skipping exons 6 and 8 (Fig. 5.2c). which has been achieved in cultured cells and in vivo in GRMD dogs (McClorey et al. 2006; Yokota et al. 2007). This skipping of additional exons is in fact similar to the double exon skipping required for some deletions (Fig. 5.2b). Exon-internal mutations by contrast can be bypassed through skipping of the mutated exon (Fig. 5.2d, e).

An example of a nonsense point mutation is present in the mdx mouse model, which carries a C to T transition in exon 23 (Sicinski et al. 1989). Since exon 23 is in-frame skipping this exon will bypass the premature truncation signal while maintaining the reading frame (Fig. 5.2d). This has been achieved in the mdx mouse in cultured cells and in vivo (Lu et al. 2003; Mann et al. 2001) as well as in patient-derived cells with small mutations in exon 41 (Surono et al. 2004) and 49 (Aartsma-Rus et al. 2003) in vitro. When the mutation is in an out-of-frame exon, skipping this exon will disrupt the open reading frame. Often the reading frame can be restored through skipping an additional exon (Fig. 5.2e). Proof-of-concept for this approach has been achieved in patient-derived cells with a single base deletion in exon 43, where exon 43 and exon 44 skipping restored dystrophin synthesis (Aartsma-Rus et al. 2004).

For duplications the exon skipping approach is more challenging as the original and duplicated exons are identical and the AONs can thus not discriminate between them. For single exon duplications, skipping either the original or duplicated exon will restore the normal transcript (Fig. 5.2f). These mutations are thus ideal targets, as they allow restoration of full-length dystrophin. In cultured cells from a patient

with an exon 45 duplication AON treatment indeed resulted in efficient skipping of either the original or duplicated exon and restoration of the dystrophin protein (Aartsma-Rus et al. 2007). Unfortunately, in cells from patients with an exon 44 duplication, exon skipping was too efficient and both exons 44 were skipped in the vast majority of transcripts, thus disrupting the open reading frame (Fig. 5.2g) (Aartsma-Rus et al. 2007). This has also been reported for other single exon duplications (Wilton and Fletcher 2008). However, in case of the exon 44 duplication, the reading frame and dystrophin production could be restored through triple exon skipping of exon 43 and both exons 44 (Fig. 5.2g) (Aartsma-Rus et al. 2007). For duplications of multiple exons the situation is more complicated, as only skipping of one or more duplicated exons will restore the reading frame, while skipping of original exons will disrupt the open reading frame (regardless of whether duplicated exons are skipped (Fig. 5.2h). So far it has not been possible to restore the reading frame multiple exon duplication in sufficient levels in cell cultures to allow detectable levels of dystrophin.

In a small subset of DMD patients, intronic mutations lead to the activation of aberrant splice sites and inclusion of aberrant exons into the transcripts (Fig. 5.2i). AONs blocking these splice sites will result in skipping of the aberrant exons, which has indeed been achieved in cell cultures from several patients (Gurvich et al. 2008). As with single exon duplications, these mutations are ideal targets for this approach as the wild type transcripts are restored.

Finally, exon skipping has also been employed in cells derived from a patient with a complex rearrangement involving an inversion of exons 49 and 50, leading to the omission of exons 49 and 50 in the transcript and the inclusion of one or more pseudoexons (Fig. 5.2j). Interestingly, treatment with an exon 51 AON resulted not only in exon 51 skipping, but also in removal of the pseudoexons, thus restoring the open reading frame and dystrophin production (Madden et al. 2009).

5.3 Exon Skipping Applicability

Antisense-mediated exon skipping is a mutation specific approach. As the incidence of the disease is quite high (1 in 3,500 newborn boys) and one in three mutations are de novo, the mutation spectrum is vast. So far an estimated 6,000 different mutations have been identified in DMD and BMD patients worldwide, close to 5,000 of which are reported in the Leiden DMD mutation database (Aartsma-Rus et al. 2009a). The majority of patients (65%) has a deletion of one or more exons, while 27 % has a small mutation, 8% has a duplication of one or more exon and less than 1% has a translocation, inversion or intronic mutation (Aartsma-Rus et al. 2009a).

The deletions tend to cluster around the major hotspot (exons 45–53, 75% of all deletions) and the minor hotspot (exons 2–20, 20% of all deletions) and thus the skipping of exons in these regions is applicable to larger patient groups (Aartsma-Rus et al. 2006b) (Table 5.1). Due to the high occurrence of deletion breakpoints in intron 50 and intron 51, exon 51 skipping is applicable to the largest number of

Table 5.1 Overview of exons applicable to the largest number of patients (Aartsma-Rus et al. 2009a)

Ranking	Exon(s)	All mutations (%)	Deletions (%)	Small mutations (%)	Single exon duplications (%)
1	51	13	19.14	0.31	2.98
2	45	8.05	11.79	0.24	2.18
3	53	7.67	11.37	0.08	1.45
4	44	6.17	8.83	0.39	2.72
5	46	4.28	6.15	0.23	1.61
6	52	4.11	5.70	0.46	2.31
7	50	3.96	5.64	0.23	1.92
8	43	3.81	5.32	0.16	2.63
9	6 and 7	2.98	3.56	0.08	6.31
10	8	2.27	2.32		7.97

patients (13% of all patients), while exon 45 and 53 skipping would both individually be applicable to 8% of the patients (Aartsma-Rus et al. 2009a). AONs to skip each individual exon have been identified and theoretically exon skipping would be applicable to 80% of deletions, 91% of small mutations and 73% of duplications, or 83% of all patients (Table 5.2).

However, mutations in the essential cysteine-rich domain invariably cause DMD, regardless of whether deletions are in-frame or out-of-frame (Aartsma-Rus et al. 2006b). Thus, for patients with mutations in the part of the transcripts that encodes the cysteine-rich domain (exons 64–70, Fig. 5.1) exon skipping will in all likelihood not be beneficial. Since there are two N-terminal actin-binding domains, and a third domain located in the central rod domain (Fig. 5.1), there is more flexibility for deletions affecting one or two actin-binding domains, as the additional domains retain some of the functionality (Aartsma-Rus et al. 2006b). Nevertheless, BMD patients with mutations in the minor mutation hotspot generally suffer from a more severe phenotype and in-frame deletions affecting all actin-binding domains result in DMD (Aartsma-Rus et al. 2006b) indicating that for out-of-frame mutations affecting all actin-binding domains exon skipping is not beneficial. Finally, exon skipping is not possible for patients with mutations that affect the first or the last exon, or large rearrangements (e.g., translocation). Fortunately, these mutations are very rare and together make up less than 5% of all mutations (Aartsma-Rus et al. 2009a).

However, even in the parts encoding the nonessential rod domain, skipping different exons for (different) mutations will lead to qualitatively different dystrophins for which the functionality will likely vary, as, e.g., exemplified by the finding that in-frame deletions in the proximal part of the rod domain are generally associated with very mild phenotypes and deletions in the distal part of the rod domain are found in typical Becker patients (Aartsma-Rus et al. 2006b). As the phenotype of Becker patients varies a lot, even within families carrying identical genetic mutations (Dastur et al. 2008), it is impossible to exactly predict the extent of the effect of reading frame restoration for DMD patients. There are some mutations that are

Table 5.2 Applicability of single and double exon skipping for different mutation types (Aartsma-Rus et al. 2009a)

Mutation type	Single skipping	Double skipping	Total	All mutations
Deletions (%)	70	8	79	54
Small mutations (%)	44	47	91	23
Duplications (%)	61	12	73	6
All mutations (%)	64	19		83

generally found in mildly affected BMD patients, such as the exon 45–55 deletion (Beroud et al. 2007). This suggests that the resulting dystrophin is likely quite functional. As exons 45–55 include a significant amount of DMD mutations (30% of the Leiden DMD mutation database and 63% of the French DMD UMD database) we and others envisaged an exon 45–55 multiexon skipping approach (Beroud et al. 2007; van Vliet et al. 2008; Aartsma-Rus et al. 2006a). This would allow treating a large group of patients with a single treatment and likely conversion into a mild BMD phenotype. Unfortunately, the simultaneous skipping of a large number of exons at a stretch is very challenging and thus far multiexon skipping levels are too low to result in detectable levels of dystrophin and are thus too low to be beneficial (van Vliet et al. 2008).

5.4 AON Design

The exon skipping approach modulates pre-mRNA splicing, a tightly regulated process coordinated by a large protein complex, the spliceosome, and many additional splicing factors (Cartegni et al. 2002). Splicing is dictated through the recognition of pre-mRNA motifs and consensus sequences by splicing factors and spliceosome components. The most important sites are the splice sites, located at the intron–exon and exon–intron boundaries and the branch point sequences, located upstream of the intron–exon boundary. In addition, exonic and intronic splicing enhancer and silencer motifs further regulate exon definition. To induce exon skipping either one of the splice sites, the branch point site or one or more splicing enhancer sites (or a combination thereof) can be blocked by AONs (Aartsma-Rus and van Ommen 2007). Targeting the splice or branch point sites directly interferes with binding of spliceosome components and seem optimal choices. Exon skipping has indeed been achieved using AONs targeting either splice sites or branch point sites (Aartsma-Rus and van Ommen 2007). However, this also has disadvantages as these sites are consensus sequences and thus there is an inherent risk of targeting other splice sites and disrupting the splicing of nontarget genes. This is less of an issue with AONs targeting exonic and intronic splicing enhancers, as these consist of weakly defined motifs that can be recognized by members of the ever increasing SR-protein family of splicing factors (Aartsma-Rus et al., 2005, 2009b). The disadvantage here is that

while splicing sites are known and can be predicted accurately, software to predict splicing enhancers is still being developed and optimized, and functional validation of predicted enhancing sites is laborious. Nevertheless, targeting exon splicing enhancers (ESEs) has proven to be very efficient for DMD exon skipping (Aartsma-Rus et al., 2005, 2009b; Aartsma-Rus and van Ommen 2007; Wilton et al. 2007). In fact, the majority of efficient DMD AONs targets exon-internal sites, while splice sites are overrepresented in the group of less efficient AONs (Aartsma-Rus and van Ommen 2007; Wilton et al. 2007). This may be in part due to the complex exon–intron constitution of the 2.4 Mb *DMD* gene, where introns make up over 99.5% of the gene, and are exceptionally large (varying from 107bp to 360 kb, averaging on 30 kb per intron). Thus, it is conceivable that DMD exons depend more on enhancer sites than regular exons. Nevertheless, it has been feasible to identify exon-internal AONs for other exons as well using guidelines derived from retrospective analysis of DMD AONs (Aartsma-Rus et al. 2009b) (Aartsma-Rus unpublished data). This implies that targeting exon-internal ESE motifs may be at least as efficient as targeting splice sites to induce exon skipping.

Since the majority of DMD AONs target ESEs and we have designed and tested over 150 DMD AONs, it was possible to do retrospective analysis comparing predicted ESE sites for effective and ineffective AONs using different software tools (Aartsma-Rus et al. 2009b). As anticipated, effective AONs targeted significantly higher and/or more ESEs than ineffective AONs as predicted with various software programs that predict ESE sites or SR protein binding sites (RESCUE-ESE, ESEfinder and PESE). Unexpectedly, ineffective AONs targeted significantly more and higher binding sites of the SR protein Tra2β. It is possible that the affinity of Tra2β for its target site is higher than for other SR proteins and that where AONs can replace the other SR proteins or prevent their binding to ESEs, they are unable to achieve steric hindrance for Tra2β. Regardless of the mechanism, AON design can most likely be optimized through selecting target sites with predicted ESE sites, but without predicted Tra2β-binding sites (Aartsma-Rus et al. 2009b).

Like all antisense oligomer applications, antisense-mediated exon skipping is dependent on thermodynamic properties of the AON and the target. The free energy of AON-target binding has to be sufficient, or the AON will not efficiently bind the target exon and no skipping will take place. This is underlined by the finding that the binding energy (difference between the free energy of AON-target and the free energy of the target) and the melting temperature of efficient AONs are significantly higher than those of inefficient AONs (Aartsma-Rus et al. 2009b). Summarizing, through our retrospective analysis of our series of exon-internal AONs we have gained more insight into the AON design, which we are currently translating into a software tool to facilitate AON design and allow AON identification. Of course optimizing AON design and elucidation of factors involved in this process is an ongoing process, as new AONs are still being designed and tested and new tools to analyze and compare AONs are becoming available as well.

5.5 From In vitro and In vivo Studies to Patients

The exon skipping approach was developed in patient-derived and mdx-mouse-derived cells in parallel (van Deutekom et al. 2001; Wilton et al. 1999) for mouse in vivo studies with local intramuscular AON injections soon followed (Fletcher et al. 2005; Lu et al. 2003; Mann et al. 2002). After some initial optimization of the AON itself and of delivery methods (Mann et al. 2002), Lu and colleagues obtained very encouraging results after intramuscular injections of mdx mice with 2'-O-methyl phosphorothioate AONs in combination with a pluronic copolymer F127 (Lu et al. 2003). High levels of dystrophin restoration (up to 20%) could be observed in treated muscles, which lasted for over 3 months. More importantly, this was accompanied by functional improvement of the treated muscle. Later studies revealed that similar results could be obtained with AONs in saline and that intramuscular injections of the phosphorodiamidate morpholino oligomer (PMO) counterpart resulted in even higher levels of exon skipping and dystrophin (Fletcher et al. 2005). Recent work suggests that conjugating arginine-rich peptides to AONs enhances uptake to an even larger extent (Jearawiriyapaisarn et al. 2008; Wu et al. 2008; Yin et al. 2008).

AONs are species specific and as the human and murine *DMD* genes are similar but not identical, human AONs generally have one or more mismatches with the mouse target sequence. Our group has developed a unique mouse model that contains the complete human DMD gene stably integrated into the mouse genome ('t Hoen et al. 2007). This model can be used to test human AONs in vivo and also allows analysis of AON specificity, since human specific AONs should not induce skipping of mouse exons when they contain mismatches. Interestingly, exon skipping levels after intramuscular injections of hDMD muscles tend to be much lower than those observed in the dystrophic mdx mouse model (Bremmer-Bout et al. 2004). This is a direct consequence of the quality of muscle fibers. The hDMD mouse has two functional DMD genes (the murine and the human), and the human dystrophin can functionally compensate for the lack of mouse dystrophin ('t Hoen et al. 2007). Thus, hDMD muscles are healthy, which forms a barrier for AON uptake. By contrast, as in patients, mdx fibers are continually damaged and thus are leaky, which allows increased AON uptake after local or systemic AON delivery and translates into enhanced exon skipping levels (unpublished data). Notably, this is thus an example where a facet of the disease actually facilitates the cure!

Due to the very encouraging results, two clinical trials were planned where the safety and efficiency of exon 51 AONs of different chemistries will be tested after intramuscular injection (Muntoni et al. 2008). Exon 51 was chosen because it is applicable to the largest group of patients (Aartsma-Rus et al. 2009a). However, while exon 51 skipping restores the open reading frame for 13% of all DMD patients, it disrupts the reading frame in unaffected individuals. It is thus unethical and impossible to first test this approach in healthy volunteers and exon skipping can therefore only be tested in patients (van Ommen et al. 2008). The first trial took place in the Netherlands and employed 800 μg PRO051, a 2′-O-methyl phosphorothioate AON, which was injected in a small area of the tibialis anterior muscle of

four prescreened DMD patients (van Deutekom et al. 2007). A muscle biopsy of the treated area, taken 4 weeks after the injection, showed dystrophin restoration for the majority of fibers in each patient (65–97%). The levels of dystrophin were between 17 and 35% and correlated with the muscle quality, i.e., patients with more muscle fibers produced more dystrophin. This was anticipated as the AONs target dystrophin pre-mRNA, and the *DMD* gene is transcribed in the muscle but not in the adipose and fibrotic tissue that replaces the muscle in later stages of the disease. A similar trial where a phosphorodiamidate morpholino oligomer targeting exon 51 will be injected locally into the extensor digitorum brevis muscle is currently ongoing in the UK (Arechavala-Gomeza et al. 2007).

While for safety reasons these first in man trials have to use intramuscular injection, for future clinical application, systemic injection is preferred (see Chap. 6). A follow up, dose finding study coordinated by Prosesna where PRO051 is injected subcutaneously in patients is currently ongoing. The primary objective is to confirm that systemic AON treatment is safe, but RNA and protein analysis will also be performed. A second study using an AON targeting exon 44 (PRO044) will be initiated soon.

5.6 Summary and Future Direction

Antisense-mediated exon skipping aims to reframe dystrophin transcripts to allow generation of partly functional proteins and conversion of a severe Duchenne into a milder Becker phenotype. The first proof-of-concept in patient-derived cells was obtained in 2001 (van Deutekom et al. 2001), and the first clinical trial in DMD patients was conducted in 2006 (van Deutekom et al. 2007). This is relatively fast, especially given that AONs are a new type of drug. There are several reasons why AONs are now closer to clinical application than some of the more traditional therapeutic approached like gene and cell therapy. The exon skipping approach modulates endogenous pre-mRNA transcripts using synthetic DNA/RNA analogs. This eliminates the risks of an immune response towards viral vectors, donor-derived or autologous ex vivo modified cells and/or insertional mutagenesis. In addition, delivery of small AONs is very efficient, especially in dystrophic muscle, while efficient delivery of genes without a viral vector system or through cell transplantation needs further optimization.

An immune response to the newly generated dystrophin is unlikely. First, in the majority of patients spontaneous exon skipping and reframing of dystrophin transcripts takes place at a very low level, resulting in occasional dystrophin positive revertant fibers (Fanin et al. 1992). The amount of these dystrophin positive fibers is not sufficient to prevent the progressive deterioration in muscle quality . However, it does mean that for most patients dystrophin will not be a neo-antigen. In addition, the N-terminal part (up to the mutation) of dystrophin will still be produced and dystrophin has several C-terminal isoforms as well and generally not all of these are affected by the mutation.

One of the disadvantages of the approach is that due to AON and protein turnover, treatment is not permanent and will have to be repeated. Nevertheless, AONs are new types of drugs, that are still being further developed and for which no long-term treatment data is yet available. Thus, it is not so bad that treatment can be stopped should there be any unforeseen toxic effects due to long-term AON exposure, or should a more efficient AON chemistry be identified the original treatment can be replaced by the more optimal treatment.

The exon skipping approach can be seen as personalized medicine, as it is tailored to the need of different patients with varying mutations (van Ommen et al. 2008). Despite the current hype about personalized medicine, the biggest challenge for clinical application of exon skipping is that regulatory offices are not yet ready for these types of drug. AONs targeting different exons are considered different drugs, but this is also the case for AONs targeting different sequences in the same exon and even for AONs targeting the same sequence but with different backbone chemistries. Given that DMD is a rare disease and that the largest group of patients (requiring exon 51 skipping) consists of only 13% of all patients, multicenter studies are needed already for early stage clinical trials to allow inclusion of sufficient numbers of patients. While it is true that each AON consists of a unique combination of nucleotides, and thus each carries the possibility to induce unique side effects, the chemistry-specific side effects will be similar for all AONs of the same backbone. For some exons the groups that benefit are very small, sometimes limited to one or two patients worldwide, making later phase clinical trials for these AONs impossible (van Ommen et al. 2008). Thus, new ways to properly test the safety of these drugs in patients have to be devised to prevent that treatment development is limited only to patients with "common" mutations due to an incompatibility with the rules and regulations.

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Chapter 6
Systemic Treatment of Duchenne Muscular Dystrophy by Antisense Oligomer-Induced Exon Skipping

Qi Long Lu and Bo Wu

Abstract Duchenne muscular dystrophy (DMD), caused by nonsense or frame-shift mutations in the dystrophin gene, is a progressive degenerative disease involving all the muscles body-wide. Antisense oligomer-mediated exon skipping has recently emerged as an effective approach for the restoration of dystrophin. A clinical trial by intramuscular delivery of antisense oligonucleotide demonstrates efficacy in principle in DMD patients, providing optimism for its clinical application as an effective treatment. However, DMD is a systemic disease and requires life-long treatment systemically with antisense therapy for the restoration and maintenance of dystrophin expression in body-wide muscles, especially cardiac muscle. Unmodified antisense oligomers are able to induce effective exon skipping systemically but with significant variation between and within muscles and failure of dystrophin induction in the cardiac muscle. Modifying phosphorodiamidate morpholino oligomer (PMO) with cell-penetrating peptide and polymers greatly improves the efficiency of the delivery and leads to the restoration of near normal levels of dystrophin in both skeletal and cardiac muscles with improved functions. Clinical trials of systemic treatment with antisense oligomers for DMD are well under way and could represent the first realization of gene therapy to muscular dystrophies.

6.1 Introduction

Frame-shift and nonsense mutations in the dystrophin gene abolish dystrophin expression in nearly all muscles body-wide in Duchenne muscular dystrophy (DMD) patients. For DMD boys, the initial clinical symptoms are weakness in the skeletal muscles with delayed onset in walking, difficulty in performing a standing jump and in getting up from the floor. These symptoms usually occur anywhere

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between ages 4 and 8, and there is no clear impairment in the boys' respiratory and heart functions. As the disease progresses, immobility is inevitable and the boys will generally lose the ability to walk between age 9 and age 14. Finally, vital organ systems such as the lung start to malfunction. The child eventually has to be placed on a ventilator to retain the ability to breathe. Malfunction of the cardiac muscle will become evident in the later stage of the disease and ultimately, failure of the heart muscles is one of the leading causes for the death of DMD boys. The organ failures are the later, but direct consequences of the lack of dystrophin expression in the muscles of these organs. During the last 2 decades, the quality and life-span of DMD patients have been improved gradually, largely due to coordinated multidisciplinary patient care. Therefore, rescue of the lung and heart functions becomes ever more critical for the quality and longevity of a patient's life (Wagner et al. 2007; Townsend et al. 2008; Matsuda et al. 1995; Silver et al. 1983; Bostick et al. 2008; Quinlan et al. 2004). More importantly, restoration of dystrophin only in the skeletal muscles may exacerbate the failure of heart function if dystrophin expression cannot be effectively restored in cardiac muscle (Townsend et al. 2008).

Clearly, fundamental treatment of DMD requires restoration of the functions of the mutated dystrophin gene in body-wide muscles. Antisense oligomer-induced exon skipping as an effective therapy therefore has to be able to restore dystrophin expression in at least the majority of skeletal muscles and critically in smooth muscles and the cardiac muscle (Foster et al. 2006). In this chapter, we will discuss the factors important for the restoration and maintenance of dystrophin expression by antisense oligomer-induced exon skipping. Specifically, we will discuss the comparison between various chemistries as antisense oligomers for systemic delivery; the levels of dystrophin required for prevention of muscle damage; the potential barriers to achieve high efficiency in the cardiac muscle and a regime to maintain therapeutic levels of dystrophin in critical organs.

6.2 Chemistry of Antisense Oligomers

Since the first attempt of using antisense oligonucleotide for exon skipping of dystrophin gene transcripts, several chemistries have been examined for their potential to achieve enhanced antisense effects (Fig. 6.1). Among them, 2-O-methyl phosphorothioate antisense oligoribonucleotide (2OMePS AON) has been the most widely used for its resistance to nuclease degradation and higher affinity to target sequence than natural oligonucleotides. The other advantage of the 2OMePS AON is that the chemistry maintains a negative charge, thus permitting effective delivery by most delivery reagents commercially available in cell culture systems (Mann et al. 2001). Efficacy of 2OMePS AON has been demonstrated by intramuscular injections (i.m.) in dystrophic mdx mice with restoration of dystrophin (Lu et al. 2003). Furthermore, 2OMePS AON has also been successfully demonstrated to induce dystrophin expression in muscles of DMD patients by i.m. injection in the clinic trial conducted in Netherlands (van Deutekom et al. 2007). The chemistry has

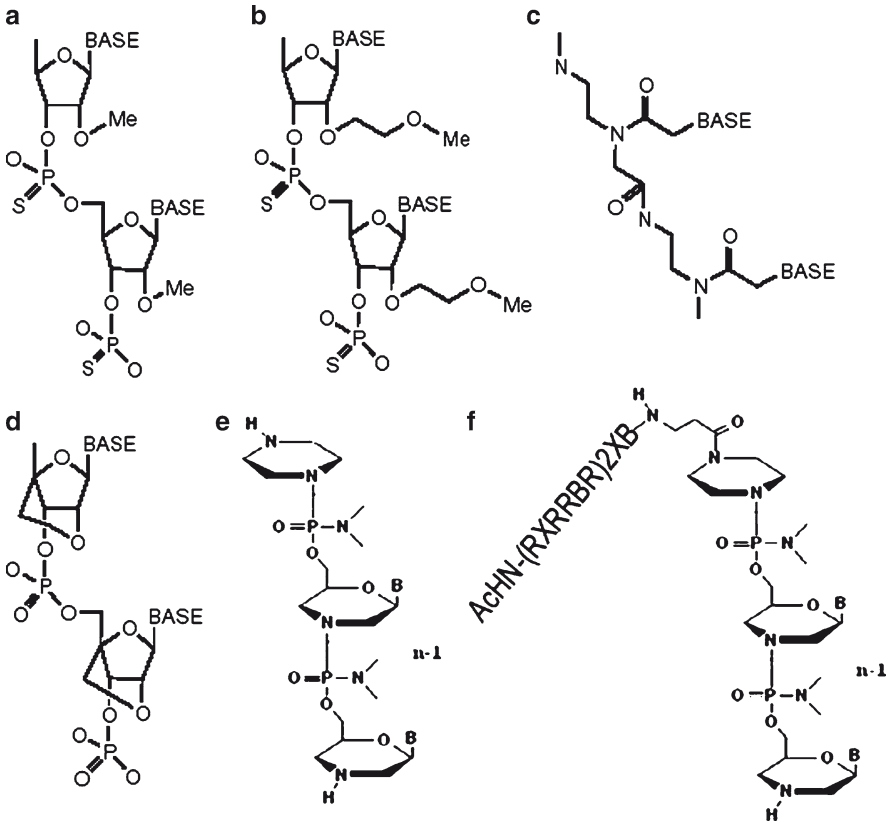

Fig. 6.1 Chemistry of antisense oligomers. (**a**) 2′-O-Methyl Phosphorothioate (2OMePS AON); (**b**) 2′-O-methoxyethyl Phosphorothioate; (**c**) Peptide nucleic acid (PNA); (**d**) Locked nucleic acid (LNA); (**e**), Phosphorodiamidate morpholino oligomers (PMO); (**f**), AcHN-(RXRRBR)2XB peptide-tagged PMO (R = arginine, X = 6-aminohexanoic acid and B = β-alanine) (PPMO)

been used in vivo without any delivery enhancer and shows to be safe in both animal models and in humans. However, delivery of 2OMePS AON systemically appears to have limited efficiency in inducing exon skipping and dystrophin induction in body-wide muscles. As we reported, intravenous (i.v.) injection of 2 mg 2OMePS AON per mouse (approximately 60–80 mg/kg) three times at weekly intervals was able to induce dystrophin expression in all skeletal muscles of the mdx mice. However, only a small proportion of muscle fibers in focal areas expressed detectable levels of dystrophin by immunohistochemistry. The amount of dystrophin was less than 5% of normal level in all muscles of the treated mice. Disappointingly, no increase in dystrophin expression was observed in the cardiac muscle after treatment. At this level of dosage, no toxicity to the liver and kidney was observed. These results appear to suggest that 2OMePS AON is safe but with low efficacy for inducing dystrophin expression systemically (Lu et al. 2005).

Phosphorodiamidate morpholino oligomers (PMO) have recently been explored for the exon skipping of dystrophin gene. In the PMO, the phosphodiester bond is replaced by phosphoroamidate linkage and the ribose replaced by a morpholino moiety (Fig. 6.1). This renders the PMO charge neutral in biological systems. PMO has higher affinity than natural oligonucleotides for their target nucleic acid sequences and much greater resistance to degradation than neutral nucleic acids (Summerton and Weller, 1997). These properties offer higher potential for the chemistry to be applied for interfering with gene expression. The chemistry was first and is still being used as effective antisense oligomers for gene silencing in zebrafish (Nasevicius and Ekker, 2000; Ding et al. 2008). This is contributed to its charge-neutral nature, allowing PMO with relative ease to penetrate into the body part of the developing fishes in the aqueous solution. The nonionic nature, however, appears to impede the delivery of PMO into cultured mammalian cells (Sazani et al. 2002), prompting the use of "scrape-loading" to create pores in the membrane for effective delivery in cell culture system as almost all transfection reagents available had been developed specifically only for the delivery of negatively charged natural or modified oligonucleotides. This method however limited its applications only to adherent cell cultures. Effort was then made to complex PMO with negatively charged complementary DNA sequences (called leash) to enhance delivery (Bruno et al. 2004; Gebski et al. 2003). The leash provides negative charge to the DNA/PMO complex, so commercially available gene/oligo delivery reagents, such as polyethyleneimine (PEI) and Lipofectin, could be used for enhanced delivery. However, the improvement in exon skipping in cell culture was limited and the toxicity of the complex was much higher than simple PMO due to the use of positively charged delivery reagents. The leash/PMO/lipofectin complex delivery method was also tested for in vivo exon skipping, but the antisense efficiency was only marginally better than the other chemistry (Gebski et al. 2003). In fact, the use of leash did not enhance antisense effect compared to the uncompleted PMO (Alter et al. 2006). In 2006, the higher antisense effect of bare PMO was demonstrated by both i.m. and i.v. injections (Alter et al. 2006). A single injection of PMO induced higher levels of dystrophin expression than the same PMO complexed with leash and Lipofectin in tibialis anterior (TA) muscles of the mdx mice. Furthermore, the complexed PMO created significant tissue damage whereas PMO alone did not cause any detectable tissue toxicity. Most significantly, toxicity with such PMO complexes prevented them from i.v. administration. In contrast, bare PMO at all dosage reported so far causes no noticeable toxicity in muscles and organs in animal models examined. Serial (seven times) i.v. injections of 2 mg per mouse (adult mice, 6 weeks of age) PMO targeting mouse dystrophin exon 23 induce up to 50% of normal levels of dystrophin in body-wide skeletal muscles in the mdx mice. Induction of dystrophin significantly reduces the central nucleation of muscle fibers. Improved muscle pathology in the treated animals was also demonstrated by the decrease in serum levels of muscle creatine kinase (MCK) and partial restoration of muscle force generation. The systemic effects of PMO therefore demonstrate the realistic potential of the antisense therapy for the treatment of DMD patient (Alter et al. 2006).

6.3 Mechanisms for the Differential Antisense Effect by Systemic Treatment of Unmodified AONs

Antisense therapy for treating DMD relies on the rescue of dystrophin expression throughout the body musculature. It was expected that systemic delivered AON could restore dystrophin expression evenly in all muscles and muscle fibers. When 2OMePS AON targeting mouse exon 23 was delivered i.v., systemic effect was evident (Lu et al. 2005). However it was a surprise to see that dystrophin expression induced by systemically delivered 2OMePS AON was highly variable with very limited number of fibers expressing near normal levels of dystrophin by immunostaining. Variation was observed between individual muscles and within muscles as groups of dystrophin positive fibers can be seen right adjacent to completely dystrophin negative fibers. The mechanism(s) responsible for such disparity is not understood, but is of great importance if we are to achieve therapeutic effect in the body-wide affected muscles in DMD patients. The focal distribution of positive fibers in the same muscle suggests that the differential induction is unlikely to be related to the fiber types and this is supported by immunohistochemistry with antibodies specific to fiber types and dystrophin (Lu et al. 2005). The efficiency of dystrophin induction is not simply related to the levels of fiber maturation as fibers of all calibers were found to express similar levels of dystrophin. This is also supported by the fact that both neonatal myosin positive and negative fibers can be either dystrophin positive or negative. Since the characteristic pathological feature of the DMD muscles and muscles in the mdx mice is the cycles of muscle degeneration and regeneration, the focal variations in dystrophin induction is therefore proposed to be related to the stages of the pathological cycles of dystrophic muscles. Studies of dystrophin induction by AON and muscle damage indicated by Evans blue staining suggested that mature fibers expressing high levels of dystrophin are most frequently seen neighboring to or in the areas with clear leakage of Evans blue dye (Fig. 6.2). Fibers of small caliber are also more frequently observed with high levels of dystrophin induction. These results suggest that higher efficiency in dystrophin induction is related to a more permeabilized vasculatures system and fiber membrane. This leads to the hypothesis that delivery efficiency of the negatively charged 2OMePS AON in vivo is closely related to the process of muscle degeneration and the delivery mechanism of 2OMePS AON is likely to be a process of passive diffusion. The repulsion between the like-charges of 2OMePS AONs and the molecules at the surface of cell membrane would be expected to hinder efficient delivery of the AON into target cells with intact membrane, and thus the lower efficiency of antisense effect in those fibers with minimum membrane permeabilization.

This together with other factors prompted the examination of the charge-neutral PMO for systemic effect of exon skipping and dystrophin induction. The lack of charge is expected to have much less impediment to cell surface contact and may thus allow PMO enter muscle fibers more efficiently, particularly those with

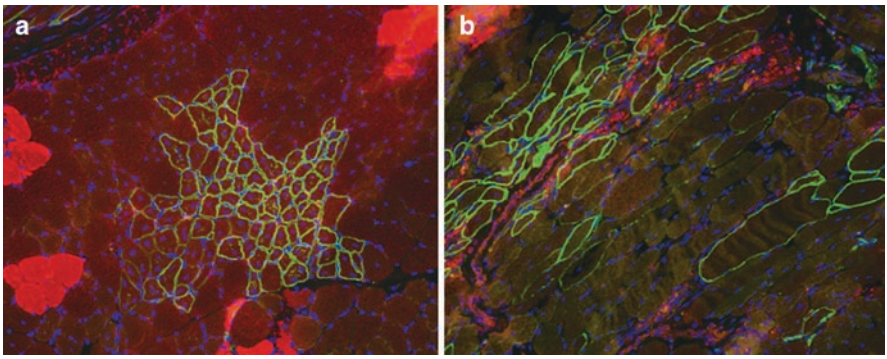

Fig. 6.2 Correlation between antisense oligomer-induced dystrophin expression (green fluorescence membrane labeling) and muscle damage indicated by Evans Blue staining (red labeling). Focal clusters of fibers expressing dystrophin localize in the areas with trace but clear observable staining with the Evans Blue. The dye is given 24 h before the experiment is terminated and 7 days after the treatment with an antisense oligomer for dystrophin exon 23 skipping in the mdx mouse. (**a**) TA muscles; (**b**) diaphragm

leaky membrane as seen in the dystrophic muscles. This hypothesis however would predict a similar pattern of dystrophin induction by PMO in dystrophic muscles. Indeed, single i.v injection of low doses of PMO produced highly variable dystrophin expression in all muscles (Alter et al. 2006). PMO is highly stable in biological system, for example, without any clear degradation in serum for at least 24 h (Arora et al. 2004). This together with the fact that most if not all fibers in DMD muscles will undergo cycles of degeneration provides the possibility that regular injections could effectively deliver PMO into majority of muscle fibers with the production of therapeutic levels of dystrophin. This was demonstrated by regular i.v. injections in the mdx mice as we discussed in the previous section. The higher efficiency in dystrophin induction by PMO compared to 2OMePS AON is most likely a combinatorial effect of the stable oligomers, the high binding affinity to target sequence and the higher mobility of the charge-neutral molecules within the extracellular matrix and through the cell membrane.

The dependence on muscle damage for effective delivery of AONs including charge-neutral PMO has an advantage of limiting the amount of AON entering untargeted and undamaged nonmuscle cells, thus diminishing possible side effects. At the same time however, this limitation presents a potential barrier for effective treatment of DMD (Alter et al. 2006). This model would predict that muscle fibers rescued earlier by PMO-induced exon skipping, might have to re-enter a myopathic state before they could again be protected by further entry of antisense oligomers. Such a requirement for recurring cycles of rescue and degeneration in treated muscles would severely limit the value of antisense therapy for DMD patients.

6.4 Exon Skipping and Dystrophin Induction in Cardiac Muscle

The requirement of muscle damage for effective delivery and AON induced dystrophin expression is further supported by the result obtained in the cardiac muscle in the mdx mice after systemic treatment of both 2OMePS AON and PMO. Cardiac muscles in mdx mice are less affected by the dystrophic process and no significant pathology and functional impairment can be obviously demonstrated until late age. Consistently, only minimum amount of dystrophin expression can be detected in the cardiac muscle even after repeated injections of both 2OMePS AON and PMO in all mdx mice aged 6 months or younger (Lu et al. 2005; Alter et al. 2006), whereas the same treatment can induce high levels of dystrophin in skeletal muscles. There is a possibility that the special tissue structure (vasculature and membrane) and pattern of metabolism or gene expression regulation could be responsible for lower efficiency of AON delivery or exon skipping inside myonuclei. However, direct injection of AON into cardiac muscles showed effective dystrophin induction, suggesting that lower delivery efficiency is perhaps the most critical factor (Vitiello et al. 2008). This is further supported by the restoration of dystrophin in cardiac muscles via AAV-mediated AON delivery (Denti et al. 2008).

6.5 Modified PMO for Dystrophin Restoration in Skeletal and Cardiac Muscles

One approach to overcome the disparity in dystrophin expression in skeletal muscles and lack of dystrophin in cardiac muscle is to employ cell-penetrating peptides or polymers for providing active transportation of AON into muscles. Various types of cell-penetrating peptides have been tested and among the most effective ones are arginine-rich peptides (Yin et al. 2008; Ivanova et al. 2008). Studies of using antisense oligomers for exon skipping in the dystrophin gene showed that arginine-rich peptide could significantly improve delivery of peptide nucleic acid (PNA) and PMO in cell cultures and in muscles by i.m. injection (Fig. 6.1) (Abes et al. 2007). However, only arginine-rich peptide-tagged PMOs have been used systemically for splicing modulation in animal models (Abes et al. 2006; Fletcher et al. 2007; Wu et al. 2008; Jearawiriyapaisarn et al. 2008). Earlier studies using arginine-rich peptide conjugated PMOs (Yuan et al. 2006) showed significant improvement in delivery and PMO-mediated antiviral effect. More recently, two studies described a significant advance in achieving high efficiency of exon splicing with peptide conjugated PMOs in animal models. Jearawiriyapaisarn et al. examined PMOs conjugated with several arginine-rich cell-penetrating peptides containing 6-aminohexanoic acid (X) and/or beta-alanine (B) for their potency, functional biodistribution, and toxicity in a transgenic mouse expressing EGFP. The GFP expression is disrupted by the insertion of a mutant β globin gene

sequence. Therefore this mouse ubiquitously expresses the aberrantly spliced EGFP-654 pre-mRNA reporter, but no EGFP is expressed unless antisense oligomers block the mutant site of the insertion. Correct splicing and enhanced EGFP upregulation serve as a positive readout for the efficiency of antisense oligomer-mediated splicing alteration. Highest efficiency in restoration of EGFP expression is observed with the PMO tagged with a peptide of (RXRRBR) 2XB (PPMO) in both skeletal and cardiac muscles (Jearawiriyapaisarn et al. 2008). At the same time and using dystrophic mdx mice as a model of DMD, we reported that systemic delivery of the PPMO restores dystrophin to almost normal levels in all skeletal muscles by a single dose of 30 mg/kg i.v. injection. Regular administrations are able to maintain the similar levels of dystrophin (Fig. 6.3). Most significantly, near normal levels of dystrophin can also be achieved in the cardiac muscle after regular i.v. injections of PPMO (Fig. 6.4). This leads to increase in the muscle strength and prevents cardiac pump failure induced by dobutamine in vivo. Muscle pathology and function continue to improve during a 12-week course of biweekly treatment with significant reduction in serum levels of creatine kinase. High efficiency of exon skipping is also achieved in smooth muscles and other muscles in other organs such as the esophagus. Treatment with the PPMO did not cause detectable toxicity. All these together highlight the feasibility of the PPMO for rescuing dystrophin in both skeletal and cardiac muscles in DMD patients (Wu et al. 2008).

6.6 Nonpeptide Polymers for Effective Delivery of PMO

One major concern that arises with the use of peptides as delivery enhancers is the immune response they might elicit, preventing from repeated administration and causing rejection of targeted tissues. This is particularly important as long-term repeated administrations are required for the treatment of DMD with antisense therapy. No signs of immune response were observed during the short-term treatment with the PPMOs (Wu et al. 2008) and previous reports also suggested the lack of immunogenicity with similar peptides in animal models (Abes et al. 2006; Fletcher et al. 2007). However, these observations are made only with short-term use of arginine-rich peptides (Abes et al. 2006; Fletcher et al. 2007; Wu et al. 2008; Jearawiriyapaisarn et al. 2008). The fact that immunogenicity varies considerably between species would argue for longer-term studies in other species. Long-term safe use of arginine-rich peptides can only be confirmed in clinical trials even if immunogenicity is not detected in animal models. It is therefore important to search for nonpeptide alternatives to enhance delivery of oligomers for inducing exon skipping and dystrophin induction. The known sequence and structure of the peptide used in the PPMO provide bases for modeling to develop possible nonpeptide polymers with similar or even improved function as effective delivery vehicles. Li et al. exploited a nonlinear, nonpeptidic dendrimer as effective transporter for delivery of PMO (Li and Morcos 2008). The design of

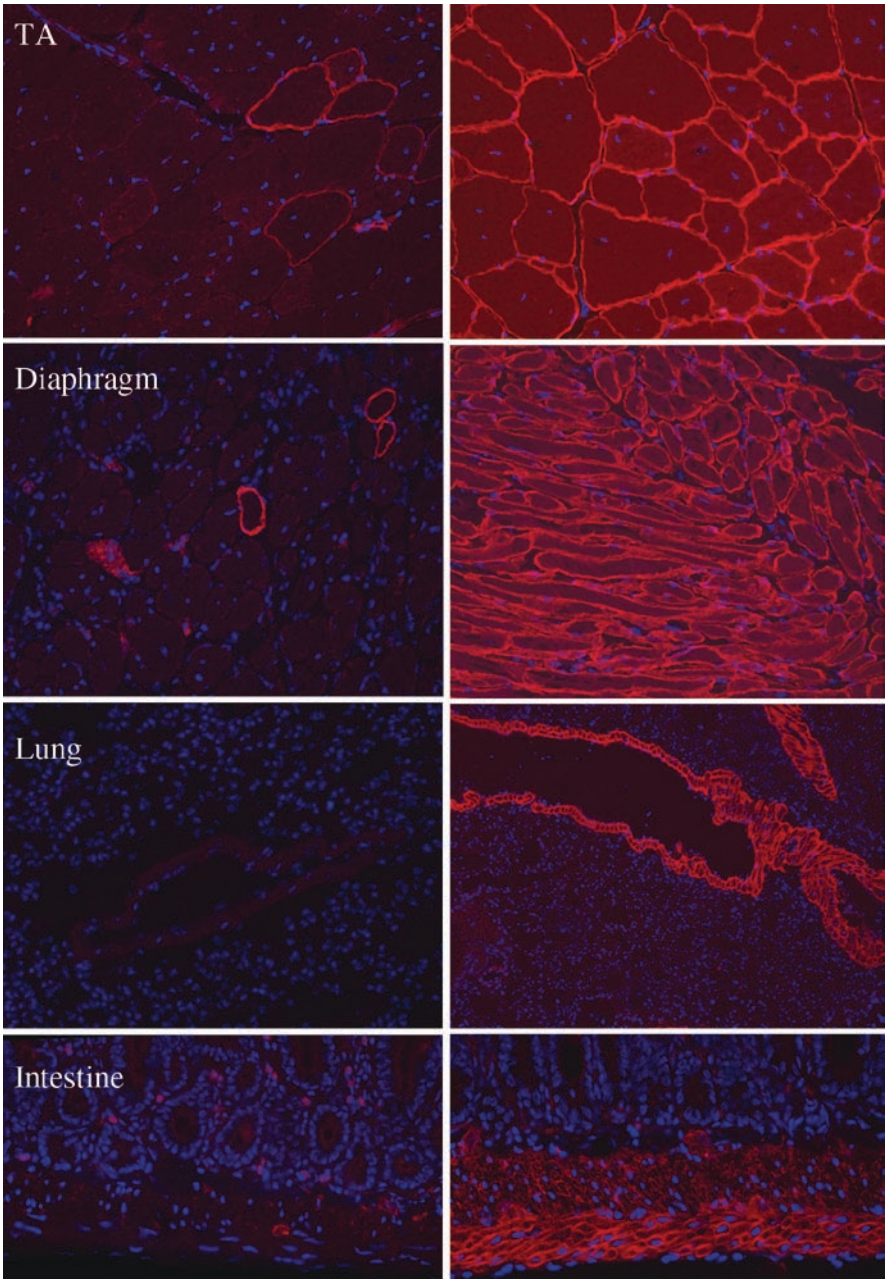

Fig.6.3 Restoration of dystrophin in body-wide muscles of mdx mice after six intravenous injections of 30 mg/kg of the PPMOE23 targeting mouse dystrophin exon 23 at biweekly interval (*right panel*). Muscles were examined 2 weeks after the last injection. *Left panel*, muscles from control scrambled (same monomer composition, but different in sequence) PPMO-treated mdx mice. Blue, nuclear staining with DAPI. Dystrophin was expressed homogenously in all muscle fibers from the PPMOE23-treated mdx mice. TA, tibialis anterior muscle. Restoration of dystrophin is observed in smooth muscles in vessel walls within the lung and in the intestine

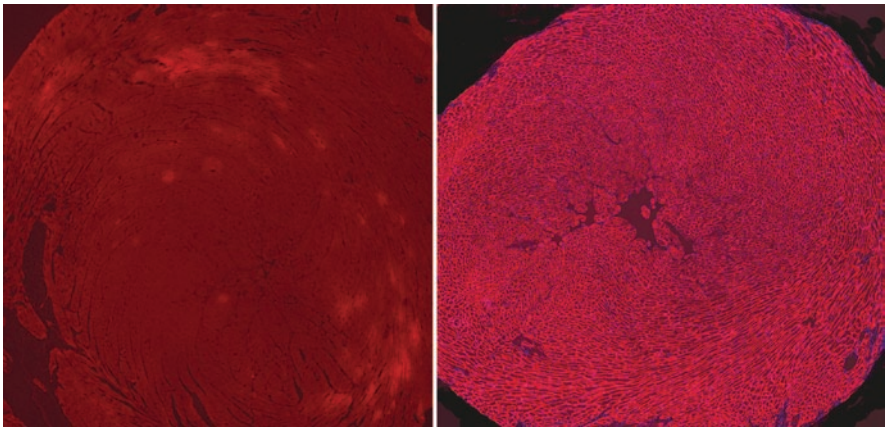

Fig. 6.4 Dystrophin expression in cardiac muscle after PPMOE23 treatment (30 mg/kg six times at biweekly intervals). Heart from scrambled PPMO-treated mdx mouse (*left*) and PPMOE23-treated mdx mouse (*right*). The muscles were examined 2 weeks after last injection of the PPMOs. Dystrophin was detected by immunohistochemistry with rabbit antibody P7 and visualized by Alexa 594 conjugated goat-anti-rabbit Igs. Blue, nuclear staining with DAPI

the transporter is based on the following factors: guanidinium head groups of arginine-rich peptides are principally responsible for uptake of PMO into cells, a tri-functional triazine was used as a core scaffold to assemble and present guanidinium head groups in a nonlinear pattern. Previous reports have suggested that a range of 7–15 arginine residues is effective for oligomer uptake (Futaki et al. 2002), with 8 arginine residues exhibiting the most efficient internalization (Lebleu et al. 2008). Therefore, they chose to install a total of eight guanidine head groups on two of the side chains of the triazine core. The polymer is then conjugated to a PMO (termed as Vivo-PMO). The polymer was shown to significantly enhance the delivery of PMO into cultured cells and to a wide variety of tissues in the EGFP-β actin transgenic mice (Li and Morcos 2008). Using a Vivo-PMO targeting mouse dystrophin exon 23 (Vivo-PMOE23), we found that this octa-guanidinium dentrimer is highly effective for the delivery of PMO into body-wide muscles even at low doses. A single intravenous injections of 6 mg/kg Vivo-PMOE23 generated dystrophin expression in skeletal muscles at the levels equivalent to the injection of 300 mg/kg unmodified PMOE23. Repeated injections of 6 mg/kg Vivo-PMOE23 achieved near normal and approximately 10% levels of dystrophin expression in skeletal muscles body-wide and in the cardiac muscle respectively, without eliciting a detectable immune response. Vivo-PMOs showed no signs of toxicity with the effective dosages and regime and the treatment reduced the serum levels of creatine kinase significantly (Wu et al. 2009). These results therefore offer prospects for the development of new nonpeptide delivery moieties with improved function and reduced toxicity as it is unlikely that this polymer structure represents the best option.

6.7 Summary and Future Direction

Antisense oligomer-mediated exon skipping has now been demonstrated as one of the most promising experimental therapies for DMD patients. With the most potent chemistry in conjunction with the application of delivery-enabling polymers, the efficacy of the therapy systemically has now been well-established in models of both dystrophic mice and dogs. While the long-term efficacy of the therapy to DMD boys remains to be established, successful restoration of dystrophin expression in both cardiac and skeletal muscles provides hope that the therapy can be effective to rescue dystrophic phenotypes of both skeletal and cardiac muscle. As clinical trials of systemic treatment with highly effective antisense oligomers are well under way, this therapy could represent the first realization of gene therapy to muscular dystrophies.

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Chapter 7
RNAi Therapy for Dominant Muscular Dystrophies and Other Myopathies

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Abstract Over the last ~15 years, muscular dystrophy gene therapy strategies have been primarily aimed at replacing defective or missing genes underlying recessive disorders, such as Duchenne muscular dystrophy. These gene replacement strategies are typically not indicated for treating dominant diseases; instead, patients bearing dominant mutations would likely benefit from reduction or elimination of the abnormal allele. Until very recently, there was no feasible mechanism to reduce or eliminate disease genes, and molecular therapy development for dominant muscular dystrophies was largely unexplored. RNA interference (RNAi) has recently emerged as a powerful tool to suppress any gene of interest in a sequence-specific manner. As such, RNAi is a leading candidate strategy to silence dominant disease genes, including those involved in muscular dystrophy and related myopathies. Here, we discuss the potential for RNAi-mediated gene therapy of dominant muscular dystrophies and other myopathies.

7.1 Introduction

Individually, all myopathies are classified as rare disorders by the NIH Office of Rare Diseases, and Orphanet, which respectively define rare diseases as those affecting less than 200,000 people in the US and 1 in 2,000 Europeans (INSERM and French Ministry of Health 2008; National Institutes of Health Office of Rare Diseases Research 2009). Among all muscular dystrophies, X-linked recessive Duchenne muscular dystrophy (DMD) is the most common (1/3,500 newborn males; Flanigan et al. 2001), followed by the dominant disorders, myotonic dystrophy type 1 (DM1; 1/8,000; Harper 1989) and facioscapulohumeral muscular dystrophy (FSHD; 1/15,000–20,000; Flanigan et al. 2001; Tawil and Van Der Maarel 2006). However, a recent Orphanet report of disease prevalence in Europe places FSHD first, followed by DMD

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and DM (Table 7.1; INSERM and French Ministry of Health 2008). Mutations in at least 29 known genes cause various dominant muscular dystrophies and other related myopathies, and there are at least seven other clinically characterized disorders that have yet to be linked to specific genes (Table 7.1). Collectively, these dominant myopathies approach a prevalence that classifies them as common disorders (~1/2,400 to ~1/3,200[1]). This is significant as similar gene silencing strategies, with modifications depending on genetic etiology, may be effective for treating most dominant myopathies. Thus, proof-of-principle demonstration of RNA interference (RNAi) therapeutic efficacy in one myopathy could broadly impact an entire class of disorders.

7.2 RNA Interference

RNAi is a cellular mechanism to control coding gene expression prior to translation (Fire et al. 1998). RNAi-controlled messenger RNAs are therefore transcribed but not translated. RNAi is mediated by small (21–25 nucleotide, nt), noncoding microRNAs (miRNAs) and several proteins involved in miRNA processing and gene silencing (Bartel 2004; Elbashir et al. 2001a, b). A key feature of RNAi is sequence specificity: miRNAs share nucleotide sequence homology and base-pair with 3′ untranslated (UTR) regions of cognate mRNAs (Lai 2002). These base-pairing interactions allow miRNAs to direct cellular gene silencing machinery to target mRNAs and prevent their translation.

Naturally occurring miRNAs arise as relatively long primary transcripts from eukaryotic genomes ranging in complexity from single-celled algae to mammals (Elbashir et al. 2001a, b; Lagos-Quintana et al. 2001, 2003; Molnar et al. 2007, 2009). Over the last several years, a large amount of work has focused on understanding how miRNAs are expressed and processed to a biologically functional form. An important consequence of this growing knowledge has been the development of RNAi therapeutics. Designed RNAi molecules can be engineered to mimic natural miRNAs and subsequently used to suppress any gene of interest. It is therefore important to understand the biology underlying natural miRNA biogenesis when developing RNAi as a therapeutic tool.

7.3 RNAi Pathway

Rationally designed RNAi molecules are based on the structure and, in some cases, the nucleotide sequence, of natural miRNAs (Figs. 7.1–7.2; Boudreau et al. 2009; Lagos-Quintana et al. 2002; Zeng et al. 2005). Like other coding and noncoding

[1]Incidence range was calculated from Orphanet 2008 Report data (INSERM and French Ministry of Health 2008) and traditional reports of FSHD and myotonic dystrophy prevalence (Flanigan et al. 2001; Harper 1989). Least prevalence was calculated using myotonic dystrophy incidence of 4.5/100,000 and FSHD 5/100,000. Highest prevalence calculations used 12.5/100,000 and 7/100,000, respectively.

Table 7.1 Mutations causing dominant muscular dystrophies and other myopathies. Mutations in at least 29 different genes cause dominant muscular dystrophies and other myopathies. The genetic defect underlying FSHD is currently unknown, but may be caused by overexpression of one or more genes. FSHD candidates include FRG1 and DUX4*. Seven other clinically characterized myopathies have not yet been linked to specific genes. Moreover, Alzheimer's disease-related genes, Tau, APP, PSEN1, GSK3B, and ApoE, are overexpressed in sporadic inclusion body myositis (IBM). RNAi-mediated reduction of each may possibly benefit sporadic IBM disease

Gene	Clinical disorder	OMIM
LMNA	Emery-Dreifuss muscular dystrophy	181350
PABPN1	Oculopharyngeal muscular dystrophy	164300
FHL1	Scapuloperoneal amyotrophy	300695
MYH7	Laing distal myopathy	160500
	Myosin storage myopathy	608358
MYH2	Hereditary inclusion body myopathy	605637
DMPK	Myotonic dystrophy type 1	160900
ZNF9	Myotonic dystrophy type 2	602668
MYOT	LGMD1A	604103
	Spheroid body myopathy	182920
	Myofibrillar myopathy	609200
CAV3	Distal myopathy	607801
	Rippling muscle disease	606072
TTN	Tibial muscular dystrophy	600334
DES	Myofibrillar myopathy	601419
CRYAB	Myofibrillar myopathy	608810
ZASP	Myofibrillar myopathy	609452
FLNC	Myofibrillar Myopathy	609524
BAG3	Myofibrillar Myopathy	Unassigned
TPM3	Nemaline myopathy	609284
ACTA1	Nemaline myopathy	161800
	Congenital myopathy w/ fiber type disproportion	255310
TPM2	Nemaline myopathy	609285
TNNT1	Nemaline myopathy	605355
RYR1	Central core disease	117000
CLCN1	Thomsen Myotonia congenita	160800
SCN4A	Paramyotonia congenita	168300
DNM2	Myotubular (or centronuclear) myopathy	160150
MYF6	Myotubular (or centronuclear) myopathy	159991
MTMR14	Myotubular (or centronuclear) myopathy	160150
CHRNA1	Congential slow-channel myasthenic syndrome	601462
CHRNA2	Congential slow-channel myasthenic syndrome	601462
CHRNA3	Congential slow-channel myasthenic syndrome	601462
CHRNA4	Congential slow-channel myasthenic syndrome	601462
FRG1*	FSHD	158900
DUX4*	FSHD	158900
chrom 2p13	Welander distal myopathy	604454
chrom 6q23	LGMD1E	602067
chrom 7q	LGMD1D	603511
chrom 7q32	LGMD1F	608423

(continued)

Table 7.1 (continued)

Gene	Clinical disorder	OMIM
chrom 4q21	LGMD1G	609115
chrom 15q	Nemaline myopathy	609273
chrom 19p13	Vacuolar neuromyopathy	601846
Tau	Sporadic inclusion body myositis	147421
APP	Sporadic inclusion body myositis	147421
PSEN1	Sporadic inclusion body myositis	147421
GSK3B	Sporadic inclusion body myositis	147421
ApoE	Sporadic inclusion body myositis	147421

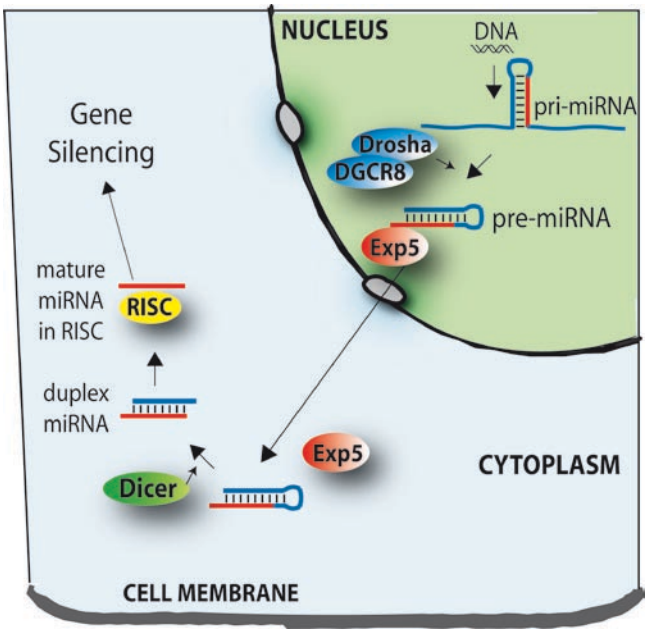

Fig. 7.1 MicroRNA biogenesis pathway. See text for details. Designed therapeutic microRNA shuttles, shRNAs, and siRNAs mimic pri-, pre-, and mature-miRNAs, respectively. Upon delivery to cells, exogenous inhibitory RNAs therefore enter the microRNA biogenesis pathway at different points, but all elicit gene silencing effects

transcripts in the cell, primary miRNA (pri-miRNA) precursors vary in size (can be several kilobases in length) and how they are transcribed: some miRNAs are RNA polymerase II (pol II) transcripts, others are RNA polymerase III (pol III), and expression may be tissue-specific (Borchert et al. 2006; Cai et al. 2004; Lagos-Quintana et al. 2002; Lee et al. 2004). Transcription of the pri-miRNA is the first step in the miRNA biogenesis pathway. The pri-miRNA is generated as a single-stranded transcript that forms an intramolecular stem-loop structure. Subsequent posttranscriptional

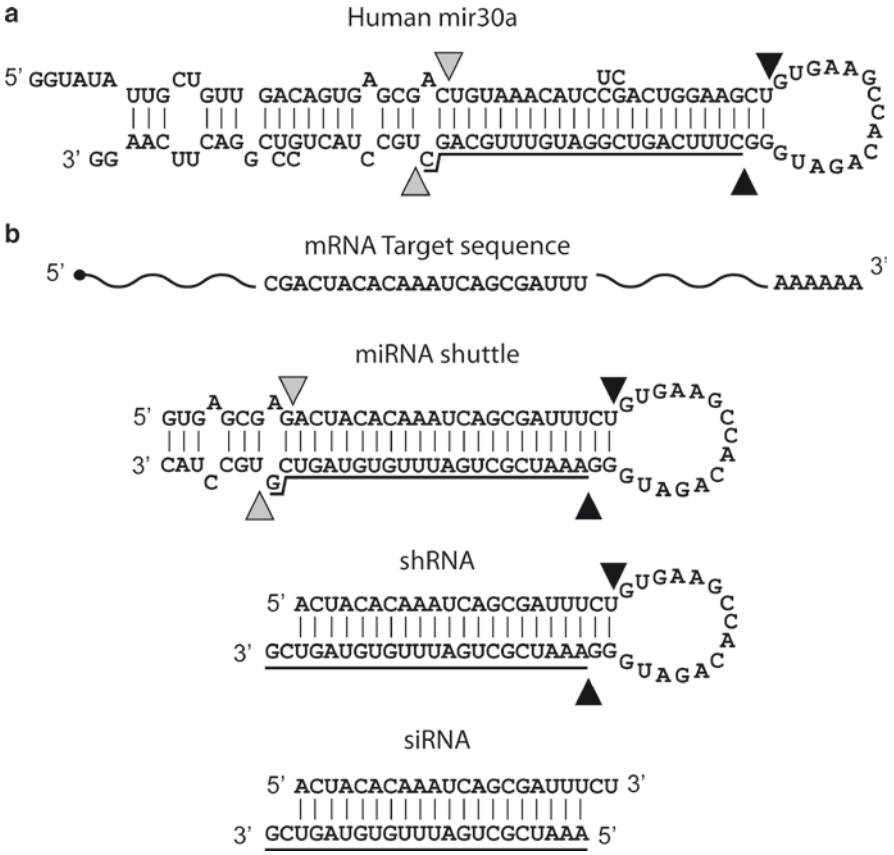

Fig. 7.2 Natural microRNA sequences and structures are used to design therapeutic inhibitory RNAs. (**a**) Human mir-30a. Gray and black triangles point to Drosha and Dicer nuclease sites, respectively. Note the staggered cuts leaving 2 nucleotide 3′' overhangs. Underlined sequence indicates the mature antisense guide strand sequence. (**b**) Example of designed inhibitory RNAs. Messenger RNA target sequence from *E. coli* LacZ gene. Mature mir-30 sequences are replaced by complementary LacZ-targeted inhibitory RNAs. In a miRNA shuttle, some mir-30 stem and loop sequences are maintained. The former help direct DGCR8/Drosha processing. ShRNAs are not Drosha processed; instead the 5′ end of the hairpin is defined by the transcription start site. An siRNA is produced in vitro and designed to mimic the final mature miRNA duplex

processing steps, catalyzed by several evolutionarily conserved proteins, serve to trim the pri-miRNA to a smaller, functional form, and ultimately create a double-stranded miRNA from the single-stranded primary transcript. Simplistically, the pri-miRNA contains important sequence and structural elements that direct a nuclear microprocessor complex composed of Drosha and DGCR8 to cleave the RNA at a specific location (Gregory et al. 2004; Han et al. 2004; Han et al. 2006; Landthaler et al. 2004; Lee et al. 2003b). DGCR8 recognizes and binds an important miRNA structural feature – a characteristic junction between the miRNA double-stranded stem and flanking

single-stranded sequences (Han et al. 2006). DGCR8 binding serves as a ruler to correctly position Drosha at the base of the miRNA stem, where it then makes a staggered cleavage to produce a characteristically shorter (~65–70 nt) hairpin pre-miRNA containing a 2 nt 3′ overhang (Han et al. 2006; Lee et al. 2003b). The nuclear export factor, Exportin-5 (Exp5) binds this overhang and then shuttles the pre-miRNA to the cytoplasm (Zeng and Cullen 2004). There, the enzyme Dicer recognizes the Drosha-generated overhang and catalyzes another staggered cleavage event ~21 nt away (~2 RNA helical turns), which removes the loop from the hairpin and produces a second 2 nt 3′ overhang at the opposite end (Provost et al. 2002; Zhang et al. 2002, 2004). The final result is the mature, 21–25 nt duplex miRNA containing 2 nt 3′ overhangs at both ends. This small range in mature miRNA size may be partly accounted for by bulged, looped-out mismatches in the miRNA stem. As a Dicer cut is ~21 nt long, stem mismatches that do not extend the length of the RNA helices may still be incorporated in the primary mature guide strand, which may, as a result, be slightly longer in some natural miRNAs. One strand of the mature miRNA duplex (the antisense "guide" strand) becomes the RNA component of the RNA induced silencing complex (RISC), which is ultimately responsible for sequence-specific gene silencing. The sense or "passenger" strand of the miRNA may be degraded or used to program a second RISC complex (Matranga et al. 2005; Ro et al. 2007). Indeed, some miRNAs are bifunctional and both strands can direct gene silencing (Ro et al. 2007). For therapeutic RNAi strategies, it is therefore important to validate that only the intended guide strand is directing gene silencing, as this will reduce risks of nonspecific, "off-target" effects.

The degree of complementarity between the guide strand miRNA and an mRNA determines (1) whether the transcript will be regulated at all by a programmed RISC complex and (2) if so, which of two gene silencing mechanisms will be induced (translational inhibition or transcript degradation). In general, incomplete pairing of an inhibitory RNA and a target mRNA will produce gene silencing through translational inhibition. In this case, target mRNA levels do not change. In some instances, as little as 7 nt of homology between the guide strand and a target mRNA (miRNA nts 2–8; called the seed match) may be required to elicit gene silencing effects (Lewis et al. 2005). Base-pairing outside the seed region may serve to stabilize the miRNA–mRNA interaction and help produce a more robust knockdown. In contrast, perfect miRNA–mRNA complementarity across the ~21–25 nt stretch results in mRNA degradation, and thus the pool of target mRNAs in the cell is depleted. The degradation mechanism is associated with more robust gene silencing.

7.4 RNAi Therapeutics

RNAi molecules can be engineered to suppress any gene. Numerous strategies to design inhibitory RNAs have been developed and all share two common features: artificial RNAi molecules are double-stranded and comprised of sequences cognate to an mRNA of interest. Artificial inhibitory RNAs can be designed to mimic

mature, pre-, or pri-miRNAs and will thus, upon delivery to cells, enter the miRNA pathway at different points (Fig. 7.1). There are three main classes of inhibitory RNAs (Fig. 7.2). (1) Small inhibitory RNAs (siRNAs) are in vitro synthesized, dsRNAs that are structurally identical to miRNA duplexes (Elbashir et al. 2001a). When delivered to cells, all siRNAs bypass the transcription and nuclear processing steps of the miRNA pathway. Some designed siRNAs are processed by Dicer (Rose et al. 2005), while others avoid this step and are immediately available to complex with RISC proteins after delivery to the cytosol. (2) Short hairpin RNAs (shRNAs) are structurally similar to stem-loop pre-miRNAs. They are typically designed to contain ~21 nt of paired stem sequence connected by an unpaired loop that is often derived from natural miRNA sequences (Paddison et al. 2002). ShRNAs are produced intracellularly, arising as transcripts from DNA expression cassettes using RNA pol III, and very rarely, pol II promoters. ShRNAs mimic Drosha-processed miRNAs and thus, following transcription, are immediately shuttled by Exp-5 to the cytoplasm for Dicer processing and incorporation into RISC. (3) Artificial miRNA shuttles resemble pri-miRNAs (Boudreau et al. 2009; Zeng et al. 2005). Like shRNAs, miRNA shuttles are transcribed from DNA expression cassettes, but are amenable to regulation by both pol II and pol III promoters. In this design, miRNA sequences required to direct Drosha and Dicer processing are maintained, but the natural, mature, 21–25 nt miRNA sequence is replaced by an inhibitory RNA sequence targeting the gene of interest. Thus, a natural miRNA is used to deliver an artificial siRNA. MiRNA shuttle transcripts are produced intracellularly and utilize all processing steps required for natural miRNA biogenesis.

Each of the three systems described above is capable of eliciting strong RNAi responses in vitro and in vivo. The key difference between siRNAs and shRNA/miRNAs is duration of expression. In vitro synthesized siRNAs are transient and long-term disease-gene suppression requires repeated administration; expressed shRNAs or miRNA shuttles are longer lasting, and if delivered via an appropriate viral vector, may produce permanent gene silencing effects. Importantly, muscle-directed gene delivery systems are well-developed, especially those using adeno-associated viral (AAV) vectors, which have been used extensively in the last few years to deliver shRNA/miRNA to numerous tissues (Fechner et al. 2008; Grimm et al. 2006; Harper et al. 2005; Xia et al. 2004).

As described above, shRNAs and miRNAs differ in the level of processing required by endogenous miRNA biogenesis machinery. This differential processing has direct implications for how each is expressed. Because shRNAs are not Drosha processed, their 5′ end must be defined by the start of transcription. This is important because Dicer binds the “Drosha-cut” end of the pre-miRNA and makes a defined cut ~21 nt downstream, which ultimately determines the sequence of the mature guide strand molecule (Fig. 7.2). As a result, shRNAs must be positioned near a promoter’s transcription start site to ensure proper processing and gene silencing function. This restriction is not necessary for miRNA shuttles because Drosha processing, not transcription, defines the critical 5′ Dicer binding site. As a result, artificial miRNAs can be expressed from any promoter. Moreover, several bifunctional expression vectors have been described, in which a coding gene and intron- or UTR-embedded miRNA

arise from the same pol II promoter-driven transcript (Du et al. 2006; Harper et al. 2006). Another difference between shRNAs and miRNAs is potential for nonspecific toxicity; miRNAs may be safer than shRNAs in vivo (Boudreau et al. 2009). ShRNAs were the first generation of plasmid- or vector-expressed artificial inhibitory RNAs used in vivo. Several studies have demonstrated shRNA efficacy for silencing disease genes and improving associated pathologies in, for example, models of neurodegenerative disease and viral infection (Grimm et al. 2006; Harper et al. 2005; Li et al. 2003; Xia et al. 2004). However, a few recent studies have raised concerns about shRNA safety. Specifically, uncontrolled, high-level shRNA expression from constitutively-active pol III promoters caused liver failure and brain striatal loss in mouse models of hepatitis and Huntington's disease (HD), respectively (Grimm et al. 2006; McBride et al. 2008). This observed toxicity seems to be related to shRNA-induced saturation of endogenous miRNA biogenesis pathways, especially at the level of nuclear export, thereby interfering with natural miRNA function (Grimm et al. 2006). Importantly, lowering the dose of vector-expressed shRNAs in the liver, or using a less-powerful miRNA shuttle system in the brain, mitigated these toxic effects (Grimm et al. 2006; McBride et al. 2008). Both strategies ultimately led to significant gene silencing without overexpression associated toxicity. Although not all shRNAs are overtly toxic, and sufficient safety data regarding long-term artificial miRNA is lacking, miRNA shuttles may be safer than shRNAs simply because they are more efficiently processed and amenable to expression by tissue-specific, regulated, or weaker RNA pol II promoters, while shRNAs are dependent upon strong, constitutively-active pol III promoter expression. Regardless of the system used, RNAi therapy has shown promise in preclinical models of neurodegenerative disease, viral infection, and cancer, supporting its potential for treating dominant muscular dystrophies and other myopathies (Fechner et al. 2008; Grimm et al. 2006; Harper et al. 2005; Li et al. 2003; Mook et al. 2009; Saydam et al. 2005; Xia et al. 2004). These studies support its potential for treating dominant muscular dystrophies and other myopathies.

7.5 Disease Allele-Specific Gene Silencing

Excepting the extremely rare cases of X-linked dominant FHL1 mutations in males (Quinzii et al. 2008), patients with dominant disorders possess one mutant and one normal copy of their specific myopathy-related gene. As the underlying pathogenic events in these disorders are dominant gene mutations, simply reducing mutant allele expression may be therapeutic. For example, myotilin (MYOT) mutations cause dominant, progressive muscle disease clinically classified as LGMD1A, myofibrillar myopathy (MFM), or spheroid body myopathy (SBM; Foroud et al. 2005; Garvey et al. 2006; Hauser et al. 2000, 2002; Schroder et al. 2003; Selcen and Engel 2004). To date, 12 distinct point mutations were associated with dominant myotilinopathies in numerous families (Foroud et al. 2005; Garvey et al. 2006; Hauser et al. 2000, 2002; Schroder et al. 2003; Selcen and Engel 2004). Myotilin is a Z-disc protein expressed predominantly in skeletal and cardiac muscle.

Mutations cause myofibrillar aggregation and muscle weakness that is recapitulated in transgenic mice expressing a dominant human mutation (T57I; Garvey et al. 2006). Importantly, MYOT null mice are normal; they show no obvious muscle pathology or weakness, Z-disk proteins are unaltered, and animals live a normal lifespan (Moza et al. 2007). These data suggest that there may be a compensatory mechanism to counteract MYOT deficiency in mice. Whether MYOT absence is well-tolerated in humans is unknown. However, because LGMD1A phenotypes are recapitulated in an available mouse model, and MYOT absence produces no overt defects in mice, it may be an ideal target to demonstrate proof-of-principle for RNAi therapy of dominant muscle disorders.

In contrast to myotilinopathies, which may be an exception, most dominant muscular dystrophies may require specific silencing of the dominant allele. As normal copies of disease genes likely encode essential proteins, normal allele haploinsufficiency may contribute to myopathic phenotypes as well. Loss-of-function contributions to dominant disease can be predicted from knockout mouse models and by examining genetic case studies, in which different mutations in the same gene give rise to dominant and recessive myopathies. For example, nemaline myopathy (NM) can arise from autosomal dominant or recessive TPM3 mutations (Corbett et al. 2005; de Haan et al. 2002; Durling et al. 2002; Ilkovski et al. 2008; Kee and Hardeman 2008; Laing et al. 1995; Lehtokari et al. 2008; Penisson-Besnier et al. 2007; Tan et al. 1999). Dominant NM patients have one mutant and one normal TPM3 gene copy, while human carriers of recessive alleles and TPM3+/− mice are normal, and TPM3−/− animals die as embryos (Lehtokari et al. 2008; Rethinasamy et al. 1998). These observations support two conclusions: only one normal TPM3 allele is required to maintain normal muscle, and gain-of-function TPM3 mutations are most likely the sole pathogenic event in dominant NM forms. Therefore, an RNAi strategy that specifically suppresses mutant TPM3 while leaving the normal allele untouched may improve myopathy in NM patients. Likewise, disease allele-specific RNAi therapies may be important for Caveolin-3-related myopathies, as normal Cav-3 gene dosage impacts muscle disease severity (Carbone et al. 2000; Galbiati et al. 2001; Minetti et al. 1998; Traverso et al. 2008). Specifically, severe LGMD1C is caused by autosomal recessive homozygous or dominant negative Cav-3 mutations resulting in complete or 97% Cav-3 loss. In contrast, different mutations resulting in 84 or 50% Cav-3 reductions produced mild hyperCKemia without muscle weakness, or normal phenotypes, respectively (Carbone et al. 2000; Galbiati et al. 2001; Minetti et al. 1998; Traverso et al. 2008). In both NM and LGMD1C examples, it would be advantageous to restrict gene knockdown to the affected allele while leaving the normal allele unperturbed. As many dominant myopathies are caused by single point mutations in one allele, the question arises: can inhibitory RNAs be designed to distinguish two transcripts differing by 1 base-pair? In short, the answer is yes. As previously discussed, perfect sequence complementarity between an inhibitory RNA and target mRNA causes message degradation; imperfect base-pairing leads to translational inhibition. However, this rule is not absolute. Complementarity does not ensure inhibitory RNA efficacy; not all inhibitory RNAs containing perfect homology with a target mRNA actually cause gene silencing. Conversely, more mismatch does not

Fig. 7.3 Hypothetical example of mutant allele-specific Cav-3 targeting. Wild-type (WT) and mutant T78K Cav-3 sequences are shown. The C to A Cav-3 mutation is located centrally within the miRNA sequence. This theoretical T78K-specific inhibitory RNA also contains a secondary peripheral mutation, as discussed in the text

necessarily reflect reduced potency; miRNAs can have several mismatches with a target mRNA and still cause gene silencing, but a single nucleotide difference may be sufficient to prevent silencing altogether (Kurosawa et al. 2005; Lewis et al. 2005; Miller et al. 2003, 2005; Rodriguez-Lebron and Paulson 2006; Schwarz et al. 2006). Thus, well-designed inhibitory RNAs can specifically silence disease genes by distinguishing between normal and mutant alleles differing by one nucleotide. Although each allele-discriminating miRNA must be uniquely designed and empirically validated, some general guidelines can be followed. Specifically, the discriminating nucleotide should be placed centrally within the inhibitory RNA duplex and if sufficient disease allele-specific silencing is not produced, optimal specificity can be achieved by including additional peripheral mismatches in the inhibitory RNA sequence (Fig. 7.3).

7.6 RNAi Therapy for the Most Common Dominant Muscular Dystrophies

DM1 and FSHD are among the top three most common muscular dystrophies and both are dominantly inherited. Therefore, DM1- and FSHD-targeted treatments would potentially have the broadest benefit for patients with dominant muscle disease, making them logical candidates for RNAi therapy development. However, both disorders have complicated and unique etiologies that make them challenging, though not impossible, targets for RNAi treatment.

7.6.1. Myotonic Dystrophy Type 1

DM1 is caused by CTG trinucleotide repeat expansion in the DMPK 3′ UTR, which causes nuclear retention of this toxic mRNA (Cho and Tapscott 2007). Patients develop myotonia leading to skeletal muscle weakness, and cardiac conduction

abnormalities that often cause death in patients. DMPK+/− and −/− knockout mice both show skeletal and cardiac muscle sodium channel gating abnormalities that recapitulate conduction defects in human DM1 (Berul et al. 1999; Lee et al. 2003a; Mounsey et al. 2000). Older (7–11 month) DMPK+/− mice also show mild, variable sarcomeric disorganization, myofiber regeneration, and decreased force production (Reddy et al. 1996). Together, these phenotypes support that DMPK haploinsufficiency may contribute to some DM1 pathologies, which could complicate RNAi therapy for several reasons: (1) wild-type and mutant DMPK alleles are identical except for the 3′ UTR trinucleotide repeat expansion. Therefore, therapeutic RNAi would theoretically knock down normal and mutant DMPK equally. Reducing normal DMPK could dampen beneficial effects caused by silencing the expanded mutant allele. (2) To target the mutant allele specifically, disease-linked polymorphisms, located outside the CTG repeat area, would have to be identified and (3) if possible, complete mutant DMPK knockdown would yield half the normal DMPK amount, potentially resulting in haploinsufficiency-related DM1 phenotypes. One strategy to circumvent these potential problems would involve knocking down endogenous mutant and wild-type DMPK and simultaneously delivering a normal DMPK cDNA engineered with base changes that prevent its regulation by the therapeutic miRNA. A final issue regarding the feasibility of DM1-targeted RNAi therapeutics relates to sub-cellular localization of RNAi processes and mutant DMPK. Mutant DMPK transcripts are nucleus-sequestered, but recent conventional thinking was that RISC-mediated gene silencing only occurred in the cytoplasm, raising doubts about whether RNAi therapy could work for DM1 (Lee et al. 2002). However, several recent studies demonstrated that nuclear RISC exists, and that RNAi can reduce nuclear-localized transcripts, including 7SK and, importantly, DMPK (Langlois et al. 2005; Ohrt et al. 2008; Robb et al. 2005; Weinmann et al. 2009). Therefore, RNAi therapy for DM1 is feasible, but some complicating factors, discussed above, may have to be addressed to make it a therapeutic option in humans.

7.6.2 Facioscapulohumeral Muscular Dystrophy

FSHD is an autosomal dominant disorder characterized by progressive and asymmetric weakness of facial, shoulder, and limb muscles. In 1992, FSHD was shown to arise from contraction of DNA repetitive elements (called D4Z4 repeats) on human chromosome 4q35 (Lemmers et al. 2007; van Deutekom et al. 1993; Wijmenga et al. 1990, 1992). Normal individuals have highly variable numbers (11–150) of D4Z4 repeats while FSHD patients have only 1–10. No genes are completely lost or mutated as a result of FSHD-associated DNA deletions. A widely-supported model for FSHD pathogenesis suggests that D4Z4 contractions alter normal chromatin structure at 4q35 leading to aberrant upregulation of genes in *cis* or *trans* (Gabellini et al. 2002, 2003). This subsequent overexpression could produce FSHD-associated muscle toxicity. Since the primary genetic mutation

was identified ~17 years ago, numerous studies have attempted to implicate specific genes in FSHD pathogenesis using global expression analysis (e.g. RNA microarray or proteomics) or by investigating candidate genes based on 4q35 proximity, such as DUX4, FRG1, FRG2, and ANT1 (Celegato et al. 2006; Dixit et al. 2007; Eisenberg et al. 2007; Gabellini et al. 2006; Laoudj-Chenivesse et al. 2005; Osborne et al. 2007; Reed et al. 2007; van Deutekom et al. 1993, 1996; Winokur et al. 2003). The former two are arguably the best FSHD candidate genes currently under investigation, but to date neither gene has been definitively linked to FSHD development in humans. Therefore, RNAi therapy may soon be a valuable therapeutic approach for FSHD as more definitive data implicating specific genes in FSHD pathogenesis accumulate.

7.7 Summary and Future Direction

RNAi therapeutics is an emerging field. Several preclinical studies demonstrated its immense potential for treating dominant neurodegenerative diseases, chronic viral infection, and cancer. Likewise, RNAi may be a leading candidate strategy to treat dominant muscular dystrophies and other myopathies. Although currently in its infancy as a technology, its potential for disease allele-specificity may someday allow RNAi therapeutics to be a tool for personalized medicine.

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Chapter 8
Combinatorial Gene Therapy Strategies for Treating Muscular Dystrophies

Catherine E. Winbanks and Paul Gregorevic

Abstract Muscular dystrophies are commonly associated with progressive loss of muscle mass and strength as a consequence of ongoing myofibril degeneration and wasting. Genetic therapies have been proposed as interventions for disorders where specific monogenic mutations have been linked to the origin of disease. For muscular dystrophies of this nature, a "single-gene therapy" intended to introduce a surrogate "gene" in lieu of the defective endogenous copy (or to ablate a dominant negative state) may prove sufficient to prevent or reverse the development of disease. However, the increasingly severe morphological disruption and depletion of functional muscle fibers observed with disease progression may prove to be difficult to halt or reverse completely depending on the severity of the condition at the time of treatment. Consequently, it may be advantageous to consider coadministration of additional genetic interventions that address not only primary genetic defect, but also exert beneficial effects via other means that minimize degeneration, enhance muscle function, and promote muscle regeneration. Using Duchenne muscular dystrophy (DMD) as a representative neuromuscular condition, this chapter will discuss the potential benefits of combining genetic interventions to prevent or reverse the loss of muscle function, and also treat the primary genetic defect.

8.1 Introduction

Like a number of severe muscular dystrophies, the disease features of Duchenne muscular dystrophy (DMD) arise from a single genetic defect but the effects contribute to the development of a complex pathophysiological phenotype. Mutations in the dystrophin gene that ablate expression of the 427 kDa dystrophin protein in the muscle are considered to be the origin of the disease in DMD (Kunkel and Hoffman 1989). However, a deficiency of dystrophin increases the susceptibility of the muscle fibers to

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mechanical strain injury, and to metabolic perturbation during contraction via direct intracellular changes, as well as impaired vasoactive regulation compromising blood flow (Tidball and Wehling-Henricks 2007; Kobayashi et al. 2008). With the ongoing muscle fiber damage and remodeling occurring as a consequence of these mechanisms, muscles frequently exhibit evidence of an overly active inflammatory system, deleterious accumulation of fibrotic and adipose content, and perturbation of regenerative processes that promote muscle fiber repair via recruitment of satellite cells. All these processes contribute to the morphological and functional deficiencies that are hallmarks of this disease. When these combined features of the DMD phenotype are considered, it becomes apparent that restoration of dystrophin expression in the remaining muscle fibers may not necessarily resolve all of the facets of disease progression, much less reverse the loss of myofibers or the perturbation of regenerative processes that occurred prior to its intervention. Thus, in the absence of appropriately early single-gene interventions that prevent the development of secondary effects, combinatorial approaches may be warranted to address specific disease attributes. This scenario has been demonstrated in the treatment of dystrophic mice with recombinant vectors carrying dystrophin-based gene expression cassettes, whereby timely expression of dystrophin "micro-genes" prevents significant progression of the disease state when administered early in life (Harper et al. 2002; Watchko et al. 2002; Reay et al. 2008), whereas the same intervention administered to much older (>20 m) mice exerts markedly reduced (albeit still valuable) benefits upon muscle mass and function (Gregorevic et al. 2008). Other muscular dystrophies may be attributable to different genetic defects and exhibit unique disease features, which require a different primary intervention strategy, but loss of muscle mass and function is a unifying characteristic of most neuromuscular disorders, and therefore coadministration of secondary interventions that enhance muscle function may be complementary across a number of conditions. For instance, myotonic dystrophies are associated with triplet repeat expansions in the DMPK genes, which create a "toxic" dominant negative transcript that sequesters RNA-binding proteins and regulate efficient transcript splicing and translation (Mankodi et al. 2001; Lin et al. 2006). Compared with DMD, these conditions require a different primary approach to treatment, which blocks generation of the aberrant transcript or its sequestration of RNA binding components (or both events). However, both dystrophies result in a significant loss of muscle mass and strength that could conceivably be treated with an identical intervention designed to promote myofiber hypertrophy. Thus, the simultaneous administration of several genetic interventions that target the consequences of dystrophin deficiency (or another primary defect) may therefore be particularly advantageous to enhance muscle function.

8.2 Combining Interventions to Restore Subcellular Structural Organization

In dystrophies such as DMD, most of the secondary pathology is attributed to the consequences of the initial destabilization of the highly ordered dystrophin–glycoprotein complex and the dispersal or loss of the various dystrophin-associated

proteins from the myofiber/myofibril sarcolemma (Batchelor and Winder 2006). Logically then, many of the genetic interventions devised to combat this condition have focussed on restoring the expression of dystrophin within affected muscles. Tireless work by many groups has demonstrated that the restoration of dystrophin in transgenic animals or via vector-mediated expression cassette delivery can ameliorate features of the dystrophic pathology (Gregorevic and Chamberlain 2003). However, strategies employing several of the more efficient platforms for postnatal gene delivery (typically recombinant viral vectors) have necessitated the configuration of miniaturized dystrophin-based genes which can be efficiently packaged and delivered in vector systems of limited carrying capacity; yet still generate a protein with significant dystrophin-like functionality. Though the findings from studies using these miniaturized dystrophin genes have been exciting and no doubt encouraging, there remains the possibility that genes that are larger than that the delivery system can carry must be manipulated, and the omission of genomic portions from these constructs to achieve a desirable size may consequentially lose sequences that encode the domains involved in protein–protein interactions and signaling. In this regard several strategies involving the coadministration of genetic interventions may hold merit for the treatment of structural defects associated with severe dystrophic conditions.

One of the best developed combinatorial intervention approaches devised to combat the effects of dystrophin deficiency concerns the codelivery of complementary dystrophin-gene expression cassettes which interact in vivo to facilitate translation of a larger protein than could be generated by a single expression cassette (Ghosh et al. 2008; Li et al. 2008). As reviewed extensively elsewhere in this book, the codelivery of two or more vectors harboring complementary portions of an expression cassette can be utilized to coerce homologous recombination, or trans-splicing in vivo, to produce a functional transgene product. This approach has been used to achieve the expression of larger dystrophin-based transgenes that have added functionality compared with shorter designs necessary for single vector packaging (Ghosh et al. 2008; Li et al. 2008). Efficiency of multiconstruct translation depends on the careful design of expression cassettes to facilitate correct interaction, but results to date have shown considerable promise as an approach to achieve expression of constructs that exceed the packaging limitations of a single vector system.

Restoration of appropriate structural organization in dystrophic myofibers may alternately utilise the codelivery of expression cassettes encoding proteins that function in lieu of the principally deficient protein. In the case of DMD, increased expression of utrophin has been proposed as a means of compensating for dystrophin deficiency (Deconinck et al. 1997; Tinsley et al. 1998). This protein is widely expressed in the muscle during embryogenesis but is constrained typically to the neuromuscular junction subsequent to increased dystrophin expression after birth. Transgenic and vector-based studies of dystrophic mice have demonstrated that elevated utrophin expression may ameliorate at least some of the pathological aspects associated with dystrophinopathies (Deconinck et al. 1997; Tinsley et al. 1998; Gilbert et al. 1999; Odom et al. 2008). Whether codelivering dystrophin and utrophin constructs might help or hinder in the treatment of DMD has not been studied.

However, the utrophin gene is comparable in size to the dystrophin gene, which therefore presents many of the same challenges concerning successful construct packaging and delivery in vivo via the established viral and nonviral delivery vectors (Odom et al. 2008). An interesting alternate strategy entails the administration of expression cassettes encoding for transcription/translation regulators that promote the expression of endogenous genes. Various iterations of novel transcriptional regulator constructs have been shown to target the endogenous utrophin promoter region and increase expression of the utrophin protein in the muscle (Mattei et al. 2007; Lu et al. 2008). Expression of these transcriptional factors in transgenic mice, or via viral-vector mediated delivery has been shown to enhance the morphological and functional properties of dystrophic muscles (Mattei et al. 2007; Lu et al. 2008). Critically, these constructs are conceivably very small compared with constructs intended to deliver the dystrophin or utrophin genes themselves, thereby offering the possibility of highly efficient packaging and improved delivery in vivo. Given that protein translation is both a function of transcriptional activity and translational efficiency, a complementary approach may be to simultaneously utilise interventions that facilitate increased translation of utrophin (or similarly functioning genes). Naturally occurring microRNAs have been implicated in the regulation of translational efficiency in a broad range of cell types, according to a process involving the engagement of a mRNA sequence-specific "RISC" silencing complex that can degrade transcripts or stall their translation (Eisenberg et al. 2009). The muscle enriched miRNA-206 has been determined to directly target utrophin, causing downregulation of its expression (Rosenberg et al. 2006). Conceivably, an intervention that can inhibit the interaction of miRNA-206 with utrophin mRNA may potentiate utrophin expression to a degree that can confer phenotypic benefits in dystrophic muscles. However this approach may exert complex phenotypic effects in vivo, as most miRNAs have the ability to target many genes simultaneously and the perturbation of miRNA-206's actions in the muscle may trigger a range of other unanticipated effects that may not necessarily be advantageous (Eisenberg et al. 2009).

A different approach to potentially compensating for structural disorganization within DMD muscle fibers concerns the codelivery of expression cassettes encoding for key proteins associated with the dystrophin–glycoprotein complex, and its interacting partners both within and outside the myofiber. Mechanical forces generated by the muscle fiber under contraction or applied under physical loading are disseminated through the muscle membrane and the extracellular matrix via the dystrophin-associated glycoprotein complex (DGC)] and associated binding proteins (Batchelor and Winder 2006). A considerable body of work has already established that the deficiencies of specific proteins are associated with distinct forms of muscular dystrophy, but there is also growing evidence that the expression of particular proteins may also contribute in part to maintaining muscle structure and function in the absence of dystrophin (Cohn and Campbell 2000; Ervasti 2007). Notably, members of the integrin family have been shown to exacerbate the phenotype of dystrophin-deficient striated muscles when also deleted (Rooney et al. 2006; Elsherif et al. 2008), whilst their over expression has been able to ameliorate aspects of the dystrophic state to a

degree (Burkin et al. 2001, 2005), though not equally effectively for all disease models. In the context of DMD, codelivery of an integrin expression cassette with a dystrophin-based construct may augment the effects of each in an additive or even synergistic manner. This approach may also hold true for other DGC proteins, as it has been shown that ablation of even very low levels of certain sarcoglycans from the muscles of dystrophin-deficient mice can markedly aggravate the dystrophic condition (Li et al. 2009). Whether the increased expression of these proteins or others (Rezniczek et al. 2007) in conjunction with a dystrophin-based approach remains unknown. In contemplating which structural proteins might be appropriate to express more strongly, strategies need to be evaluated on a disease-by-disease basis. As a demonstration, it has been noted that the expression of alpha-7 integrin did not recapitulate the phenotypic improvements observed in dystrophin deficient mice when applied to mice that lack delta-sarcoglycan in a fashion designed to model human limb girdle muscular dystrophy 2F (Milner and Kaufman 2007). An interesting possibility not yet explored in depth also concerns the idea of configuring expression cassettes to encode for key structural proteins engineered to include additional binding regions that may add to their functionality. In the case of DMD, the intactness of the DGC structure is thought to rely on the localization of the dystrophin protein. Potentially, strategic engineering of additional binding regions into the existing conformation of various DGC members (which are subsequently expressed following gene delivery) may provide a means of supporting aspects of DGC assembly and functional interactions even if a primary intervention consisting of a miniaturized dystrophin-based construct fails to restore the native structure optimally. Little has been established concerning the validity of such an approach for DMD at this stage, but it is hoped that future studies will explore this concept further.

As reviewed in brief earlier, other forms of muscular dystrophy may require a different complementary approach to address the primary defect depending on the mechanisms underlying the disease. In the case of the myotonic dystrophies, the generation of mutant transcripts containing extra triplet repeats creates a structure for sequestering proteins such as splicing regulators, thereby compromising the myofibers' abilities to appropriately express specific isoforms of important structural proteins (Wheeler and Thornton 2007). Using gene delivery technologies to introduce several cDNA-based expression cassettes may provide a means of restoring the expression of the necessary protein isoforms in a manner that does not rely on accurate splicing control. However more elegant and effective solutions may be achieved in combining approaches that inhibit the formation of the errant transcripts, restore correct splicing of key transcripts, or by increasing expression of the RNA binding proteins themselves such that they are supplied in quantities that enable them to undertake their typical roles in spite of ectopic sequestration also taking place (Kanadia et al. 2006; Wheeler et al. 2007). Groups are working on all three of these concepts, and have shown promising early data, especially with regards to ameliorating aspects of the disease by increasing expression of proteins such as MNBL1 to compensate for their sequestration by toxic transcripts. Combining interventions may yield even more promising outcomes in time.

Reviewing the various strategies that could be employed to restore the subcellular architecture in dystrophin-deficient muscles, it is apparent that there is significant scope to explore the coadministration of expression cassettes for combinations of structural proteins as a means of improving muscle structure and function. Especially in muscles where disease progression is significant, combining interventions may be a legitimate approach to quickly halt further deterioration of the condition, or to stand the best chance of ameliorating the disease. However, in circumstances where significant loss of muscle fibers or muscle mass has taken place, incorporating interventions designed to enhance muscle function may also be warranted.

8.3 Promotion of Muscle Fiber Hypertrophy

Given that degenerative neuromuscular disorders lead to a progressive loss of muscle fibers, one strategy to enhance muscle function is to increase the force producing capacity of the remaining muscle fiber populations within affected muscles, using interventions that promote muscle hypertrophy. In older, more affected patients whose muscles might conceivably comprise fewer than half the number of muscle fibers compared with the muscles of an unaffected sibling, such an approach may reduce the deficiency in force producing capacity by enabling each muscle fiber to generate increased force. It should be kept in mind that promoting an increase in the size and contractile capacity of dystrophic muscle fibers may be counterproductive in the absence of a primary intervention when the mechanisms of the disease include an increased susceptibility to mechanical injury (such as in DMD). However as a complementary strategy supporting a primary intervention targeting the primary defect implicated in muscle fiber degeneration (e.g. dystrophin gene replacement in DMD), the controlled promotion of myofiber hypertrophy is an exciting prospect to enhance muscle function.

One approach being explored as a means to promote muscle fiber hypertrophy is the locally elevated expression of insulin-like growth factor-I (IGF-I). This growth factor is synthesized by skeletal muscle and is particularly abundant in response to muscle stress, regeneration and repair (Marsh et al. 1997). IGF-I exerts its effects upon muscle fiber size by interacting with the membrane-bound IGF-I receptor to initiate PI3K/Akt/mTOR–mediated signaling which positively influences protein transcription and translation (Russell-Jones et al. 1994; Stitt et al. 2004). Additionally, IGF-I mediated signaling appears to inhibit the expression of muscle-specific ubiquitin ligases that are associated with muscle protein breakdown (Bodine et al. 2001). Several groups have observed increased muscle fiber size and function following IGF-I peptide administration, or via transgenic IGF-I over-expression in mice (Vlachopapadopoulou et al. 1995; Musaro et al. 2001; Barton et al. 2002; Song et al. 2005; Gehrig et al. 2008). However, IGF-I stimulates pathways that are associated in cancer development and progression, making a muscle-targeted genetic approach more attractive from a safety perspective

(Rommel et al. 2001). Fewer studies have considered the effects of postnatal genetic interventions to increase IGF-I expression in the muscle (Barton-Davis et al. 1998; Abmayr et al. 2005; Schertzer and Lynch 2006; Schertzer et al. 2008). Outcomes vary between studies considering the effects of IGF-I depending on the mode of action used to increase local IGF-I levels, and the nature of the disease model used, although there is a general trend towards increased functional capacity and interestingly, also the potential for increased fatigue resistance under repeated contraction. The variability in findings may reflect the complexity of IGF-1 signaling in vivo as much as reflect on the different experimental models used (Shavlakadze et al. 2005). However, it appears that the timing of IGF intervention also plays a role in determining beneficial outcomes. Increasing IGF-I expression early in life (typically preceding significant muscle degeneration) elicits more substantial improvements in muscle strength and morphology (Barton et al. 2002; Shavlakadze et al. 2004), whereas interventions provided at a later point of time exert lesser effects on myofiber/myofibril damage and turnover (Abmayr et al. 2005). Some groups propose that this may be related to a declining sensitivity of muscle satellite cells to activation over lifespan and disease progression, as these cells play a critical role in the formation of new muscle fibers, and are also recruited into the existing muscle fibers to promote hypertrophy. These and other findings may indicate that the merit of IGF-I based interventions is greatest when prescribed early in disease progression.

As an alternate strategy to promote muscle hypertrophy, considerable interest has centered on the inhibition of myostatin (or GDF-8), a member of the TGF-β super family. Myostatin is secreted by muscle, and initiates a host of SMAD-dependent signal transduction responses subsequent to interaction with membrane-bound activin receptors. Much like other TGF-β family members, the full scope of its mechanism of action remains unclear. Mutations of the myostatin gene increase the number and size of the muscle fiber during embryogenesis, creating a hypermuscular phenotype in a range of mammals including humans (Grobet et al. 1997; Kambadur et al. 1997; McPherron et al. 1997; McPherron and Lee 1997; Schuelke et al. 2004; Mosher et al. 2007). Several studies have noted that crossing a myostatin-null genotype to a mouse model of neuromuscular disease may enhance muscle function, although it appears that some disease states may even be exacerbated by this intervention (Wagner et al. 2002; Li et al. 2005). This highlights the need to carefully consider the role of prospective interventions as part of a combination therapy in conjunction with an intervention to target the primary defect, and the mechanisms underlying each particular disease. Efforts to therapeutically inhibit myostatin have stimulated the development of soluble myostatin receptors, dominant-negative dimerizing propeptides, endogenous and artificial binding proteins, and blocking antibodies (Zhu et al. 2000; Lee and McPherron 2001; Bogdanovich et al. 2002; Whittemore et al. 2003; Lee et al. 2005; Bartoli et al. 2007). Most of these reagents have been tested upon dystrophic-mouse models, although the effects have varied significantly. Typically, increases in muscle mass and muscle fiber size are observed to some degree, although as with IGF-I the age of the animal (and therefore the severity of the disease at the time of treatment) appear to negatively

influence the scope for phenotypic benefit (Ohsawa et al. 2006; Parsons et al. 2006). In some instances, benefits observed do not necessarily delay premature death associated with the disease model (Holzbaur et al. 2006) which demonstrates that where disease progression and prognosis is influenced by the severity of architectural disruption, myostatin based interventions which don't always affect muscle structure may not always be indicated.

In comparing myostatin-targeting interventions, it should be considered that the active component of myostatin protein shares significant amino acid sequence homology with other TGF-β family proteins, known to act upon nonmuscle cells (such as GDF-11). Consequently, systemically circulating myostatin inhibitors may present a risk of generating off target effects in other cell types. To this end, muscle-directed genetic treatments may provide a more appropriate way of influencing in vivo effects of myostatin in the muscle. Genetic strategies that have shown potential for reconfiguration into vector-mediated delivery so far include the local expression of the myostatin propeptide, and various native and mutant peptides that can bind and inhibit myostatin (Bartoli et al. 2007; Haidet et al. 2008; Nakatani et al. 2008; Qiao et al. 2008). Different approaches have been explored with varying degrees of beneficial outcome, although in general it appears that the approaches hold considerable potential for promoting muscle growth. Nevertheless, the validity of each approach needs to be considered in the context of the disease of interest, as interventions can sometimes achieve increases in muscle mass and strength, yet still fail to significantly enhance lifespan. One such example is AAV-mediated delivery of follistatin, which was shown to increase muscle mass (presumably through its effects upon myostatin), fiber number and diameter in an ALS model of muscle wasting, but did not enhance the survival (Miller et al. 2006). Comprehensive studies need to be carried out to fully elucidate the potential effects (therapeutic and otherwise) of these prospective interventions to establish their value in the treatment of muscular dystrophies.

8.4 Reducing Inflammation and Oxidative Stress in Muscular Dystrophy

Because primary defects in muscular dystrophies often contribute to increased incidence of myofiber/myofibril damage and degeneration, elevated activity of the acute inflammatory response is a prevalent attribute of dystrophic muscles. It is well established that a functioning inflammatory cell population is essential for timely elimination of the damaged and degenerating muscle fibers prior to initiation of myofiber/myofibril regeneration. However, it is also clear that overactive inflammatory cell responses may adversely affect muscle structure and function in severe dystrophies (Nguyen and Tidball 2003a; Tidball and Wehling-Henricks 2005; Dudley et al. 2006). In particular, the local generation and release of reactive oxygen species as a consequence of inflammatory cell activation within dystrophic muscles imposes increased oxidative stress upon myofibers, which in conjunction

with metabolic stress as a consequence of compromised microvascular circulation can increase the potential for further myofiber/myofibril damage (Tidball and Wehling-Henricks 2007).

In regard to reducing inflammation and oxidative stress in dystrophic muscles, the role of nitric oxide synthase (NOS) and its production of nitric oxide (NO) have received considerable attention. In DMD, the loss of dystrophin is associated with reduced levels of neuronal NOS in muscle (Brenman et al. 1995; Kasai et al. 2004), which in turn is thought to potentially contribute to increased oxidative stress (Brenman et al. 1995). Furthermore, because NO acts as a vasodilator, its loss in muscular dystrophy can render vessels less responsive to vasodilatory mechanisms leading at least to reduced exercise performance, and at worst, damage-inducing ischemia (Thomas et al. 1998; Sander et al. 2000; Dudley et al. 2006; Kobayashi et al. 2008). Importantly, the reduction of NO levels appears to reduce the anti inflammatory signals within dystrophic muscles, which may cause undesirable escalation of acute inflammatory processes subsequent to muscle damage (Wehling et al. 2001; Wehling-Henricks et al. 2005). Analyses of dystrophic mice bred to express a nNOS transgene in muscle demonstrate decreases in macrophage activity, and circulating levels of muscle enzymes, indicative of reduced muscle degeneration (Wehling et al. 2001; Nguyen and Tidball 2003b), as well as improvements in neuromuscular junction organization (Shiao et al. 2004). Comparatively little has been established concerning the use of vector-based NOS expression in muscles, although it is exciting to consider that such an approach could potentially recapitulate the elevated expression of NOS in a postnatal intervention strategy. Recent developments in dystrophin expression cassette design have established a configuration that can restore NOS expression levels in dystrophic muscles subsequent to dystrophin expression (Lai et al. 2009). However, a coadministration approach to elicit increased NOS levels may yet prove even more effective in managing pathological aspects of severe dystrophies, including those where NOS levels are not reduced as a result of the primary defect.

Also of interest as a strategy to reduce local inflammation and metabolic stress within muscles, is the attenuation of tumor necrosis factor (TNF) levels. In dystrophic muscles featuring ongoing muscle degeneration, increased TNF levels are thought to negatively affect muscle structure and function by several mechanisms (Grounds et al. 2008). Most prominent of these is the role of TNF to contribute to elevated inflammatory cell accumulation and activity, with the aforementioned consequences for increased breakdown of myofibers (Hodgetts et al. 2006). However, TNF (and other proinflammatory cytokines) are increasingly being regarded as potential negative regulators of signal transduction events that are associated with the control of protein synthesis and breakdown within myofibers themselves (Grounds et al. 2008). Thus TNF is gaining more attention as a prominent player in the pathology of degenerative dystrophic states. Dystrophic mice cross-bred with TNF-knockout animals have been shown to exhibit reduced evidence of local inflammation, with a consequent improvement in muscle morphology and function (Gosselin et al. 2003). These data have supported investigations into intervention-based strategies to reduce TNF levels. Based on initial findings in other forms of inflammatory diseases, several

studies have reported encouraging evidence of reduced inflammation and myofiber/myofibril necrosis in mouse models of muscular dystrophy following the administration of either TNF-specific antibodies or doses of soluble TNF receptor (Hodgetts et al. 2006; Pierno et al. 2007; Radley et al. 2008). These interventions have established the potential for benefit in controlling TNF levels but utilize systemically circulating agents that could conceivably exert nonmuscle effects as well. With regards to a genetic approach that might sustainably reduce TNF levels in skeletal muscles in a constrained fashion, less has been accomplished presently. Other groups have developed recombinant viral vectors that express soluble TNF receptor subunits for the prospective treatment of inflammatory conditions, with promising early outcomes (Sandalon et al. 2007). These approaches could be adapted for the treatment of muscular dystrophies, whereby a muscle-selective vector platform carrying a TNF receptor expression cassette under the control of a regulatable muscle-specific transcriptional promoter/enhancer configuration could afford controlled muscle-localized expression.

8.5 Improving Muscles' Fatigue Resistance

Various forms of muscular dystrophies frequently exhibit increased susceptibility to fatigue during repeated recruitment/contraction (Frascarelli et al. 1988; Wineinger et al. 1998). Reduced fatigue resistance may be a consequence of the reduced number of functional fibers present within a given muscle being tasked with a comparatively greater work requirement compared with the myofiber/myofibril population in a non dystrophic muscle, although increased inflammatory cell activity and altered metabolic status also appear to contribute to this deleterious phenotype. Therefore, intervention strategies with the potential to reduce the susceptibility of muscles to fatigue could be valuable in increasing/sustaining muscle function. One component of muscle fatigue is the role of oxidative stress upon ATP production. As mentioned earlier, supplementation of muscle NO levels via increased expression of NOS could be a viable approach to enhance fatigue resistance. Several groups have shown that nNOS deficient mice (dystrophic and otherwise) exhibit reduced force-producing capacity with repeated contraction (Kobayashi et al. 2008; Percival et al. 2008). Possible mechanisms by which increased NOS expression may reduce fatigue relate to cGMP stimulated mitochondrial biogenesis (in the longer term), and also through enhancing cGMP-based signaling that contributes to the vasomodulation of the muscle's circulatory system (Nisoli et al. 2003, 2004; Kobayashi et al. 2008). That pharmacological restoration of cGMP-mediated signaling can counter the increased susceptibility to fatigue observed in dystrophic muscles supports this argument, and serves as justification for further investigation into the potential to ameliorate fatigue susceptibility in dystrophic muscles via interventions that can increase NOS expression (Kobayashi et al. 2008).

Interestingly, although IGF-I has been traditionally regarded as a promoter of skeletal muscle hypertrophy, several studies have observed that IGF-I based interventions can also increase the oxidative metabolism of dystrophic muscles, and

increase contractile capacity over the course of a repetitive recruitment test (Gregorevic et al. 2004; Schertzer et al. 2006). The mechanisms associated with this effect of IGF-I expression remain a point of continued investigation, but reflect the complexity of biological effects exerted by a single factor depending on its mode of administration. Improving fatigue resistance of existing muscle cells by using combined IGF and NOS therapy would also have the beneficial consequences of improving aspects of muscle strength, and reducing oxidative stress and inflammation, thereby highlighting the potential of codelivery based strategies.

A different intervention that could enhance the abilities of muscles to sustain contractile capacity with repeated exertion may be based around the cellular mechanisms influenced by the expression and activation of the peroxisome proliferator activated receptor (PPAR) family of transcription regulators. Of the various PPAR isoforms, PPAR-delta is particularly strongly expressed in the skeletal muscle, and especially in the more fatigue-resistant and oxidative metabolism-favoring type I fiber population (Wang et al. 2004). PPAR-delta null mice have been shown to exhibit reductions in oxidative metabolism which correlate with decreased running performance in a treadmill-based assay (Wang et al. 2004). Transgenic mice expressing an activated PPAR-delta construct, and animals administered a PPAR-delta activating agonist have been shown to demonstrate varying degrees of increasingly induced gene expression associated with a "fast-to-slow" muscle fiber type transformation which encompasses increased oxidative metabolism and importantly, considerably improved fatigue resistance under exercise (Wang et al. 2004; Narkar et al. 2008). Animals that express increased levels of the PPAR coactivator peroxisomes-proliferator-activated receptor-gamma coactivator 1 alpha (PGC1-alpha)-1a have also been shown to exhibit increased fatigue resistance attributes, further substantiating the importance of the PPAR-associated mechanisms as key regulators of metabolism and muscle fiber phenotype (Lin et al. 2002; Handschin et al. 2007a). So potent are some of the adaptations observed following stimulation of the PPAR-mediated processes, that it has been proposed that PPAR-delta agonist interventions may even be able to impart some of the benefits of regular endurance exercise without the need for the exercise component itself (Narkar et al. 2008). Such a statement should be qualified by acknowledging that exercise adaptations affect multiple organs as well as the nerve–vasculature–tendon–muscle interactions, though it is plausible to suggest that at least a degree of functional benefit could be derived via an intervention designed to increase the activation of PPAR-delta in the muscles of dystrophic mice and patients. Further investigation is required to explore this fascinating possibility, and also the prospects of further enhancing muscle function by combining interventions that could enhance fatigue resistance.

8.6 Reducing Muscle Fiber Degeneration and Atrophy

In dystrophic muscles, excessive mechanical strain and/or sustained metabolic challenge by virtue of local inflammation and oxidative stress contribute to the damage of muscle fibers. However a number of studies have also begun to establish

a basis for the existence of growth regulating signaling processes that operate through or are reliant upon, interactions with the DGC. Taken together, these findings suggest interventions that inhibit the degeneration/atrophy of muscle fibers may be valid intervention to consider when devising a multifactorial approach to the treatment of muscular dystrophies.

Calpains are a family of nonlysosomal calcium-dependent proteases which are thought to cleave particular muscle proteins subsequent to sustained elevations in cytosolic calcium, which directs ensuing degradation by the proteasome. A degree of calpain activity may be important for timely regulation of sarcomeric protein turnover to ensure that partly damaged proteins are eliminated from the sarcomeric structure (Beckmann and Spencer 2008). However, dysregulation of specific calpains may contribute to excessive or inappropriate protein degradation in some forms of muscular dystrophy (Vainzof et al. 2003), and perturb other signaling pathways in a manner which may disrupt maintenance of cell size and function (Baghdiguian et al. 1999). Efforts to attenuate calpain activity in the muscles of dystrophic mice via administration of calpain-inhibiting compounds, or by transgenic overexpression of calpastatins have generally demonstrated the potential to reduce the evidence of muscle necrosis and myofiber/myofibril turnover, although results vary between studies depending on the mode of intervention, the condition of the animals, and the types of muscles studied, and the indices used to evaluate benefit (Spencer and Mellgren 2002; Burdi et al. 2006). The results typically support the concept of calpain inhibition as potentially therapeutic, although it should be remembered that complete ablation of calpain activity would likely also prove deleterious in skeletal muscles, and a muscle-focused intervention that could provide regulation of calpain activity could be a preferable longer-term strategy.

An emerging strategy to counter the loss of muscle mass and function in muscular dystrophy concerns that of regulating the activity and expression of PGC1-alpha. In forms of muscle wasting associated with nondegenerative myofiber/myofibril atrophy, significant transcriptional repression of this transcription coactivator has been observed (Sandri et al. 2006). Efforts to increase PGC1-alpha expression in vivo using transgenic mouse cross breeding have established that elevated PGC1-alpha levels can inhibit models of muscle wasting and atrophy (Sandri et al. 2006). The mechanisms of action underlying these results are likely to be associated with multiple effects, although the repression of muscle-specific ubiquitin ligases that facilitate the proteasomal degradation of particular muscle proteins is prominently involved. Interestingly, it appears that the muscles of dystrophin-deficient mice exhibit altered transcriptional profiles consistent with dysregulation of a subset of the genes influenced by PGC1-alpha (Handschin et al. 2007b). Moreover, transgenic expression of PGC1-alpha in dystrophin deficient mouse muscles has been shown to improve the histological attributes of muscles, and reduce serum creatine kinase levels (indicating reduced muscle fiber breakdown) (Handschin et al. 2007b). Improvements in muscle function observed with PGC1-alpha expression in dystrophic mice may in part be attributed to changes in the fiber type composition of treated muscles similarl to that discussed in the context of PPAR-delta, as the two factors coordinately interact to regulate transcription of specific genes associated

with fiber phenotype. Whether increased expression of PGC1-alpha and simultaneous activation of PPAR-delta may have synergistic effects upon dystrophic muscles by way of reducing degeneration and enhancing fatigue resistance is less clear but exciting to speculate upon. That stated, caution should be exercised in interpreting the appropriate course of PGC1-alpha related intervention, as there is evidence that its overexpression can lead to significant mitochondrial uncoupling and ATP consumption with deleterious effects (Miura et al. 2006). These findings again support the concept that preferred intervention strategies would be well served to incorporate mechanisms for controlling or regulating transgene expression, in order to "titrate" expression to the appropriate level for optimal therapeutic outcomes. Nonetheless, proof of this concept has been established to support the development of interventions that can reduce the incidence of myofiber/myofibril degeneration and wasting in forms of muscular dystrophy. Conceivably, incorporating one or more of these approaches into a cohesive therapeutic strategy may augment the prognosis of halting and/or reversing loss of muscle mass and functional capacity for severe muscular dystrophies, especially if administered early in the disease progression.

8.7 Enhancing Muscle Regeneration

In mouse models of degenerative muscular dystrophies, and in the muscles of patients themselves, it is clear that myofiber/myofibril breakdown and progressive depletion with inadequate regeneration are key contributors to the loss of muscle mass and strength. Satellite cells residing between intact muscle fibers are tasked with the responsibility of repeatedly creating populations of myoblasts that will ultimately form replacement myofibers. However, in dystrophic muscles where myofiber/myofibril turnover is significant and frequent, various groups have suggested that satellite cells may become depleted, or less capable of sustaining the production of myogenic progeny. In these scenarios, prospective interventions that can enhance muscle regeneration by promoting satellite cell activation or expansion of the satellite cell pool within dystrophic muscles are extremely valuable as a means of halting or reversing the loss of functional muscle mass.

Interventions intended to enhance the regenerative potential likely need to target the satellite cells (or progeny) themselves, or must be directed to neighboring cells (e.g. mature myofibers), which in turn will secrete appropriate regulatory factors that can act upon the myogenic cell pool. This is an important consideration, as many of the most established gene delivery technologies typically transduce quiescent satellite cells in vivo with low efficiency compared to the adjacent cells. Moreover, because satellite cells undergo cycles of mitotic activity in response to myofiber damage, gene delivery to the satellite cells will become diluted with cell division unless the gene delivery entails integration of the expression cassette into the genome of satellite cells and therefore enables continued effects in the descendent cell populations. Most often, these challenges have been circumvented by transducing

myogenic cells harvested from muscle samples with an integrating viral vector configuration, prior to injecting the treated cells into recipient muscles. While this is a comparatively efficient mode of achieving sustainable gene transfer into satellite cells, the approach is not without its own challenges, which are particularly concerned with sourcing appropriate quantities of cells to treat, and then achieving successful dissemination and survival of the treated cell population upon transplantation. Therefore, one strategy to improve the regenerative potential of dystrophic muscles is to administer interventions to the recipient muscles that may "condition" the environment to enhance efficient engraftment of transplanted cells.

A number of interventions that promote myofiber hypertrophy have been shown to induce a program of transcriptional expression that also facilitates the secretion of factors which recruit satellite cells. These factors may also play roles by acting upon satellite cells directly. Using interventions to increase IGF-I levels and to ablate myostatin signaling as mentioned earlier, have both shown promise as methods of enhancing satellite cell recruitment in vivo both from endogenous pools and also from introduced cells (Chakravarthy et al. 2000a, b; Benabdallah et al. 2005, 2008; Schertzer et al. 2007). IGF-I has been shown to enhance local cell populations expressing stem cell markers (Engert et al. 1996) and guide the homing of bone marrow-derived cells to muscle niches (Musaro et al. 2004). Some groups suggest that IGF-I may act preferentially on activated as opposed to quiescent satellite cells, which could explain discrepancies in the degree of improvement observed in animals of different age and disease severity following IGF-I treatment (where younger animals with larger and more sensitive satellite cell pools appear to derive greater benefit).

With regards to promoting expansion of the satellite pool in vivo, inhibiting the actions of myostatin upon satellite cells has also proven worthy of investigation. Active myostatin binds as a ligand to the activin receptor 2B (Acvr2b), a subclass of the TGF-ß type II (TbRII) receptor, which then heterodimerises with the TbRI subclasses ALK 4 or ALK 5 which are predominantly expressed by satellite cells or myoblasts moving through proliferative and differentiation phases. Formation of this trimeric complex then engages the downstream Smad2 and Smad3 cytoplasmic proteins, which undergo phosphorylation, complex with Smad4 and translocate to the nucleus. This has the effect of blocking MyoD activation [which plays a key role in the differentiation of muscle cells (Langley et al. 2002; Bradley et al. 2008)], which means that myostatin can inhibit proliferation (Thomas et al. 2000). In addition, noncanonical TGF-ß signaling pathways including Erk/MAPK and p53 have been implicated in the negative regulation of proliferation, differentiation and resistance to apoptosis (Rios et al. 2002; Joulia et al. 2003; Philip et al. 2005; Yang et al. 2006). These findings may in part explain why myostatin knockout mice possess increased myofiber numbers [i.e., added proliferation during embryonic myogenesis (McPherron et al. 1997)], and why expression of myostatin inhibiting factors such as follistatin appears to enhance myogenic cell engraftment after transplantation (Benabdallah et al. 2005, 2008).

A number of studies have also established the existence of other mechanisms that may influence the regenerative/proliferative capacity of satellite cells in dystrophic muscles. For instance, the related cytokines leukemia inhibitory factor (LIF) and ciliary

neurotrophic factor (CNTF) have been shown to potentiate the regenerative capacity of stem cells (Wolf et al. 1994), and LIF in particular has been explored as an approach to stimulate satellite cell activation/expansion in vivo with generally positive findings (White et al. 2001, 2002). These two factors act upon membrane-bound gp130 receptors so potentially it is sufficient for them to be secreted from skeletal muscle cells transduced in vivo, in order to act upon adjacent myogenic cells.

Another potentially very interesting therapeutic approach concerns that of the Notch receptor/Delta ligand signaling system. Of note, with increased aging, there is a decline in Notch activity accompanied by concurrent decreases in the Delta ligand and Numb antagonist (Conboy et al. 2003). Cumulatively, these changes cause increases in the phosphorylation of the downstream TGF-β target Smad3 amongst other effects (Carlson et al. 2008). Because it has been shown that inhibition of Smad3 phosphorylation in old injured muscle, restored regenerative capacity, identification of this mechanism in dystrophic stem cell niches could establish a therapeutic basis for enhancing Notch activation in vivo (Carlson et al. 2008). Inhibition of Notch activity has been demonstrated to inhibit muscle regeneration, while forced Notch activation in aged muscles has been able to enhance muscle regeneration (Conboy et al. 2003). Whether forced Notch activation could also potentiate regeneration in young dystrophic patients' muscles is less well defined presently, but worthy of consideration. Finally, recent advances in our understanding of Wnt-mediated signaling as a regulator of myogenic cells suggest that interventions that can modify Wnt isoform expression may potentiate regeneration and inhibit fibrotic accumulation in dystrophic muscles. Studies examining the regenerative potential of old muscles have determined that reduced satellite cell activity and increased mis-differentiation correlates with elevated circulating Wnt levels, and that administration of Wnt inhibitors can reverse this phenomenon (Brack et al. 2007). Potentially, utilizing a genetic intervention that can increase the expression of Wnt receptors or Wnt binding proteins (such as Frizzled family members) may effectively sequester circulating Wnt in a manner that can enhance the regenerative and myogenic capabilities of satellite cells and their progeny in dystrophic muscles (Brack et al. 2007). However, other studies have suggested that the story is more complex, and that different Wnt isoforms can exert positive and negative effects on satellite cell proliferation (Polesskaya et al. 2003; Otto et al. 2008). Combined, these new findings open up exciting new avenues of research for novel therapeutic strategies to enhance regeneration in dystrophic muscles.

8.8 Summary and Future Directions

This review hopefully serves to highlight a variety of potential avenues for developing new genetic interventions for the treatment of severe neuromuscular disorders, and the muscular dystrophies in particular. Importantly, while many of the proposed strategies have already been considered as prospective interventions for dystrophies in at least preliminary studies, very little has been accomplished with regards to

exploring coadministration of interventions in order to treat several facets of the disease state concurrently. This is a significant point, as most forms of muscular dystrophy result in a complex phenotypic profile which compromises maintenance of muscle mass and function by several mechanisms. It should be stressed that the development of interventions that target the primary genetic defect of specific conditions should be pursued and advocated wherever possible in the hope of preventing or halting the ensuing events of the onset of the disease and progression. However, the progression of pathology and deterioration of muscle function often is accompanied by changes in muscle morphology, which may be difficult to reverse or account for by a "single-gene" treatment modality later in a patient's life. Therefore, careful consideration of the mechanisms and perturbations underlying individual disease states could dictate strategic approaches to codelivering interventions that could collectively exert significantly greater improvements upon muscle structure and function than any one treatment alone. It is logical to suggest that treatment of diseases characterized by complex phenotypic changes may warrant the development of multimodality interventions for the greatest benefit, and with continued development of the aforementioned approaches and other strategies it is hoped that viable and practical approaches to the prevention and treatment of severe muscular dystrophies will become achievable in the near future.

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Chapter 9
Duchenne Cardiomyopathy Gene Therapy

Jin-Hong Shin, Brian Bostick, Deborah M. Fine, Yongping Yue, and Dongsheng Duan

Abstract Duchenne cardiomyopathy is a heart disease resulting from the loss of cardiac dystrophin. It significantly reduces the life quality and shortens lifespan in Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD) and X-linked dilated cardiomyopathy patients and carriers. Gene replacement therapy with adeno-associated viral vector (AAV) and gene repair therapy with exon skipping hold great promise for restoring dystrophin expression and ameliorating cardiomyopathy. The last few years have witnessed tremendous advances towards Duchenne cardiomyopathy gene therapy. The infrastructure (animal models and functional assays) is now available for comprehensive preclinical studies. Essential parameters, such as the therapeutic threshold, have also been defined. Together with the recent developments in novel AAV vectors and modified antisense oligonucleotides, clinical application of Duchenne cardiomyopathy gene therapy may become a reality in the near future.

9.1 Duchenne Cardiomyopathy

Duchenne cardiomyopathy refers to the heart disease seen in patients with Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD) and X-linked dilated cardiomyopathy (XLDC) (Duan 2006a). The fundamental problem in Duchenne cardiomyopathy is absent or abnormal dystrophin expression in the heart. DMD, BMD and XLDC are all caused by mutations in the dystrophin gene (Kunkel 2005). DMD is the most severe form where dystrophin expression is completely erased from all muscles (Hoffman et al. 1988). In BMD, gene mutations result in reduced dystrophin expression or expression of a truncated dystrophin. Clinical manifestations are usually mild in BMD patients because of the residual

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dystrophin protein in muscle (Beggs et al. 1991; Hoffman 1993; Koenig et al. 1989). In XLDC, dystrophin is selectively removed from the heart but not from the skeletal muscle (Cohen and Muntoni 2004).

Dilated cardiomyopathy is the classic clinical manifestation of Duchenne cardiomyopathy. Early symptoms are usually nonspecific such as pronounced fatigue, palpitations, dyspnea, chest pain, sleeping alteration and unexplained weight change (Markham et al. 2005; Perloff et al. 1966). Clinical examination often reveals regional myocardial hypertrophy, ventricular wall motion abnormalities, sinus tachycardia and conduction problems. At the end-stage, patients die from ventricular arrhythmia-associated sudden death and/or congestive heart failure (Finsterer and Stollberger 2003; Markham et al. 2005). Characteristic pathological findings include an abnormally enlarged ventricular chamber, a thin ventricular wall, excessive trabeculation, sporadic atrophy, calcification and marked fibrosis in the posterobasal region of the free wall of the left ventricle. In severe cases, fibrosis spreads throughout the entire heart (Frankel and Rosser 1976; Heymsfield et al. 1978; Perloff et al. 1967; Politano et al. 1996). Current treatment for Duchenne cardiomyopathy is limited to symptom-relieving medicines (such as angiotensin-converting enzyme inhibitors and β-blockers) and heart transplantation (American Academy of Pediatrics 2005; Bushby et al. 2003; Finsterer et al. 1999; Melacini et al. 1998, 2001; Patane et al. 2006; Quinlivan and Dubowitz 1992; Ruiz-Cano et al. 2003). Gene therapy (replacement or repair) holds great promise for restoring cardiac dystrophin expression and alleviating heart disease.

9.2 Dystrophin, Minidystrophin and Microdystrophin

The 2.4 mb dystrophin gene is located on the short arm of the X-chromosome and it consists of 79 exons. The use of different promoters and alternative splicing yield numerous dystrophin isoforms expressed in a variety of tissues (Ahn and Kunkel 1993; Blake et al. 2002). The main isoform expressed in striated muscle (skeletal muscle and the heart) is a 427 kD protein called Dp427m, or full-length dystrophin, or simply, dystrophin. By crosslinking intracellular γ-actin with the extracellular matrix protein laminin, dystrophin stabilizes the sarcolemma during the repeated cycles of muscle contraction and relaxation (Ervasti and Sonnemann 2008; Petrof 2002). Two γ-actin binding domains have been identified in dystrophin and are named ABD and ABD2. ABD is located at the N-terminus of dystrophin and ABD2 is located in the middle of the dystrophin protein between spectrin-like repeats 11 and 17 (Fig. 9.1). Dystrophin indirectly interacts with laminin through dystroglycan via a dystroglycan binding domain (DgBD). The DgBD starts at the end of the hinge 4 region of dystrophin and continues through the cysteine-rich (CR) region. Besides protecting the sarcolemma, dystrophin also plays a role in muscle cell signal transduction (Rando 2001). In this signaling role, the dystrophin C-terminal domain has been shown to interact with scaffolding proteins syntrophin and dystrobrevin. We have recently found that the combined efforts of syntrophin and

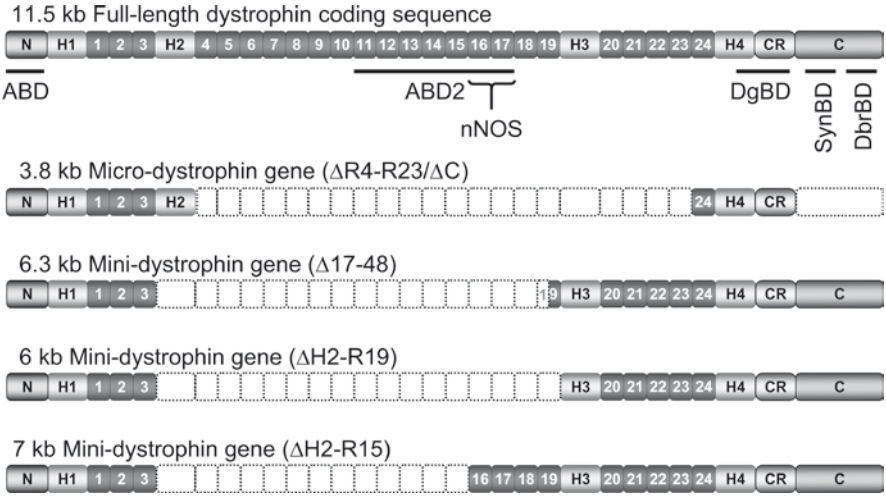

Fig. 9.1 Schematic outlines of the full-length dystrophin coding sequence and the representative mini- and microdystrophin genes (Not drawn to scale). *N*, N-terminal domain; *H*, hinge; *CR,* cysteine-rich domain; *C,* C-terminal domain; *ABD*, actin-binding domain; *ABD2*, the second actin-binding domain; *nNOS*, dystrophin neuronal nitric oxide synthase recruiting domain; *DgBD*, dystroglycan binding domain (including part of H4 and the CR domain); *SynBD*, syntrophin binding domain; *DbrBD*, dystrobrevin binding domain. *Numeric numbers* refer to spectrin-like repeats. *Dotted box*, regions deleted

spectrin-like repeats 16 and 17 (R16/17) recruit the signaling molecule neuronal nitric oxide synthase (nNOS) to the sarcolemma (Lai et al. 2009).

A major challenge for Duchenne cardiomyopathy gene therapy is the large size of the dystrophin gene. As one of the largest genes known, dystrophin is beyond the packaging capacity of most gene transfer vectors. To overcome this hurdle, investigators have developed massively abbreviated, but at least partially functional versions of the dystrophin gene termed, minigenes and microgenes (Fabb et al. 2002; Harper et al. 2002; Lai et al. 2009; Sakamoto et al. 2002; Wang et al. 2000; Yuasa et al. 1998).

Minidystrophins are about half the size of the full-length protein (~200–240 kD) (Clemens et al. 1995; England et al. 1990; Harper et al. 2002; Lai et al. 2009). The first minidystrophin was discovered in 1990 in a patient exhibiting very mild BMD (England et al. 1990). Despite a large deletion that eliminated ~46% of the coding sequence in the middle of the dystrophin gene (from exon 17 to 48), the patient was able to walk with the help of a stick at the age of 61 (Fig. 9.1) (England et al. 1990). Transgenic expression of this 6.3 kb Δ17-48 minigene recovered the dystrophin-associated glycoprotein complex (DGC) and reduced histopathology (Phelps et al. 1995; Wells et al. 1995). Harper et al. (2002) removed the partial spectrin-like repeat in the Δ17-48 minigene and generated a perfectly phased version called the 6 kb ΔH2-R19 minigene (Fig. 9.1). Notably, this slightly smaller but functionally

improved minigene was able to normalize skeletal muscle strength and completely prevent eccentric contraction-induced injury (Harper et al. 2002; Lai et al. 2009).

The latest development in minidystrophin design was reported by Lai et al. (2009). Based on the finding that R16/17 is required for anchoring nNOS to the sarcolemma, Lai et al. (2009) generated a 7 kb ΔH2-R15 minigene. In addition to retaining all the functional features of the 6 kb ΔH2-R19 minigene, this new minigene was able to reduce muscle fatigue and enhance exercise performance.

Microdystrophins (such as ΔR4-23/ΔC, Fig. 9.1) are ~30% of the size of the full-length protein (125–150 kD). Although the first set of synthetic microdystrophins yielded little or no protection (Yuasa et al. 1998), newer versions are quite effective in ameliorating histopathology (Fabb et al. 2002; Harper et al. 2002; Lai et al. 2009; Sakamoto et al. 2002; Wang et al. 2000). The major limitation of microdystrophins is that they cannot fully restore diaphragm force to wild type levels. Furthermore, they cannot fully protect skeletal muscle from eccentric contraction-induced injury (Abmayr et al. 2005; Foster et al. 2008; Gregorevic et al. 2004, 2006; Harper et al. 2002; Liu et al. 2005; Rodino-Klapac et al. 2007; Wang et al. 2008a, b, 2000; Watchko et al. 2002; Yue et al. 2006).

Both the mini- and microdystrophin genes have been evaluated for treating Duchenne cardiomyopathy. Microdystrophin has been shown to restore the DGC and improve sarcolemma integrity in cardiomyocytes (Yue et al. 2003). Furthermore, microdystrophin can partially correct both ECG and hemodynamic defects in dystrophin-deficient mice (Bostick et al. 2008a; Townsend et al. 2007). Based on the fact that the 6 kb ΔH2-R19 minidystrophin gene can fully restore skeletal muscle force (in particular, the diaphragm force) to wild type levels (Harper et al. 2002), we recently evaluated whether cardiac-specific expression of this minidystrophin in transgenic mice could normalize heart function (Bostick et al. 2009). Similar to microdystrophin, minidystrophin also restored DGC and enhanced sarcolemmal strength in the heart. Furthermore, minidystrophin prevented myocardial fibrosis. Surprisingly, cardiac-specific minidystrophin expression failed to completely normalize ECG and hemodynamic abnormalities. In ECG assay, the PR interval was normalized but the heart rate remained abnormal. In cardiac catheter assay, the ejection fraction was corrected but the stroke volume and the cardiac output remained suboptimal (Bostick et al. 2009). Taken together, the strategies that have been developed for treating Duchenne skeletal muscle disease may have to be carefully re-evaluated for Duchenne cardiomyopathy gene therapy.

9.3 Animal Models for Evaluating Duchenne Cardiomyopathy Gene Therapy

9.3.1 Young and Adult Mdx Mice

The most widely used mouse model for DMD is the dystrophin-deficient mdx mouse (Bulfield et al. 1984). Mdx mice show minimal clinical signs and usually live up to 22–23 months of age (Chamberlain et al. 2007; Li et al. 2009).

Numerous studies have examined the mdx heart (Bridges 1986; Cohn et al. 2001; Coulton et al. 1988; Grady et al. 1997; Hainsey et al. 2003; Kamogawa et al. 2001; Nakamura et al. 2002; Quinlan et al. 2004). Despite intensive interrogations, only mild cardiac histopathology and some ECG abnormalities are found in mdx mice that are younger than 12 months of age (Bostick et al. 2008a; Chu et al. 2002; Heymsfield et al. 1978; Markham et al. 2005; Nigro et al. 1990; Sadeghi et al. 2002). External stress (such as administration of positive inotropic drug β-isoproterenol or dobutamine) can induce some, but not all, aspects of cardiomyopathy in adult mdx mice (Danialou et al. 2001; Duan 2006a; Yasuda et al. 2005; Yue et al. 2004).

9.3.2 Double Knockout (dko) Models

Based on the hypothesis that inactivating the compensatory pathways in mdx mice may lead to anatomical and/or histological changes similar to human patients, several double knockout (dko) models have been created (Deconinck et al. 1997; Grady et al. 1997; Guo et al. 2006; Hainsey et al. 2003; Megeney et al. 1996; Megeney et al. 1999; Rooney et al. 2006). The first reported dko model is the utrophin/dystrophin double null model (u-dko) (Deconinck et al. 1997; Grady et al. 1997). Utrophin is a structural and functional homologue of dystrophin. U-dko mice show severe skeletal muscle disease and their lifespan is reduced to ~2.5 months (Deconinck et al. 1997; Grady et al. 1997, 1999; Li et al. 2009). Two u-dko strains have been reported (Deconinck et al. 1997; Grady et al. 1997). In the Grady strain, all utrophin isoforms are inactivated (Grady et al. 1997). In the Deconinck strain, only the largest utrophin isoform is inactivated (Deconinck et al. 1997). Interestingly, severe histopathology and abnormal magnetic resonance imaging (MRI) have been observed in the heart of 8 to 10-week-old Grady strain u-dko mice (Duan 2006a; Gaedigk et al. 2006; Grady et al. 1997).

Enhanced regeneration also contributes to the mild skeletal muscle disease in mdx mice. MyoD is an essential myogenic transcriptional factor in the skeletal muscle but is not expressed in the heart. MyoD/dystrophin double null mice (m-dko) develop severe skeletal muscle disease and die prematurely at ~12 months of age (Megeney et al. 1996). Surprisingly, the hearts of 5-m-old m-dko mice appear enlarged on visual examination and they also show other anatomical and histological changes such as increased heart weight to body weight ratio, cardiac fibrosis and calcification (Duan 2006a; Megeney et al. 1999).

Similar to dystrophin, α7β1-integrin also protects muscle cell from contraction-induced injury. α7-integrin/dystrophin double null mice have an extremely short lifespan, living only up to 1 month of age (Guo et al. 2006; Rooney et al. 2006). Although the hearts of these mice appear normal on histological examination, electron microscopy studies do show ultrastructural abnormalities (Guo et al. 2006). Furthermore, necropsy suggests that these mice die from congestive heart failure (Guo et al. 2006).

9.3.3 *Aged Mdx Model*

Although adult mdx mice and different dko mice are useful models, none of them fully recapitulates cardiac defects in patients. In an effort to develop a mouse model that is genetically as well as phenotypically identical to human patients, we examined old mdx mice. We first performed an echocardiography (echo) study in 12-m-old mice (Fig. 9.2). Consistent with a previous report (Quinlan et al. 2004), we observed a 29.5% reduction in fractional shortening. Left ventricular posterior wall thickness in systole was also significantly reduced, a finding consistent with dilated cardiomyopathy. Nevertheless, the stroke volume was not significantly decreased in 12-m-old mdx mice (Fig. 9.2). In summary, signs of dilated cardiomyopathy were detected, however, overall cardiac performance is not affected at 12 months of age.

Severe heart disease is not observed in DMD patients until the final stages of their illness. The average lifespan of mdx mice is 22–23 months (Chamberlain et al. 2007; Li et al. 2009). So we performed a comprehensive examination in 21-m-old mdx mice, an age that matches with that of end-stage DMD patients (Bostick et al. 2008b). We demonstrated that the hearts of aged mdx mice display the same structural

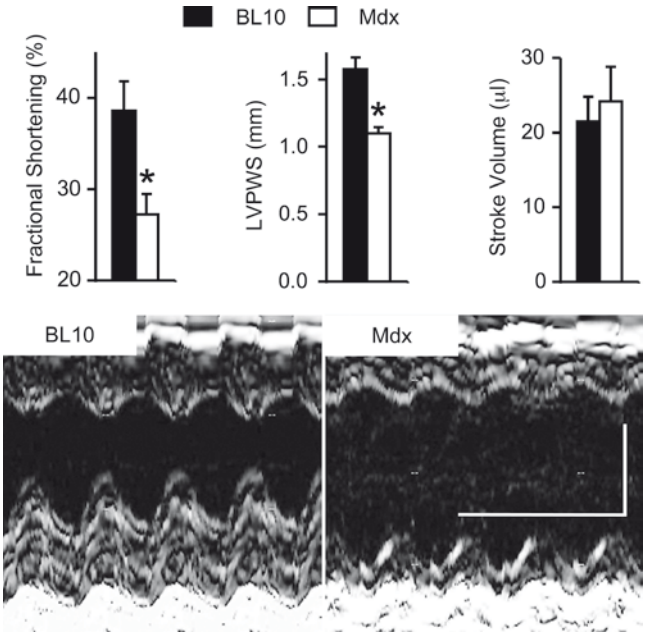

Fig. 9.2 Echocardiographic assessment of cardiac function in 12-month-old BL10 and mdx mice. *Top panels*, mdx mice show significantly reduced fractional shortening and reduced left ventricle posterior wall thickness in systole (LVPWS). However, the stroke volume is maintained. *Asterisk*, significantly different from that of the other group. Mean ± standard error of mean; $N = 4$ for BL10, $N = 5$ for mdx. *Bottom panels*, M-mode tracings of representative transthoracic left ventricle echo in BL10 and mdx mice. *Vertical scale bar*, 2 mm; *Horizontal scale bar*, 250 ms

abnormalities as seen in human patients such as prominent myocardial fibrosis, calcification and irregularly enlarged ventricular chamber (Fig. 9.3a) (Bostick et al. 2008b). An ECG examination, revealed changes like those found in patients, such as the appearance of R' waves, deep Q waves, PR interval reduction and QT interval prolongation (Bostick et al. 2008b). Most importantly, cardiac catheter assay revealed a significant increase in the end-diastolic volume. Cardiac dysfunction was further confirmed by a reduction in the maximal pressure and the stroke volume (Fig. 9.3b). In summary, the hearts of very old mdx mice show all characteristic changes seen in the end-stages of human patients. Thus, aged mdx mice represent an excellent model for developing Duchenne cardiomyopathy gene therapy (Bostick et al. 2008b).

9.3.4 Canine Model for Duchenne Cardiomyopathy

Dystrophin-deficient dogs are exceedingly valuable to DMD gene therapy studies. The dog has a body size closer to that of humans. Additionally, dystrophin-deficient dogs faithfully recapitulate the clinical course of the human disease (reviewed in (Shelton and Engvall 2005; Wieczorek et al. 2006)). The most well studied dog model is the Golden Retriever Muscular Dystrophy (GRMD) model (Cooper et al. 1988; Kornegay et al. 1988). Similar to human patients, GRMD dogs develop severe cardiomyopathy (Chetboul et al. 2004a, b; Moise et al. 1991; Valentine et al. 1989; Yugeta et al. 2006). On necropsy, the ventricular chamber of GRMD dogs is clearly enlarged compared to that of the age and sex-matched normal golden retriever dogs (Fig. 9.3c). Also evident in the heart of affected dogs are thin ventricular walls, prominent trabeculations, fibrosis and sporadic calcification (Fig. 9.3c). There is no doubt that dystrophin-deficient dogs will be extremely valuable to define the parameters for Duchenne cardiomyopathy gene therapy before human trials are initiated.

9.4 The Therapeutic Threshold for Duchenne Cardiomyopathy Gene Therapy

There is no doubt that 100% transduction is not possible with any gene therapy approach. Will partial transduction be therapeutically relevant? In skeletal muscle, 20–60% dystrophin positive myofibers have been shown to increase muscle force in mdx mice (Liu et al. 2005; Watchko et al. 2002; Yoshimura et al. 2004). Encouragingly, patients with 50% mosaic expression in skeletal muscle were found to have mild muscle weakness (Arahata et al. 1989; Hoffman et al. 1988). Approximately 50% of the cardiomyocytes express dystrophin in the hearts of DMD carriers (Bostick et al. 2008b; Duan 2006a; Yue et al. 2004). However, for a

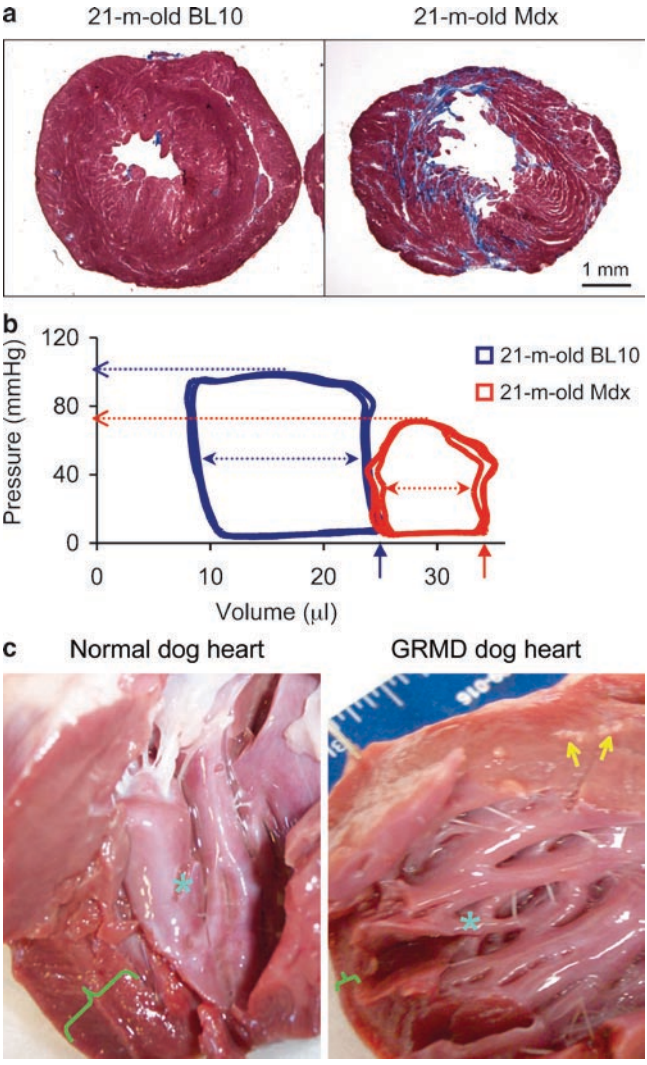

Fig. 9.3 Animal models for Duchenne dilated cardiomyopathy. (**a**) Representative photomicrographs of cardiac Masson trichrome staining from 21-m-old BL10 and mdx mice. Fibrotic tissue is in *blue color*. (**b**) Representative pressure–volume loops from 21-m-old BL10 and mdx mice. *Vertical arrow*, end-diastolic volume; *Horizontal arrow*, maximal pressure; *Double arrow*, stroke volume. (**c**) Representative necropsy photomicrographs of sex matched 2-year-old dog hearts. *Left panel*, normal golden retriever dog heart; *Right panel*, golden retriever muscular dystrophy (GRMD) dog heart. *Bracket*, ventricular free wall thickness; *Asterisk*, ventricular free wall trabeculation; *Arrow*, sporadic calcification seen only in the GRMD heart. (Panels **a** and **b** were previously published in Bostick et al. 2008b)

long time it was thought that 50% of the dystrophin positive cells are not sufficient to protect the heart. This assessment was supported by frequent case reports that showed cardiomyopathy as the major clinical manifestation in the referred carrier patients (Emery 1969; Hoogerwaard et al. 1999; Kamakura 2000; Kamakura et al. 1990; Lane et al. 1980; Melacini et al. 1998; Mirabella et al. 1993; Ogata et al. 2000; Walcher et al. 2008). A survey published in 1996 by Pilitano and colleagues (1996) even suggested that 90% of adult DMD carriers have signs of cardiac involvement. Collectively, these observations give an impression that one may need to treat all cardiomyocytes in order to reduce heart disease.

To determine whether 50% mosaic expression can protect the heart, we evaluated heart function in heterozygous carrier mice. To thoroughly address this issue, we examined both young (3-m-old) and very old (21-m-old) carrier mdx mice (Bostick et al. 2008; Yue et al. 2004). Surprisingly, the hearts of carrier mice were almost identical to those of normal mice except for occasional focal inflammation in ~20% of 21-m-old carrier mice. More importantly, cardiac electrophysiology and hemodynamics were completely within the normal range (Bostick et al. 2008b; Yue et al. 2004). Our results strongly suggest that 50% mosaic dystrophin expression is sufficient to correct both anatomical and physiological defects in the heart.

To resolve the difference between our mouse data and clinical reports, in particular, the survey by Politano et al. (1996), we reviewed data from several large sample studies published recently (Grain et al. 2001; Holloway et al. 2008; Hoogerwaard et al. 1999a; Nolan et al. 2003). Interestingly, less than 10% of the carriers were found to have cardiomyopathy in these carefully designed studies. Furthermore, no causal relationship was established between the carrier status and heart disease. A report published last year examined 397 carriers, the largest study of DMD carriers to date. Consistent with our mouse results, the investigators did not see increased cardiac death in carriers (Holloway et al. 2008). Taken together, both mouse data and human results suggest that expressing dystrophin in 50% cardiomyocytes may very likely meet the therapeutic threshold.

9.5 Treating the Heart Versus Treating Skeletal Muscle

In DMD and many other types of muscular dystrophies, cardiac and skeletal muscles are both compromised. The interplay between heart disease and skeletal muscle disease remains to be fully appreciated. It is generally agreed that normalizing skeletal muscle function alone cannot halt heart disease (Muntoni et al. 1995; Towbin et al. 1993; Townsend et al. 2007; Zhu et al. 2002). Limb muscles and respiratory muscles (in particular, the diaphragm) are auxiliary pumps that promote venous return. When skeletal muscle contractility is severely compromised (such as in m-dko mice), cardiac output will decrease as a consequence of reduced venous return. This seems to suggest that treating skeletal muscle alone should increase cardiac output and improve heart function. If left unchecked, severe skeletal muscle disease will aggravate cardiomyopathy. This argument is supported by findings

from two clinical studies (Hunsaker et al. 1982; Matsuda et al. 1977). Hunsaker et al. (1982) followed nine ambulant DMD patients for 10 years. Interestingly only the patients who became wheelchair-bound (suggesting a more severe skeletal muscle disease) developed heart disease. The remaining patients showed no clinical symptoms of heart disease and their heart function was significantly better, according to echocardiographic examinations. Matsuda et al. (1977) found similar results in a larger group of patients (57 patients). Experimental evidence for this hypothesis comes from m-dko mice (Megeney et al. 1999). MyoD is involved in skeletal, but not cardiac, muscle regeneration. As expected, m-dko mice develop much severer skeletal muscle disease (Megeney et al. 1996). Surprisingly, they also show anatomical and histological signs of dilated cardiomyopathy (Duan 2006a; Megeney et al. 1999). Hence, both mouse results and clinical observations seem to suggest that exaggerated skeletal muscle disease induces and/or precipitates hidden heart disease.

On the other hand, patients with mild skeletal muscle disease are generally capable of greater physical activity. It is thus quite conceivable that these physical activities may place more stress on the heart and worsen cardiomyopathy. Based on this theory, ameliorating skeletal muscle disease may encourage more physical activities, and consequently, lead to untoward heart damage. When patients are immobilized by severe skeletal muscle disease, the reduced physical activity will also reduce cardiac demand. Hence, heart disease will not get worse in these patients. In support of this view, several clinical studies have found no increase in severity of Duchenne cardiomyopathy in patients who suffered from more severe skeletal muscle disease (Angelini et al. 1996; Brockmeier et al. 1998; Nigro et al. 1995; Takenaka et al. 1993; Utsunomiya et al. 1990). A recent mouse study also found that transgenic amelioration of skeletal muscle disease alone increased physical activity and worsened heart function (Townsend et al. 2008).

Taken together, these findings suggest that worsening of skeletal muscle disease may promote heart deterioration in untreated patients. However, if patients were only treated for the skeletal muscle problem, they may end up facing more severe heart disease. Similarly, if patients were only treated for cardiomyopathy, they may not achieve full cardiac recovery (Bostick et al. 2009). We believe that an effective therapy for Duchenne cardiomyopathy should treat both skeletal muscle disease and cardiomyopathy simultaneously.

9.6 Adeno-associated virus (AAV)-mediated Gene Replacement Therapy for Duchenne Cardiomyopathy

AAV is currently the leading muscle gene delivery vector. It is the only vector that is capable of transducing all muscles in the body. Wild type AAV carries an approximately 4.8 kb single stranded DNA genome (reviewed in (Duan et al. 2002; Yan et al. 2006)). AAV vectors are generated by replacing the wild type viral genome with a therapeutic expression cassette.

Two issues have been baffling AAV-mediated myocardial transduction. The first is the low transduction efficiency. To overcome this hurdle, investigators have screened a number of different AAV serotypes including AAV-1, 2, 5, 6, 7, 8 and 9. AAV-2 results in limited transduction after direct myocardial injection (Su et al. 2000; Svensson et al. 1999). *Ex vivo* coronary perfusion, increases AAV-2 transduction efficiency to more than 50% in rodent hearts (Li et al. 2003; Svensson et al. 1999). Unfortunately, this method is not feasible for the vast majority of patients. Using an invasive trans-coronary approach, Hoshijima et al. (2002) achieved 60–80% transduction efficiency in the hamster heart with AAV-2. We have also achieved limited success with AAV-5 via intracavity injection in neonatal mice (Yue et al. 2003). Because of the invasive nature of these methods (direct myocardial or intracavity injection and trans-coronary delivery), their clinical application will be limited. From a practical standpoint, the more appealing method would be intravenous delivery. In this regard, AAV-6, 8 and 9 have emerged as the most promising serotypes for systemic gene delivery in rodents. AAV-9 is particularly attractive because it preferentially transduces the murine heart (Bostick et al. 2007, 2008a; Ghosh et al. 2007; Inagaki et al. 2006; Pacak et al. 2006). Additionally, recently developed *in vivo* evolution strategies offer the hope of further improving cardiac transduction efficiency with AAV vectors (Yang et al. 2009). In summary, transduction efficiency is no longer a problem in the murine heart.

In contrast to rodent muscle, dog muscle has been notoriously difficult to transduce with any gene therapy vector. Strong cellular immune response essentially eliminates AAV transduction in adult dog muscles unless appropriate immune-suppression is applied (Herzog et al. 2001; Wang et al. 2007a, b; Yuasa et al. 2007; Yue et al. 2008). Interestingly, we recently found that local muscle injection of AAV-9 does not provoke cellular immune reaction when delivered to neonatal puppies. Studies from several groups suggest that AAV-9 is the best serotype for the mouse heart (Fig. 9.4) (Bostick et al. 2007, 2009; Ghosh et al. 2007; Inagaki et al. 2006; Pacak et al. 2006). Based on these observations, we tested whether systemic AAV-9 injection in newborn puppies can transduce the canine heart (Yue et al. 2008). Despite the robust and persistent expression throughout all skeletal muscles in the body, barely any expression was observed in the heart (Fig. 9.4) (Yue et al. 2008). This unexpected result highlights the difficulties in achieving efficient AAV transduction of the canine heart.

The other hurdle for AAV-mediated DMD gene therapy is the small viral packaging capacity. In general, transduction competent AAV vectors are produced only when the vector genome is ≤5 kb (reviewed in (Duan et al. 2006; Ghosh and Duan 2007)). To overcome this hurdle, investigators have developed the highly abbreviated mini- and microgenes. However, only the suboptimal microgene can fit into a single AAV virion (reviewed in (Blankinship et al. 2006; Duan 2006b)). Innovative strategies are needed to deliver the larger, more functional minigene.

Several single or dual vector approaches have been explored to increase AAV packaging capacity. With the single vector approach, investigators were able to deliver a larger genome in a single AAV virion. A classic AAV capsid is composed of three viral proteins (VP1, VP2 and VP3) (Rose et al. 1971; Xie et al. 2002).

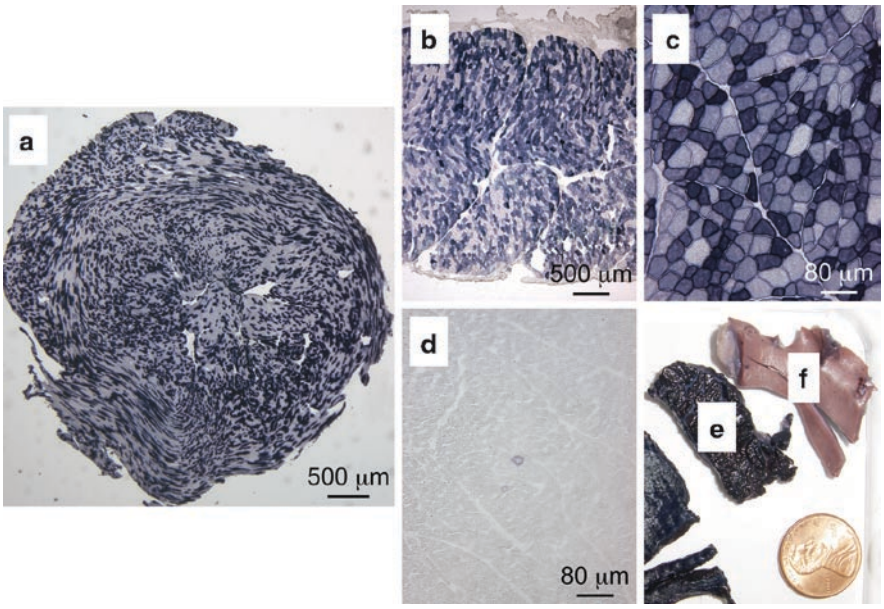

Fig. 9.4 Differential response of systemic AAV-9 infection in the mouse and dog hearts. **(a)** Representative alkaline phosphatase (AP) staining of a mouse heart at 12 weeks after neonatal facial vein injection (3.5×10^{11} vg particles/mouse, AV.RSV.AP). (**b**) to (**f**) Representative AP staining of dog muscles at 6 months after neonatal jugular vein injection (2×10^{11} vg/g body weight, AV.RSV.AP). Panels (**b**) to (**d**) are cross sections and (**e**) and (**f**) are whole mount tissue blocks. (**b**) The diaphragm; (**c**), the pectoralis muscle; (**d**), left ventricle; (**e**), the intercostal muscle; (**f**), left atrium. (Panels **b** to **f** were previously published in Yue et al. 2008)

Interestingly, infectious AAV-2 particles can be assembled from VP1 and VP3 (Warrington et al. 2004). Surprisingly, VP2-null AAV can accommodate a 6 kb genome (Grieger and Samulski 2005). However, this is still too small for the minidystrophin expression cassette. Recently, Allocca et al. (2008) reported a very intriguing observation suggesting that AAV-5 can package a 9 kb vector genome. However, the viral yield was nearly two logs lower than that of a regular AAV-5 virus (≤5 kb genome) (Allocca et al. 2008). Furthermore, this strategy did not work for other AAV serotypes such as AAV-1, 2, 3, 4, 7, 8 and 9 (Allocca et al. 2008). Due to the poor cardiac transduction efficiency of AAV-5 and the low viral titer, this strategy is unlikely to be useful for minigene-based Duchenne cardiomyopathy gene therapy.

To cope with the packaging size challenge, a series of dual vector approaches have also been developed (reviewed in (Duan et al. 2006; Ghosh and Duan 2007)). These include the cis-activation, trans-splicing, overlapping, and hybrid vector approaches. The last three methods can be used to express the minidystrophin gene. In these dual vector approaches, the entire expression cassette is split into two parts and each part is carried by one AAV virion. The complete expression cassette is reconstituted *in vivo* by either viral inverted terminal repeat-mediated recombination and/or homologous recombination (Duan et al. 2001; Halbert et al. 2002; Sun

et al. 2000; Yan et al. 2000). In the overlapping approach, the transcript from the recombined genome can be directly translated into protein products. In the other two approaches, the cellular splicing machinery is required to remove the intervening junctional sequences.

Several trans-splicing and hybrid dual AAV vectors have been generated to express the 6 kb minigene (Ghosh et al. 2008; Lai et al. 2005, 2008). Lai et al. engineered a set of extremely efficient trans-splicing vectors by splitting the minigene between exons 60 and 61 (Lai et al. 2005, 2008). Local injection in mdx skeletal muscle transduced approximately 50% and 80% myofibers in the extensor digitorium longus and tibialis anterior muscles, respectively (Lai et al. 2005). Ghosh et al. (2007) recently reported that intravenous injection of a set of the alkaline phosphatase *trans*-splicing vector can transduce ~50% of cardiomyocytes in normal neonatal mice. It remains to be determined whether similar transduction efficiency can be achieved with the minigene *trans*-splicing vectors in the hearts of dystrophin-deficient animals. Nevertheless, these results provide reason for optimism.

9.7 Restore Cardiac Dystrophin Expression with Exon Skipping

In the vast majority of DMD patients, mutations disrupt the open reading frame (Koenig et al. 1989; Monaco et al. 1988). Restoring the reading frame by exon skipping holds great promise in ameliorating muscle disease (van Deutekom et al. 2007). Despite body-wide correction in the skeletal muscle, traditional exon skipping strategy has thus far had minimal success in the heart (Alter et al. 2006; Yokota et al. 2009). This hurdle is now resolved with modified antisense oligonucleotides (AON). In one approach, investigators conjugated AON with cell penetrating peptide to achieve efficient cardiac correction (Jearawiriyapaisarn et al. 2008; Wu et al. 2008; Yin et al. 2008a; b). In another approach, nonpeptide polymers, such as an octa-guanidine-conjugated morpholino AON, were found to result in widespread dystrophin restoration in the heart (Wu et al. 2009).

9.8 Summary and Future Directions

Cardiomyopathy is a leading health concern for DMD, BMD, XLDC patients and carriers. The development of Duchenne cardiomyopathy gene therapy has made significant strides in the last few years. Notably, a phenotypic mdx mouse model has been generated and the therapeutic threshold has also been determined. In terms of gene replacement therapy, the newly developed mini- and microdystrophins as well as dual AAV vectors have shown promising preclinical evidence in the mouse model. In terms of gene repair therapy, the modified AONs for cardiac exon skipping

have finally open the door to test this exciting strategy for Duchenne cardiomyopathy therapy. Although there are still hurdles (such as poor transduction of AAV-9 in the canine heart), we have every reason to believe that the progresses made in the last few years will lead to clinical trials for Duchenne cardiomyopathy gene therapy in the near future.

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Chapter 10
Systemic Gene Delivery for Muscle Gene Therapy

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Abstract The muscular dystrophies (MDs) are a heterogeneous group of monogenetic disorders that affect striated muscles, often throughout the body. A promising approach to treating the MDs is to use gene therapy to replace, repair, or modify expression of the mutant gene. Accomplishing such a goal requires that gene expression cassettes, which comprise a gene regulatory element driving expression of an effecter DNA sequence and are followed by a transcriptional terminator, be delivered to target cells to counteract the effects of the mutation. For the MDs, this type of therapy will require delivering expression cassettes to all the striated muscles in a patient's body. Striated muscle forms a significant percentage of total body mass, so the prospects for achieving such large spread delivery had seemed like a remote goal. Recent studies using a variety of vector systems indicate that genes can be delivered to muscles body wide by infusing them into the blood stream under appropriate conditions. The simplest and one of the most promising methods for systemic delivery involves infusion of vectors derived from adeno-associated virus (AAV). These recombinant AAV vectors can target muscles throughout the body of small, adult mammals and may be adaptable to larger animals and humans. Consequently, the use of rAAV vectors has great potential to lead to a therapy for the MDs and other disorders of striated muscle.

10.1 Introduction

The potential for correction of human disorders through gene therapy has created significant interest in academia and industry, as well as in the public sector. Gene therapy refers to the replacement, modification, or repair of a defective or mutated gene or its transcript in order to restore normal function. Gene therapy offers the

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potential for administering therapeutic gene expression cassettes as a method to treat muscular dystrophies (Blankinship et al. 2006; Chamberlain 2002; Chamberlain and Rando 2006), as well as other muscle disorders, such as cardiomyopathies (Hajjar and Zsebo 2007) and sarcopenia (Gregorevic et al. 2004b). Ideally, gene therapy for diseases of the muscle would involve targeting all the muscles of the body with a therapeutic gene cassette. Muscle tissue represents ~40% of the body weight (Liu et al. 2001), and achieving gene transfer to the entire musculature poses a considerable challenge. However, recent studies in a variety of labs have established that body wide gene transfer can be achieved in mouse models for several human genetic disorders, including Duchenne muscular dystrophy (DMD; Gregorevic et al. 2004a; Odom et al. 2008), congenital muscular dystrophy (Qiao et al. 2005), Limb-girdle muscular dystrophy (LGMD) 2A (Bartoli et al. 2007), 2D (Rodino-Klapac et al. 2008), 2F (Goehringer et al. 2009; Zhu et al. 2005), and Pompe disease (Sun et al. 2008). Muscle gene targeting has also been used for the production of secreted therapeutic proteins like erythropoietin (Kessler et al. 1996), human blood clotting factor VIII and IX (Hasbrouck and High 2008; Hoffman et al. 2007), and plasma apolipoprotein E (Evans et al. 2008). These exciting studies in a small mammalian model (mouse) raise considerable hope that such methods could be scaled up to larger animals and eventually applied in the clinic to treat a variety of neuromuscular disorders. Nonetheless, considerable difficulties remain in scaling these approaches and demonstrating safety, efficiency, and efficacy in humans. Ultimately, what is needed is a delivery system that enables re-administration, without immunological or toxic complications and which results in long-term expression of a therapeutic gene cassette.

10.2 Advantages and Limitations of Systemic Gene Transfer

There are many advantages to systemic gene delivery for muscle gene therapy. Systemic gene delivery allows targeting all muscles rather than isolated muscles, and avoids the need for hundreds, if not thousands, of repeated injections to saturate larger muscles; systemic delivery is also the only practical method for treating cardiac muscle and deeper less accessible muscles such as the diaphragm. Though intramuscular (IM) delivery has shown successful muscle transduction, transgene expression is restricted to relatively small areas within approximately 1 cm of the injection site (Howell et al. 1997). Recently, systemic routes including intravenous, intra-arterial and intra-peritoneal injection have been successful in generating widespread gene transfer to muscles (Fig. 10.1; Goyenvalle et al. 2004; Gregorevic et al. 2004a; Townsend et al. 2007; Su et al. 2005; Wang et al. 2005; Yue et al. 2008). However, as systemic delivery uses the vascular system, it is accompanied by certain complications, including delivery to nonmuscle tissues (Gregorevic et al. 2004a; Grimm et al. 2003a, b; Nakai et al. 2005; Wang et al. 2005). The use of vectors highly tropic for muscle coupled with muscle-specific gene regulatory elements could prevent or minimize gene expression in non-target tissues (Salva et al. 2007). Given the large volume of muscle tissue, uniform transduction of skeletal and cardiac muscle may be difficult to achieve with a single systemic injection, and multiple deliveries at a variety of sites may be required.

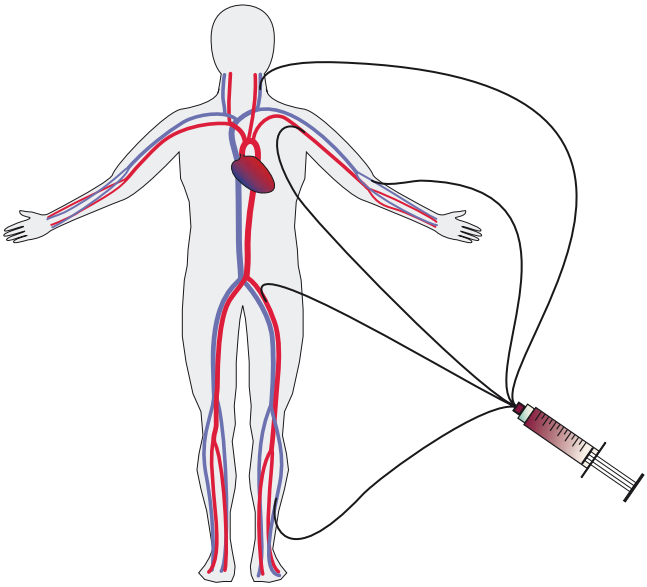


Fig. 10.1 Approaches to scale systemic gene delivery methods to larger animals and patients. Unlike mice, larger juvenile and adult animals and humans may not be treatable by a single intravascular injection of a rAAV vector. More efficient targeting of striated musculature can be achieved instead by infusing vector into multiple veins and arteries, enabling higher local concentrations of vector to trigger extravasation from capillaries downstream of the infusion site, leading to transduction of underlying myofibers. Candidate infusion sites include the jugular vein to target the heart, as well as the major veins and arteries in each limb

10.3 Delivery Vehicles

Genes can be delivered to muscle tissue using either nonviral or viral vectors, and there are potential and real advantages and disadvantages to each. The most promising nonviral delivery vehicle is naked plasmid DNA, which is easy to handle, cost effective to produce, and presents few safety concerns (Patil et al. 2005; Prud'homme et al. 2006). Plasmid DNA is delivered into muscle via mechanical or electrical techniques (Mir et al. 1999; Patil et al. 2005; Wolff et al. 1990). Studies using hydrodynamic delivery procedures show that transgene expression can be obtained in widely dispersed muscles throughout an isolated limb without association with significant muscle or tissue damage (Hagstrom et al. 2004; Toumi et al. 2006). Plasmid DNA infusion also has a major advantage in allowing repeated administration of vector without elicitation of an immune response against the plasmid. A concern, however, is that these intravascular methods require enormous volumes of fluid, and could potentially present safety issues in frail patients such as those with advanced muscular dystrophy. Also, it remains unclear whether high-pressure infusion of plasmid DNA could be applied to achieve efficient targeting of cardiac, diaphragm, or intercostal muscles. Studies of liposome mediated delivery of plasmid DNA to mice showed that regenerating muscle is more efficiently transfected,

favoring use in degenerative disorders such as the muscular dystrophies (Pampinella et al. 2000). Nonetheless, concerns with toxicity (Hofland et al. 1997) and efficiency of transduction (Patil et al. 2005) remain for systemic liposome based gene delivery technologies. Other approaches to enhancing efficiency depend on using electroporation or ultrasound to increase plasmid DNA uptake by muscle fibers (Danialou et al. 2002; Lawrie et al. 2000; Lentacker et al. 2008; Taniyama et al. 2002; Wang et al. 2008). Such methods could be applied to limb muscles after intravascular infusion of DNA, but questions remain as to applicability to internal muscles such as heart and diaphragm. Efficient electroporation often results in significant bystander damage to myofibers, raising safety concerns for human trials. Thus, despite the favorable issues of cost, production, and low immunogenicity generally associated with nonviral gene transfer methods, residual concerns related to safety and efficacy suggest that significant improvements in these technologies are needed before systemic delivery methods could be of use for the muscular dystrophies.

Some of the limitations of nonviral delivery methods can be overcome by using viral vectors, although these delivery shuttles present their own unique set of concerns that must be addressed. Much of the early work on developing viral-derived delivery shuttles for the muscular dystrophies focused on adenoviral vectors. Adenoviruses are nonenveloped DNA viruses with a 36 kb double stranded genome that can be harnessed to carry up to 40 kb of exogenous DNA (DelloRusso et al. 2002; Doerfler 1986; Dudley et al. 2004; Hartigan-O'Connor et al. 2002). Unfortunately, the transgene expression resulting from adenoviral vector delivery is typically short lived because of silencing and clearing of the transduced cells by host immune responses against viral proteins, particularly with first generation vectors that retained numerous viral genes (Schagen et al. 2004; Tripathy et al. 1996; Yang et al. 1996). Systemic delivery of high doses of first generation adenoviral vectors also led to profound activation of an innate host immune response (Schnell et al. 2001). A number of important improvements to adenoviral vector technology have been made, including elimination of genes essential for replication, which shuts down viral gene expression (second generation vectors) and elimination of all viral genes from gutted or helper-dependent, (third generation) vectors (Amalfitano et al. 1998; Hartigan-O'Connor et al. 2002; Jiang et al. 2004; Xiong et al. 2007). These gutted vectors have the great advantage of not expressing viral antigens and they also are capable of packaging large genes defective in certain muscle diseases, such as dystrophin/DMD (DelloRusso et al. 2002; Dudley et al. 2004). Gutted adenoviral vectors have demonstrated reduced immunogenicity, high transduction efficiency, and prolonged gene expression (Alba et al. 2005; Espenlaub et al. 2008; Jiang et al. 2004; Kawano et al. 2008; Morral et al. 1999). However, concerns remain about the potential for long-term (many years) gene expression from even the gutted adenoviral vectors, and toxicity related adverse events have been triggered following high dose systemic administration (Brunetti-Pierri et al. 2004). Another disadvantage of the adenoviral vectors is their tremendous affinity for liver, such that intravascular delivery methods typically result in high level gene transfer to liver with minimal transduction of muscle, at least when infused at low pressure (Raper et al. 1998).

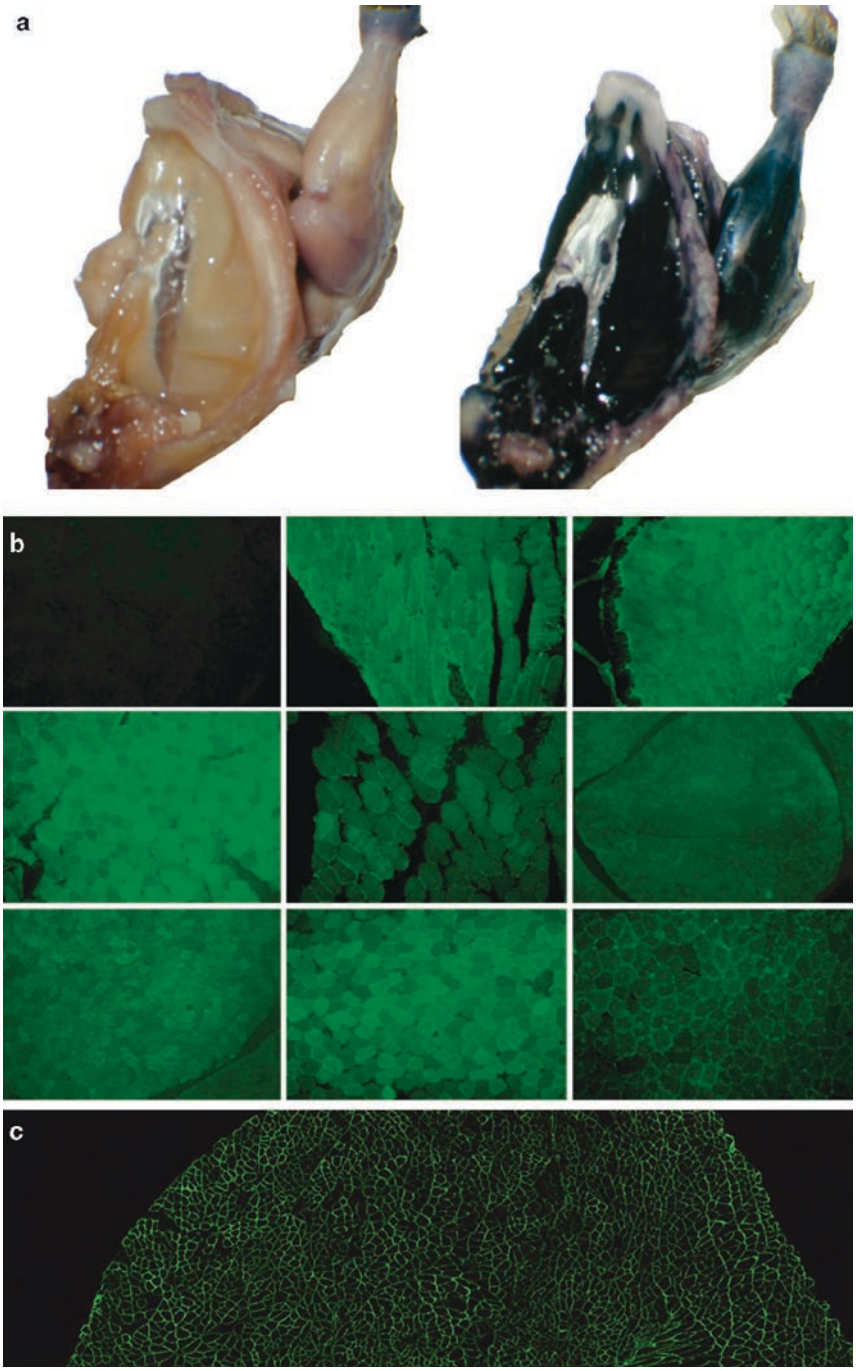
Recent studies suggest that the most promising vector for systemic delivery of gene to muscle is based on adeno-associated virus (AAV). AAV is a single stranded DNA virus, a member of the *Parvoviridae* family, and belongs to the genus *Dependovirus* (Muzyczka 1992). An increasing number of AAV serotypes are being identified, with more than 110 unique capsid sequences being recovered from human and nonhuman primate tissues (Gao et al. 2004, 2005). A growing number of AAV serotypes, such as AAV1-9, have been described and characterized as vectors for gene transfer, and each displays somewhat unique tropisms (Zincarelli et al. 2008). The AAV genome is 4.7 kb, with *rep* and *cap* open reading frames. The *rep* region codes for replication related proteins, and the *cap* region encodes the capsid proteins VP1, 2, and 3 (Muzyczka 1992). Inverted terminal repeats (ITRs) flank the *rep* and *cap* gene and are required for packaging DNA into capsids. Recombinant AAV may be produced by replacing wild type coding regions with a DNA sequence of interest up to ~4.5 kb (Muzyczka 1992). Vector genomes, containing ITRs from one serotype, can usually be packed into the capsid of another serotype, producing a recombinant pseudotyped vector (Rabinowitz et al. 2002). Comparative tropisms have been evaluated in a number of studies (Grimm and Kay 2003; Schultz and Chamberlain 2008; Zincarelli et al. 2008) even though route of delivery has been shown to affect the tissue distribution. Serotype 2 is the most studied AAV vector in transgene expression in vivo in mice (Li et al. 2007; Manno et al. 2003; Mori et al. 2006; Nicklin et al. 2001; Niemeyer et al. 2009). Recombinant AAV vectors have demonstrated great transduction efficiency and long-term expression from muscle in several systems. Following IM injection of AAV2 vectors into mice, secreted transgene expression was observed for at least 40 weeks (Kessler et al. 1996). IM expression of *lac*Z was observed for at least 19 months in a similar study (Xiao et al. 1996). A number of the pseudotyped vectors have shown a particularly high tropism for skeletal muscle when injected intramuscularly (Blankinship et al. 2004; Chao et al. 2000; Duan et al. 2001; Gao et al. 2002; Xiao et al. 1999). Long-term muscle expression has also been observed when AAV vectors have been delivered via systemic routes (Gregorevic et al. 2006; Odom et al. 2007; see below). Comparing serotypes from 1 to 9 systemically in mice, using bioluminescence imaging, showed high expression for AAV, 7 and 9, followed by moderate expression with AAV 1, 6, and 8 and low expression for AAV 2–5, although large differences were found in their relative transduction levels depending on which muscle type was examined (Zincarelli et al. 2008). AAV6 has high cardiac tropism in canines compared with AAV 8 and 9, although AAV7, 8, and 9 work well in rodents (Bish et al. 2008; Hajjar and Zsebo 2007). For skeletal muscle gene therapy AAV 1, 6, 8, and 9 all have high transduction efficiency (Blankinship et al. 2004; Gregorevic et al. 2004a; Inagaki et al. 2006; Wang et al. 2005; Yue et al. 2008; Zincarelli et al. 2008).

rAAV is a replication defective vector that does not usually integrate into the host genome (Schultz and Chamberlain 2008). Thus, AAV transduction permits long-term transgene expression with fewer potential complications related to insertional mutagenesis compared with retroviral vectors, although some concerns have been raised (Cavazzana-Calvo and Fischer 2007; Donsante et al. 2007). The mechanisms of AAV vector transduction, including the rate limiting steps involved, have been recently reviewed (Schultz and Chamberlain 2008). Given the aforementioned

ability of various AAV vectors to deliver transgenes to muscle for sustained expression and with reduced immunogenicity compared with other viral vector systems, we feel that rAAV vectors are the most promising for achieving clinically relevant delivery of therapeutic gene cassettes to striated muscles. Consequently, the remainder of this chapter will focus on rAAV.

The initial studies using rAAV for muscular transduction used IM injections to deliver the vector (Bohl et al. 1998; Fisher et al. 1997; Kessler et al. 1996; Xiao et al. 1996). However, delivery of rAAV vectors via IM injection transduces relatively small regions, typically less than 1 cm^2, such that targeting the entire musculature is not practical (Howell et al. 1997). However, subsequent results using intra-arterial delivery of rAAV vectors to isolated limbs and heart raised the possibility of establishing whole body systemic gene delivery methods (Greelish et al. 1999). These techniques initially relied on potentially toxic cofactors to enhance muscle targeting by inducing vascular constriction and permeability, and as such were not practical for human application. Subsequent refinements to these approaches included rAAV delivery under high pressure into isolated limb without added cofactors using arterial (Gonin et al. 2005) or venous routes (Su et al. 2005). High-pressure injections might not be a good option for frail patients awaiting muscle gene therapy, but they have not resulted in adverse effects in animal models (Toumi et al. 2006). A breakthrough in systemic delivery to muscles was reported in 2004 with the demonstration that rAAV6 vectors could achieve whole body muscle gene transduction when injected intravenously into young and adult mice (Fig. 10.2; Gregorevic et al. 2004a, 2006). Transduction was widespread throughout both skeletal (including diaphragm) and cardiac muscles, although smooth muscle is targeted less efficiently. An increased transduction could be obtained

Fig. 10.2 Systemic delivery of genes to striated muscles of adult mice using rAAV6. Examples are shown where three different expression cassettes were delivered to adult mice by tail vein injection of purified rAAV6 vectors. (**a**) Shown is the upper quadrant of mice injected with saline (*left*) or rAAV6 vectors expressing human placental alkaline phosphatase (AP; *right*) from the CK7 promoter (Salva et al. 2007). After whole mount staining for AP activity, the vector-injected animal is seen to express high levels of AP in all skeletal muscles, including those in the forearm and the diaphragm. No AP staining was seen in the saline injected mouse. (**b**) Shown are cryosections of various muscles from a wild type mouse injected via the tail vein at 6 weeks of age with 1×10^{12} vector genomes of rAAV6/CMV-*lac*Z and examined 11 days postinjection. Staining is with an anti-*lac*Z antibody. As can be seen, all muscles examined uniformly expressed the *lac*Z transgene, unlike those from uninjected controls. *Upper row*, *left* to *right*: gastrocnemius muscle from an uninjected control; diaphragm; forelimb muscle. *Middle row*, *left* to *right*: gastrocnemius muscle; masseter muscle; quadriceps (low power image). *Bottom row*, *left* to *right*: higher power image of the quadriceps; soleus; and tibialis anterior. (**c**) Long-term, uniform and widespread expression of microdystrophin following intravenous injection into dystrophin:utrophin double knockout mice (mdx:utrn$^{-/-}$). 3×10^{12} vector genomes of rAAV6/CMV-microdystrophin were injected into the tail vein of 4-week-old mdx:utrn$^{-/-}$ mice. The mouse was sacrificed 12 months later and the gastrocnemius muscle was cryosectioned and then stained with antibodies against the N-terminal domain of dystrophin. Examined 1 year after the injection. All myofibers display robust expression of microdystrophin and a normal morphology


a
b
c

if vector was co-administered with the vascular permeabilizing agent, vascular endothelial growth factor. However, at higher doses required for whole body gene transfer there was no increase in transduction using VEGF. Though these initial studies used rAAV6 pseudotyped vectors, subsequent studies have shown impressive results using rAAV8 and 9 (Ohshima et al. 2009; Rodino-Klapac et al. 2007; Wang et al. 2005; Yue et al. 2008).

10.4 Systemic Delivery of rAAV Vectors in Models of Duchenne Muscular Dystrophy

The ability to deliver genes systemically to muscle using rAAV vectors has raised hope for a gene therapy treatment for DMD, the most common form of muscular dystrophy (Chamberlain and Rando 2006). DMD results from mutations in the dystrophin gene, which expresses a 14 kb mRNA in all muscle cells (Koenig et al. 1987). However, the development of truncated mini- and microdystrophins that fit within the rAAV cloning capacity (<5 kb) has enabled studies on the phenotypic impact of rAAV gene transfer to the mdx mouse models for DMD (Crawford et al. 2000; Harper et al. 2002; Phelps et al. 1995; Sakamoto et al. 2002; Wang et al. 2000). Expression of microdystrophin has a profound ability to halt ongoing myofiber necrosis and improve histopathological parameters in dystrophic mdx mouse skeletal muscles, including diaphragm, although normal muscle contractility is not fully restored (Gregorevic et al. 2004a, 2006; Harper et al. 2002; Sakamoto et al. 2002; Wang et al. 2000; Watchko et al. 2002). Most microdystrophins reported to date do not localize neuronal nitric oxide synthase to the sarcolemma, impacting the ability of exercising muscles to trigger vasodilation to increase blood flow and prevent hypoxia and ischemia (Kobayashi et al. 2008; Lai et al. 2009). As there are numerous ways in which to design a microdystrophin, efforts to develop truncated clones with improved function remain an important focus of research (Lai et al. 2009; Odom et al. 2008).

An important feature related to systemic delivery of genes using rAAV vectors is the efficiency with which cardiac muscle can be transduced (Gregorevic et al. 2004a; Su et al. 2005; Bostick et al. 2008; Wang et al. 2005). The initial IM injection studies using rAAV had raised concerns that cardiac muscle might not be treatable using gene transfer because of accessibility issues. However, several serotypes of rAAV capsids transduce cardiac muscle more efficiently than skeletal muscles when delivered via the vasculature (Gregorevic et al. 2004a; Wang et al. 2005; Zincarelli et al. 2008). Nearly all patients with DMD develop cardiomyopathy (Emery and Muntoni 2003), and systemic delivery of rAAV vectors expressing microdystrophin has resulted in a significant amelioration of the dystrophic phenotype in hearts of young and adult dystrophic mdx mice (Bostick et al. 2008; Townsend et al. 2007). This ability to transduce cardiomyocytes and correct dystrophic properties is critical for the success of DMD gene therapy, as correction of skeletal muscle pathology without also correcting the cardiac problems can exacerbate

the cardiac phenotype (Towbin et al. 1993; Townsend et al. 2008). Of the numerous candidate approaches to therapy, rAAV-mediated gene transfer appears to be one of the only methods being tested that has potential to improve both cardiac and skeletal muscle pathophysiology (Bostick et al. 2008; Townsend et al. 2008).

10.5 Limitations of rAAV Vectors for Muscle Gene Therapy

Gene transfer of rAAV vectors to rodents has rarely been associated with elicitation of a cellular immune response against the AAV capsid proteins (Gregorevic et al. 2004a; Jooss and Chirmule 2003; Snyder et al. 1997; Xiao et al. 1996). In contrast, studies using IM injection of rAAV2 and 6 capsids in dogs elicited a strong cell-mediated immune response against the vector (Wang et al. 2007a; Yuasa et al. 2007). However, a brief course of immune suppression was found sufficient to allow long-term expression of microdystrophin when delivered to the canine model of DMD using rAAV6 (Wang et al. 2007b). Several studies have used rAAV vectors for systemic gene transfer to muscles of dogs (Arruda et al. 2005; Greelish et al. 1999; Ohshima et al. 2009; Yue et al. 2008; Chamberlain et al. unpublished observations). A recent study used hydrodynamic limb vein injection of rAAV8 vectors to achieve widespread expression of the canine myostatin propeptide in limb musculature. Nevertheless, cardiomyocytes and diaphragmatic muscle were not targeted (Bartoli et al. 2007; Qiao et al. 2008). A single intravenous injection of rAAV9 into neonatal dogs resulted in whole body skeletal muscle transduction, although canine cardiac muscle, unlike rodent muscle, was not transduced by rAAV9 (Yue et al. 2008). Also, it appears that skeletal muscle transduction was not maintained in the absence of immune suppression when rAAV was administered to more mature dogs (Wang et al. 2007b; Yue et al. 2008; Ohshima et al. 2009), although some studies suggested that this may depend on serotype and/or route of administration (Arruda et al. 2005). More importantly, it has become clear from several human clinical trials involving systemic and IM injection of rAAV vectors that a cellular immune response can be generated against both rAAV2 and rAAV1 (Manno et al. 2006; Mingozzi and High 2007; Stroes et al. 2008). These cellular immune responses are presumably dose dependent and could be influenced by vector pseudotype. While the best choice of AAV serotype for systemic skeletal muscle therapy is not clear from animal studies, clinical studies could potentially benefit from the use of multiple serotypes or modified capsids to escape prior immunity or to enhance transduction of multiple muscle types and groups. Nonetheless, it appears increasingly likely that systemic gene transfer to human patients will require careful safety studies and the use of transient immune suppression to avoid immune mediated destruction of transduced tissues (Hasbrouck and High 2008; Wang et al. 2007b).

Several other limitations will influence the use of rAAV vectors for systemic gene transfer to muscle. As noted earlier, the ~5 kb cloning capacity of these vectors hinders transfer of large genes such as dystrophin, dysferlin, and laminin (Blankinship et al. 2006). Whether the mini-gene strategies being developed for

dystrophin can be applied to other large genes will need to be addressed individually for each gene (Warner and Chamberlain 2002). An alternative approach to delivering mini-genes is to use multiple vectors to carry portions of a larger gene. Such a dual vector approach has led to surprisingly efficient generation of larger expression cassettes that were generated in vivo using either a trans-splicing method (Ghosh et al. 2007, 2008; Lai et al. 2005) or a homologous recombination (Duan et al. 2000; Halbert et al. 2002). Vector dose and production issues will also complicate systemic delivery to humans. While an entire mouse can be transduced with $1–4 \times 10^{12}$ vector genomes (vg; ~$4–16 \times 10^{13}$ vg/kg) (Gregorevic et al. 2004a; Grimm et al. 2003b; Salva et al. 2007; Wang et al. 2005), the same dosage, when scaled for humans application would require ~1,000 times more vector. Further, for large-scale clinical trials production yield and vector batch quality need to be improved. At present rAAV is typically produced by direct transfection of cells with plasmids (Grimm et al. 2003a; Xiao et al. 1998), a tedious and difficult to scale procedure. In contrast, baculovirus-based production methods have much greater potential for high titer production in smaller volumes and may be amenable to use in bioreactors (Merten et al. 2005; Negrete et al. 2007).

The high vector doses needed for systemic gene delivery also present safety issues beyond those associated with immune rejection of transduced tissues. It will be critical to monitor any potential innate immune responses and acute or chronic toxicity resulting from high dose rAAV administration, which will require careful dose escalation studies in animal models and in the clinic (Raper et al. 2003). As vector genomes transduce multiple tissues, muscle gene transfer will likely be facilitated by the use of muscle-specific promoters (Goehringer et al. 2009; Gregorevic et al. 2004a; Mori et al. 2006). Finally, while rAAV vectors integrate into transduced cells at a very low frequency, the high doses needed for systemic gene transfer will likely lead to a significant number of integration events in both muscle and nonmuscle tissues (Chamberlain et al. 2004; Donsante et al. 2007; Inagaki et al. 2007). Despite these various limitations, rAAV vector remains the most efficient vector for muscle gene therapy, as evidenced by the number of ongoing preclinical and clinical studies for muscle gene therapy (Mueller and Flotte 2008).

10.6 Summary and Future Directions

Systemic delivery for muscle gene therapy using rAAV vectors is showing great promise in preclinical models for muscular dystrophy and is close to being tested in the clinic. However, given the amount of muscle tissue in humans compared with that in mice and small dogs, a number of safety concerns need to be addressed and questions remain as to the best serotype(s) to use for vector encapsidation as well as the optimal expression cassette to deliver. The immune response to rAAV after systemic delivery should be given special attention given the fact that most humans have neutralizing antibodies and memory T-cells against various AAV serotypes (Halbert et al. 2006; Manno et al. 2006; Mingozzi and High 2007). However, it is

clear that highly functional dystrophin genes can now be delivered to all the striated muscles of adult mammalian models of muscular dystrophy, raising hopes for a treatment for these disorders in the coming years.

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Chapter 11
Modulating Immune Responses in Muscle Gene Therapy

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Abstract One of the major safety issues of gene therapy for muscular dystrophy remains the control of unwanted immune responses to the transgene and/or recombinant vector. The challenges of gene transfer for dystrophin-deficient patients consist of effective body-wide transfer of the therapeutic gene in the context of the ongoing tissue inflammatory and repair processes. Preventive strategies based on the delivery of vector with minimal tissue damage, the regulation of transgene expression using tissue-specific promoters, and the use of alternative transgenes to which the host is tolerant, are highly desirable to minimize immune responses. Transient drug-induced immune tolerance to the transgene product is likely to be an attractive therapeutic strategy for sustained transgene expression. Current immunosuppressive drugs nonspecifically induce deletion of activated/effector T cells by depleting antibodies, or generate T cell apoptosis or anergy by costimulation blockade. Recently, pharmacological therapies aimed at increasing regulatory T cells provide a novel area for the modulation of immune responses against the transgene product. The development of safe transient immunomodulatory strategies will be fundamental to the success of translational studies of promising gene- and cell-based therapies for these debilitating muscle diseases.

11.1 Introduction

Over the last decade, the gene therapy field has evolved rapidly from its focus on the efficacy of several viral and nonviral gene transfer systems to the safety of these strategies, culminating in several early-phase clinical trials, including studies of muscular dystrophy. The major safety issues identified from these preclinical and clinical studies involve the risk of insertional mutagenesis, inadvertent germline

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transmission of vector sequences, and the unwanted immune responses to the vector and therapeutic transgene product.

It is well known that avoidance of immune responses to a neoantigen is a better approach than eradication of B or T cell mediated responses, after activation. Therefore, in gene therapy, every effort should be made to avoid immune responses prophylactically. For this, viral gene therapy vectors have been designed to contain few or no viral coding genes to prevent expression of pathogenic genes (Thomas et al. 2003). Factors influencing the host immune response against the vector, such as route of vector administration, dose of vector, choice of promoter/enhancer, alterations to vector genome sequence and/or structure, and the status and nature of the target tissue (e.g. underlying disease or immune privileged sites) are important. Patient-related factors (age, gender, immune status, drug intake, co-morbid pathology, and others) are all critical to the development of a clinically-relevant gene-based strategy to treat human diseases.

Preventive strategies are not always sufficient to avoid immune responses, thus more potent alternatives are required. One of these alternatives is the use of drug-induced immunosuppression (IS), a very well-established strategy for organ transplantation that has been recently translated to the gene therapy field. The concept of drug-based immune tolerance induction emerged half a century ago in the context of organ transplantation. Immunological tolerance can be defined as a state in which the immune system, in the absence of the ongoing immunosuppression, does not mount a pathological response against specific antigens, even after new challenges with these antigens, while responses to other antigens are maintained (Strom 2007). Recent developments in immunology and drug discovery provide the basis for a paradigm shift from using nonspecific, toxic, wide-ranging IS toward a more specific, refined approach to attain the optimal balance of naïve cells, effector cells, memory cells, and regulatory cells. Notably, the benefits of standard and novel immunosuppressive drugs can now be reevaluated in the context of achieving an effective immunosuppressive effect while maintaining or enhancing key regulatory cells governing successful tolerance induction.

11.1.1 Mechanism of Immune Responses and Tolerance Induction

The immune system's reaction to antigen depends on (a) the relative frequencies of responding T and B cells and on the thresholds of the binding affinity that their receptors display, (b) the levels of antigen present, and (c) the period during which the antigen remains in secondary lymphoid tissue, where primary immune responses are initiated.

For successful activation of naïve T cells, signals derived by antigen-presenting cells (APCs) (Fig. 11.1) are required. The initial signal (Signal 1) is triggered by the antigen displayed on the surface of APCs in the form of peptides bound to histocompatibility molecules that trigger T cells with cognate T-cell receptors, and is

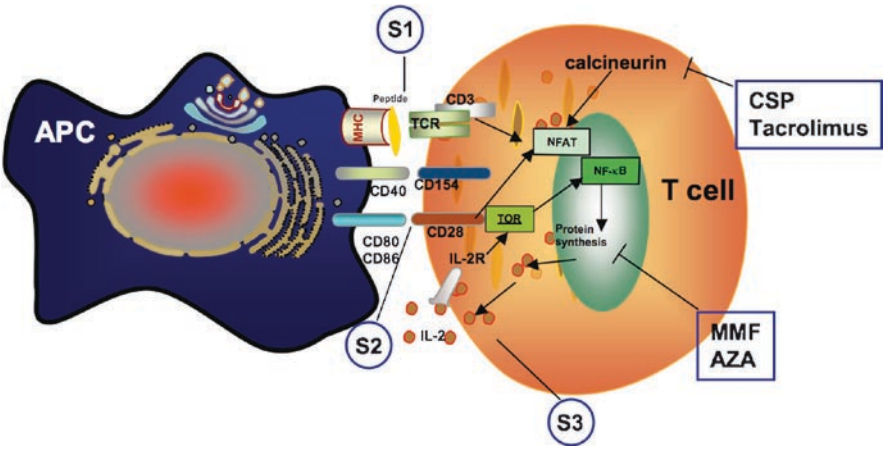

Fig. 11.1 Scheme of T cell activation and targets of immunosuppression drugs. Activation of naïve T cell signals derived by antigen-presenting cell (APC). The initial signal (S1) is triggered by the antigen displayed on the surface of APC in the form of peptides bound to major histocompatibility molecules (MHC) that trigger T cell with cognate T cell receptor (TCR), and is transduced through the CD3 complex. Signal 2 (S2) is derived from ACP's co-stimulation in response to the interaction of CD80 (B7-1) and CD86 (B7.2) with the engagement of CD28 on the surface of T cell. Signals 1 and 2 activate signal transduction pathways that trigger the expression of other molecules, including interleukin-2 (IL-2), CD154, and CD25. Signal 3 (S3) triggers cell via activation of target of rapamycin (TOR) pathway. *MMF* mycophenolate mofetil, *AZA* azathioprine, *CSP* cyclosporine, Tacrolimus (FK506), monoclonal antibodies to CD3 (muromonab), anti-IL2R (basixilimab, daclizumab), anti-CD154 (or CD40L) and CTLA4-Ig (blocking CD28-B7 interaction)

transduced through the CD3 complex. The recognition of antigen by T cell receptors provides specificity to the response. Signal 2 is derived from APC's costimulation in response to the interaction of CD80 (B7-1) and CD86 (B7-2) on the surface of dendritic cells (DC), with the engagement of CD28 on the surface of T cells. This is one of the best characterized T cell costimulatory pathways. Signals 1 and 2 activate signal transduction pathways that trigger the expression of others molecules, including interleukin-2 (IL-2), CD154, and CD25. An additional signal (Signal 3) triggers cell proliferation, which is provided by activation of the "target of rapamycin" pathway by IL-12 and IL-15, or by type I interferon. Lymphocyte proliferation also requires nucleotide synthesis. Proliferation and differentiation lead to a large number of effector and memory T cells. When antigens engage with B cells via the B cell receptor, a variety of intracellular signaling pathways are initiated, which ultimately lead to B cell activation and the production of antibodies. Recognition of peptides bound to class I or class II MHC molecules leads to the clonal expansion, activation, and maturation of T lymphocytes, resulting in effector populations of either cytotoxic ($CD8^+$) or helper ($CD4^+$) T cells, respectively. Thus, within days, the immune response generates the agents that recognize tissue damage – activated T cells and antibodies. If only signal 1, but not signals 2/3 are provided, the cell becomes unresponsive to antigen, a state originally called anergy,

which can often be overcome by exogenously provided IL-2. Costimulatory blockade may induce anergy and has been successfully applied for peripheral tolerance induction in rodents. The most frequently used agents are anti-CD154 antibody (blocking the CD40–CD154 interaction) and CTLA4-Ig (blocking the CD28–B7 interaction). Peripheral T cell deletion, anergy and regulation are mechanisms of tolerance induction in these models.

Another mechanism of immune tolerance is derived from regulatory T cells (Treg). These cells are well-characterized T cell subsets that play a central role in inducing and maintaining immunologic tolerance (Sakaguchi et al. 2008). One subset of Treg cells is present in 5–10% of unstimulated $CD4^+$ T cells and expresses the IL-2 receptor α-chain (CD25). $CD4^+$ $CD25^+$ Treg cells are generated in the thymus as a functionally mature subpopulation of T cells. However, these cells can also be induced from naïve T cells in the periphery as a part of the normal peripheral T cell repertoire where they potently suppress proliferation and cytokine production by both $CD4^+$ and $CD8^+$ T cells (Kang et al. 2007). Naturally occurring Tregs specifically express the transcription factor Foxp3 (forkhead box P3), a member of the forkhead/winged-helix family of transcription factors (Mays and Chen 2007). Foxp3 is a master regulator of Treg development and function. Naïve T cells in the periphery can also acquire Foxp3 expression, and consequently Treg function in several experimental settings. T cells are indispensable for controlling unresponsiveness to self-antigen and suppressing excessive immune responses (Sakaguchi et al. 2008). Notably, evidence for the induction of antigen-specific $CD4^+CD25^+$ T regulatory cells by hepatic gene transfer and its importance in maintaining the tolerance to the neoantigen have been demonstrated in in vivo models (Cao et al. 2007b; Mingozzi et al. 2007). These data open a possibility of using gene-based strategy not only for the treatment of diseases, but also for immune tolerance induction.

Skeletal muscle accounts for more than 30% of the human body mass and has important roles in survival of the species. However, little is known about the mechanisms of immunological tolerance to muscle autoantigens. Skeletal muscle fibers do not express MHC molecule I, preventing direct presentation of antigen peptides to self-reactive T cells, although regenerative myogenic cells may express low levels of MHC class I transiently during muscle repair. Therefore, it has been tempting to consider that ignorance is one of the main mechanisms that confer tolerance to this tissue. Ignorance is defined as the lack of antigen detection by the immune system due to absence of presentation, or the lack of appropriate T cell activation conditions. Recent data demonstrated two mechanisms for muscle tolerance (Calbo et al. 2008). The first is ignorance of antigen-specific $CD4^+$ T cells that showed vigorous humoral responses upon antigen-specific immunization. The second was the identification of antigen-specific $CD8^+$ T cells that lost their cytotoxic activity due to upregulation of programmed death 1 (PD-1) that favors tolerance. In this model, there was no evidence that Treg cells participated in muscle immunotolerance. However, the potential role of Treg cells in suppressing B and T cell responses was recently demonstrated in a model of gene transfer in skeletal muscle by AAV vectors encoding a highly immunogenic transmembrane transgene (influenza hemagglutinin protein). Adoptive transfer of antigen-specific $CD4^+CD25^+$ Treg

cells resulted in sustained local transgene expression abolishing cellular toxicity and humoral responses (Gross et al. 2003). Therefore, it is possible that drug-induced and/or cellular therapies aimed at increasing the pool of functional Tregs may prove beneficial in gene therapy for muscular dystrophy.

Modern research into mechanisms of immune tolerance offers the promise of reprogramming the immune system to harness the natural tolerance mechanism of the body. There is growing evidence that pharmacological therapies that induce immune tolerance by diverting the T cell activation pathways augment the apoptosis rates of activated T cells; or by up-regulating Tregs provide a novel area for the treatment of immune-mediated diseases (Roncarolo and Battaglia 2007; Tao et al. 2007). Thus, preservation of these natural Tregs as well as augmentation of inducible subsets may provide an alternative for successful immune modulation, without the side effects of immunosuppressive drugs (Kang et al. 2007).

11.1.2 Muscular Dystrophy: Human Disease and Animal Models

Duchenne muscular dystrophy (DMD) is a severe X-linked recessive, progressive muscle-wasting disease affecting approximately 1:3,500 boys. A milder form of the disease, Becker muscular dystrophy (BMD), has a later onset and longer survival rates. Both disorders result from mutations in the dystrophin gene. Approximately 65% of DMD and BMD patients have gross deletions of the dystrophin gene resulting in the absence of full-length molecules of dystrophin. In humans the gene is expressed in all muscles, with the highest dystrophin levels in skeletal and cardiac muscles (Blake et al. 2002). Dystrophin is an essential component of a multiprotein complex, the dystrophin-glycoprotein complex (DGC), which links the cytoskeleton of the muscle fiber to the extracellular matrix which protects the muscle cell from stress caused by mechanical force. Thus, dystrophic muscle is susceptible to damage induced by muscle contraction and undergoes progressive degeneration and necrosis. Current strategies designed to reduce the severity of muscular dystrophy are based on reducing muscle necrosis, enhancing muscle degeneration, and combating fibrosis using a variety of drugs and nutritional interventions (Nowak and Davies 2004). For most of the patients, curative treatments will involve cell therapy and/or gene replacement, and for a subgroup of patients, corrections of specific mutations are envisioned.

The main goal of gene replacement therapy for DMD is the production of endogenous dystrophin protein in a previously dystrophin-deficient muscle. Immunostaining of muscle sections is essentially negative in DMD patients; whereas in BMD muscle samples, sporadic cell fibers are positive (Hoffman et al. 1992). By western blot, DMD patients have little or no detectable dystrophin, and those with BMD have reduced levels of dystrophin with an abnormal size due to deletions of a specific coding region. Thus, it is possible that expression of the curative dystrophin will be perceived by the immune system as a neoantigen (foreign antigen), which might induce destruction of recently transduced cells and accelerate

the loss of muscle fibers in patients who have already lost many of their muscles due to the dystrophic pathological process.

The replacement of the full-length dystrophin gene is challenging due to its large coding sequence (14-kb mRNA transcript). Structure–function analyses of dystrophin variants expressed in the milder form of the disease (BMD) demonstrate that large in-frame deletions in the central rod domain minimally affect the functional capacity of dystrophin (Blake et al. 2002). The development of truncated dystrophins (μ-dystrophins) has enabled the design of expression cassettes to be highly effective at preventing muscle degeneration in models of DMD using gene therapy. However, it is possible that the formation of novel-junction sequences within the μ-dystrophin protein could potentially be presented as neoantigens with increased risk of immune responses.

Most of the gene therapy trials for genetic diseases are aimed at sustained expression of therapeutic genes by introducing the vector in the target tissue with minimal or no tissue damage. Activation of T cells is dependent on the "danger" or inflammatory signal. Thus, the context of antigen presentation for the treatment of DMD has a significant role in T cell activation, since the health status of target tissue, the nature of the vector, and tissue injury associated with the vector delivery all impose additional risks of unwanted T cell activation. Early studies on the prevalence of the expression of MHC class I in skeletal muscle of DMD revealed a remarkable variation. More recently, muscle biopsies of humans with muscular dystrophy or idiopathic inflammatory myopathies revealed upregulation of MHC class I in the sarcolemma in 11% and >60% of patients, respectively (van der Pas et al. 2004). Interestingly, prolonged immunosuppression was associated with significant reduction in MHC class I detection. Studies demonstrated that MHC class I was upregulated in Golden Retriever muscular dystrophy (GRMD) dogs following IM injection of a viral vector (Yuasa et al. 2007). Therefore, it is possible that several underlying host- and vector-dependent factors may influence the MHC class I status on the skeletal muscle fibers of DMD, and potentially the modulation of cellular immune responses. Further preclinical studies are imperative to address the safety profile of such IS regimens and a careful evaluation of the data has to take into consideration the evolutionary level of the immune system of the model (rodent vs dogs vs nonhuman primates vs humans), the disease-specific model availability (normal animals vs. animals with underlying-specific disease relevant to humans), and lastly, the possibility of testing drugs developed specifically for humans that recognize therapeutic targets in the disease model. Preclinical studies for DMD are favored by the homologues of the disease identified in several animals, including mice and dogs (Banks and Chamberlain 2008; Wang et al. 2009; Wells et al. 2002). The mdx mouse is the most widely used model of DMD, and displays some features of moderate muscle degeneration. Apart from the diaphragm, mechanical function and cardiomyopathy are less affected than in humans, and this model displays only a 20% reduction in lifespan. There are five mdx models characterized by distinct underlying mutations which have resulted in premature stop codons; RNA splicing defect, mutation at splice acceptor site, nonsense mutation, and the creation of a new splice donor site that generates a premature stop

codon in RNA transcript (Banks and Chamberlain 2008). Thus, this model is easily accessible and is versatile for the testing of several alternative therapies for DMD. However, the intrinsically inbred nature and the small size of the mouse model preclude the translation of studies on the immune responses and the determination of efficacy of delivery techniques to achieve widespread transduction of the disease target. The X-linked canine muscular dystrophy model resulted in a spontaneous mutation in the dystrophin gene identified in several breeds of dogs; the best characterized is the GRMD. The disease results from a mutation in the 3′ consensus splice site of exon 6 of the dystrophin gene, which leads to skipping of exon 7, and consequent disruption in the open reading frame and premature termination of translation with no detectable protein (Cooper et al. 1988). The pathogenesis of GRMD is similar to humans with DMD already evident at the time of birth, such as extensive necrosis of the muscles of the limbs, and severe fibrosis and joint contractures developed by six months of life. Similar to humans, young GRMD models frequently die from cardiac or respiratory failure, although some survive to reach several years of age (Collins and Morgan 2003; Cooper et al. 1988). Since these dogs are immunocompetent animals, and at adult age they have a body mass that is comparable to DMD patients, they represent an ideal model to determine the immunogenicity and the ability of scaling up to novel therapeutic platforms for translational studies for humans. However, these dogs are fragile and require frequent and close surveillance that precludes studies on large numbers of animals (Collins and Morgan 2003). Nevertheless, studies on immunosuppression coupled with cell- and/or gene-based strategies in the GRMD dogs are of fundamental importance for translational studies (Liu et al. 2004; Wang et al. 2009).

11.1.3 Preventive Strategies

11.1.3.1 Tissue-Specific Promoters

Systemic delivery of vectors for DMD gene therapy has the risk of spreading to non-targeted tissues and overall toxicity and/or the initiation of a host immune response against tissues expressing the transgene or gene vector (Wells et al. 2002). Transgene expression restricted to the target tissue by using tissue-specific promoters has been extensively utilized to avoid immune responses to the transgene. Thus, the use of muscle-specific promoters is highly desirable in the development of gene therapy vectors for DMD to minimize the harmful effects of ectopic transgene expression (Cordier et al. 2001) and to prevent transgene expression within antigen-presenting cells (APCs), such as dendritic cells (DC) or macrophages. However, the uptake of exogenous protein by APC and presentation in the context of MHC class I, as well as class II, does not require direct transduction of DC by the recombinant vectors. Plasmid DNA appears to generate cytotoxic $CD8^+$ lymphocytes using a cross-priming mechanism (Wells et al. 2002). Therefore the use of muscle-specific promoters would not prevent immune responses if cross-priming is involved, even

if the parental vectors do not transduce APCs. Moreover, muscle-specific promoters are typically less active than viral promoters; subsequently, high vector doses will be required. Hence, it seems likely that the balance between vector dose and tissue specificity will define the optimal strategy. Hauschka and colleagues (Salva et al. 2007) developed a series of muscle-specific promoters by modification and optimization of a muscle creatine kinase (MCK)-based construct to fulfill the desirable tissue-specificity and high efficacy. The size of these promoter sequences may overcome the capacity of some promising vectors with restricted packing capacity. Further modifications resulted in new cassettes that drive high-level tissue-specific transgene expression in both cardiac and skeletal muscle with a packaging size constraint for accommodating the μ-dystrophin cDNA in AAV vectors (Salva et al. 2007; Wang et al. 2008). Thus, tissue-specific promoters may provide an alternative to avoid immune responses but its effect is rather limited if high vector doses are required.

11.1.3.2 Alternative Therapeutic Transgenes

An alternative approach for gene transfer for DMD is the upregulation of therapeutic transgenes that are endogenously expressed (nonforeign proteins) and can substitute dystrophin function or increase muscle growth. These approaches have the potential to circumvent the risk of immune responses to dystrophin in most DMD patients with null mutations in the dystrophin gene. Pharmacological strategies also are able to further enhance the expression of these proteins as an alternative or coadjuvant therapy for DMD. Two alternative transgenes are discussed below.

Utrophin

Utrophin is the autosomal paralogue of dystrophin that might be able to serve as a surrogate for the dystrophin in DMD muscle fibers (Miura and Jasmin 2006). These proteins present a high degree of sequence identity and functional redundancy. Utrophin is also expressed in the muscle of DMD patients and animal models of the disease, albeit at very low levels (Karpati et al. 1993). Studies in mdx mice demonstrate that pre- or post-natal upregulation of utrophin in dystrophic muscle fibers can restore sarcolemmal expression of the DGC and alleviate the dystrophic pathology (Banks and Chamberlain 2008). An increase in expression levels of utrophin correlates with a delay in wheelchair use in DMD patients, validating this type of therapy in humans (Kleopa et al. 2006). In the GRMD model, expression of a synthetic truncated form of human utrophin was accompanied by local upregulation of the DGC with reduced fibrosis (Cerletti et al. 2003), which further supports this alternative for the treatment of DMD. In this model, the use of cyclosporine was required to sustain transgene expression with no cellular or humoral immune responses to the non-species specific transgene. Unfortunately, utrophin delivery to GRMD dogs using other vector systems is currently lacking.

Inhibitor of Myostatin

Myostatin is an endogenous negative regulator of muscle growth that belongs to the transforming growth factor beta superfamily. Myostatin normally exists in a latent complex in blood circulation, and the myostatin propeptide (MPRO) is one of the major negative regulators of the protein (Patel and Amthor 2005). The function of myostatin is conserved in several species, including mice, dogs and humans. In the absence of myostatin, muscle regeneration has been shown to occur earlier and more robust after tissue damage. Therefore, endogenous enhancement of expression of the MPRO gene offers the opportunity to treat the skeletal muscle disease of DMD without the hurdles imposed by immunological responses against a neotransgene in subjects with a null mutation in the dystrophin gene. The *mdx* mouse lacking myostatin exhibited increased muscle mass and strength, and decreased fibrosis. Notably, Whippet dogs lacking myostatin are muscular, healthy and have a typically faster racing speed. Therefore, the down-regulation of myostatin by MPRO has several potential advantages as a treatment for DMD: (1) the muscle wasting phenotype can be delayed or prevented; (2) muscle fibrosis can be reduced, and (3) as myostatin is a secreted protein, local expression of MPRO to the affected muscle is not required. Finally, ongoing clinical studies of the use of MYO-029, a neutralizing antibody to myostatin, have initially demonstrated safety in limited studies of adults with muscular dystrophy (Wagner et al. 2008). Xiao and colleagues (Qiao et al. 2008a) showed that a myostatin blockade in both normal and dystrophin-deficient *mdx* mice by systemic delivery of MPRO gene with an AAV serotype 8 (AAV-8) vector, could enhance muscle growth and ameliorate dystrophic lesions. Further studies in normal dogs showed that expression of MPRO resulted in enhanced muscle growth without immune responses as evidenced by the lack of $CD4^+$ and $CD8^+$ T-cell infiltration in the vector-injected limbs. MPRO-based strategies are not effective in the treatment of the underlying cardiac disease.

11.1.4 Induction of Central Tolerance

The induction of hematopoietic mixed chimerism, defined as the coexistence of donor and recipient hematopoietic cells, could be an approach to induce robust central tolerance. This strategy capitalizes on the use of nonmyeloablative hematopoietic stem cell transplantation (HSCT) prior to injection of myogenic stem cells from the donor HST (Parker et al. 2008). In this study two irradiated GRMD dogs with established full or partial chimerism first achieved at 11–26 months, were injected with donor-specific muscle-derived cells. The recipient dogs were transiently immunosuppressed with cyclosporine for 40 days. Local dystrophin expression, up to 6.5% of normal levels, was sustained for periods up to 24 months post injection. This strategy is promising, as novel developments in the area of nonmyeloablative HSCT are ongoing for other cell-based therapies for benign diseases. Induction of central tolerance by intrathymic injection of viral vectors has

been tested in rodents, and offers alternatives to antigen-specific tolerance (Ilan et al. 1996; Marodon et al. 2006).

11.1.5 Immunosuppression Strategies

Since preventive strategies are not always sufficient to avoid immune responses, immunosuppressive drugs will be required. Current treatment for immunological disorders are nearly all empirical in origin, using immunosuppressive drugs identified by screening large numbers of natural and synthetic compounds.

Therapeutic immunosuppression (IS) can be achieved by depleting lymphocytes, blocking lymphocyte response pathways, or diverting lymphocyte traffic. The most commonly used drugs in clinical studies include glucocorticoids, small-molecule drugs, depleting and nondepleting protein drugs (polyclonal and monoclonal antibodies), fusion proteins, and intravenous immune globulin (Table 11.1). In the majority of IS protocols for organ transplants, IS drugs are given in combination because many of the classes of IS drugs are able to act synergistically. This allows for greater efficacy from lower doses of drugs, an important consideration when trying to avoid unwanted dose-dependent side effects (Halloran 2004). The regimen and the duration of the IS required to prevent or ameliorate undesirable immune responses following gene therapy is not yet defined. There is evidence in several large animal models of disease, suggesting that transient (short-duration) immune modulation would allow sustained transgene expression and correction of the disease phenotype.

Early studies in DMD patients using IS drugs alone or in combination with myoblast implantation revealed a beneficial role of IS in the clinical management of the disease. The use of anti-inflammatory and IS drugs can reduce the severity of muscular dystrophy by producing short-term functional improvement or slow disease progression. Clinical trials with cyclosporine in DMD subjects revealed a significant increase in muscle force after 8 weeks of treatment (Sharma et al. 1993). The beneficial effect of IS was also observed with prednisone in boys with DMD, data similar to those obtained in the GRMD model (Liu et al. 2004). Ongoing clinical studies of cyclosporine and prednisone in DMD patients will define the potential role of IS in the disease phenotype. A series of small-scale clinical trials testing the feasibility of myoblast transplantation were performed in the last decades. In some of these studies, humoral responses to the cell and/or the dystrophin protein were detected (Wells et al. 2002). Early phase studies of allogeneic myoblast transplantation in patients with DMD were typically of limited efficacy due to immune responses, rapid cell death and poor migration of the therapeutic cell. Long-term studies using cyclosporine combined with myoblast therapy resulted in increased muscle strength after 7 months, probably due to the use of IS (Miller et al. 1997). Successful cell transplants were also obtained by sustained administration of tacrolimus (FK506) or cyclophosphamide. The addition of IS in this setting was beneficial, but resulted in low efficacy due to non-immunological toxicity of the transplanted myoblasts

Table 11.1 Classification of immunosuppression drugs and their effect on T regulatory (Treg) cells

General class	Drug	In vivo effects on Tregs
Corticosteroids	Prednisone	Positive
	Methylprednisolone	Positive
	Dexamethasone	Positive
Calcineurin inhibitor	Tacrolimus (FK-506)	Negative
	Cyclosporine (CsA)	Negative
Antimetabolites	Azathioprine (AZA)	N/A
	Cyclophosphamide	Negative
	Mycophenolate mofetil (MMF)	No effect
Target of Rapamycin inhibitors	Sirolimus (rapamycin)	Positive
	Everolimus	Positive
Polyclonal antibodies	Rabbit Antithymocyte globulin	Positive
	Horse Antithymocyte globulin	Negative
Monoclonal antibodies	Muromonab-CD3 (anti-CD3)	Positive
	Alemtuzumab (anti-CD52)	Positive
	Basiliximab (anti-IL-2 receptor)	Negative
	Daclizumab (anti-IL-2 receptor)	Negative
	Rituximab (anti-CD20)	N/A
Others	FTY720	Positive
	CTLA4-IG (LEA29Y)	Positive
	Intravenous immune globulin (IVIG)	Positive

(Peault et al. 2007). Nevertheless, these studies provide evidence on the safety of some IS drugs on the DMD phenotype that could be adapted for preclinical and clinical studies based on gene- and/or cell-based therapies.

11.1.5.1 Route of Vector Administration

Proof of concept studies of intramuscular (IM) or intravascular (IV) injection of vectors encoding a series of therapeutic genes in murine models for DMD significantly improve muscle membrane integrity and muscle function (Odom et al. 2007; Wang et al. 2009). Before translating these discoveries into clinical therapy, a major limitation is the formidable scale-up from mouse to dog to human. Skeletal muscle contains one of the highest capillary densities in the body that can be chemically and/or mechanically modified to ensure vascular leakage of fluid containing vectors. The capillaries, consisting of a single layer of endothelial cells, permit rapid exchange with the interstitial fluid. To achieve scale-independent dosing, one needs to take advantage of intravascular delivery techniques aimed at widespread vector dissemination. Regional perfusion utilizing the vascular tree of the isolated limb as the delivery network affords such a solution to the problem of scaleable delivery. It is important to take into consideration that the vascular permeability of large vertebrates is substantially less permeable than their homologues in mice (Williamson et al. 1971).

Therefore, the delivery of vector to the muscle in the large adult animal has relied upon transient alteration in endothelial permeability by using vasoactive drugs (Arruda et al. 2005; Greelish et al. 1999), or by increasing the hydrostatic pressure (Hagstrom et al. 2004; Su et al. 2005). Thus, based on the existing knowledge, it is clear that the combination of vector system, route of delivery, vascular-modulating drugs and the animal model will define the clinical applicability of the many distinct approaches for the treatment of DMD.

11.1.5.2 Non Viral Vectors

Direct intramuscular injection of naked plasmid DNA into the GRMD dog muscles was also attempted with profoundly low efficiency (Howell et al. 1997; Howell et al. 1998). Newly developed hydrodynamic delivery through peripheral vein injection of plasmid DNA into nonhuman primate and canine muscles has shown much improved efficiencies (Hagstrom et al. 2004) with no major local or systemic toxicity (Toumi et al. 2006). However, the possibility of using nonviral vectors for transduction of widespread areas of the skeletal muscle and heart has not been tested in large animals.

11.1.5.3 Adenoviral Vectors

Initial studies on gene transfer show that adenoviral (Ad) vectors were among the most commonly used vectors for gene therapy due to their large cloning capacity, ability of transducing post-mitotic cells, and the low risk for insertional mutagenesis and germline transmission. (Verma and Weitzman 2005). However, the ability of adenoviral vectors to induce long-term transgene expression has been hampered by both the host immune response and the nonimmune loss of vector genomes. The use of IS drugs have been attempted to circumvent the immunological barriers. In mdx mice (Jiang et al. 2004), coblockade of both CD28/B7 and CD40L/CD40 costimulatory pathways was required for effective inhibition of the Ad vector-induced humoral immune response in DMD mice, whereas blockade of CD28/B7 alone by murine CTLA4Ig would be sufficient for prolonged dystrophin expression in treated muscles. Transient IS with tacrolimus (FK506) efficiently suppressed both cellular and immune responses in Ad-injected mdx mice (Lochmuller et al. 1996). In GRMD dogs, expression of human dystrophin by adenoviral vectors was sustained by continuous use of cyclosporine, whereas in dogs without IS both humoral responses and robust infiltrates of $CD4^+$ and $CD8^+$ T cells prevented transgene expression (Howell et al. 1998). Efforts from several investigators resulted in the development of helper-dependent (HDAd) or gutless adenoviral vectors (Verma and Weitzman 2005). These engineered vectors are deleted of all viral genes from the parental vector backbone, whereas maintaining the inverted terminal repeats to keep them intact. Encouraging data obtained in mice without IS (Dellorusso et al. 2002) motivates further studies of HDAd in

large animal models. However, short-term gene transfer studies using the HDAd in the muscle of GRMD newborn dogs showed that this vector has limited efficacy (Gilbert et al. 2001). Systemic administration of HDAd vector is further complicated by the potential liver toxicity and transient thrombocytopenia as observed in canine models of hemophilia (Arruda 2006). Thus, therapies based on repeated doses of HDAd vectors for widespread sustained transduction of the skeletal muscle for the treatment of DMD at this point are not supported by the safety profile of these preclinical studies.

11.1.5.4 Retroviral Vectors

Oncoretroviral vectors are attractive for the treatment of genetic disease when stable long-term integration in the genome is required. These vectors are efficient in gene transfer for dividing cells and they can accommodate up to 11 Kd transgene cassettes (Verma and Weitzman 2005). However their efficacy in DMD models is limited and the results in *mdx* mice showed poor transduction of skeletal muscle. Moreover, safety concerns of the risk of leukemia development by insertional mutagenesis in patients enrolled in a phase I clinical study for severe combined immune deficiency further diminished the use of this vector system for genetic diseases (Hacein-Bey-Abina et al. 2003).

In contrast, lentiviral vectors based on the human immunodeficiency virus has the ability of transducing dividing and non-dividing cells; the insert capacity is 7.5–9 Kb, and early data showed stable expression of transgenes in muscle cells, muscle stem cells or early precursors. Data from the use of lentiviral for ex vivo transduction of autologous mesangioblast stem cell with μ-dystrophin gene or heterologous mesangioblast stem cells harvested from normal dogs and injected into the GRMD model are encouraging (Sampaolesi et al. 2006). Following the delivery of the mesangioblasts by intra-arterial (femoral) injection, dystrophin expression was associated with remarkable improvement of both muscle morphology and function. There was improvement of the disease phenotype in both groups of treated dogs. The group of dogs receiving heterologous cells required transient IS with cyclosporine or cyclosporine with rapamycin to avoid immune responses to the donor cells (containing the wild-type canine dystrophin derived from normal young donor dogs). These animals presented improved strength and gait of skeletal muscles and reduced serum creatine kinase levels, compared to lentiviral-transduced mesangioblasts. One potential explanation is that the IS was playing a co-adjuvant role in the improvement of the disease phenotype, and further studies will be required to confirm the potential of this strategy. Nevertheless, this study provides evidence that intravascular delivery of stem cell and/or gene therapy coupled with IS has potential therapeutic use in DMD. In a nonhuman primate model, autologous or allogenic (with IS with FK-506) transplantation of myoblast modified by lentiviral-mediated gene transfer was also associated with sustained, yet low, local gene expression (Quenneville et al. 2007).

11.1.5.5 Adeno-Associated Viral (AAV) Vector

AAV is single-stranded DNA virus belonging to the Parvovirus family. Recombinant AAV vectors are advantageous for human gene therapy because of their ability to transduce dividing and nondividing cells, including skeletal muscle and cardiac muscle fibers. To date there are 12 serotypes characterized and more derivatives identified. The use of AAV vectors as a gene delivery vehicle to skeletal muscle has shown promise both in preclinical studies and early-phase clinical trials (Brantly et al. 2006; Kay et al. 2000; Stroes et al. 2008). Overall the safety profile was excellent with no serious sustained adverse effects. Moreover, the therapeutic potential of AAV serotype 2 (AAV-2) following local delivery to skeletal muscle is attested to by documented long-term local transgene expression (Jiang et al. 2006b; Manno et al. 2003), which is an attractive property for the treatment of genetic diseases.

However, two major drawbacks associated with IM injection of AAV vectors are the risk of immune responses and the restricted transduction of muscle area. Studies in mice and dogs had previously shown that local immune responses in skeletal muscle could cause activation of T and B lymphocytes in draining lymph nodes following IM injection of AAV-2 (High 2005). In the case of a coagulation factor IX (FIX) transgene, the underlying mutation in the dog FIX gene predicts the risk of immune responses to the transgene. Dogs with missense mutation had a lower risk than dogs with null mutation. In the later model, a relatively mild transient suppression protocol using cyclophosphamide was often sufficient to prevent this response in hemophilia B dogs (Herzog et al. 2001). The identification of novel potent AAV vectors offers the opportunity to further increase AAV transduction efficiency (Gao et al. 2002). In the hemophilia B dog model, IM injection of AAV-1 resulted in > tenfold higher levels of FIX compared to AAV-2 vector, but immune response to FIX limited the duration of expression and required IS for long periods (Arruda et al. 2004). Thus, by increasing the amount of local FIX synthesis, the risk for antibody formation increased. In other large animal models, IM AAV vectors of alternate serotypes (−1, −5, −7, −8) encoding erythropoietin in nonhuman primates resulted in an anemia due to inadvertent immune response, following supraphysiological levels of transgene expression (Chenuaud et al. 2004; Gao et al. 2004). In a model of limb-girdle muscular dystrophy due to deficiency of α–sarcoglycan (SGCA), overexpression of the therapeutic transgene by AAV under the control of the CMV promoter resulted in cellular toxicity (Dressman et al. 2002). Thus, immune responses following IM injection of AAV prevent the clinical translation of the full potential of these vectors.

In the GRMD model, Storb and colleagues demonstrated T cell-mediated immune responses following IM injection of AAV-2 or AAV-6. This prompted the authors to use short-term IS (up to 3 months post vector injection) to prevent immune responses (Wang et al. 2007). The regimen, containing cyclosporine, MMF and antithymocyte globulin was effective in sustaining expression of canine μ-dystrophin after discontinuation of the drugs without local T cell infiltrates (Wang et al. 2007). Some lymphocytic infiltrates were still observed at later timepoints (4 months after immune suppression was discontinued). Recently, studies in

mice demonstrated that antigen-specific $CD8^+$ T cells in AAV-transduced skeletal muscle were associated with programmed death of effector cells; the cellular infiltrates were considered immunologically silent (Lin et al. 2007; Velazquez et al. 2009). Moreover, strong $CD8^+$ cytotoxic T cell response against a β-galactosidase transgene with severely limited expression in AAV-2-transduced canine muscle (normal dog) and IS with mycophenolate mofetil (MMF) and cyclosporine partially blocked this immune response, resulting in improved transgene expression (Yuasa et al. 2007). Interestingly, in this study no cellular immune response to the vector capsid was observed. The use of AAV-8 in this dog model was also associated with both cellular and humoral immune responses in the absence of IS (Ohshima et al. 2009). Thus, further characterization of these cells are required to assess whether in GRMD dogs the residual cellular infiltrates at the injection site are directed to the AAV capsid (see below) and/or the transgene and notably, whether these cells are immunologically functional or not. These data in dogs and nonhuman primates demonstrate that immune responses following IM injection of early or novel AAV remain the main obstacle in translating these studies to humans. It is possible that AAV vectors more readily yield undesired gene transfer to antigen presenting cells in large immunocompetent animals than in mice, or that antigen presenting cells and/or T cells respond more strongly to inflammatory signals. Therefore, optimized immune suppression protocols will be required in such gene therapies in humans, if the canine model is a good predictor of the response.

Although access to the skeletal muscle is easily performed by direct IM injections, achievement of AAV therapeutic target doses in humans has proved impractical because of the large number of injections required. Moreover, it is an ineffective strategy for the treatment of DMD that requires widespread transduction of the skeletal muscle, diaphragm, and heart. Thus, the development of intravascular delivery of vector for regional or systemic tissue transduction is critical for translation to humans. In early trials on intravascular delivery of AAV vectors, immune responses to the vector capsid and the risk of germline transmission were recognized as critical challenges to the safety of this strategy. These observations underlie the importance of translational studies in large animals, which have proven a more stringent screen and more accurate predictor of success in humans. Preclinical studies in the GRMD model are critical in terms of defining the feasibility and safety of a given strategy.

Greelish et al. showed that regional intra-arterial delivery of rAAV to the skeletal muscle, with either adenovirus or AAV vectors, resulted in extensive transduction of skeletal muscle in rats and hamsters (Greelish et al. 1999). This procedure required the use of histamine and papaverine to enhance vector extravascular dissemination to the target muscle. Studies in hemophilia B dogs demonstrate that this technique was also successful in widespread transgene expression in a large animal. Regional vascular delivery of AAV-2 canine FIX to the muscle tissue in a single limb perfusion model resulted in long-term expression (> 4 years) of the transgene (Factor IX) at therapeutic levels (ranging from 4 to 15% of normal). In this study, transient IS with weekly injections of cyclosphosphamide (total 6 doses) prevented the formation of antibody to the transgene. In the hind limb that received the direct

intramuscular injection, cF.IX expression was confined to the sites of injection, with a radius of diffusion of ~0.5 mm, whereas the hind limb that received vector by the intravascular infusion process showed transduction throughout the muscle groups supplied by the injected vessel (Arruda et al. 2005). In nonhuman primates, intrafemoral delivery of an isolated limb of AAV-8-GFP vector resulted in transduction of > 65% of the muscle area perfused three weeks after injection with no acute toxicity (Rodino-Klapac et al. 2007). Although it will be necessary to address details of the immune response to the transgene product in a GRMD model, and to identify an approved pharmacologic agent that can induce a vascular leak (i.e., a drug other than histamine), these studies have established the efficacy of the strategy.

Stedman and colleagues developed an alternative noninvasive delivery method aimed at ensuring widespread gene transfer to the skeletal muscle named afferent transvenular retrograde extravasation (ATVRX). This delivery method is achieved by an intravenous injection of vector through a peripheral vein of an isolated limb under elevated hydrostatic pressure to ensure vascular leakage in large animals (Su et al. 2005). The increase in venous pressure induces passive capillary dilation and changes in transmural pressure, which facilitates tissue perfusion (Williams 1999). Thus, a sudden increase in the venous pressure by injecting enough volume of a solution-containing vector will forcibly deliver vector to large areas of the skeletal muscle. This strategy has been successful in AAV-mediated gene transfer to the skeletal muscle of normal dogs or nonhuman primates through the superficial saphenous vein under elevated hydrostatic pressure. ATRVX delivery of AAV-1 or AAV-8 vectors encoding lacZ gene in the normal dog resulted in widespread transduction of the injected limb, but the expression is short-lived due to immune responses to the transgene (Su et al. 2005; Ohshima et al. 2009). ATRVX delivery of AAV-1 or AAV-8 was well tolerated in nonhuman primates with sustained transgene expression for the duration of the experiment with transient IS with MMF and prednisone (Toromanoff et al. 2008). Transgene expression was detected in most of the muscle of the injected limb, but the local gene copy number is lower when compared to the IM injection of a similar vector. Vector biodistribution revealed that vector sequences were found outside the ATRVX-treated limb and these findings raise safety concerns. ATRVX-mediated human μ-dystrophin in two GRMD dogs was effective in ensuring transgene expression in muscle biopsies collected at week 4, but the number of μ-dystrophin positive cells decreased by week 8, probably, in part due to the use of non-species specific transgene (Ohshima et al. 2009). In normal young dogs, ATRVX delivery of AAV-8 of canine myostatin propeptide gene (MPRO) resulted in muscle growth, increased muscle myofiber size in multiple muscles of the injected limb, and muscle volume (Qiao et al. 2008b). Notably, no T cell infiltrates were observed for the duration of the experiment (3 months). The data on ATRVX delivery to skeletal muscle in large animals are encouraging, and a comprehensive study on the rates of cellular and humoral immune responses to the vector and/or transgene need to be addressed in adult GRMD dogs using species-specific μ-dystrophin transgene. Moreover, vector dissemination in this model will require further refinement since the use of appropriate tourniquets are critical to restrict regional delivery of the vector.

11.1.6 Systemic Delivery of Therapeutic Genes in DMD Models

To date efficient widespread expression in skeletal and cardiac muscles has not been demonstrated in large animal models of DMD. Intravascular delivery of AAV vectors demonstrated the proof of concept in mdx mice by restoring functional correction of skeletal muscle, diaphragm and heart disease (Bostick et al. 2009; Gregorevic et al. 2006; Wang et al. 2005). In normal neonate dogs, injection of AAV-9 resulted in expression of a reporter gene, in a dose-dependent manner, in widespread skeletal muscle but not in the heart (Yue et al. 2008). In another study, AAV-6 was the most efficient vector serotype in cardiac gene transfer following percutaneous transendocardial delivery to normal adult dogs (Bish et al. 2008). In both studies, no cellular immune responses were observed. More studies are required to determine whether these results could address safety scale-up issues in DMD dogs using a relevant therapeutic transgene, and whether the potential risk of immune responses to the vector and/or transgene may be detrimental to the diseased heart.

11.1.7 Immune Responses Related to the AAV Capsid

Findings in a clinical trial in which AAV-2 vector introduced into the liver of hemophilia B subjects highlighted a new safety issue, characterized by an immune rejection of transduced hepatocytes mediated by AAV-2 capsid-specific $CD8^+$ T cells resulting in transient transgene expression (Manno et al. 2006). This phenomenon was not observed in preclinical studies in several species from rodents to nonhuman primates. The hypothesis formulated here is that these human $CD8^+$ T cells expanded upon re-exposure to the vector capsid; i.e. upon AAV-2 hepatic gene transfer and clearance of AAV epitope-bearing transduced hepatocytes. Possible solutions for this problem include the administration of an IS regimen concomitant to gene transfer, and/or using alternate serotypes of AAV vectors. Moreover recently, cellular immune responses to the AAV capsid were also observed in another clinical trial for LPL deficiency based on intramuscular injection of AAV-1-LPL (Stroes et al. 2008). In one subject in the high dose cohort, $CD8^+$ T cell responses to the vector capsid were associated with transient transgene expression in the absence of immune responses to the transgene product (Mingozzi et al. 2009). In an attempt to avoid vector capsid-mediated immune responses, a short course of MMF and cyclosporine was administered for 12 weeks (Hui et al. 2008). In this study, transient IS was safe and effective in preventing or delaying anti-vector T cell responses. There is no animal model to test the efficacy of IS therapy in preventing capsid-mediated immune responses, but the safety of distinct therapeutic regimens with vector administration was determined in nonhuman primates. These studies consisted of intravascular delivery of AAV-8 or AAV-2, coupled with, of MMF and tacrolimus or MMF and rapamycin, respectively. These IS regimens were safe with no adverse effects of gene transfer, transgene expression or vector biodistribution noted. However, in a group of animals receiving daclizumab (anti-CD25),

in addition to MMF and rapamycin, there was an increased risk of neutralizing antibody formation to the FIX transgene (Mingozzi et al. 2007). In this group of nonhuman primates (NHP) , daclizumab induced significant reduction of $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cell population. Early data demonstrate that sustained transgene expression by AAV-mediated, liver-directed gene transfer induces antigen-specific tolerance, and in mice this effect is mediated by a subset of $CD4^+$ $CD25^+$ T cells (Cao et al. 2007a). Thus, it is possible that the pool of Treg cells involved in inducing and/or sustaining immune tolerance to FIX was severely affected by the anti-CD25 regimen. The role of these Tregs in muscle-directed gene transfer by AAV vectors is not yet determined. Further studies to define whether drug-induced interference of T regulatory cells is safe for muscle gene therapy are needed.

The presence of neutralizing antibodies to wild-type viruses imposes significant limitations on the in vivo transduction efficacy of the cognate recombinant vector. The use of AAV vector in NHP with neutralizing antibodies to AAV capsid proteins, at titers equal to or higher than 1:5, failed to induce transgene expression in comparison with animals with low or undetectable antibody titers (Jiang et al. 2006a). In humans, AAV-2 hepatic gene expression was ablated in the presence of neutralizing antibodies against the AAV-2 capsid at titers of 1:17 (Manno et al. 2006). In contrast, in humans subjects with hemophilia B, the presence of neutralizing antibodies to AAV-2 did not prevent local FIX gene transfer and transgene expression following IM injection of AAV-2 encoding human FIX at muscle biopsies collected at early and late time points (Jiang et al. 2006b; Manno et al. 2003). Thus, the intravascular delivery route of AAV vector to skeletal muscle could be potentially more susceptible to the presence of neutralizing antibodies than by IM vector injection into the solid tissue.

11.2 Summary and Future Directions

The control of immune responses to the transgene and/or vector is critical for the development of gene-based strategies for DMD. Preclinical studies in large and immunocompetent animal models of the disease provide the best platform for assessment of both efficacy and safety of several gene therapy protocols. The goals of gene therapy for muscular dystrophy are to improve muscle strength, reduce muscle necrosis, enhance muscle regeneration, and in turn, reduce fibrosis. The development of efficient, nontraumatic intravascular gene delivery for widespread transduction of the skeletal muscle, and the upregulation of alternative transgenes may minimize immune responses. Notably, the use of GRMD dogs demonstrate that transient immunosuppression has the potential to overcome unwanted immune responses and allow sustained transgene expression. Several immunosuppressive regimens have been tested in preclinical models with acceptable safety profiles. However, low numbers of animals in such studies preclude conclusive evidence of a specific therapeutic IS protocol. These data, together with cumulative experience of the immunosuppression in early-phase gene therapy clinical trials for genetic

diseases, will provide the basis for the development of optimal protocols for the treatment of DMD.

11.3 Future Directions

The success of gene therapy for DMD depends on the development of systemic gene transfer to striated muscle with no detrimental immune responses to the transgene and/or recombinant vectors. The assessment of the potential therapeutic of emerging and promising gene and cell-base therapies for DMD requires the systematic testing in relevant animal models of the disease. Further refinement of clinically relevant endpoints in canine models is certainly desirable. Continuous development of novel immunosuppressive drugs aimed at specific cellular targets and at the upregulation of regulatory cells are attractive strategies to improve the control of unwanted immune responses, while minimizing the side effects of current immunosuppressive drugs. The ability of cytotoxic T cells to recognize viral vector-capsid and to prevent long-term expression of the transgene needs further investigation in GRMD models. In addition, induction of antigen-specific immunotolerance by non-myeloablative mixed chimerism and central tolerance need further investigation. For cell-based therapy using lentiviral vectors, the confirmation of safety of these promising integrating vectors will be required to further support translational studies in DMD patients. Overall, ongoing and planned early-phase clinical studies of gene transfer for genetic diseases, coupled with immunosuppression, will be informative for the design of future therapeutic strategies to overcome the immunological hurdles of the DMD models.

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Chapter 12
Delivering Large Therapeutic Genes for Muscle Gene Therapy

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Abstract Gene replacement therapy holds great promise for treating inherited muscle diseases. Unfortunately, the size of the therapeutic genes often exceeds the packaging capacity of commonly used viral vectors. This poses a tremendous challenge for muscle gene therapy. To overcome this hurdle, investigators have been forced to use minimized synthetic genes that are often not fully functional. Novel vector engineering technologies have now brought the hope of ending this size dilemma. The development of gutted vectors with minimal viral sequences allows the accommodation of large therapeutic expression cassettes. Creative single and dual vector strategies have also been developed to expand the packaging capacity of the adeno-associated viral vector, currently the most efficient vehicle for muscle gene transfer. Collectively, these newly developed technologies have greatly expanded the realm of muscle gene therapy.

12.1 Introduction

Many muscle proteins are encoded by large genes. Mutations in these genes have been associated with various forms of myopathy afflicting the skeletal muscle and/or the heart (Table 12.1). Currently, there is no cure for these devastating muscle diseases. Replacing the defective gene with a functional gene offers the hope for correcting inherited muscle diseases at the DNA level. The challenge lies in delivering these large therapeutic genes to the affected muscles throughout the body. Due to their excellent transduction efficiency, viral vectors are often the top choice for *in vivo* gene delivery. Among different viral vectors, adeno-associated virus (AAV) is particularly attractive for muscle gene therapy. AAV is the only vector capable of whole-body muscle transduction after single intravascular injection (Bostick et al. 2007;

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Table 12.1. Examples of large genes invovled in muscle diseases

Gene size	Gene name	Coding sequence	Protein size	Disease
6 mb	Titin	82 kb	4,200 kD	Dilated cardiomyopathy 1G T Tibial distal myopathy Limb-girdle muscular dystrophy 2J Hypertrophic myopathy Hereditary myopathy with early respiratory failure
2.3 mb	Dystrophin	11.1 kb*	427 kD	Duchenne muscular dystrophy Becker muscular dystrophy X-linked dilated cardiomyopathy
260 kb	α-2 Laminin (merosin)	10 kb	342 kD	Congenital muscular dystrophy
150 kb	Dysferlin	6.2 kb	237 kD	Limb-girdle muscular dystrophy 2B Miyoshi myopathy Anterior tibial distal myopathy
22.9 kb	Myosin heavy chain 7	5.8 kb	212 kD	Familial hypertrophic cardiomyopathy 1 Dilated cardiomyopathy 1S Midventricular hypertrophic cardiomyopathy Myosin storage myopathy Laing distal myopathy

*Highly abbreviated mini- and micro-genes are currently being developed for dystrophin-deficient muscle diseases.

Gregorevic et al. 2004; Wang et al. 2005; Yue et al. 2008). For this reason, there has been a great interest in developing AAV-mediated gene therapy for muscle diseases. In this chapter, we will review different strategies used to deliver large therapeutic genes with AAV vectors. We will also discuss vectors based on other large DNA viruses, including adenovirus and herpes simplex virus.

12.2 AAV Vector

12.2.1 Overview

AAV is a single-stranded DNA virus. Wild-type AAV genome is ~4.8 kb and it contains three components, including the rep gene, the cap gene, and the inverted terminal repeats (ITRs). Both rep and cap genes are deleted from AAV vectors. The only viral sequences left in recombinant AAV are two ITRs flanking the vector genome. A mature AAV virion is ~20 nm (Duan et al. 2002; Yan et al. 2006). The nano-range

size imposes a physical constraint on the maximal genome that can be packaged in a single AAV vector. It is generally believed that the vector genome in infectious AAV particles is equal to or less than 5 kb (Dong et al. 1996). Over the last few years, a series of creative strategies have been developed to expand AAV packaging capacity. These include two single vector approaches and four dual vector approaches.

12.2.2 Single Vector Approach Based on VP2-Null AAV Viruses

An assembled AAV capsid contains three viral proteins (VP1, VP2 and VP3). These proteins are produced from a single open reading frame by alternative splicing and/or translation initiation. In each AAV capsid, there are approximately three VP1s, three VP2s and 54 VP3s in a 1:1:18 ratio (Rose et al. 1971; Xie et al. 2002). VP2 is 65 amino acids longer than VP3 at the N-terminus. It starts from a noncanonical translation initiation codon ACG. Despite the fact that the first amino acid of VP2 (a threonine residue) maps to the viral outer surface (Wu et al. 2000), the rest of the VP2 N-terminus seems to localize inside virion (Warrington et al. 2004; Wobus et al. 2000; Wu et al. 2000; Xie et al. 2002). Deleting VP2 may therefore leave more internal space for viral genome packaging.

Warrington et al. (2004) first demonstrated that infectious AAV particles can be assembled without VP2. The possibility that a VP2-null AAV can carry a larger genome was soon confirmed (Grieger and Samulski 2005). Grieger and Samulski (2005) showed that VP2-null AAV serotypes 1 through 5 can encapsidate a 6-kb viral genome. Since AAV serotype 6 is one of the most potent serotypes for muscle transduction (Ghosh et al. 2006; Wang et al. 2005), we generated VP-2 null AAV serotype 6 (AAV-6) by mutating the VP2 translation-starting site coding sequence from ACG to ACA. We then compared VP2-null (5.8-kb genome) and traditional (4.9-kb genome) AAV-6 LacZ vectors. We obtained similar yield for both vectors (Fig. 12.1a). To evaluate vector genome packaging and transduction efficiency, we co-infected Hela cells with AAV and Ad.dl802 at an moi of 6,000 vector genome particles/cell for AAV and 5 particles/cell for Ad.dl802 (Duan et al. 1999, 2001). LacZ expression and viral genomes were examined at 24 h post co-infection. Transduction efficiency was substantially lower in VP2-null AAV-6 infected cells (Fig. 12.1b). VP2-null AAV-6 also showed less efficient single-strand to double-strand conversion (Fig. 12.1c). Nevertheless, the replicative monomer (Rm, 5.8 kb), dimer (Rd, 11.6 kb) and trimer (Rt, 17.4 kb) were all seen at the expected sizes for VP2-null AAV-6 (Fig. 12.1c). Next, we generated VP2-null AAV-6 expressing a 5.7-kb ΔR4-23 micro-dystrophin gene expression cassette. We delivered 10^9 VP2-null AAV-6 particles to the tibialis anterior muscle of adult dystrophin-deficient mdx mice. Two months later, we detected moderate micro-dystrophin expression (Fig. 12.1d). Although the overall transduction efficiency is much lower than what we have observed with a traditional AAV-6 vector (Ghosh et al. 2006; Lai et al. 2005; Yue et al. 2006), our results suggest that VP2-null AAV may still be useful for certain muscle gene therapy applications, especially if the therapeutic genes encode secreted products.

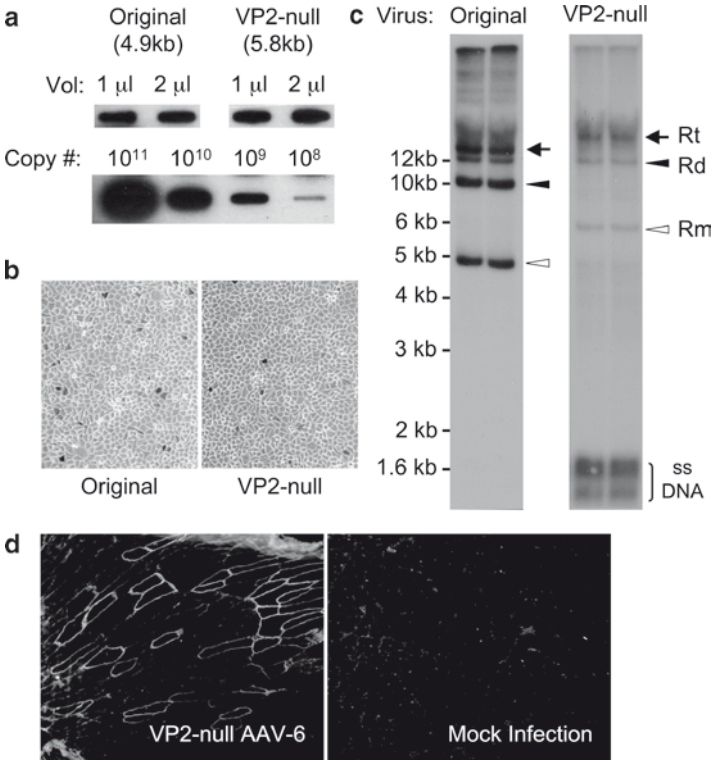

Fig. 12.1 Development of VP2-null AAV-6 vector. (**a**) Slot blot titration of VP2-null and original AAV-6 LacZ vectors. (**b**) A comparison of original and VP2-null AAV-6 LacZ virus transduction efficiency in Hela cells in the presence of a helper adenovirus Ad.dl802. (**c**) Southern blot analysis of replicative forms of the original and VP2-null AAV-6 genomes. *Open arrow head*, replicative form monomer (Rm); *Filled arrow head*, replicative form dimer (Rd); *Arrow*, replicative form trimer (Rt); ssDNA, single-strand viral DNA. (**d**) In vivo evaluation of VP2-null ΔR4-23 micro-dystrophin vector expression in the anterior tibialis muscle in mdx mice. Dystrophin expression was revealed with a human dystrophin-specific antibody

12.2.3 Single Vector Approach Based on AAV Serotype 5

Allocca et al. (2008) recently reported a very intriguing observation suggesting that AAV serotype 5 (AAV-5) can package an 8.9-kb vector genome. Interestingly, this strategy did not work for other AAV serotypes, including AAV-1, 2, 3, 4, 7, 8 and 9 (Allocca et al. 2008). This unexpected finding suggests that the AAV-5 capsid may be fundamentally different from that of other AAV serotypes. In support of this notion, it has been shown that AAV-5 is the most divergent serotype sharing only ~60% sequence homology with other AAV serotypes (Bantel-Schaal et al. 1999). Nevertheless, the AAV-5 structure, determined by cryo-electron microscopy, is remarkably similar to that of AAV-2 (Walters et al. 2004; Xie et al. 2002). It is important to point out that the viral

yield is several logs lower when a large vector genome is used for packaging (Allocca et al. 2008; Wu et al. 2009). Further, it remains to be confirmed that AAV-5 can indeed package an intact vector genome that is larger than 5 kb (Dong et al. 2009; Lai et al. 2009; Wu et al. 2009). Considering the inefficiency of AAV-5 for systemic transduction and the low yield, this approach may be of very limited use for muscle gene therapy.

12.2.4 Dual Vector Approach via Cis-activation

Dual vector approaches are based on the intermolecular recombination of two independent AAV genomes. In the *cis*-activation approach, one AAV is used to carry the transgene while the other is used to carry the regulatory elements such as an enhancer. Through ITR-mediated intermolecular concatamerization, the regulatory elements are joined to the transgene cassette. Essentially, *cis*-activation provides a means of regulated gene expression (Duan et al. 2000). However, this approach is not applicable for transgenes that are larger than 5 kb.

12.2.5 Dual Vector Approach via Trans-Splicing

This approach also exploits the ITR-mediated inter-AAV genome recombination. Specifically, a large therapeutic gene is split into two mini-exons, one carrying the splicing donor signal and the other carrying the splicing acceptor signal. Each half of the gene is packaged into its own separate AAV virion. After co-infection, the intervening viral ITR junction is removed by the cellular splicing machinery and a full-length transgene expression is established (Fig. 12.2) (Duan et al. 2001; Nakai et al. 2000; Sun et al. 2000; Yan et al. 2000).

The transduction efficiency of the first few sets of the published *trans*-splicing AAV vectors was quite low (Duan et al. 2001; Sun et al. 2000; Yan et al. 2000). It was soon discovered that mRNA accumulation is a rate-limiting factor (Xu et al. 2004). To overcome this obstacle, Lai et al. developed a strategy to rationally design the *trans*-splicing vectors based on the splicing value and mRNA yield (Lai et al., 2005, 2006, 2008). With this approach, Lai et al. (2005) and Ghosh et al. (2007) generated highly efficient *trans*-splicing vectors to express a 6-kb ΔH2-R19 mini-dystrophin gene and an alkaline phosphatase (AP) reporter gene, respectively. Transduction efficiency of these vectors reached that of a single intact AAV virus. The latest development in *trans*-splicing technology is the demonstration of whole-body muscle transduction (Ghosh et al. 2007). After a single intravenous injection of the AP *trans*-splicing vectors in normal neonatal mice, Ghosh et al. observed robust AP expression in all skeletal muscles throughout the body (including limb muscles, body wall muscles, the tongue and the diaphragm). Approximately 50% of the cardiomyocytes were also efficiently transduced (Ghosh et al. 2007).

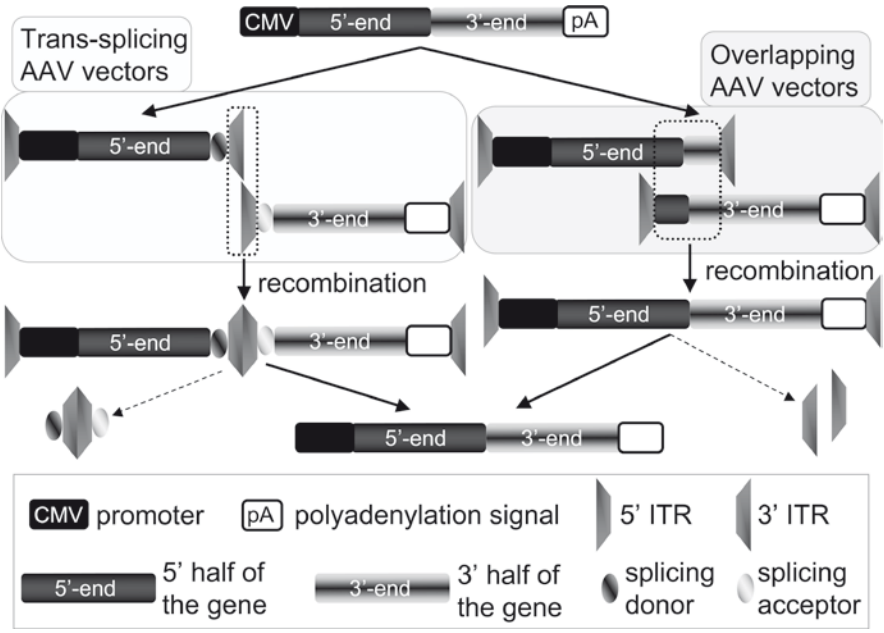

Fig. 12.2 Schematic outline of transgene reconstitution in the *trans*-splicing and overlapping dual AAV vectors

12.2.6 Dual Vector Approach via Overlapping

The overlapping approach regenerates the full-length transgene through a homologous recombination between the overlapping regions in two independent AAV vectors (Fig. 12.2) (Duan et al. 2001; Halbert et al. 2002). Briefly, a large therapeutic gene is split into an upstream vector and a downstream vector. The upstream and downstream vectors share a common region. Transgene reconstitution is achieved through homologous recombination between the shared region in the upstream and downstream vectors (reviewed in (Duan et al. 2006; Ghosh and Duan 2007)). Initial attempts with this strategy (AAV-2, LacZ gene) yielded nominal transduction in the skeletal muscle (Duan et al. 2001). However, a subsequent study with a different AAV serotype and a different reporter gene (AAV-6, AP gene) resulted in a remarkable expression in the murine airway (Halbert et al. 2002). To determine the relative contribution of the transgene, AAV serotype and the target organ, Ghosh et al. (2006) compared LacZ and AP overlapping vectors in three different serotypes including AAV-2, 5 and 6. Interestingly, the AP overlapping vectors significantly outperformed the LacZ overlapping vectors. This result suggests that the transgene sequence plays an important role in overlapping AAV vector transduction. If a gene carries a highly recombinogenic sequence, it will likely yield good expression when delivered by the overlapping vectors.

12.2.7 Hybrid Dual Vector Approach

The *trans*-splicing vectors can effectively deliver a large expression cassette if an optimal gene-splitting site can be identified and used. Similarly, the overlapping vectors also have the potential for efficient expression of a large gene should there exist a highly recombinogenic region in the target gene. A generic approach that can meet the need of any transgene would certainly be more appealing. With this in mind, we developed a novel hybrid vector system (Fig. 12.3) (Ghosh et al. 2008). In the hybrid system, transgene reconstitution can be accomplished by both ITR-dependent and ITR-independent pathways. A highly recombinogenic foreign DNA sequence is engineered into the intron regions in the *trans*-splicing vectors. The foreign DNA sequence can then mediate efficient homologous recombination (Fig. 12.3a, the overlapping pathway). As a result, transgene reconstitution is guaranteed irrespective of the gene-splitting site or the recombinogenic potential of the transgene. Alternatively, transgene reconstitution can also occur via ITR-mediated recombination (Fig. 12.3a, the *trans*-splicing pathway). Using the LacZ reporter gene as a template, we found that the performance of the hybrid vectors significantly surpassed that of other dual vector systems (Fig. 12.3b) (Ghosh et al. 2008). Preliminary studies with the 6-kb ΔH2-R19 mini-dystrophin gene also yielded promising results (Ghosh et al. 2008).

12.3 Adenoviral Vector

Adenovirus is a double stranded DNA virus with an approximately 36-kb genome. First generation adenoviral vectors are created by replacing the viral E1 gene with a reporter or a therapeutic gene expression cassette. The maximal packaging capacity of the first generation adenoviral vector is 8 kb. The biggest problem with adenoviral vectors is the host immune response. In general, transgene expression reaches the peak within one week and then rapidly reduces to the preinfection levels in 2–4 weeks. In an effort to reduce immune response and also to further increase the packaging capacity, gutted adenoviral vectors were developed (Chen et al. 1997; Clemens et al. 1996; Kochanek et al. 1996; Mitani et al. 1995; Morsy et al. 1998; Parks et al. 1996; Schiedner et al. 1998). The only viral elements in gutted adenovirus are the viral ITR and the packaging signal. All viral genes are deleted.

Several laboratories have explored gutted adenovirus for muscle gene therapy, in particular, for delivering the full-length dystrophin coding sequence to mdx mice (Bilbao et al. 2005; Clemens et al. 1996; DelloRusso et al. 2002; Dudley et al. 2004; Gilbert et al. 2002, 2003 ; Haecker et al. 1996; Jiang et al. 2004a, b; Kochanek et al. 1996; Matecki et al. 2004; Reay et al. 2008). Collectively, these studies reveal full-length dystrophin expression, reduced host immune response, amelioration of muscle pathology and enhanced muscle contractility. However, because of immune elimination, long-term expression remains a challenge.

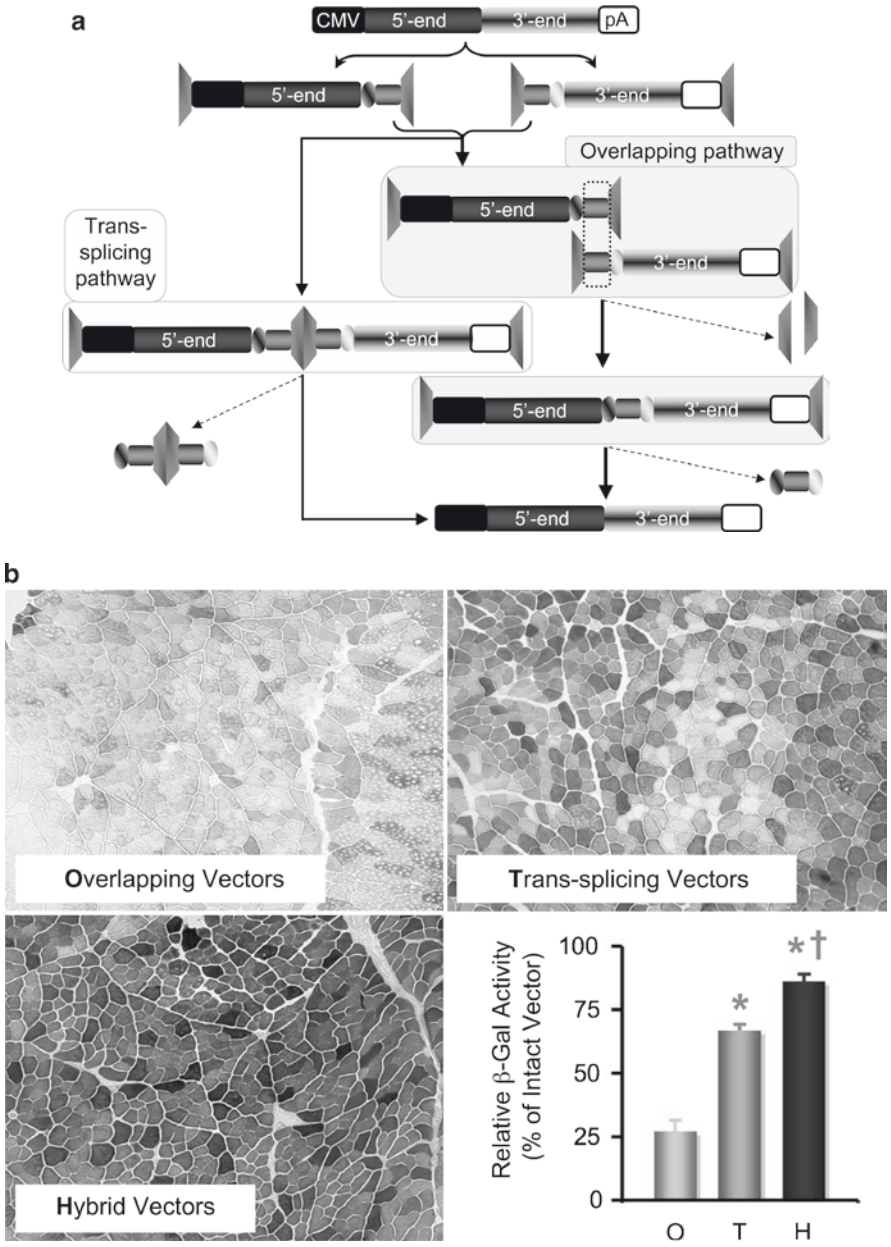

Fig. 12.3 (continued) The bar graph shows the relative transduction efficiency of the dual vectors compared to that of the single intact LacZ vector. Data are mean ± standard error of mean, $N=4$. O, overlapping vectors; T, *trans*-splicing vectors; H, hybrid vectors. Asterisk, the relative β-gal activities in *trans*-splicing or hybrid vector infected muscles were significantly higher than that in overlapping vector infected muscles. Cross, the relative β-gal activity in hybrid vector infected muscles is significantly higher than that in *trans*-splicing vector infected muscles

To further limit the cellular immune reaction, several novel strategies have been explored. These include the use of the utrophin gene (an endogenous homologue of dystrophin), species-specific dystrophin gene, muscle-specific promoter, immune-modulating strategies and neonatal or in utero gene delivery (Bilbao et al. 2005; DelloRusso et al. 2002; Deol et al. 2007; Jiang et al., 2004a, b; Reay et al. 2008). Together, these approaches have significantly extended transgene expression from the gutted adenovirus. Admittedly, systemic delivery remains to be developed for body-wide muscle delivery. However, the improved versions of gutted adenoviral vector may have the potential for local muscle gene therapy to improve life quality.

12.4 Herpes Simplex Viral Vector

The wild-type herpes simplex virus type-1 (HSV-1) is an enveloped, double-stranded DNA virus. Nearly half of its 152-kb viral genome sequence is nonessential for viral replication. Theoretically, a HSV-1 vector can be built to carry a 76-kb foreign DNA sequence without compromising viral replication. In the laboratory, recombinant HSV-1 vectors with a 38-kb packaging capacity have been generated (reviewed in (Burton et al. 2002; Glorioso et al. 1997; Goins et al. 2004; Huard et al. 1997b)). Early experimentation with HSV-1 vectors demonstrated quite efficient myoblast transduction *in vitro*. However, direct muscle injection only yielded transient, restricted expression (Akkaraju et al. 1999; Huard et al., 1995, 1996, 1997a).

A variant of the HSV-1 vector called an amplicon has also been investigated for gene transfer. HSV-1 amplicons only contain ~1% of the wild-type viral sequences encoding the HSV replication origin and the packaging signal (reviewed in (Hibbitt and Wade-Martins 2006; Link et al. 2003; Wang 2006)). HSV-1 amplicons are generated by supplying the essential viral replication machinery and structural proteins *in trans*. Although *in vivo* muscle transduction efficiency is quite disappointing, HSV-1 amplicons offer a unique opportunity to deliver some of the largest therapeutic genes (such as titin, see Table 1) for *ex vivo* muscle gene therapy application (Bujold et al. 2002).

Fig. 12.3 Expanding AAV packaging capacity with the hybrid dual vector strategy**.** (**a**) Schematic outline of transgene reconstitution in the hybrid AAV vectors. (**b**) LacZ dual AAV vector transduction in muscle. The tibialis anterior muscles of 6-week-old BL10 mice were either infected with an intact LacZ vector (10^{10} vg particles/muscle) or co-infected with dual AAV vectors. In co-infection, a total of 2×10^{10} vg particles were delivered to each muscle (10^{10} vg particles/vector/muscle). Transgene expression was determined 6 weeks after infection by histochemical staining in muscle section and β-galactosidase activity assay in muscle lysate. Photomicrographs are representative images from dual vector co-infected muscles.

12.5 Summary and Future Directions

The considerable size of many therapeutic genes is a critical limitation to muscle gene therapy. For some muscle diseases, moderate success has been achieved with gene repairing and/or gene truncation strategies to bypass the need for a full-length gene. However, such approaches cannot completely meet the clinical need. Delivering the full-length coding sequence or a minimally abbreviated gene will undoubtedly offer a more effective therapy for many muscle diseases. For some diseases, full-length gene transfer may even represent the only therapeutic option. Tremendous progress has been made in expanding the viral vector packaging capacity over the past decade. Yet, we are still in the infantile stages of delivering a large gene for muscle gene therapy. For AAV vectors, the next set of milestones will be to (1) demonstrate systemic correction in a disease model with the dual vectors, and (2) further expand the packaging capacity with multimer vectors (such as the use of tri-AAV vectors to carry the full-length dystrophin coding sequence). *In vivo* application of gutted adenovirus and HSV vectors face more challenges. Some of these may include strategies to (1) produce ultra-pure vector stock without the helper virus or a helper viral sequence contamination, (2) achieve body-wide muscle transduction, and (3) further reduce untoward immune reaction.

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Chapter 13
Muscle as a Metabolic Factory for Gene Therapy

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Abstract Since the identification of gene transfer vectors that can efficiently transduce skeletal muscles, the muscle has been targeted in gene therapy strategies to act as a factory of therapeutic gene expression for the treatment of metabolic diseases. The easy accessibility of the skeletal muscle for vector delivery procedures and the ability of the muscle cells to both express and secrete proteins encoded in gene therapy vectors makes the muscle an ideal platform on which to develop gene therapy strategies for the treatment of metabolic diseases of the muscle as well as those diseases that do not involve muscle pathology. We discuss herein current gene therapy strategies focused on using the muscle as a biosynthetic factory. In particular, we will discuss preclinical work for two metabolic myopathies, McArdle and Pompe diseases, and updates on muscle-targeted gene therapy clinical trials for the treatment of other inborn errors of metabolism, alpha-1-antitrypsin deficiency and hemophilia B.

13.1 Introduction

In this chapter, we will discuss gene therapy strategies in which the muscle is targeted to act as a factory for therapeutic transgene expression to provide correction for metabolic diseases. There exist numerous classifications and hundreds of forms of inborn errors of metabolism, many of which are caused by a single gene defect, thus making them attractive candidates for gene therapy strategies. Gene therapy studies for inherited diseases of metabolism have widely varied; however, small subsets of studies have focused on the muscle as a metabolic factory for gene therapy. Due to the identification of gene transfer vectors that efficiently transduce skeletal muscles, the ease of accessibility for delivery procedures, and the ability of muscle cells to express and secrete proteins, the muscle provides an ideal target in

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gene therapy strategies for the treatment of myriad metabolic diseases, including metabolic myopathies as well as other diseases that are not associated with any muscle pathology (Kessler et al. 1996). Herein we will focus on the preclinical studies of muscle-directed gene therapy for two metabolic myopathies as well as examples of strategies using the muscle as a metabolic factory that have resulted in clinical studies.

Metabolic myopathies are inborn errors of metabolism that result in exercise intolerance and/or progressive skeletal muscle weakening. Gene therapy strategies have been assessed for two such diseases thus far, McArdle and Pompe disease, both of which are forms of glycogen storage diseases (GSDs) and will be discussed in the following sections.

13.2 McArdle Disease

McArdle disease (Myophosphorylase deficiency; GSD V) results from a deficiency in the muscle-specific isozyme of glycogen phosphorylase (PYGM). There exist 65 known mutations in the PYGM gene and although there is a great deal of genetic heterogeneity in the McArdle disease, it generally presents itself with clinical and biochemical homogeneity (Dimaur et al. 2002; McArdle 1951; Nogales-Gadea et al. 2007).

McArdle disease was first described by Dr. Brian McArdle in 1951 after he evaluated a 30 year old male patient who was experiencing pain followed by weakness and stiffness after exercise. McArdle's observations that cramps experienced by this patient were electrically silent and that the patient's venous lactate level failed to increase after ischemic exercise led to the conclusion that his patient's myopathy was caused by a defect in muscle glycogen breakdown (McArdle 1951).

Patients with McArdle disease experience exercise intolerance, developing severe muscle pain and fatigue in the first few minutes of exercise. Interestingly, people with McArdle disease often experience a "second wind" phenomenon (Dimaur et al. 2002). The first few minutes of exercise are usually anerobic and include intense, sustained exertions. During anaerobic exercise, muscle phosphorylase converts glycogen to glucose which can then enter the glycolytic pathway to ultimately produce ATP for energy. In McArdle disease the defective glycogen phosphorylase blocks this ATP formation. In contrast, during aerobic exercise (such as walking or gentle jogging) free fatty acids carried in the blood stream provide the main source of energy. These fatty acids enter the oxidative phosphorylation pathway in the mitochondria where ATP is then produced. When individuals with McArdle disease rest briefly after experiencing pain in the first few moments of exercise they often find that they can then continue the exercise, pain free. This second wind is likely due to a switch from their defective glycolytic pathway to their fully functional oxidative phosphorylation pathway as a source for ATP (Muscular Dystrophy Association 2009).

There are currently two known naturally occurring animal models of McArdle disease. The bovine myophosphorylase gene was found to have a missense mutation in Charolais cattle (Angelos et al. 1995). Additionally, a splice junction mutation was identified in the ovine myophosphorylase gene that results in premature truncation of the protein and was associated with cramps and exercise intolerance in merino sheep (Tan et al. 1997). A mouse model for the disease does not yet exist.

There is no established therapy for McArdle disease at this time. However, several treatments have been tested in their ability to alleviate the pain associated with exercise in McArdle patients over the years. Current disease management includes both regular, moderate exercise, and dietary management (Haller 2000; Phoenix et al. 1998; Vorgerd et al. 2000). Specifically, a high protein-low carbohydrate dietary regimen appears to be particularly effective when combined with daily submaximal aerobic exercise (Dimaur et al. 2002).

Several investigators have begun to explore various gene therapy schemes to deliver the myophosphorylase gene. The first of these studies was designed to establish the feasibility of using adenovirus as a vector for delivery of the myophosphorylase gene to either differentiated or nondifferentiated C2C12 myoblasts in vitro (Baque et al. 1994). The study found that as compared to controls, cells exposed to an adenovirus-based vector, Ad-CMV-MGP (adenovirus with a cytomegalovirus promoter driving expression of muscle glycogen phosphorylase), expressed increasing levels of muscle phosphorylase for 6 days. For up to 15 days post-infection the muscle phosphorylase levels in the Ad-CMV-MPG infected cells were higher than in untreated controls. The authors noted that gene delivery to differentiating myoblasts was less successful than delivery to fully differentiated myotubes. They surmised that these data indicate that direct delivery of the muscle gene into mature muscle cells may be a more suitable strategy for treatment of this disease than implantation of adenovirus-engineered myoblasts (Baque et al. 1994).

Another group of investigators was able to show that adenovirus-mediated delivery of the human myophorylase gene under control of the Rous sarcoma virus promoter was able to restore phosphorylase activity in deficient primary myoblast cell lines from both human McArdle patients and the sheep model of the disease (Pari et al. 1999). While still an in vitro study, this investigation importantly demonstrated the ability of gene delivery to provide a fully functional form of the gene in a diseased background.

More recently, Howell et al. published a study in which the human myophosphorylase cDNA was delivered using either adenovirus (Ad) or recombinant adeno associated virus serotype 2 (rAAV2) as the vehicle for gene delivery (Howell et al. 2008). Myophosphorylase deficient sheep were injected in the semitendinosus muscles with either rAAV2 or Ad expressing the human myophosphorylase gene. The results showed that while both vectors were capable of transducing ovine skeletal muscle, the treatment was limited to muscle fibers immediately surrounding the injection site. Although the transduced area was small, the investigators were able to identify some myophosphorylase positive fibers in animals treated with either vector at 90 day post administration (Howell et al. 2008). Differences observed between the two vectors used in the study can be attributed to the different volumes,

titres and the varying number of injections used between animals in the experimental groups. In conclusion, this study is the first to demonstrate gene delivery in an animal model of the McArdle disease and has laid the necessary groundwork for future studies designed to optimize delivery routes, vector serotypes and to carefully assess any potential immune response to either the gene product or the delivery vehicle.

13.3 Pompe Disease

Pompe disease (Acid-Maltase deficiency; GSD II) is an autosomal recessive form of muscular dystrophy and metabolic myopathy caused by mutations in the acid alpha-glucosidase (*GAA*) gene. GAA is important for the breakdown of glycogen in lysosomes through hydrolysis of α-1, 4 and α-1, 6 glycosidic bonds. Deficiency in the GAA enzyme leads to the accumulation of glycogen in lysosomes of a variety of tissues and consequent cellular dysfunction (Hirschhorn and Reuser 2000; Raben et al. 2002).

In human patients, there is a direct correlation between the amount of GAA enzyme produced and severity of disease (Kishnani et al. 2006; Raben et al. 2002). The relationship between the amount of GAA enzyme activity and the disease severity in humans has resulted in a continuum of severity and broad range of age-of-onset amongst the patient population. In the adult-onset population, patients generally have residual levels of functional GAA and the primary disease complications include progressive skeletal muscle weakening and wasting and respiratory insufficiency. In the most severe form, infantile-onset Pompe, patients are born with a complete or nearly complete deficiency of the protein and therefore lack GAA enzyme activity. In such cases, the disease presentation occurs within the first few months of life with hypotonia (severely decreased muscle tone), severe hypertrophic cardiomyopthy, and mild hepatomagaly. Without treatment, cardio-respiratory failure typically occurs in the early onset patients within the first 2 years of life.

A variety of treatment strategies have previously been performed in an attempt to ameliorate various aspects of Pompe disease. In humans, the first treatment ever approved for Pompe disease or any other inherited muscle disorder is the enzyme replacement therapy (ERT) product *alglucosidase alpha* or (Myozyme®) that has been developed by Genzyme Corporation (Cambridge, Massachusetts). Myozyme® consists of the human acid alpha glucosidase enzyme and was encoded by the most dominant of 9 observed haplotypes of this gene (Myozyme 2009). Patients being treated with this therapy receive intra-venous (IV) infusions of the enzyme once every 2 weeks. While improvement in the survival of patients has been observed, the Myozyme® treatment does not substantially diminish the disease complications nor halt disease progression to the extent necessary to be considered a true cure for all patients (Kishnani et al. 2007; Schoser et al. 2008).

A gene delivery approach for the treatment of Pompe disease is therefore an attractive alternative for several reasons. A single administration of gene therapy

could provide a sufficient amount of the gene necessary to supply the quantity of enzyme needed for the treatment of the disease, thus eliminating the need for bi-weekly enzyme infusions. Additionally, in several *Gaa*$^{-/-}$ mouse studies, investigators have shown that while the ERT method is effective in clearing glycogen in the cardiac tissue and type I skeletal muscle fibers, it is not as effective at glycogen clearance in type II skeletal muscle fibers (Raben et al. 2001; Raben et al. 2005). The authors suggest that this may be a result of the low abundance of proteins involved in endocytosis and trafficking of lysosomal enzymes present in type II fibers. Many investigators have hypothesized that this limitation may be overcome through delivery of the *GAA* gene instead of the fully modeled protein because the protein derived from transgene delivery would undergo natural folding and trafficking within cells and hence be handled within cells in a more native manner.

Two primary gene therapy strategies have been investigated for the treatment of Pompe disease; direct correction of the affected tissues and cross-correction of tissues in which a depot organ synthesizes the enzyme and distal tissues are corrected via a secretion/uptake process. While the majority of synthesized GAA enzyme is targeted to the endosomal system via binding of the enzyme to the mannose-6-phosphate receptor (M6PR) followed by intracellular trafficking of the complex, a small percentage of expressed enzyme is shunted toward secretion from the cell. The secreted enzyme can then be taken up by distant cells via cell-surface M6PRs and traffic to the lysosome to perform its enzymatic function (Hoefsloot et al. 1990; Wisselaar et al. 1993). Current ERT therapy takes advantage of this secretion/uptake process. A challenge for cross-correction-based strategies is the efficient penetration of the circulating enzyme into the affected tissue(s), however gene therapy strategies have the potential to both simultaneously correct affected cells and also provide an endogenous system for cross-correction.

In one of the earliest in vitro experiments designed to test the feasibility of gene therapy to treat Pompe disease, a retroviral vector was used to carry cDNA encoding GAA into enzyme deficient myoblasts and fibroblasts (Zaretsky et al. 1997). The authors found that enzyme activities were 30-fold higher than wild-type levels in transduced myoblasts and remained fivefold higher upon differentiation. Transduced cells were able to provide enzyme to the surrounding deficient cells upon fusion and in all cells containing the GAA enzyme glycogen levels in lysosomes decreased. Another study by Pauly et al. utilized an E1 deleted recombinant adenovirus encoding human (GAA Ad-GAA) to assess the possibility of gene replacement therapy (Pauly et al. 1998). Following intracardiac and intramuscular injections of Ad-GAA into newborn rats, GAA enzyme activity levels were found to be ten- and six-fold (respectively) above wild-type levels approximately 1 week after vector administration. Another paper by the same group showed that at 3 months post single intramuscular injections of Ad-GAA into immunodeficient mice enzyme, activity levels were increased to 20-fold above wild-type levels in the injected tissue (Pauly et al. 2001).

Another group was able to achieve successful in vivo transduction of the skeletal muscle by injecting Ad-GAA into a Japanese quail model of the Pompe disease (Tsujino et al. 1998). Later they also showed that adeno-associated virus (AAV) can

be used as a vehicle for GAA gene delivery to the skeletal muscle in this disease model and showed the presence of GAA, lack of glycogen accumulation in lysosomes and improved wing flapping movement on the treated side as compared to the contra-lateral control side (Lin et al. 2002). Fraites et al. showed that AAV could efficiently transduce mouse skeletal muscle and demonstrated Gaa enzymatic activity and concomitant glycogen clearance as well as partial restoration of skeletal muscle contractility in the AAV-Gaa treated soleus muscles of *Gaa*$^{-/-}$ mice (Fraites et al. 2002). Ding et al. revealed the potential for a single muscle to behave as a secretory organ by injecting [E1-]Ad-GAA into the gastrocnemius muscle of SCID and *Gaa*$^{-/-}$ mice. In both mouse strains, circulating and distal GAA protein, respectively, could be detected; however, no biochemical cross-correction could be demonstrated (Ding et al. 2002). Martin-Touaux et al. administered [E1-E3-] Ad-GAA to the gastrocnemius of neonate *Gaa*$^{-/-}$ mice (Martin-Touaux et al. 2002). They detected strong GAA expression in the injected muscle, secretion into plasma, and uptake by the peripheral skeletal muscle and the heart with a concomitant decrease in glycogen content within these tissues. These results were in contrast with earlier studies suggesting that secretion of GAA from transduced muscle did not occur (Pauly et al. 2001; Tsujino et al. 1998). Transgenic mouse studies, in which conditional GAA expression was muscle-restricted, showed that while secretion from the muscle was possible, it was an inefficient process and required very high levels of expression (Raben et al. 2001). It is possible that further optimization of current vectors or the development of other novel vectors as well as increasing transgene expression to direct high levels of muscle-derived GAA without elicitation of immune response can lead to effective cross-correction strategies using muscle as a depot organ. Ever since these studies, however, efforts have focused more on either cross-correction using liver as a depot organ (Cresawn et al. 2005; Sun et al. 2005; Ziegler et al. 2008). Furthermore with the development of vectors that can efficiently transduce multiple muscle groups from a single systemic injection, in contrast to the aforementioned studies in which an isolated muscle group was targeted for correction, other studies have focused more on the direct correction of the metabolic deficiency within the affected skeletal muscles themselves.

Recombinant adeno-associated virus pseudotype 1 (rAAV2/1) vectors have been shown to transduce skeletal muscle with high efficiency (Gao et al. 2002; Hauck and Xiao 2003; Rabinowitz et al. 2002; Xiao et al. 1999). Mah et al. administered therapeutic rAAV2/1 vector via a simple intravenous injection into a peripheral vein into *Gaa*$^{-/-}$ mouse neonates (Mah et al. 2005). Assessment of GAA enzyme activity levels, glycogen clearance within cells and soleus force mechanics measurements showed sustained correction of Pompe disease for up to 11 months of age. They later established that in addition to biochemical and histological correction, rAAV2/1 vectors can mediate sustained physiological correction of both the cardiac and respiratory function (Mah et al. 2007). The treated mice showed a significant improvement in cardiac conductance, a significant decrease in cardiac left ventricular mass as well as an increased diaphragmatic contractile force of approximately 90% of wild-type peak forces with corresponding improved ventilation. Whether correction stemmed solely from direct correction

of individual cells or that cross-correction of adjacent untransduced cells also occurred is not clear, though both possibilities are plausible. Further studies investigated the potential of other rAAV pseudotyped vectors (in particular types 2/8 and 2/9) which have more recently been shown to transduce muscles at even greater levels (Pacak et al. 2006; Sun et al. 2008). Work by Pacak et al. demonstrated preferential transduction of cardiac tissues by rAAV2/9 vectors but also showed highly efficient transduction of other skeletal muscle groups as well. However as these vectors have also been shown to tranduce a wide-variety of nonmuscle tissues as well, and as such, further measures to restrict expression to only the affected tissue types becomes more important in avoiding any untoward effects. Sun et al. found that muscle-restricted expression of GAA through the use of a hybrid alpha-myosin heavy chain enhancer/muscle creatine kinase enhancer-promoter provoked only a blunted immune response, in the context of an rAAV pseudotyped 8 vector administered intravenously (Sun et al. 2008). As immune response to vector and/or transgene has been a complicating factor in muscle gene therapy strategies for numerous disease models, as well as traditional enzyme replacement therapy for Pompe disease, it is likely that further examination of this relationship is warranted.

More recently, Douillard-Guilloux et al. showed that either lentivirus or AAV mediated RNA interference can be employed to modulate glycogen synthesis in Gaa-deficient mice. This deprivation approach to managing substrate overabundance led to a significant reduction in glycogen accumulation following a single intramuscular injection of AAV-shGYS (short hairpin ribonucleic acid designed to target glycogen synthase) (Douillard-Guilloux et al. 2008).

Each advance has further increased the clinical relevance of gene therapy for Pompe disease. Since the deficient protein in the disease (GAA) can be secreted, every cell in the body may not require transduction to achieve overall therapeutic success. Certainly, the native or intracellular route of GAA trafficking is most efficient to reach the lysosomal compartment. The various administration routes that have been tested (intramuscular, intravenous, and intracardiac) each result in a different transduction pattern which can then be combined with tissue specific promoters and/or a gene delivery vehicle with a high natural tropism for a particular tissue. Together, these tools allow for a great deal of control over transgene expression at specific GAA enzyme factory sites within the body. The greatest challenge in the spectrum of Pompe disease patients are those with complete deficiency of GAA where anti-transgene immune response will be a critical factor in achieving clinical efficacy. Immune modulation strategies will undoubtedly be required to effectively manage such patients.

13.4 Clinical Trials Targeting Muscle as a Metabolic Factory

Adeno-associated virus (AAV)-based vectors have been shown to transduce skeletal muscle with high efficiency and result in sustained transgene expression. Preclinical studies using muscle-targeted therapeutic rAAV vectors for the treatment of other forms of metabolic disease including hemophilia B and alpha-1-

antitrypsin (AAT)-deficiency have been very successful and have lead to testing in Phase I clinical trials.

Hemophilia B is an X-linked recessive disorder resulting from a deficiency of functional coagulation factor IX and results in prolonged bleeding and spontaneous bleeding episodes into joints and soft tissue and increased risk of intracranial and intraperitoneal bleeding. Treatment of hemophilia B consists of repeated therapeutic and prophylactic infusions of recombinant factor IX (F.IX) protein. As little as 1% of wild-type levels of circulating factor IX, can mediate significant therapeutic benefits in affected individuals. Factor IX is normally synthesized in the liver; however, preclinical studies in both murine and canine models of the disease have shown that skeletal muscles can also act as a depot organ for factor IX expression and secretion and provide long-term therapeutic levels of correction.

A phase I dose-escalation clinical study was performed in 8 subjects with hemophilia A, testing a therapeutic rAAV serotype 2-based vector (rAAV2-CMV-F.IX) delivered via multiple intramuscular injections. 1.2×10^{12} vector genomes were injected per site with the numbers of injection sites ranging from 10 to 12 (low dose), 30–50 (middle dose), or 80–90 (high dose). No significant toxicities were noted and the highest dose of 1.8×10^{12} vg/kg was well-tolerated. Circulating F.IX levels increased above baseline levels in 4 of the 8 subjects, however levels remained below the therapeutic threshold of 1% normal, except in one patient who demonstrated a transient level of 1.4% normal at 12 weeks post-injection. F.IX expression was noted in the regions of injection sites for all subjects at 2 months post-injection. Although all but 2 subjects opted out of subsequent muscle biopsy procedures after the first one, both samples showed continued F.IX expression at 6 and 10 months post-injection (Kay et al. 2000; Manno et al. 2003). In one subject, local F.IX expression in the injection site could still be detected 3.7 years post-treatment (Jiang et al. 2006). Since the initiation of the clinical studies, improvements in muscle-targeted gene therapy strategies have emerged. In one study, Arruda et al. demonstrated that an intravascular method of vector delivery could increase circulating F.IX levels by almost tenfold as compared to multiple intramuscular injections of the same vector (Arruda et al. 2005). In another study, Arruda et al. demonstrated that approximately 50-fold higher levels of F.IX could be expressed in the canine model of the disease using a pseudotyped rAAV2/1 vector (Arruda et al. 2004). Of note, with the higher levels of transgene expression, new complications such as immune response to the expressed therapeutic protein, not previously seen at the lower transduction levels, emerged, thus highlighting the complexities in developing effective therapies for metabolic diseases. However, the overall improvements in transduction levels achieved with the newer vectors or delivery mechanisms may eventually translate into better clinical outcomes.

Alpha-one antitrypsin is a protease inhibitor produced normally in the liver and plays a role in the maintenance of structure and function of the lung. AAT-deficiency results in the disruption of the normal balance of proteases and antiproteases and affected individuals become prone to lung disease. Protein replacement therapy is provided to prevent progression of lung disease in AAT-deficient individuals. Initial Phase I dose escalation studies utilized a therapeutic rAAV2 vector

which was administered intramuscularly to 12 subjects. Similar to the findings from the hemophilia B trial, the rAAV2 vector was found to be safe with minimal toxicities (Brantly et al. 2006). More recently, a rAAV2/1-based dose escalation Phase I clinical trial was initiated and is currently active (ClinicalTrials.gov Identifier: NCT00430768). Nine subjects were enrolled in which rAAV2/1 vector encoding normal (M) AAT was injected intramuscularly at doses ranging from 6.9×10^{12} to 6×10^{13} vector genome particles per subject. Initial results indicate that the vector is well-tolerated and safe and furthermore, that 6 of 6 subjects within the two highest dose cohorts had M-specific AAT expression levels above the background, and sustained at least 90 days, indicating that more efficient vector-mediated transduction can result in improved outcomes (Brantly et al. 2009; Mueller and Flotte 2008).

13.5 Summary and Future Directions

Preclinical and clinical studies have established the feasibility of the muscle as a factory for the treatment of metabolic diseases; however it is evident that improvements in transduction levels may be necessary for this approach to be successful clinically, especially for those diseases that require circulating protein for correction. Refinements such as improved vector packaging and purification systems as well as modifications within the vectors themselves to increase relative bioactivity are the focus of current and future studies.

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Chapter 14
Muscle as a Target for Genetic Vaccine

Yan Zhi and Guangping Gao

Abstract The complexity of microbe infections requires novel approaches to vaccine design. It is particularly challenging to develop a safe and effective vaccine against human immunodeficiency virus (HIV)-1. The versatility of DNA vaccination provides new perspectives. Recombinant subunit vaccines derived from adenoviruses and adeno-associated viruses are under extensive development as HIV-1 vaccines. For all of those vaccine vector platforms, muscle appears to be a practical, effective, and safe target. A vaccine strategy based on initial priming with DNA vaccine and/or viral vector vaccine and then boosting with a second viral vector vaccine has shown promise in HIV-1 vaccine development. The recent progress in DNA vaccines and viral vector subunit vaccines, particularly those derived from novel serotypes of adenovirus and adeno-associated virus, is reviewed here.

14.1 Introduction

Prevention of diseases by vaccination is one of the most significant and cost-effective medical interventions at our disposal. Immunity is a result of coevolution between the immune system and pathogens. Microbes have evolved multiple strategies to evade, confuse, or inhibit the immune defence mechanism in order to proliferate in a biological shelter. The immune system must counter microbial invasions by eliciting protective immunity without inducing autoimmune disorders. Vaccination activates the protective immunity by mimicking a microbial invasion without the harmful, sometimes fatal, consequences of an infection.

Vaccination was first implemented on a wide scale more than 200 years ago with the introduction of the smallpox vaccine. Today, many infectious diseases caused by various viruses and bacteria can be prevented by vaccination with inactivated organisms, live attenuated organisms, or recombinant or partially purified components of the organisms. The host immune response after these vaccines is typically

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a robust induction of antibodies directed against antigens of the pathogen. Effective antibodies can neutralize or mediate killing of the organism, or inactivate toxins produced by the organism, thereby preventing infection or disease caused by infection. This antibody-directed approach has been successful either against the pathogens, whose antigens do not undergo antigenic variation, or against the pathogens, which undergo significant variability by frequently updated vaccines in order to match the circulating strains, such as influenza. In contrast, vaccines, which can effectively elicit the antigen-specific T-cell and B-cell immunities, are most likely required to protect host against pathogens which undergo rapid change in their antigenic structures (such as HIV and pathogens) and establish chronic infections, such as HCV and *M. tuberculosis*. While B-cell vaccine can prevent the host from the invasion of pathogens, T-cell vaccine can alter an ongoing immune response and consequently treat diseases. So far, few vaccine technologies have been successfully developed to induce protective T-cell responses in humans.

Classical vaccine strategies include the development of attenuated organisms, whole killed organisms, and protein subunits, followed by empirical optimization and iterative improvements. While these strategies have been remarkably successful for a wide variety of viruses and bacteria, these approaches have proven more limited for pathogens that require T-cell immune responses for their control. Largely because of technical advances in genome characterization, antigen identification, understanding the molecular bases of protective immune responses, and rational design of adjuvant and successful development of various delivery platforms, genetic vaccines consisting only of DNA as plasmids and recombinant subunit vaccines delivered by replicating and non-replicating viral vectors for complex organisms have been accelerated in the past decade. The skeletal muscle is a clinically accepted target for vaccination and its stable post-mitotic nature allows the use of both viral and non-viral vectors to induce the antigen-specific humoral and cellular immunity.

In this chapter, we will review the progress of muscle as a target for DNA vaccines and for recombinant subunit vaccines based on adenoviral and adeno-associated viral vectors. In the end, we will summarize these vaccine strategies in the context of HIV vaccine developments.

14.2 Muscle as the Target for Vaccine

The most common route of immunization used in DNA vaccine studies is the intramuscular route. Following intramuscular immunizations, the predominant cell type transfected with the DNA vaccines is myocytes (Danko et al. 1997; Wolff et al. 1990). However, muscle is not considered an immunologically relevant tissue as myocytes lack the characteristics of antigen-presenting cells (APCs) such as MHC-II expression, costimulatory molecules, or marked cytokine secretion. The insignificant role of muscle cells in intramuscular DNA-immunization is further supported by a study in which the surgical removal of the injected muscle within

10 min of injection of DNA plasmid did not affect the magnitude or longevity of the antibody response to the encoded antigen (Torres et al. 1997). Therefore, the muscle is not likely to be the essential immune activating component after intramuscular immunizations. Furthermore, there is little or no local inflammatory infiltrate at the DNA injection site, especially after the acute effects of the vaccination have disappeared (Levitsky 1997).

There are two possible scenarios that might explain the mechanism of immune-priming by intramuscularly injected genetic vaccines. First, antigen produced by transfected myocytes can be taken up by professional APCs. Alternatively, professional APCs can be transfected directly and the antigen expressed by transfected myocytes is an irrelevant side-product. In either case, the antigen-expressing APCs then migrate to the draining lymph nodes where they present antigenic peptide-MHC complexes in combination with signaling by costimulatory molecules to naïve T-cells. This interaction provides the necessary secondary signals to initiate an immune response and to activate and expand T-cells or to activate B-cell and antibody production cascades. In addition, transfected myocytes may also serve as plasmid depots for continued APC-transfection. Because myocytes expressing the antigen are subject to CTL-lysis (Davis et al. 1997), plasmid released from these myocytes may be picked up by monocytes migrating through the muscle.

14.3 DNA Vaccine

DNA vaccination has become the fastest growing field in vaccine technology following reports at the beginning of the 1990s that plasmid DNA induces an immune response to the plasmid-encoded antigen after intramuscular injection into mice (Wolff et al. 1990). In theory, this conceptually safe, non-live vaccine approach is a unique and simple way to induce not only humoral but also cellular immunity. Whereas traditional vaccines rely on the production of antibodies through the injection of live attenuated virus, killed viral particles, or recombinant viral proteins, DNA vaccines are non-live, non-replicating, and non-spreading such that there is little risk of either reversion to a disease-causing form or secondary infection. In addition, DNA vaccines are highly flexible to encode viral or bacterial antigens, and immunological or biological proteins. DNA vaccines are stable, easily stored, and can be manufactured on a large scale. Furthermore, DNA vaccine avoids the risks associated to the manufacture of killed vaccine, as exemplified by the tainting of a polio vaccine with live polio virus owing to a production error (Offit et al. 2005). The earliest phase I clinical trial for a DNA vaccine was an HIV-1 candidate tested in individuals infected by HIV-1, followed by studies in volunteers who were not infected by HIV-1 (MacGregor et al. 1998). Subsequently, other therapeutic DNA vaccine trials against cancer, influenza, malaria, hepatitis B, and other HIV-1 candidates followed (Mincheff et al. 2000; Tacket et al. 1999; Le et al. 2000; Liu and Ulmer 2005; Ulmer et al. 2006). These trials demonstrated that the DNA vaccine platform is well tolerated and clinically safe. Some potential safety concerns

including the integration into cellular DNA, the development of autoimmunity, and antibiotic resistance have been raised for DNA vaccines. DNA vaccines currently being tested do not show relevant levels of integration into host-cellular DNA (Kurth 1995; Manam et al. 2000; Ledwith et al. 2000; Temin 1990; Pal et al. 2006; Sheets et al. 2006). Furthermore, the preclinical or clinical evaluation of DNA vaccines have not indicated any insertional mutagenesis through the activation of oncogenes or the inactivation of tumor suppressor genes or any chromosomal instability through the induction of chromosomal breaks or rearrangements. To date, there has been no convincing evidence of anti-nuclear or anti-DNA autoimmunity developing in association with a DNA vaccine (MacGregor et al. 1998; Le et al. 2000; Klinman et al. 2000; MacGregor et al. 2000; Bagarazzi et al. 1997). As the antibiotic resistance genes contained by DNA vaccines are restricted to those antibiotics not commonly used to treat human infections, the risk of introducing antibiotic resistance into participants of clinical trials is very low. Alternative strategies to omit antibiotic selection in large-scale manufacture of DNA vaccines are being explored (Mairhofer et al. 2007; Cranenburgh et al. 2001). However, the first-generation DNA vaccines failed to demonstrate high levels of vaccine-specific immunity in large animals and humans.

On the basis of the notion that strong immune response can be elicited by the high level of antigen expression, significant efforts are ongoing to maximize gene expression and subsequent antigen expression from DNA vaccines. Firstly, for most recent DNA vaccines, the human CMV promoter is a common choice because it promotes high-level constitutive expression in a wide range of mammalian cells and does not suffer from downstream read-through (Donnelly et al. 1997). Alternatively, the use of muscle-specific promoters, such as promoters for creatine kinase or desmin, avoids constitutive expression of antigens in inappropriate tissues and also leads to the induction of antibody and T-cell responses, although levels of the antigen expressions are at least tenfold less than those driven by the CMV promoter (Bojak et al. 2002; Cazeaux et al. 2002). Secondly, many DNA vaccines use the bovine growth hormone terminator sequence or endogenous terminators to ensure proper transcriptional termination and efficient export of the mRNA from the nucleus (Montgomery et al. 1993). Thirdly, both enhancer elements and transcriptional transactivators, when placed either upstream or downstream of the open reading frame (ORF), can enhance promoter activity to further increase the expression of antigens (Barouch et al. 2005; Ito et al. 2003; Garg et al. 2004). Fourthly, “Kozak” consensus sequence (Kozak 1987; Kozak 1997) and codon optimization (Ikemura 1982; Ikemura 1985) are important to increase antigen production leading to enhanced T-cell and antibody responses by DNA vaccines (Garmory et al. 2003).

Additional efforts are ongoing to optimize the DNA vaccine platform in order to increase vaccine immunogenicity. Firstly, antigens can be targeted to the class I or class II processing pathways with the addition of sequences designed to direct intracellular trafficking. The immunogenicity of CD8+ T-cell epitope encoded by DNA vaccine was significantly enhanced when an adenovirus leader-sequence was fused in frame to target this CD8+ T-cell epitope to the endoplasmic reticulum (Ciernik et al. 1996). In some cases, the secretory signal leader sequence of tissue

plasminogen activator (TPA) was fused with the N terminal of the antigens of interest to direct the antigens to the secretory pathway, which seemed to be another effective strategy to enhance the humoral immune response against the antigens (Li et al. 1999; Qiu et al. 2000; Ashok and Rangarajan 2002; Delogu et al. 2002). Secondly, antigenic proteins can be maximally truncated, leaving only defined epitopes for T-cells to induce immune response (Casares et al. 1997). This may be especially useful in cases where "full-length" proteins are toxic for the host (Barry and Johnston 1997) or immunosuppressive (Levy 1993). Thirdly, to overcome MHC restriction of individual epitopes or to induce a broader range of effector cells, multiple contiguous minimal epitopes in the form of a "polytope" can be delivered by DNA vaccine (Thomson et al. 1998). The immune potency of this strategy can be improved by including helper T-cell sequences to supply novel T-cell help (Stevenson et al. 2004). Fourthly, one current trend in DNA vaccine is the use of biodegradable polymeric microparticle-based and liposome-based delivery systems to induce cellular and humoral immunity (O'Hagan et al. 2006; Kutzler and Weiner 2008). Both polymer and liposome vehicles can protect DNA from degradation by serum proteins during transfer of DNA across membranes and after the release of generic material following fusion with endosomes. Lastly, multiple studies have shown that co-injections of DNA vaccine cocktails including plasmids encoding cytokines, chemokines, and/or costimulatory molecules along with antigens can have a substantial effect on the immune response to plasmid-encoded antigen (Leitner et al. 2000). Furthermore, synthetic oligodeoxynucleotides containing unmethylated CpG motifs can act as effective adjuvants that enhance both humoral and cellular immunity with minimal toxicity (Higgins et al. 2007).

14.4 Viral Vectors for Vaccine Development

Viral vectors provide a convenient and efficient way to deliver vaccine antigens to muscle. A broad spectrum of replicating and nonreplicating vectors is available for vaccine development. Several viral vectors, including both replicating and non-replicating forms of adenovirus and poxviruses, primary non-replicating forms of adeno-associated virus, alphavirus, and herpesvirus, and primary replicating forms of measles virus and vesicular stomatitis virus, are currently under development as HIV/AIDS vaccines (Robert-Guroff 2007). In this chapter, only adenoviral and adeno-associated viral vectors as vaccine carriers will be further discussed.

14.4.1 Adenoviral Vector for Vaccine Development

Adenoviral vectors were initially investigated for years as vehicles for gene therapy. However, attempts to replace missing or faulty genes by adenoviral vector gene transfer were largely unsuccessful in experimental animals and human volunteers

most likely because of innate and adaptive immune responses induced by the adenoviral antigens. Currently, adenovirus is known to interact with several different extracellular, intracellular, and membrane-bound innate immune sensing systems (Hartman et al. 2008). Adenovirus expresses pathogen-associated molecular patterns (PAMPs), which bind to pathogen recognition receptors (PRRs) on host cells, including those of the innate immune system, in order to produce the proinflammatory chemokines and cytokines and to differentiate the immature dendritic cells into professional antigen-presenting cells (Hartman et al. 2008; Medzhitov and Janeway 2000). Adaptive immune responses are directed to both early and late viral antigens of adenovirus. Adenovirus-neutralizing antibodies induced after adenoviral infection or upon adenoviral vector administration are primarily directed against the surface loops of the viral hexon (Wohlfart 1988), even though antibodies targeted to the penton base or the fiber can also neutralize adenovirus (Hong et al. 2003). In addition, a highly conserved human CD4+ T-cell epitope has been identified within adenovirus capsid protein hexon (Olive et al. 2002).

So far, adenoviruses are among the most heavily exploited vectors for vaccine development. Adenovirus can grow to high titer in vitro with physical and genetic stability. In addition, adenovirus infects both dividing and non-dividing cells without integration in the host genome and yields high levels of antigen expression. More importantly, adenovirus infected dendritic cells upregulate costimulatory molecules and induce cytokine and chemokine responses, thus effectively presenting antigens to the immune system and eliciting potent antigen-specific immune responses (Banchereau and Steinman 1998; Morelli et al. 2000; Molinier-Frenkel et al. 2003; Philpott et al. 2004).

Currently, non-replicating adenoviral vaccine vectors are created by the deletion of E1 region genes, which are essential for replication. Such vaccine vectors generally also have the non-essential E3 region deleted in order to provide more space for the completed expression cassette for antigen, including exogenous promoter, a transgene for antigen, poly (A) signal, etc. It has been shown that non-human primates intramuscularly immunized with non-replicating adenoviral vaccine vector expressing SIV Gag gene were protected against experimental infection with a chimeric SIV/HIV virus better than those intramuscularly immunized with plasmid DNA or the modified vaccinia Ankara (MVA) recombinant encoding the same antigen (Shiver et al. 2002). The prime and boost approaches, especially priming with improved plasmid DNA vaccines to focus the immune responses on the foreign antigen followed by a boost with an adenoviral vaccine vector expressing the same antigen, were shown to enhance the efficacy of protection (Casimiro et al. 2005; Mattapallil et al. 2006). In addition, non-replicating adenoviral vaccine vector encoding rabies virus glycoprotein can induce protective neutralizing antibody titers against rabies virus rapidly after only a single application. The efficacy of this non-replicating adenoviral vaccine was far superior to that of a well-characterized vaccinia rabies glycoprotein recombinant (Xiang et al. 1996). A recent study suggested that non-replicating adenoviral vaccine vectors were transcriptionally active at low levels for long periods of time after intramuscular immunization in mice (Tatsis et al. 2007). Continuously produced small amounts of antigen help to maintain

fully active antigen-specific effector CD8+ T-cells, which can further differentiate into central memory cells. The long-term persistence of adenoviral vaccine vectors might be highly beneficial against pathogens, which require both fully activated effector CD8+ T-cells for immediate control of pathogen-infected cells and central memory CD8+ T-cells for elimination of pathogen-infected cells that escaped the initial wave of effector CD8+ T-cells.

Non-replicating adenovirus vectors with deletion of E1 and E4 regions have been explored mainly for gene therapy (Mizuguchi and Kay 1999). E4-deleted adenovirus vectors produce fewer late viral gene products than only E1-deleted vectors. Therefore, it will be interesting to test whether E1- and E4-deleted adenovirus vaccine vectors can indeed induce less vector-specific and more focused antigen-specific immune responses than only E1-deleted vectors.

Replicating adenoviral vaccine vectors contain the deletion only in the E3 region. Therefore, replicating adenoviral vaccine vectors have a more limited cloning capacity for transgenes. The main scientific advantage of replicating adenoviral vaccine vectors is that they can elicit more potent immune responses, including innate immunity, as well as humoral, cellular, and mucosal immune responses. The wild-type adenoviral vaccine vectors were proven to be safe after they were used for over 26 years in the US military (Robert-Guroff 2007). In replicating adenoviral vaccine vectors, expression of the encoded antigen is incorporated into the viral replication cycle so that lower immunization doses can achieve longer and higher expression levels of antigen in vivo than non-replicating adenoviral vaccine vectors. In addition, adenoviral replication in vivo can stimulate production of proinflammatory cytokines, further augmenting immune responses. Apoptotic cells arising from adenoviral replication can provide DC with exogenous antigens for initiation of T-cell responses through cross-presentation (Fronteneau et al. 2002). Although replicating adenoviral vaccine vectors may compete with transgenes for induction of immune responses, strong immune responses to adenoviral antigens may paradoxically enhance immunity to transgene-encoded antigen via CD8-T-cell-mediated autocrine help (Truckenmiller and Norbury 2004), whereby CD8+ T-cells can provide help for other responding CD8+ T-cells if present in sufficient numbers (Wang et al. 2001). A comparative study in chimpanzees after immunization with either replicating or non-replicating adenoviral vaccine vector encoding the same antigen demonstrated both the greater induction of cellular immune responses and the ability to prime more potent antibody responses by the replicating adenoviral vaccine vector (Peng et al. 2005).

Virus-specific neutralizing antibodies even at moderate titers can significantly reduce uptake of the adenoviral vaccine vectors by cells, including antigen-presenting cells, after intramuscular immunization, which in turn impacts the transgene product-specific immune responses (Fitzgerald et al. 2003). Pre-existing immunity in general human population due to natural infections of the common serotypes of human adenovirus, such as serotype 5 (Ad5) and serotype 2 (Ad2), results in sustained virus-neutralizing antibody titers. Therefore, a way to circumvent the impact of pre-existing adenovirus-specific immunity in humans is essential for the success of adenoviral vaccine vectors in the clinical setting. Simple dose escalation is

unlikely to be a realistic option because of the associated toxicity. Studies in Ad5-preexposed nonhuman primates showed that a 1,000-fold increase in dose is needed to achieve frequencies of transgene product-specific CD8+ T-cells comparable to those obtained in animals that have not been pre-exposed (Casimiro et al. 2003). Currently, alternative serotypes and strains of adenovirus from different species are being developed as vaccine carriers. Adenovirus of rare serotype such as serotype 11 (Ad11), serotype 26 (Ad26), serotype 35 (Ad35), and serotype 49 (Ad49) can be used in sequential prime-boost regimens of intramuscular immunization in order to avoid or lessen the impact of pre-existing immunity as well as cross-reactivity between adenovirus serotypes (Thorner et al. 2006). In addition, vaccine vectors based on the non-human adenoviruses of chimpanzee origin (Farina et al. 2001; Tatsis et al. 2006), and the engineered chimeric vectors in which the hypervariable regions of the hexon protein of Ad5 are replaced with corresponding regions of a rare adenovirus serotype 48 (Ad48) (Roberts et al. 2006), have been shown to effectively overcome the pre-existing anti-vector immunity. Simian E1-deleted adenoviral vectors, such as AdPan9, AdPan7, and AdPan6, showed comparable yields upon propagation on HEK 293 cells, indicating that they can grow in the presence of E1 of Ad5. As the sequences flanking the E1 region show limited homology between different serotypes of adenoviruses, the risk of contamination of E1-deleted simian adenoviral vaccine vectors with replicating adenovirus due to the homologous recombination of simian vaccine vectors with Ad5 E1 of the packaging cell line is virtually absent. Overall, these alternative adenoviral vaccine vectors provide the flexibility in primer-boost strategy and focus the immune response on the inserted antigen while avoiding anti-vector immunity induced by prior natural infection or immunizations. However, these alternative adenoviral vaccine vectors will require extensive safety testing and their relative immunogenicity in comparison to Ad5 will need to be clearly established.

14.4.2 Adeno-associated Viral Vector for Vaccine Development

Adeno-associated viruses (AAVs) are non-pathogenic, transduce muscle cells well, and provide long lasting expression from primarily episomal molecular forms (Schnepp et al. 2003). As a non-enveloped virus, AAV exhibits high physical stability. Vectors derived from AAV have emerged as highly promising ones for use in gene therapy (Carter and Samulski 2000; Monahan and Samulski 2000). All virus-encoded genes are replaced with the gene of interest by flanking between the inverted terminal repeats (ITRs). The production of AAV vector presents some difficulties, as both AAV rep and cap functions and “helper” viral functions, typically adenovirus E1, E2, and E4, and VA RNAs, must be provided in trans. Integration of AAV vector is also a potential safety concern. However, a recent report suggests that AAV vector integrates no more frequently than naked DNA (Johnson et al. 2005).

The removal of all virus-encoded genes in AAV vectors results in comparatively low intrinsic immunogenicity against the viral antigens. In contrast to the adenoviral

vector, AAV vector does not induce the expression of multiple inflammatory chemokines, including RANTES, interferon-inducible protein 10 (IP-10), interleukin-8 (IL-8), MIP-1beta, and MIP-2, above baseline levels despite 40-fold-greater titers than the adenoviral vector and greater amounts of intracellular AAV vector genomes according to Southern and slot blot analysis (Zaiss et al. 2002). Nevertheless, recent studies have shown that AAV vectors can elicit both cellular and humoral immune responses against the transgene product, depending on a number of variables, including the nature of transgene, the promoter used to drive the transgene expression, the route and site of administration, vector dose, and host factors (Sun et al. 2002; Zaiss and Muruve 2005).

AAV vectors have been evaluated as vaccine carriers for multiple antigens. Intramuscular injection of mice with an AAV serotype 2 (AAV2) vector expressing herpes simplex virus type 2 glycoprotein B (gB) led to the generation of both gB-specific major histocompatibility complex class I-restricted cytotoxic T lymphocytes and anti-gB antibody. AAV-mediated immunization was more potent than plasmid DNA or protein in generating antibody responses (Manning et al. 1997). A single intramuscular injection of AAV vaccine vectors expressing HIV-1 env, tat, and rev genes induced strong HIV-1-specific serum IgG and fecal secretory IgA antibodies as well as MHC class I-restricted CTL activity in BALB/c mice. The titer of HIV-1-specific serum IgG remained stable for 10 months. When AAV HIV vaccine vectors were co-administered with AAV vector expressing interleukin 2 (IL2), the HIV-specific cell-mediated immunity was significantly enhanced (Xin et al. 2001). A promising study in rhesus macaques showed that a single high dose of intramuscular injection of AAV serotype 2 vaccine vector encoding SIV antigens could elicit both SIV-specific T-cells and neutralizing antibodies. Furthermore, the immunized animals were able to significantly restrict replication of a live and virulent SIV challenge (Johnson et al. 2005). These data suggest that AAV vaccine vectors induced biologically relevant immune responses and thus warrant continued development as a viable HIV-1 vaccine candidate. A phase I human study of an AAV-based vaccine vectors encoding HIV Gag, protease, and deleted reverse transcriptase genes has been completed. The AAV vaccine vectors proved to be safe but only minimally immunogenic, with a 20% positive T-cell response rate in the group receiving the highest immunization dose. Although AAV vaccine vectors were able to elicit potent B-cell response, no HIV-specific antibodies were observed (Van, L.J., Mehendale, S. Clumeck, N. Bets, E., Rockstroh, J., Johnson, P. Schmidt, C., Excler, J., Kochhar, S., and Heald, A. A phase I study to evaluate the safety and immunogenicity of a recombinant adeno-associated virus vaccine. Poster 474; presented at the 14th Conference on Retroviruses and Opportunistic Infections, Los Angeles, CA, February 25–28, 2007).

In humans, the presence of serum antibodies directed against AAV2 is very common. The pre-existing immunity to wild type AAV2 in human is predominantly humoral, with a minority of subjects demonstrating marginal lymphocyte proliferation and interleukin 10 (IL-10) secretions in response to AAV2 proteins (Chirmule et al. 1999). Therefore, efforts to modify AAV2 capsid and to develop AAV vaccine vectors based on alternative serotypes are currently ongoing in order to address the poor immunogenicity of AAV2 vaccine vectors in humans exhibiting prior

immunity. Several mutational analyses of AAV2 capsid proteins have been performed to both map areas of cell receptor binding and to identify points for insertion of peptides to modify vector tropisms (Girod et al. 1999; Shi et al. 2001). These studies laid the foundation of possible modification of AAV2 capsid, which elicits neutralizing responses. In addition, six peptides that were able to block human serum neutralizing activities against AAV2 were identified by screening human sera against a peptide library (Moskalenko et al. 2000). Such information may allow the design of reverse genetic approaches to circumvent the pre-existing immunity against AAV2. AAV serotype 5 (AAV5) is different from AAV serotypes 2, 3, 4, and 6 at the nucleotide level and at the amino acid level (Bentel-Schaal et al. 1999). It has been shown that AAV5 vaccine vector encoding HIV-1 Env gp160 exhibited higher tropism for both mouse and human dendritic cells and elicited more potent antigen-specific cellular and humoral responses in mice after intramuscular immunization than AAV2 vaccine vector encoding the same antigen (Xin et al. 2006). Furthermore, an expanded family of AAVs from human and nonhuman primates was discovered on the basis of recovery of latent forms of the genome using PCR techniques (Gao et al. 2003; Gao et al. 2004; Gao et al. 2002). Subsequently, AAV2 inverted terminal repeat was used to generate the pseudotyped AAV2-based novel AAV vectors. Mice were passively transferred with pooled human immunoglobulin at various doses to simulate the pre-existing antivector humoral immunity in humans. After intramuscular immunization, inhibition of antigen-specific immune responses induced by AAV2 vaccine vector encoding HIV-1 Gag occurred at doses of human immunoglobulin 10- to 20-fold less than that required to inhibit antigen-specific immunogenicity elicited by AAV2/7 and AAV2/8 pseudotyped vaccine vectors encoding the same antigen (Lin et al. 2008). These data suggested that the vaccine vectors based on these novel AAVs might be able to overcome the pre-existing immunity in human population. Furthermore, after intramuscular immunization in mice, AAV2/8 pseudotyped vaccine vector encoding HIV-1 Gag induced robust antigen-specific cellular and humoral responses. However, no CD8+ T-cell response generated by AAV2/8 vaccine vector was effectively boosted with a simian adenoviral vaccine vector expressing the same antigen. These results were consistent with the finding that most of CD8+ effector cells were quickly contracted and yielded few central memory cells. In contrast, the B-cell response to HIV-1 Gag encoded by AAV2/8 vaccine vector was quite vibrant and easily boosted with the simian adenoviral vaccine vector expressing the same antigen (Lin et al. 2007). Compared to Ad vaccine vectors, AAV vaccine vectors can induce largely comparable antigen-specific antibody responses after intramuscular immunization. However, the antigen-specific T-cell responses induced by AAV vaccine vectors might be different from that induced by Ad vaccine vectors after intramuscular injection.

Possible mechanisms that might explain the difference in antigen-specific T-cell responses induced by AAV or Ad vaccine vectors include little activation of innate immunity, insufficient CD4+ T-cell help, or T-cell exhaustion by possible long-term expression of the antigen following immunization with AAV vaccine vector compared to Ad vaccine vector. These results need to be better understood in order to take full advantage of the platform of AAV vaccine vectors.

14.5 Challenges and Future Directions in Genetic Vaccine Development: A Case Study of the Genetic Vaccine for HIV

The development of a safe and effective HIV-1 vaccine is a critically important global health priority. The goal of an HIV-1 vaccine is either to prevent infection or to reduce viral loads and clinical disease progression after infection. However, the challenges in the development of HIV-1 vaccine are unprecedented. The extraordinary worldwide diversity of HIV-1 poses a major obstacle to AIDS vaccine development (Gaschen et al. 2002). Ideally, antigens in any successful HIV-1 vaccine will need to elicit broad humoral and cellular immune responses to cross-react with highly heterologous viruses. However, it has not been possible to induce such broad neutralizing antibodies by vaccine so far, while the breadth of vaccine-elicited cellular immune responses may be limited by immunodominance constraints and by the inherent tendency of CD8+ T-cell responses to be highly focused on a limited number of epitopes. Another key challenge is the lack of clear immune correlates of protection in human, as patients cannot fully recover from HIV-1 infection.

Recently, the Merck HIV-1 vaccine candidate, formulated as a trivalent mixture of adenoviral vaccine vectors expressing HIV-1 clade B Gag, Pol, and Nef, was unexpectedly terminated in phase 2b "proof-of-concept" efficacy studies because of failure to achieve its primary end points after the first planned interim data analysis. More surprisingly, in subjects with pre-existing Ad5-specific neutralizing antibody titers, higher rates of HIV-1 infection were observed in the vaccine group than in the placebo group.

Post-hoc multi-variate analysis further suggested that the greatest increased risk was in men who had pre-existing Ad5-specific neutralizing antibodies and who were uncircumcised. It is currently unclear whether the lack of efficacy in the Merck HIV-1 vaccine candidate simply indicates the failure in this particular vaccine product or completely overturns the T-cell vaccine concept. However, it will be interesting to determine whether HIV-1 vaccine candidates based on Ad vaccine vectors derived from novel and/or rare serotypes can potentially prevent a similar failure.

HIV-1 vaccine development is still in its infancy. It is most likely that a combination vaccine consisting of separate vaccine components that can induce both humoral and cellular immunity will be needed for optimal HIV-1 vaccines. Even though the traditional vaccine technologies, including live attenuated viruses, whole killed viruses, and protein subunits, have been successful in developing vaccines against other diseases, they all have substantial limitation to become the ideal choices of HIV-1 vaccines. Live attenuated viruses pose a significant safety concern in humans, while whole killed viruses and protein subunits are unable to elicit CD8+ T-cell responses. Therefore, new vaccine strategies such as DNA vaccines and genetic vaccines based on recombinant viral vectors will most likely be the choices for HIV-1 vaccines. DNA vaccines are simple and versatile, and multiple injections of high doses of DNA vaccines can induce detectable immune responses

in non-human primates and humans. Subsequently, the immune responses could be further boosted with recombinant viral vaccine vectors. Viral vaccine vectors based on the rare and/or novel serotypes that can circumvent the pre-existing neutralizing antibodies in humans have hope of offering immunogenicity as well as limiting safety concerns.

14.8 Conclusion

Taken together, a wealth of published pre-clinical studies in small and large animal models appears to suggest that muscle is a practical, effective, and safe target for genetic vaccines based on different vector platforms. However, complexity of vaccine immunology, interactions between host protective immunity and viral immunopathology as well as the lack of full correlations between vaccine efficacy and safety data generated from currently available animal models and human trials strongly argue for the necessity to go back to the drawing board and basic research to further delineate all of those aspects for genetic vaccines before rushing into human trials. Depending on the pathogenesis of microbial infections and route of infectious disease transmission, some alternative routes of vaccination such as mucosal directed genetic vaccines should also be further explored for which AAV based vaccine may be advantageous because of its relative hardness and resistance to temperature, pH extremes, and solvents (Berns and Hauswirth 1979; During et al. 2000).

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Chapter 15
Combining Stem Cells and Exon Skipping Strategy to Treat Muscular Dystrophy

Mirella Meregalli, Andrea Farini, and Yvan Torrente

Abstract Muscular dystrophies are a group of diseases characterized by the primary wasting of skeletal muscle. Mutations in the dystrophin gene cause Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD). The characterization of the dystrophin gene and the evidence that adult stem cells are capable of participating into regeneration of more than its resident organ has lead to the development of potential gene therapy and stem cells treatments for this disorder. The combination of gene therapy and stem cell therapy may represent a very promising strategy. In this chapter, we describe an example of such combined therapy. We first corrected mutation in DMD pateints' stem cells with antisense oligonucelotide mediated exon skipping. The corrected stem cells were then delivered to the mdx mouse model via intra-arterial injection. This approach has several advantages. Intravascular delivery distributes the stem cells to the whole body musculature. The use of the autologous transplantation minimizes the risk of immunological graft rejection.

Keywords Adult stem cells • AON • DMD • exon skipping • muscular dystrophies • viral vectors

15.1 Introduction

Muscular dystrophies are a group of diseases characterized by the primary wasting of skeletal muscle; in many cases they are caused by mutations in the proteins that form the link between the cytoskeleton and the basal lamina (Cossu et al. 2007). Mutations in the dystrophin gene cause Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD). In DMD patients, the complete lack of dystrophin causes progressive degeneration and muscle wasting that lead to death in the second

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and second/third decade of the patient's life. Although both disorders are characterized by similar patterns of mutations such as large deletions and point mutations, BMD patients show a very mild phenotype, often asymptomatic primarily due to the expression of shorter dystrophin mRNA transcripts that maintain the coding reading frame. The identification and characterization of the dystrophin gene has led to the development of potential treatments for this disorder (Bertoni et al. 2008).

Gene therapy for muscular disorder embraces several concepts such as replacing and/or repairing a defective gene itself, modifying and/or enhancing cellular performance with cellular genes that are not directly related to the underlying defect (Shavlakadze et al. 2004). We have used a gene repair approach through exon skipping. In the exon skipping approach, specific antisense oligonucleotides (AON) are designed to mask the putative splicing sites of exons in the mutated region of the primary RNA transcript. Removal of such exon(s) would re-establish a correct open reading frame. During last few years, new generation of AON such as 2′-O-methyl phosphorothioate, morpholino, and peptide nucleic acids (PNA) have been chemically engineered to exhibit higher affinity for target sequences and extraordinary resistance to nucleases (Aartsma-Rus et al. 2004).

Stem cells have received 6much attention because of their potential use in cell-based therapies for human disease such as leukaemia (Owonikoko et al. 2007), Parkinson's disease (Singh et al. 2007), stroke (Gilman, 2006) and muscular dystrophies (Nowak et al. 2004; Endo, 2007). Stem cells are unspecialized cells that have been defined in many different ways but they have two important characteristics that distinguish them from other cells in the body (Bertoni et al. 2008). First, they can replenish their numbers for long periods through cell division. Second, after receiving some chemical signals, they can produce progenies that can differentiate into multiple cell lineages with specific functions (Bertoni et al. 2008). Embryonic stem (ES) cells are derived from mammalian embryos in the blastocyst stage and they are capable of differentiating into any tissue of the body (Peault et al. 2007). Ethical and political issues surrounding ES cells, however, make their use difficult. Adult stem cells, on the other hand, are intrinsic to various tissues of the body and they are capable of maintaining, generating, and replacing terminally differentiated cells within their own specific tissue. Adult stem cells have also been found in most organs tissues including the bone marrow (BM) (Jiang et al. 2002; Mitchell et al. 2003), the central nervous system (Seaberg et al. 2003; Toma et al. 2001), skin (Zuk et al. 2001), cardiac muscle (Bischoff, 2007), and skeletal muscle (Charge et al. 2004; Hughes et al. 1990). Recently, it has become evident that these adult stem cells are capable of participating in regeneration of more than just its resident organ. These cells, together with hematopoietic and muscle stem cells (Torrente et al. 2004; Gavina et al. 2006; Torrente et al. 2007), are suitable for cell-based therapy to treat DMD. Stem cell therapy is an attractive method to treat muscular dystrophy because only a small number of cells, together with a stimulatory signal for expansion, are required to obtain a therapeutic effect (Price et al. 2007). The clinically relevant candidate stem cell population must be easily extracted, remain capable of efficient myogenic conversion, and when transplanted, integrate into the muscles allowing the functional correction of the dystrophic phenotype (Price et al. 2007).

15.2 AON-Mediated Exon Skipping to Treat DMD

The use of AON to redirect splicing of the dystrophin gene has the advantage to transform a severe DMD to a much milder BMD phenotype. This theory is supported by clinical findings in BMD patients. In these patients a much shorter but in-frame dystrophin is expressed in muscle (Bertoni C, 2005). The discovery of the highly functional mini- and micro-dystrophin genes further supports the exon skipping strategy (Gregorevic et al. 2003).

Briefly, in exon skipping strategy, AON was used to block intron/exon splice site boundary or regulatory mechanisms such as splicing regulatory elements that control exons recognitions of the target gene. Through deletion of untoward exon(s), AON restores the coding reading frame of the protein (Fig. 15.1). The first paper on the potential applications of AON to restore dystrophin expression was published in 1995. Takeshima *et al.* used a 2′-O-methyl RNA oligonucleotide complementary to the first 31 nucleotides of human dystrophin exon 19. The AON was specifically designed to induce skipping of exon 19 (Takeshima et al. 1995). Over the last decade, many exon skipping studies have been published in the mdx mouse model. The mdx mouse is characterized by a single point mutation in exon 23 of the dystrophin gene. The nonsense mutation creates a stop codon aborting protein expression. In 1998 Dunckley *et al.* reported the ability of a 2′-O-methyl oligoribonucleotides to target and redirect splicing of the dystrophin gene in mdx muscle cells in culture (Dunckley et al. 1998). The AONs were designed to anneal to the acceptor splice site of exon 23 of the dystrophin gene, resulting in the expression of truncated forms of dystrophin detectable by immunostaining. Subsequent studies showed the possibility to target and exclude exon 23 by targeting the donor site of the intron/exon boundary of the mouse dystrophin exon 23 (Graham et al. 2004).

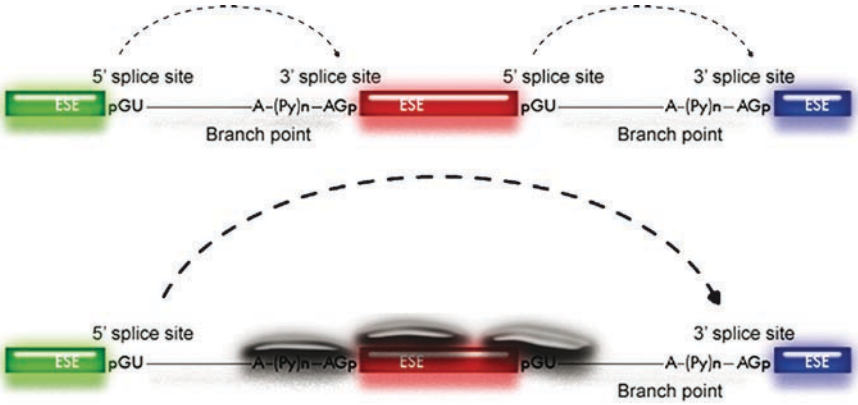

Fig. 15.1 The exon skipping has been proposed as a molecular strategy to prevent transcription of the exon containing the mutation. Skipping can be achieved by oligonucleotides or by small RNAs, which hybridize with the donor and/or acceptor site(s) of the mutated exon causing its exclusion from the transcript that starts again one or few exons downstream. Antisense sequences target the potential mutated exon internal splicing enhancer (ESE) in order to hide the splicing consensus sequence

15.3 Cell-Based DMD Therapy with Muscle Derived CD133+ Stem Cells

Cell-based therapies involve the delivery of normal cells to the damaged muscle with the hope that the host cells will fuse and repopulate the muscle, improving muscle function and pathology (Chakkalakal et al. 2005).

We recently demonstrated that circulating CD133+ cells may be used for stem cell therapy of muscular dystrophy (Torrente et al. 2007). We isolated CD133+ cells from normal and DMD biopsies. We demonstrated that the muscle-derived CD133+ cells were present in both normal and DMD muscles. However, dystrophic muscle biopsies of young patients (5-14 year-old) contained a higher number of these cells than muscle biopsies of healthy age-matched donors (Torrente et al. 2007). CD133+ cells represented 2% of the total dystrophic muscle-derived nucleated cells. Our data showed that more than 79% of muscle-derived CD133+ cells coexpressed CD34 and Thy-1 antigens. Instead ~5% of the normal muscle-derived CD133+ cells coexpressed the CD34 antigen and ~53% of these cells coexpressed Thy-1. We found that both normal and dystrophic muscle-derived CD133+ cells expressed the CD45 antigen (around 4%), indicating their hematopoietic commitment. The proliferation rate of these cells was approximately 20 population doubling with a doubling time around 36 hrs (Fig. 15.2).

We also analyzed the ability of normal and DMD muscle-derived CD133+ cells to express myogenic markers and/or to be able to differentiate into myogenic lineage in vitro (Torrente et al. 2007). RT-PCR revealed the expression of Pax-7, Myf-5, MyoD, m-cadherin, MRF-4, and myogenin in both normal and dystrophic muscle-derived CD133+ cells after 24 days of culture in the proliferation medium suggesting a myogenic commitment. Moreover we observed that CD133+ cells differentiated into multinucleated myotubes that expressed MyHCs.

To further establish the muscle-derived CD133+ cells for DMD therapy, we recently investigated the safety and feasibility after transplantation in DMD muscles. Our data showed that intramuscular transplantation of muscle-derived CD133+ cells in DMD patients was safe (Torrente et al. 2007).

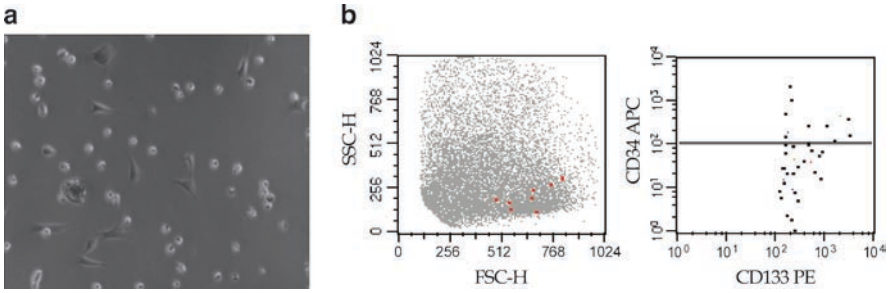

Fig. 15.2 Characterization of CD133+ stem cells. **(a)** CD133+ and CD34+ stem cells were isolated from human skeletal muscle. The proliferation rate of these cells was approximately 20 population doubling with a doubling time around 36 hrs. **(b)** Around 5% of the normal muscle-derived CD133+ cells coexpressed the CD34 antigen

15.4 Combining Exon-Skipping Therapy and CD133+ Cell Therapy to Treat DMD

To further extend our success with CD133+ cell therapy, we recently explored a combinatory therapy. We propose to correct the mutation in dystrophic CD133+ myogenic cells by exon skipping and then deliver the corrected cells back to dystrophic muscle. Use of the patient's own cells would reduce the risk of implant rejection.

As a proof-of-principle, we isolated CD133+ cells from DMD patients. We then initiated exon-skipping with a lentiviral vector. Two distinct CD133+ cell populations were used, one from blood and the other from skeletal muscle. The lentivirus carried a U7-based construct designed to skip exon 51 (Goyenvalle et al. 2004; Denti et al. 2006). CD133+ cells were infected with the lentiviral vector and then transplanted into scid/mdx mice via intra-arterial injection. We found that human genetically engineered DMD blood and muscle-derived CD133+ located beneath the basal lamina and distributed along freshly isolated fibers where they expressed M-cadherin, suggesting that they have differentiated into satellite cells within the recipient dystrophic skeletal muscle (Benchaouir et al. 2007). We also found that the muscle-derived CD133+ stem cells had the potential to differentiate towards both the muscle lineage and the endothelium lineages. Importantly, the exon-skipping corrected cells were able to fuse with regenerative fibers and express a functional human dystrophin. However, muscle-derived CD133+ cells showed a better muscle regeneration in term of spreading and the number of positive fibers in comparison with the results obtain with blood-derived stem cells. Finally, we demonstrated muscle force and endurance recovery in treated mice.

15.5 Summary and Future Directions

A promising possibility for treating DMD is a combination of different approaches. In this chapter, we reviewed our experience on combining exon-skipping gene therapy and CD133+ stem cell therapy. AON-mediated exon-skipping has shown promising results in human patients. Our phase I clinical trial also demonstrated the safety of CD133+ stem cells (Torrente et al. 2007). Combining these two strategies may likely yield beneficial outcome in DMD patients. In perspective of future clinical trials, we speculate that CD133+ stem cells (or other stem cells such as masoangioblasts) purified from DMD patients could be engineered ex vivo with exon-skipping and re-injected to the same donor intra-arterially. The intra-arterial injections of the patient's own stem cells may allow the distribution of the cells to the whole body musculature with minimal immune rejection. This strategy may be applicable to severely affected patients who have lost most of the muscle.

One of the most important problems to solve in the future is the safety issue. In our study, we used lentiviral vector to induce exon-skipping. However, hazardous integration of the provirus may disturb the control of the house keeping genes and/or tumor-suppressor genes. Other approaches need to be developed to further increase the safety threshold (Fig. 15.3).

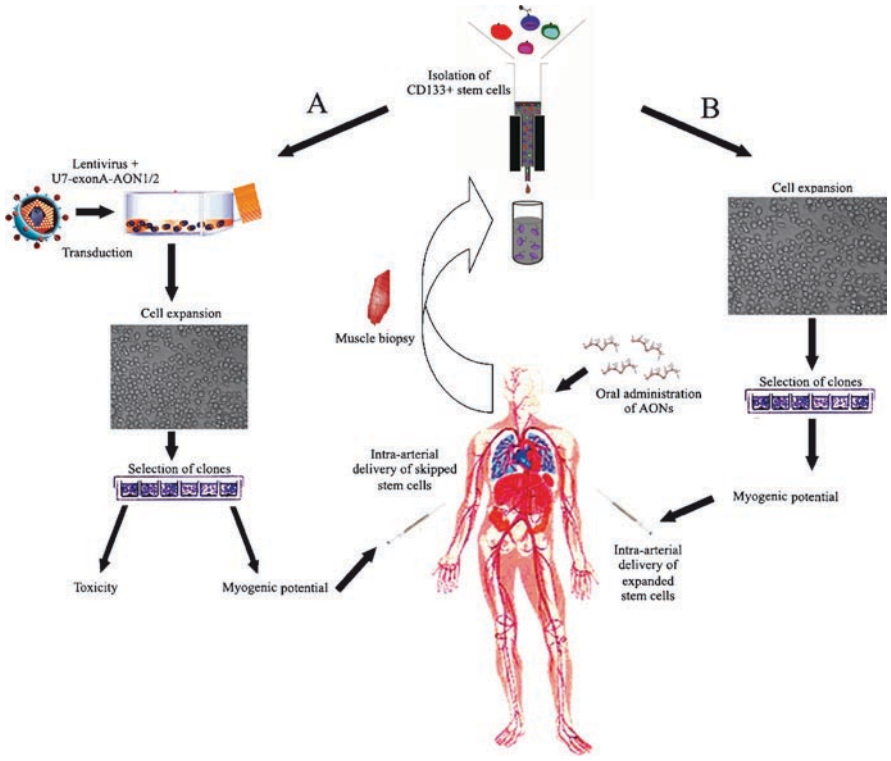

Fig. 15.3 A schematic representation of the combination of CD133+ stem cells and gene therapy to treat muscular dystrophy. Clinical steps may start with the purification of CD133+ stem cells isolated from dystrophic muscle biopsy followed by **(a)** an *ex vivo* cell engineering, based on the transfection of cells by lentiviral vectors promoting the exon skipping, and re-injection of the selected rescued clones in the same donor patient by intra-arterial delivery; **(b)** The dystrophic patients will be systemically treated with AONs after the autologous intra-arterial transplantation of "non corrected" myogenic stem cells. By this second approach we can avoid the use of lentivirus and obtain dystrophin rescue by AONs and muscle regeneration by autologous stem cells of all dystrophic muscles

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Chapter 16
Gene Therapy Clinical Trials for Muscular Dystrophies

Dominic J. Wells

Abstract The major challenge for therapy of Duchenne and related muscular dystrophies is the need to treat multiple muscles for clinical benefit. After many years of laboratory studies developing systems for gene transfer into muscle, the last 5 years have seen major steps forward with the generation of genetic treatments that can be administered systemically. Consequently, a number of different approaches have been taken through to clinical trials, including adeno-associated viral vector mediated gene delivery, plasmid mediated gene delivery, and the use of antisense oligonucleotides to modify splicing of the primary transcript to restore the reading frame. Cell transfer can also be used as a means of gene delivery. Associated with clinical trials has been the consideration of appropriate pre-clinical safety testing and outcome measures for assessing efficacy for regulatory approval. Although many of the trials look promising and offer hope for the future, it is important to understand that therapeutic approval can take considerable time and requires significant funding and input from biotechnology/pharmaceutical companies.

16.1 Introduction

Since the discovery of the gene responsible for DMD (Koenig et al. 1987), and the subsequent identification of the genes responsible for other forms of muscular dystrophy, there has been intense effort in the development of methods for restoring gene function, commonly referred to as gene therapy (Foster et al. 2006; Cossu and Sampaolesi 2007; Muntoni and Wells 2007). The availability of a range of spontaneous and engineered animal models has allowed extensive pre-clinical testing (e.g., Wells and Wells 2005; Banks and Chamberlain 2008; Spurney et al. 2009;

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Willmann et al. 2009) and the subsequent translation of many of these novel therapies into early clinical trials. A key step has been the pre-clinical demonstration of the potential for systemic administration and delivery of therapeutic agents to all of the affected muscles. Regional or systemic treatment will be essential as local treatment by intramuscular injection, while useful for first in man studies, results in very limited diffusion from the site of injection and therefore, clinical benefit is highly unlikely. Examples of pre-clinical data for approaches now in clinical trial include the use of replication-deficient viral vectors to transfer recombinant versions of the affected gene, the transfer of recombinant genes in simple bacterial plasmids, the transfer of normal or recombinant genes via cell transfer, and the use of antisense oligonucleotides to modify the splicing of the primary transcript to generate mRNA and by excluding specific exon(s) to restore the reading frame in frame shift mutations.

16.2 Regulatory Issues for Gene Therapy Trials in Muscular Dystrophy

In conventional drug development, treatments are initially tested in healthy volunteers to investigate safety (Phase 1a) before similar safety trials in patients (Phase 1b). Once safety of the new drug has been confirmed, trials proceed to efficacy studies, initially small scale and often open label (Phase 2a), before extending to larger scale placebo controlled double blind trials (Phase 2b). If these trials show promise, very large much larger trials are performed, often in comparison to the current best available treatment in order to fully assess the efficacy of the treatment and any previously undetected side effects (Phase 3). Approval to market the treatment is usually only given when this full range of studies has been completed.

In contrast, there are a number of limitations to the above scheme when considering the development of treatments such as gene therapy for the muscular dystrophies. First and foremost is the relatively small numbers of patients available for trials where safety concerns require tight exclusion criteria, especially in the case of treatments such as antisense mediated exon skipping that are sequence specific. Secondly, some forms of therapy, such as the antisense approach, have the potential to cause harm to healthy individuals. Skipping an exon in normal dystrophin may disrupt the reading frame potentially leading to a dystrophin deficiency and hence muscular dystrophy. Finally, the relatively small number of candidates for treatment will reduce the potential profit and therefore interest for biotech and pharmaceutical companies. Such problems are recognized by the regulatory authorities and special provision is made under “orphan drug” legislation. Because initial studies are often done with patients where it is possible to detect gene transfer or the gene product, such trials are often referred to as Phase 1/2. Likewise double-blind placebo controlled trials are often referred to as Phase 2/3 as larger scale trials will be impractical.

Another consideration is the approach to toxicology and safety pharmacology. Conventional drug studies include toxicity studies in at least two species (often one in rodents such as mouse or rat and the other in large animals such as dog or primate) to assess first acute toxicity and then, as trials progress, chronic toxicities. Safety pharmacology is commonly performed in telemetered larger species. While an understanding of safety pharmacology and toxicity is clearly very important, in many cases the conventional approach may be less than optimal when considering the gene therapies currently proposed in muscular dystrophies. For example there are species differences in the response of the innate immune system to specific DNA and RNA sequences (Bauer et al. 2001; Heil et al. 2004). In addition, with oligonucleotide chemistries such as the phosphorodiamidate morpholino oligomer (PMO), which do not demonstrate sequence-specific activation of the innate immune system, testing of different sequences in the nontarget species will not reliably detect off-target effects. Therefore, it is imperative to have specific discussions with the regulatory authorities in the very early stages of planning a clinical trial for muscular dystrophy.

16.3 The Step from Pre-clinical Experimental Studies to Clinical Trials

It is important to recognise that there are fundamental differences between pre-clinical experimental studies and early clinical trials. The former studies are often designed to produce the most effective treatment whereas initial studies in humans are primarily focused on doing no harm. This is particularly true of the initial proof-of-concept studies that are currently the focus of the majority of gene therapy clinical trials in muscular dystrophy. There are also problems associated with different immune responses and issues of scale between mouse and humans. Consequently, if expectations are not carefully managed, particularly on the part of the patient/parent community, early trial results may be viewed as very disappointing.

16.4 Methods of Monitoring Effects of Treatment

Monitoring of the efficiency of gene transfer and expression is a significant challenge for clinical trials. Muscle biopsy is the most common method used but is limited by the number of samples that can be taken. In addition, muscle biopsy is essentially a random process and although there can be some degree of certainty of representative sampling when the biopsy is taken at the site of local injection, it is much less certain when assessing the effects of systemic treatment. While it is clearly important to be able to treat all the affected muscles, it appears from the pre-clinical studies that different muscles can show different rates of responses either because of intrinsic properties such as fiber type, or because of differences in use and blood

flow. This is clearly the case with intravenous delivery of antisense oligonucleotides in both mouse and dog models of DMD (Alter et al. 2006; Yokota et al. 2009). Assessment of levels of dystrophin per muscle fiber is also likely to be an important measure as pre-clinical and clinical data suggest that approximately 20–30% of normal will be sufficient to provide functional benefit (Wells et al. 1995; Neri et al. 2007).

One very promising approach to monitoring the effects of treatment is the use of repeated magnetic resonance imaging (MRI). This has been used to assess the effects of steroid treatment in DMD patients (Mavrogeni et al. 2009), the results of AAV-γ-sarcoglycan gene transfer in a mouse model of LGMD2C (Walter et al. 2005), and exon-skipping in the dog model of DMD (Yokota et al. 2009). However, it is clear that much work remains to further validate and extend the use of MRI as a monitoring methodology.

16.5 Outcome Measures

A major issue when planning any clinical trial is the outcome measures. As a consequence of the pressure to provide statistically quantifiable results in pre-clinical studies in order to achieve high impact publications, the gene therapy community as a whole tends to look for similar quantitative measures in clinical trials. In addition, the relatively small number of patients means that many trials will need to be conducted in a multi-centric manner. Consequently, a lot of effort has gone into designing and validating a range of measures including quantitative muscle testing and biomarkers (Mayhew et al. 2007). However, it is questionable how relevant such measures are to the patients themselves and it will be important to develop additional methods that more accurately reflect the benefit that the patients obtain from the therapeutic intervention. Measures of general activity (e.g., McDonald et al. 2005), and quality of life will be important in seeking final approval by the regulatory authorities (Mendell et al. 2007; Mercuri et al. 2008). Timed activities such as the 30 foot walk test or climbing standard stairs appear highly reproducible and correlate well with predicted disease progression (Mayhew et al. 2007).

16.6 Past and Current Clinical Trials

16.6.1 Plasmid Gene Transfer

The first published gene therapy clinical trial for muscle disease was conducted by Transgene (Romero et al. 2004). In this trial naked plasmid DNA was used to introduce the full-length dystrophin cDNA into the muscle of 3 DMD and 6 BMD patients in a dose escalation protocol. Weak levels of expression were detected in

six of the treated individuals. No adverse events were noted. The lack of an immune response, while encouraging, may well have been due to the very low levels of expression of full-length dystrophin. It is also possible that the lack of an immune response was due to the expression of revertant or internally truncated dystrophin, as previously suggested from animal studies (Ferrer et al. 2000; Ferrer et al. 2004). The next development of plasmid based treatment for muscular dystrophies will likely involve regional intravascular delivery using high volumes and pressures to drive the plasmid into contact with the target muscle fibers. Pre-clinical studies show this to be a feasible, safe, and moderately effective approach (Hagstrom et al. 2004; Toumi et al. 2006), but as yet no clinical gene therapy trial using this approach has been initiated.

16.6.2 AAV Based Gene Transfer

Several investigators have used adeno-associated viral (AAV) vectors to carry sarcoglycans into the muscle of patients with DMD and limb-girdle muscular dystrophy (LGMD). In 1999, Mendell and colleagues (Columbus Children's Research Institute and Ohio State University) treated two patients with LGMD2D by injecting AAV2 carrying α-sarcoglycan cDNA into the extensor digitorum brevis (EDB) muscle. In 2006 Herson and coworkers (Pitié-Salpêtrière Hospital, Paris) started intramuscular injections of AAV1 carrying γ-sarcoglycan into the muscles of patients with LGMD2C. In the same year Mendell and colleagues started double blind treatment of patients with LGMD2D by injection AAV1 carrying α-sarcoglycan into the tibialis anterior muscle (Mendell et al. 2009). Finally, a double-blind trial of AAV transfer of a microdystrophin cDNA into the biceps of boys with DMD conducted at Columbus Children's Research Institute and Ohio State University in collaboration with Asklepios Biopharmaceutical should report soon.

Pre-clinical studies have identified the failure to achieve effective repeat administration because of the generation of neutralizing antibodies to viral capsid proteins as a major limitation for clinical application, but recent studies suggest that transient immunosuppression may be able to circumvent this problem (Wang et al. 2007).

Therefore, the AAV-microdystrophin trial noted above will be of particular interest in the assessment of immune responses against the viral capsid proteins and the microdystrophin protein. For clinical benefit a systemic or regional delivery will be required and this may be enhanced by the use of other AAV serotypes as shown in pre-clinical studies in non-human primates (Rodino-Klapac et al. 2007).

AAV based gene transfer may be particularly useful for patients with different forms of LGMD as the cDNAs of the mutated genes are relatively small and can be packaged into AAV vectors. To use AAV to transfer dystrophin it has been necessary to substantially modify the cDNA to produce microdystrophins. These are artificial constructs that have no direct BMD counterpart and it is unknown whether they will be as functional in DMD patients as they appear to be in the mdx mouse

model of DMD. Indeed, a form of microdystrophin appeared to be nonfunctional in the GRMD dog model of DMD when used to transduce autologous mesoangioblasts (Sampaolesi et al. 2006). Many microdystrophins do not localize nNOS to the sarcolemma where it is normally retained by interactions with syntrophin and the spectrin like repeats 16 and 17 of the rod domain of dystrophin (Lai et al. 2009). Reductions in nNOS activity have been linked to a failure to vasodilate efficiently when exercising both in the mdx mouse and DMD patients (Thomas et al. 1998; Sander et al. 2000) and to abnormal patterns of histone modifications in both species (Colussi et al. 2009). Restoration of nNOS to the sarcolemma corrects the exercise deficiency and normalizes the histone patterns in mdx mice; therefore, it will be important to re-engineer the existing microdystrophins to include repeats 16 and 17 in order to optimize the effects of AAV based gene therapy for DMD.

16.6.3 Cell Based Gene Transfer

Cell-based therapies for muscular dystrophy can also be considered as a form of gene therapy either because allogenic cells will carry a normal copy of the DMD gene or because autologous cells will have been genetically modified to carry a functional version of the gene before readministration to the patient. Promising results from congenic myoblast transfer from wild-type C57Bl/10 mice into mdx mice (Partridge et al. 1989) or from other sources with immunosuppression (e.g., Chen et al. 1992), commonly into irradiated or artificially regenerated muscle, demonstrated some of the potential benefit of myoblast transfer as a treatment for DMD. Unfortunately the clinical trials conducted in the early 1990s were disappointing (Grounds 1996). A number of explanations were proposed for these poor results including the rapid death of injected myoblasts and the limited migration from the site of injection (Fan et al. 1996).

Some groups have continued to pursue this approach to genetic treatment with a variety of modifications to enhance the contribution of the exogenous cells to muscle repair and the restoration of dystrophin expression. For example Skuk and colleagues used 25 injections of 3×10^7 allogenic muscle precursor cell (MPC) into 1 cm^3 of muscle in immunosuppressed DMD patients in order to detect significant dystrophin expression (Skuk et al. 2004). By raising this to high density (100) injections per cm^2 in nine DMD patients under immunosuppression they achieved 3.5–26% dystrophin positive muscle fibers local to the site of injection (Skuk et al. 2006). A single patient exhibited 27.5% positive fibers in the area of a treated gastrocnemius, 1 month post-transplantation and 34.5% positive fibers at 18 months (Skuk et al. 2007).

Cell transfer approaches to date have only involved local delivery and this is unlikely to provide any clinical benefit although it has been argued that preservation of finger or thumb muscles might maintain the ability to use a computer pointer. Myoblasts do not engraft efficiently when delivered systemically but some promising pre-clinical observations suggest that mesoangioblasts (pericytes) have potential for systemic delivery (Sampaolesi et al. 2003; Sampaolesi et al. 2006).

16.6.4 Antisense Mediated Exon-skipping

Antisense-induced exon skipping to restore the reading frame is not considered as a gene therapy in most countries as no gene is added as a result of the therapy. However, as the antisense modifies the mRNA product of the endogenous gene using specific gene sequences, it is commonly included in a list of potential gene therapies.

The first antisense oligonucleotide (AO) treatment in DMD was conducted by Matsuo and colleagues (Takeshima et al 2006) with the administration of 0.5 mg/kg phosphorothioate AO targeting exon 19 weekly for 4 weeks in a single patient. No adverse events were noted and the authors showed exon skipping in the white blood cells and some evidence of low level skipping in muscle. Another study treated 4 patients with intramuscular injection of 0.8 mg of a 2-O-Methyl phosphorothioate AO targeting exon 51 (PRO051) into the tibialis anterior (van Deutekom et al. 2007). Treatment was not associated with any apparent adverse effect and analysis of the treated muscle showed that 64% to 97% of muscle fibers were dystrophin positive in the injected area. In addition, RT-PCR analysis showed specific exon skipping and there was evidence of dystrophin on western blot. The same group, a collaboration between the University of Leiden and Prosensa B.V., has now conducted a dose escalation open label trial of five weekly subcutaneous treatments in DMD boys and the results are expected to be published soon. The treated individuals are likely to enter into an extended treatment arm to assess the longer term consequences of AO administration.

A related single blind placebo controlled trial has been conducted by the UK MDEX consortium in collaboration with AVI BioPharma Inc. This trial involved intramuscular injection of a PMO targeting exon 51 into the extensor digitorum brevis (EDB) of seven DMD patients. No adverse events related to the drug were reported and the results show specific skipping as assessed by RT-PCR and substantial restoration of dystrophin expression by immunocytochemistry and western blot (Kinali et al. 2009). Importantly, this study compared the PMO treated side to the contralateral saline treated muscle and was analyzed by members of the team blind to sample identity. The same partnership has now started an intravenous systemic delivery trial with treatment for 12 weeks on a weekly basis.

Although the results from the recent exon-skipping trials appear promising, there are a number of issues that remain to be resolved. The first question relates to the optimal administration regime that will give long-term dystrophin expression. Although dystrophin appears to have a long half-life, it is likely that at least some forms of internally deleted dystrophin will be less stable. In addition, some internally deleted dystrophins may be more functional than others. The available BMD patients do not allow accurate prediction of the functionality of such dystrophins as most of the databases contain only the DNA mutations and few patients have been fully characterized at the mRNA and protein level. As the antisense treatments only modify the RNA, life-long treatment will be necessary and there are no data regarding the chronic toxicity of the 2-O-Methyl phosphorothioate and PMO molecules. Finally, the most recent trials have only targeted exon 51, which will treat approximately

13% of DMD patients. To treat a greater proportion of the DMD population, additional antisense sequences will be required to target different exons. Fortunately, the majority of patients have mutations that cluster around two hotspots and this means that a number of mutations can be treated by skipping a single specific exon. From the Leiden DMD database it has been calculated that 3 antisense sequences will potentially treat 27% of the DMD population and 10 sequences would treat 41%. Up to 75% of the DMD population could be treated with the antisense technology targeting one or two exons at a time to produce a protein with some likely functional benefit (Aartsma-Rus et al. 2009).

16.7 Summary and Future Directions

The last 3 years have seen a rapid increase in the number of gene therapies entering clinical trial for the muscular dystrophies. The muscular dystrophy community faces a number of challenges in responding to the early proof-of-principle results. There is a clear need to agree on standardized outcome measures that will satisfy the regulatory bodies that the treatment makes a significant difference to the life of a patient with muscular dystrophy. The development and production of a clinically applicable treatment will require substantial further investment and the involvement and expertise of biotechnology and pharmaceutical companies. It will also be important to manage the expectations of the patient community as the process of trials and product registration will likely take longer than many currently anticipate. Despite these concerns, it now seems quite possible that gene therapy may indeed become a reality for at least some patients with muscular dystrophy.

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