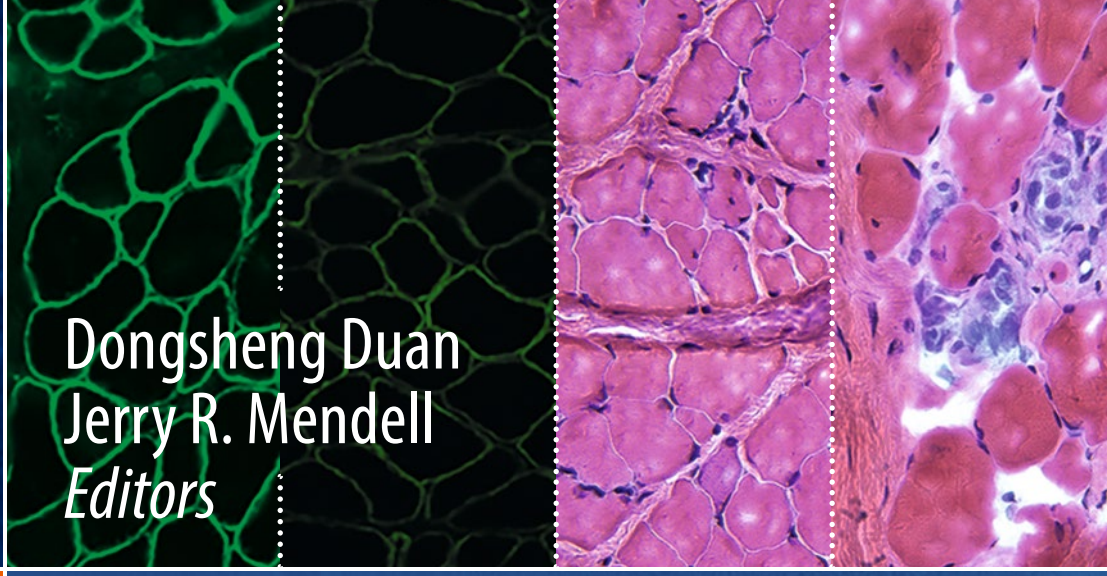

Dongsheng Duan
Jerry R. Mendell
Editors

Muscle Gene Therapy

Second Edition

Springer

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Dongsheng Duan • Jerry R. Mendell
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Editors
Dongsheng Duan
Department of Molecular Microbiology and Immunology
School of Medicine
University of Missouri
Columbia, MO, USA

Department of Neurology
School of Medicine
University of Missouri
Columbia, MO, USA

Department of Biomedical Sciences
College of Veterinary Medicine
University of Missouri
Columbia, MO, USA

Department of Bioengineering
University of Missouri
Columbia, MO, USA

Jerry R. Mendell
Department of Pediatrics
Nationwide Children's Hospital and Research Institute
The Ohio State University
Columbus, OH, USA

Department of Neurology
Nationwide Children's Hospital and Research Institute
The Ohio State University
Columbus, OH, USA

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Cover illustration: Top panels (from left to right): (1) AAV1 gene therapy restored α-sarcoglycan expression in a type 2D limb girdle muscular dystrophy patient; (2) Nominal α-sarcoglycan was detected in patient's muscle before AAV1 gene therapy; (3) AAV9 micro-dystrophin gene therapy improved muscle histology in the dog model of Duchenne muscular dystrophy; (4) Untreated dystrophic dog muscle showed degeneration, necrosis, inflammation and fibrosis. Bottom panel background: Transmission electron microscope image of purified recombinant adeno-associated virus (AAV) particles.

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To patients and their families and friends who have fought fiercely to defeat muscle diseases
To investigators who work diligently to develop gene therapy for neuromuscular diseases

Preface

> "It is like watching a car crash in slow motion. Your child is inside the car. You are outside the car and there is nothing you can do about it"— the frustration on the lack of a curative therapy from a mother whose child is suffering from muscular dystrophy. *Jen Portnoy, Hope for Javier, April 10, 2017*

It is estimated that approximately seven million people are affected by neuromuscular diseases worldwide. Majority affected are children. Almost all neuromuscular diseases are caused by genetic mutations. According to the gene table of neuromuscular disorders (www.musclegenetable.fr/), among ~900 neuromuscular diseases, nearly 500 disease genes have been identified. Contemporary gene therapy technology brings in a hope of treating these diseases at their genetic roots by correcting the mutated gene or introducing a normal one to replace the defective gene.

The first disease gene for a neuromuscular disease was discovered in 1987 by Louis Kunkel and colleagues. This gene was called the DMD gene because its mutations cause Duchenne muscular dystrophy (DMD), the most common childhood lethal muscle disease. The DMD gene encodes dystrophin, an essential muscle survival protein. In the absence of dystrophin, muscle undergoes degeneration and necrosis. The discovery of the DMD gene immediately generated euphoria and excitement among patients, their families and friends, researchers, and the general public. Optimism for a DMD cure by gene therapy appeared to be a realistic expectation. However, early attempts to transfer the DMD gene did not bring an immediate cure. To review the lessons learned from these early studies, the first edition of *Muscle Gene Therapy* was published in 2010. This was the first book entirely dedicated to muscle gene therapy. At the time of the publication of the first edition, the proof of principle for neuromuscular disease gene therapy had been demonstrated in rodent models, and a few clinical trials had just been initiated to test the safety and feasibility of directly administering a candidate muscle gene therapy vector to human patients. Yet, there was no gene therapy drug approved by a regulatory agency for any inherited disease, not to mention neuromuscular diseases. This situation is changed now. Gene therapy drugs have been marketed, including one gene expression modification therapy (exon skipping) for DMD and gene replacement therapies to treat a rare inherited lipid disease (lipoprotein lipase deficiency), a form

of blindness affecting children and adults (Leber congenital amaurosis). Cell-based gene therapies have also been approved to treat acute lymphoblastic leukemia and non-Hodgkin lymphoma. The field of gene therapy has entered a new phase and begun to produce measurable clinical benefits for some patients, including patients suffering from certain neuromuscular disorders. New approaches have been developed to expand the scope of neuromuscular disease gene therapy from the original gene replacement to gene knockdown, gene expression modulation, gene therapy with noncoding sequences (such as microRNA), gene therapy with disease-modifying genes, and, more recently, with the CRISPR technology-based gene editing. Creative new gene therapy strategies and encouraging animal study results are emerging targeting neuromuscular diseases. Preclinical rodent studies are now being scaled up in large animal models. New vector production and purification technologies are developed to meet the ever-increasing needs for both preclinical and clinical studies. Several promising bodywide therapies are on the horizon and in clinical trials for treating spinal muscular atrophy, X-linked myotubular myopathy, and DMD. In view of these advances in translational science, this new edition of *Muscle Gene Therapy* provides a comprehensive review of recent developments and ongoing progress.

In the second edition of *Muscle Gene Therapy*, we have structured the book into three major sections. Part I provides a review of the foundation for muscle gene therapy; Part II describes the importance of preclinical studies in the development of muscle gene therapy for clinical translation; Part III demonstrates the essence of translation by illustrating examples of progress from preclinical to clinical muscle gene therapy. In Part I of the book, we start with an overview of muscle biology and physiology, then a chapter on the molecular basis of neuromuscular diseases and a chapter on animal models. In subsequent four chapters, stem cells, microRNA, and immunology in muscle disease and gene therapy are discussed. The success of gene therapy hinges on our understanding of the gene delivery vector. Hence, five chapters are devoted to this topic. These include one chapter on the design of the muscle gene therapy expression cassette, one chapter on nonviral vectors, one chapter on viral vectors, and two chapters on vectors based on adeno-associated virus (AAV). AAV vectors are currently the most promising gene delivery platform for muscle gene therapy. Strategies that can improve the existing AAV vector system and AAV manufacture methods are essential to bring muscle gene therapy to every patient. Hence, one of the AAV chapters is on the development of the next-generation AAV vectors and the other on large-scale clinical grade AAV production. Outcome measures for testing efficacy of muscle gene therapy are addressed in three chapters, including one devoted to histological and biochemical evaluation of muscle gene therapy, another on biomarkers, and a chapter devoted to the newly developed imaging technology called optical polarization tractography. Part I of the book is wrapped up with a chapter dedicated to the use of genome editing to treat neuromuscular diseases.

Most chapters in the first edition of *Muscle Gene Therapy* focus on preclinical development of muscle gene therapy for various neuromuscular diseases. In the second edition, all preclinical animal studies are grouped in Part II. The design and

implementation of a preclinical muscle gene therapy study are a very important but rarely discussed topics in the literature. As a unique feature of the new edition, we introduce Part II of the book with a chapter on preclinical study considerations. The DMD gene was the first neuromuscular disease gene discovered. Consistently, DMD is also the most studied disease in muscle gene therapy. Seven chapters are devoted to different aspects of DMD gene therapy including gene replacement, exon skipping, genome editing, and gene therapy approaches to treat brain dysfunction in DMD. Two chapters are given to innovative approaches, one for alternative translation initiation and one for sarcolipin knockdown. Remaining chapters in Part II of the book review the latest gene therapy developments for treating other neuromuscular diseases such as dysferlinopathy, dystroglycanopathies, facioscapulohumeral muscular dystrophy, myotonic dystrophy, myotubular myopathy, mitochondrial myopathy, Charcot-Marie-Tooth inherited neuropathy, and other dominantly inherited muscular dystrophies and myopathies. Since sarcolemma weakness/damage is a common feature in many types of muscular dystrophies, we include one chapter to specifically discuss therapies based on muscle cell membrane repair. The last chapter of Part II discusses muscle as a target for genetic vaccination.

The ultimate goal of muscle gene therapy research is to benefit patients. In the first edition of the book, only a single chapter was devoted to clinical translation consistent with limited numbers of clinical trials largely focused on proof-of-principle studies. Recently, the field has made a quantum leap forward with highly promising clinical data from bodywide systemic AAV therapy in patients with type I spinal muscular atrophy. For the first time in history, a gene therapy has significantly changed the disease course, reduced symptoms, improved quality of life, and increased survival in a neuromuscular disease. Conditional approval of an exon-skipping therapy drug for DMD by the FDA, though still being hotly debated, marks another important milestone as the first molecular-based genetic modifying therapy approved by a regulatory agency. There is no doubt that many more candidate muscle gene therapy drugs will progress from bench to bedside in the upcoming years. In the view of the editors of the second edition of the book, there is a need to bring researchers, trainees, funding agencies, and the patient community up to date on the clinical progress of neuromuscular disease gene therapy. There is also a need to review and reflect on experiences and lessons learned from completed and ongoing trials. With this backdrop, we devote nine chapters in Part III of the book to clinical muscle gene therapy. We start this section of the book with a chapter on patient and family perspective. This is followed with two chapters on clinical trial design. Of particular interest is the discussion on the practical and regulatory issues pivotal to the development of a muscle gene therapy product from the initial hypothesis to early preclinical studies, investigative new drug application, clinical trials, and regulatory approval. One chapter provides a comprehensive discussion on magnetic resonance imaging (MRI). The noninvasive and quantitative nature of this imaging technology makes it especially appealing for monitoring neuromuscular disease gene therapy. The next three chapters are devoted to clinical gene therapy trials for DMD and limb-girdle muscular dystrophy, with a special focus on gene replacement therapy and exon skipping. These chapters touch on important issues encountered in

human studies such as the immune response and expression levels of the therapeutic protein. This is followed by a chapter on clinical gene therapy trials for the metabolic glycogen storage disease type II, commonly referred to as Pompe disease. The final chapter of the book explores muscle-directed gene therapy for treating alpha-1 antitrypsin deficiency.

The first edition of the book has a total of 16 chapters. In the second edition, we have a total of 45 chapters. The book is not only expanded greatly in its length but also on its quality and content. We are very grateful to chapter authors for their outstanding contributions. We would like to thank Springer for giving us the opportunity to compile this new edition. We would also like to thank Michael Nance for his assistance in the preparation of this book. Special thanks are extended to dedicated basic scientists and clinical researchers, the patient community, and funding agencies for taking neuromuscular disease gene therapy from a paper concept to a reality for patients.

We would also like to acknowledge the support from the National Institutes of Health; Department of Defense; Parent Project Muscular Dystrophy; Jesse's Journey, The Foundation for Gene and Cell Therapy; the Jackson Freel DMD Research Fund; the Muscular Dystrophy Association; Hope for Javier; Coalition to Cure Calpain 3; Solid Biosciences; and Sarepta Therapeutics and Avexis, Inc., for funding our neuromuscular gene therapy studies.

Columbia, MO, USA Dongsheng Duan
Columbus, OH, USA Jerry R. Mendell

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Contents

Part I
Foundations for Muscle Gene Therapy

Chapter 1
An Overview of Muscle Biology and Physiology for Muscle Gene Therapy

Paul M. L. Janssen and Jonathan P. Davis

Abstract The body's musculature is both quantitatively and qualitatively of critical importance to the body. In an average human, the muscle takes up a third to half of all the body mass. Qualitatively, it is critical to all aspects of life; even the brain has virtually no other means of expressing its thoughts other than by contraction of muscle fibers. Two main distinct muscle tissues are present in the body, smooth and striated muscle tissue. Striated muscle tissue is subdivided into two major parts: skeletal muscle tissue and cardiac muscle tissue. In the muscular dystrophies, both skeletal and cardiac muscle tissues are part of the pathological manifestation of disease. In this chapter, we will discuss the basic mechanism of contraction at the molecular level, as well as the regulatory mechanisms that make the muscle function in vivo. We will focus on skeletal muscle and cardiac muscle, briefly describing the extent to which muscular dystrophy impacts muscle contraction in these two different muscle tissues.

Keywords Contraction · Relaxation · Twitch · Tetanus · Sarcomere

1.1 Skeletal Muscle

1.1.1 Skeletal Muscle Structure Overview

Derived in large part from the myotomes of the embryo, the skeletal (or voluntary striated) muscle forms the flesh of the body. The individual muscle fibers are extremely large cells, typically cylindrical, with lengths that can range from about 1 mm to many tens of centimeters. Multiple muscle fibers are aligned in parallel to form individual muscles. These muscle fibers are connected with connective tissues and are typically highly vascularized. With the focus on the muscular dystrophies,

P. M. L. Janssen (✉) · J. P. Davis
Department of Physiology and Cell Biology, The Ohio State University, Columbus, OH, USA
e-mail: janssen.10@osu.edu; davis.812@osu.edu

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we will limit most of this chapter to the muscles most impacted and most researched in this disease—striated muscle.

The primary property of the muscle is to produce force. This force is used for locomotion but also for non-locomotion, i.e., force is produced to maintain posture by opposing external forces on the body, such as gravity. The force production of the muscle fibers originates at the molecular level by protein–protein interactions. Microscopically, each muscle fiber is composed of a large number of sarcomeres, both in series (i.e., along the length of the fiber) and in parallel. Within these sarcomeres reside the myofilament proteins whose interaction generates force. The repetitive nature of the arrangement of proteins within the sarcomere, and in-series arrangement of sarcomeres, causes the striated pattern that is observed when viewing skeletal muscle under a light microscope. Alternating bands of darker and lighter striations are caused by the arrangement and overlap of the thick and thin filaments that compose the sarcomere. These thick and thin filaments interact and slide past each other when a muscle shortens against a load, lending the name to the "sliding filament theory" [1] that currently remains the widely accepted working theory of muscle contraction.

1.1.2 Sarcomere Organization

The functional unit of muscle contraction is the sarcomere (Fig. 1.1) or, technically even more accurate, the half-sarcomere. The sarcomere in a skeletal muscle is approximately 2 μm long and stretches from Z-line to Z-line. Since the myofibril is a multidimensional cell, it is often also referred to as the Z-disk. The Z-disk is a protein-dense structure containing many structural and regulatory proteins and overlaps with the region where excitation is initiated in each sarcomere.

The thick filaments originate at the center of the sarcomere and span a length of approximately 1.6 μm. The thick filament is mainly composed of the multimeric protein myosin. The myosin molecule consists of two large heavy chains and four light chains. The myosin molecule is organized into three distinct regions, the tail, the neck, and the head. The tail of myosin (part of the heavy chains) forms the backbone of the thick filament. The globular heads of the myosin molecule (two heads per myosin) are also part of the heavy chains and protrude out of this backbone. Each myosin head contains the binding sites for ATP, the fuel for contraction as well as for actin (described below), and the partner protein needed to generate force. Each myosin head is connected to the tail by a neck that acts as a lever arm for force generation. Each neck (or lever arm) is stabilized by the binding of two small light chains. Some light chains possess regulatory functions that can modulate the extent and speed of contraction. It is the head of the myosin molecule that undergoes a conformational change during force development, when it binds to actin on the thin filament. The myosin molecule is ultimately connected to the Z-disc via the giant protein titin, originally named connectin [2], the largest protein in the body. Titin has several distinct regions and provides much of the passive forces and elasticity of

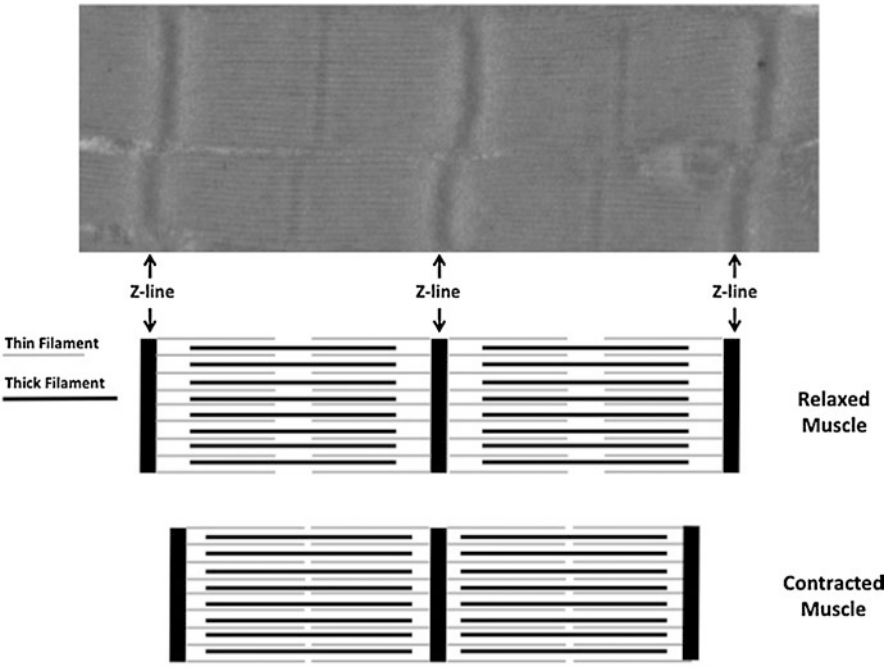

Fig. 1.1 Top: representative electron microscopic photograph of sarcomeres. Middle: arrangement of the thick and thin filaments in a sarcomere of a muscle in the relaxed state. Bottom: arrangement of the myofilaments in a contracting muscle. Photograph courtesy of Dr. Maegen Ackermann

the muscle cell. Titin runs from the Z-disk to the myosin backbone and connects all along the thick filament ending at the center of the thick filament (called the M-line). A third protein located on the thick filament is myosin-binding protein-C (MyBP-C). This protein is located in several distinct bands along the thick filament, and its N-terminal region can interact with both the thin and thick filament.

The thin filament, also called the regulatory filament, is mainly composed of actin proteins. Single actins (G-actin or globular actin) form a double-stranded helical string, resulting in filamentous actin (F-actin). The thin filament is about 1 μm in length. Each actin protein contains a myosin binding site allowing for the head of the myosin molecule to attach during the contraction process. Additional regulatory proteins on the thin filament control the availability of the myosin binding sites on actin. These include tropomyosin (Tm), another double-stranded protein complex that runs in or near the groove of the double-stranded actins, and the troponin complex, which acts as a molecular switch that controls tropomyosin's position on the double-stranded actin. This troponin (Tn) complex has three subunits, the calcium-binding subunit (TnC), the tropomyosin-binding subunit (TnT), and the inhibitory subunit (TnI).

1.1.3 Sarcomere Function

When the intracellular calcium level increases upon stimulation of a muscle fiber, TnC binds calcium and will set in motion a series of conformational changes of the regulatory proteins [3]. This results in tropomyosin being translocated into the groove of the thin filament, uncovering the myosin binding sites on actin. Myosin is now able to bind to actin and form what is called a cross-bridge. This cross-bridge can undergo a power stroke that pulls the thin filament toward the center of the sarcomere. This power stroke costs energy, which is supplied in the form of ATP. Myosin, when not bound to actin, is typically energized (has obtained mechanical strain from the hydrolysis of ATP into ADP and inorganic phosphate). Thus, upon binding to actin, it already possesses the (chemical) energy to be transformed into mechanical energy (work). Upon completion of the power stroke, ATP needs to bind to myosin, allowing for re-energizing and release from the thin filament. Lack of energy to do so will result in permanently attached cross-bridges, called rigor cross-bridges. Upon release from actin, it can now reattach and undergo the next cross-bridge cycle. Once the intracellular calcium levels decline, i.e., when the fiber is no longer stimulated and calcium levels in the cell decline, calcium comes off of TnC, resulting in reversal of the conformational changes in the regulatory proteins that ultimately translocate tropomyosin once again over the myosin binding sites of actin. New cross-bridges are no longer formed, and the muscle ceases to contract.

1.1.4 Twitch Contraction

The increase in intracellular calcium that sets in motion the cross-bridge cycle takes place when a muscle is electrically stimulated. A single stimulation of a fiber results in a twitch contraction. From a motor neuron, an electrical signal arrives at an anatomically specialized potion of the muscle fiber termed the neuromuscular junction (NMJ). At this NMJ, the motor neuron releases acetylcholine, opening muscle membrane-bound ion channels that result in an action potential that propagates at great speed across the length of the muscle fiber. When this action potential arrives at the t-tubules, which are membrane invaginations at the sarcomere level near the Z-line, it causes an interaction between the dihydropyridine receptor on the muscle membrane and the ryanodine receptor on the sarcoplasmic reticulum (SR). This voltage-dependent action triggers the opening of the ryanodine receptor, which releases calcium from the SR into the sarcoplasm. This calcium then diffuses toward the middle of the sarcomere where it binds to TnC and sets in motion the contractile apparatus (described above). This burst of calcium is short-lived, since SR-bound calcium pumps (SR calcium ATPase) constantly reuptake calcium back into the SR. As a result, during a twitch contraction (singe excitation), the rise in calcium concentration in the sarcoplasmic reticulum does not generate enough calcium to

fully activate the fiber. Typically, about 30–40% of maximal force is reached during a single twitch contraction.

1.1.5 Tetanic Contraction

A twitch contraction only lasts about 100–200 ms. However, most of the body's movements last much longer than a single twitch contraction. Moreover, many body movements and positioning actions require a steady level of force development. A steady level of force development in a fiber can be reached by a process called summation. Summation occurs when a muscle fiber is excited prior to it fully relaxing form the previous stimulus. When a muscle is not completely relaxed, and a second neural impulse causes a muscle action potential, a second burst of calcium is released from the SR. This calcium release is now in addition to the calcium still in the sarcoplasm from the previous twitch and thus reaches a higher peak level. When neuronal pulses follow in such rapid succession that the muscle has no time to relax, i.e., the next pulse arrives prior to the muscle reaching peak force, a tetanus occurs. In this condition, the frequency of stimulation is so fast that the calcium concentration in the cytoplasm reaches a high pseudo-steady-state level (i.e., the release by each action potential equals the reuptake into the SR), and the cross-bridge binding sites are maximally exposed. This tetanic contraction mode is a common activation of a muscle fiber, i.e., a muscle fiber received a high-frequency train of neural pulses that last as long as the muscle needs to be activated.

1.1.6 Motor Units

Each muscle or muscle group consists of many motor units. A motor unit is composed of an innervating neuron plus all the fibers it innervates. Per muscle, many motor units exist, and these motor units can be of different sizes. Some motor units only contain a few fibers, where other motor units contain many 100 s of muscle fibers. When a certain force development of a muscle is required, a number of motor units are activated in order to produce the desired force. Maximal force of the whole muscle is generated when all motor units within a muscle are stimulated to contract. The number of motor units that need activation mainly stems from lifelong learned behavior. The senses give input to the brain, and the brain initially determines how many, and which, motor units to switch on. When the senses are "tricked," for instance, when an object is significantly heavier or lighter than it looks, initially too few or too many motor units are activated. Feedback loops between brain, body-positioning, and load perceptions help fine-tune movements. The ability to modulate force production through activating a different number of motor neurons is called recruitment. Recruitment typically occurs in a specific order from the weakest motor neurons to the strongest motor neurons.

1.1.7 Fiber Sub-Types

Not all skeletal muscle fibers are the same. There are two main classifications of muscle fibers. The first classification is based on whether or not the muscle is "fast" or "slow." Fast fibers, also called type II fibers, express a fast myosin isoform that has a fast cross-bridge cycle, roughly four times faster than the slow myosin isoform. These fast fibers have a faster shortening velocity, although the force per cross-bridge cycle is not significantly different from the slow isoform. Slow fibers (type I fibers) express a slow myosin isoform, resulting in slower cross-bridge cycling, and a slower shortening velocity.

The second classification is based on how these fibers generate ATP to fuel the force production. Fast glycolytic fibers (type IIb) possess a high concentration of enzymes involved in glycolysis and have a large store of glycogen. These fibers use little oxygen and are typically surrounded by only a few blood vessels. They are also known as "white fibers," because they contain a low concentration of myoglobin. Anatomically, these glycolytic fibers typically have large diameters. These fibers are also typically the strongest of muscle fibers. Fast oxidative-glycolytic fibers (type IIa) have an intermediate glycolytic activity but also possess a high oxidative capacity. These fibers contain more mitochondria and more myoglobin. Also, to supply the oxygen needed, they are more vascularized. These muscle fibers are often referred to as red muscle fibers. The third type of fiber is the slow-oxidative fibers (type I). These fibers rely almost exclusively on oxygen-mediated burning of fuel and are highly vascularized.

Due to the different myosin isoforms and ATP-generating strategies, there are important functional implications of the fiber type. The fast fibers are typically organized in large motor units and are used for events that require short bursts of a lot of force, like weight lifting or sprinting. The generation of ATP in the muscle is much slower than the maximal usage rate, and this large power comes at the cost of endurance resulting in fast fibers exhausting rapidly (often within 10 s when used at full capacity). On the other hand, the rate of ATP generation can be kept up by oxidative phosphorylation in slow fibers, and thus they can function for many hours. Examples are body posture maintenance or slow running or walking.

1.1.8 Modes of Contraction

When a muscle is activated, i.e., "contracts," it does not necessarily mean that the muscle shortens. Shortening of the muscle only occurs if the opposing force, or load, on the muscle is lower than the generated force. The speed at which the muscle can shorten depends on the balance between activation of the muscle and the opposing load. With a high muscle activation (i.e., switching on all motor units), and an absence of load, maximal shortening velocity is reached. When the load on a muscle is equal to the opposing force, the muscle contracts, i.e., cross-bridges are activated

and undergo their power strokes, but the muscle stays at the same length. This is an isometric contraction. These isometric contractions are the most common form of contractions, as they are used to keep our body in a certain position. During standing, sitting, and even laying down, many of the muscles in our body are contracting isometrically to maintain the body's position. Sometimes, the load on a muscle can exceed the force generated by the muscle, and the muscle will lengthen, while it is still actively contracting. The latter form is particularly damaging, as the muscle tries to shorten (i.e., pull the actin toward the center of the sarcomere), while the opposing load is pulling actin away from the sarcomere's center. Eccentric contractions typically result in some degree of muscle damage and occur while one is walking downhill or attempting to sit.

1.1.9 Length Tension Relationship

The sarcomere length during a contraction has a small modifying impact on force development [4]. Typically, skeletal muscle works at the optimal length, i.e., a length of the sarcomere that promotes the highest level of force development when stimulated (i.e., optimal thin and thick filament overlap). The anatomical fixed location of skeletal muscle attached to bones keeps the sarcomere length in the optimal or very close to optimal range. In the laboratory, smaller than in vivo muscle length can be reached, resulting in depressed force development. Likewise, an overstretched muscle also produces less force (i.e., nonoptimal thin and thick filament overlap).

1.1.10 Lever Action

Almost all skeletal muscles attach around a joint. This mean that one end of the muscle is attached via a tendon onto a bone, while the other tendon wraps around a joint, and attaches to a different bone. When a muscle shortens, it only shortens by a small amount, typically 10% or less. However, a 10% shortening, often less than an inch, can through lever arm actions result in moving the end of a limb by several feet. For each muscle that acts on a specific joint, there is typically at least one other muscle located at the opposite side of the joint. These are referred to as antagonist muscle pairs. For instance, contraction of the biceps muscle closes the elbow joint, while contraction of the triceps muscle opens this joint.

1.1.11 Muscular Dystrophy and Skeletal Muscle Contraction

Muscular dystrophies ultimately result in weaker contractions of the muscle. The main reason is, as the name suggests, dystrophy. This dystrophy is typically characterized by a replacement of muscle tissue by fibrotic tissue and fat. Deterioration

of muscle function is seldom the result of a primary myofilament impact. The most common dystrophies develop due to a compromised muscle fiber membrane and due to either an increase in membrane fragility or a decrease in membrane repair. When this membrane insufficiency is large, it can lead to a chronic calcium overload of the muscle, resulting in muscle fiber death. Initially, these muscle fibers are replaced, but the regenerative capacity of skeletal muscle is limited, and, once depleted, the muscle can no longer be repaired and will deteriorate. From a contraction standpoint, the remaining myofilaments are typically capable of producing normal levels of force; it is the lack of quantity, not quality, that is the most common cause of overall muscle weakness in muscular dystrophy. In the most common mouse model of muscular dystrophy, the mdx mouse, the force-per-cross-sectional-area is lower, and the muscle is more fibrotic and has more fat accumulation. In order to compensate, the total muscle is typically larger, and as a result the amount of total force is not depressed in the mdx mouse. When calculated by cross-sectional area of the myofilaments, force is again not different from wild-type muscles. Thus, it stands to reason that gene therapy, other than prevention of the disease occurring in the first place, is directed at maintaining, or returning muscle mass, not necessarily altering muscle function. Currently, many efforts are underway to combat muscular dystrophy. A more stable membrane, for instance, by reintroducing lost components of the membrane dystrophin-dystroglycan complex, would help membrane integrity and reduce or prevent damage. Also, better membrane repair machinery would reduce the deleterious impact of weak membranes.

However, not only the force of contraction is important, but the speed at which contraction and relaxation occur also can have functional consequences. Much less is known regarding these dynamic features of the muscle contraction, as the vast majority of end points in laboratory experiments are levels of force and not speed of contraction and relaxation. Much less is known regarding the contraction and relaxation kinetics, as they are generally thought to play an insignificant role. If a striated muscle needs to stop contracting, typically the antagonist muscle is activated to counter the agonists' impact of contraction. Hence, if the active contraction (i.e., stimulation) of the muscle has stopped, the antagonistic muscle will be much stronger than the residual force of the agonist muscle, and the intended movement will occur. However, if the relaxation of a muscle is substantially impaired, it could have significant residual tension that is now (a) potentially impairing the force of the antagonistic muscle and, possibly clinically more important, (b) causing this muscle to undergo an eccentric stress. Thus, if relaxation kinetics were impaired, it may lead to excessive eccentric stress, possibly contributing to the pathology. Thus, although force of contraction may not necessarily be an applicable target, kinetics of relaxation could potentially be improved with therapy of the myofilaments or calcium sequestration.

1.2 Cardiac Muscle

1.2.1 Cardiac Muscle Structure Overview

The heart muscle is a specialized striated muscle that has a large number of similarities with skeletal muscle. However, its specialized function requires also some very significant differences in regulation of contraction. Unlike skeletal muscle, the individual muscle cells of the heart or cardiomyocytes are all connected. The individual myocytes are about 150 μm long and about 20–25 μm in diameter. Functionally, the cardiomyocytes need to contract simultaneously, for optimal pumping performance. Thus, the connections between myocytes, in the form of gap junctions, allow for the passage of the action potential that initiates a heartbeat.

1.2.2 Cardiac Muscle Function

The excitation of the muscle differs in several important ways from skeletal muscle. First, the action potential has a very long plateau phase (150–300 ms), during which the heart is unresponsive to any subsequent action potential. This delay in repolarization causes a refractory period, which is essential in allowing the heart muscle to relax prior to the next stimulation (i.e., the heart cannot tetanize). Second, the intracellular calcium increase that activates the myofilament is regulated differently. The SR calcium release is not triggered by a voltage-mediated release but by a calcium-induced release [5]. The L-type calcium channel, upon stimulation by action potential, opens and allows calcium entry into the myocyte. This calcium triggers an additional release of calcium from the SR. Combined, these two sources of calcium form the activating calcium transient. In humans, at rest, about 30% of the calcium transient comes from the L-type calcium current, and the remaining 70% is released from the SR. Conversely, to promote relaxation, 70% of the calcium release is taken back up into the SR, while the remaining 30% is extruded via the Na/Ca exchanger. The contractile machinery is almost identical to skeletal muscle, with only minor isoform changes in some of the myofilament proteins. The cross-bridge cycle occurs virtually identically too.

A notable difference is however that, unlike skeletal muscle that operates at optimal sarcomere length, the cardiac sarcomere operates on the ascending limb of the force-tension relationship. When the sarcomere is stretched, i.e., at the end of the ventricular filling phase, it is around 2.2 μm and close to optimal (i.e., highest force). When the ventricle ejects, sarcomere length shortens to well below optimal, and maximal force production is lower. This is an intrinsic mechanism, also known as the Frank-Starling law of the heart [6, 7], where the larger the volume (or sarcomere length) in the heart, the higher the developed pressure (or force).

Unlike skeletal muscle, within the ventricle there are no different classes or types of muscle; all myocytes practically behave the same regarding isoform expression

and ATP generation. Because the heart needs to beat continuously, it has to generate ATP at a rate that at least keeps up with ATP usage. Hence, the heart is extremely rich in mitochondria; up to 25–30% of the volume of a myocyte is occupied by mitochondria, as well as heavily vascularized. Each myocyte borders a capillary that supplies oxygenated blood and carries away waste products. The heart almost exclusively uses oxidative phosphorylation to generate ATP, with fatty acids as the primary fuel.

1.2.3 Muscular Dystrophy and Cardiac Muscle Contraction

Damage to the cardiac muscle occurs in most types of muscular dystrophy, albeit with typically a later onset compared to skeletal muscle pathology. Although skeletal limb muscle weakness is typically the most prominent phenotypical pathology, death in muscular dystrophy patients is mainly due to respiratory failure and heart failure. The heart does not possess significant regeneration capacity. Once a cardiac myocyte dies, it is not replaced. This means that the remaining cells of the heart have to work harder to pump blood. The heart becomes progressively weaker, to a point where it can no longer pump the minimal required amount of blood. Like skeletal muscle, membrane weakness and impaired membrane repair are at the basis of the eventual dysfunction. Small amounts of eccentric stress occur, even in a regular heartbeat, and this cumulatively leads to cell death, remodeling, and ultimately cardiac pump failure.

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Chapter 2
Molecular Basis of Muscle Disease

Ning Liu and Rhonda Bassel-Duby

Abstract Skeletal muscle is responsible for body movement, ranging from maintaining posture to dancing to running a marathon race. The heterogeneity in size, shape, and arrangement of fibers coupled with a variety of metabolic, contractile, and endurance properties gives skeletal muscle the ability to perform a wide range of functions. Over the years, our understanding of the molecular basis of muscle formation, growth, adaptability, and disease has dramatically expanded. Much of our understanding stems from studies of the pathology of skeletal muscle. To date, 840 neuromuscular disorders have been identified and attributable to mutations in 465 different genes. More genes are expected to be discovered with the advances in molecular diagnostics and next-generation sequencing. Here we focus on congenital myopathy and muscular dystrophy to highlight our understanding of the molecular basis of skeletal muscle disease. Elucidating the molecular basis of skeletal muscle disease offers the ability to use gene therapy approaches to correct genetic mutations and ameliorate skeletal muscle disease.

Keywords Satellite cells · Nemaline myopathy · Congenital myopathies · Kelch proteins · Muscular dystrophy · Dystrophin · Dystrophin-glycoprotein complex

2.1 Introduction

Muscle diseases, myopathies, are debilitating illnesses that impair the function of skeletal muscle. To date, there are 840 recorded neuromuscular disorders caused by mutations in 465 different genes, with the expectation that more muscle disease-related genes will be discovered [1–3]. An online gene table (http://www.musclegenetable.fr) has been

N. Liu (✉) · R. Bassel-Duby
Department of Molecular Biology, Senator Paul D. Wellstone Muscular Dystrophy Cooperative Research Center, Hamon Center for Regenerative Science and Medicine, University of Texas Southwestern Medical Center, Dallas, TX, USA
e-mail: Ning.Liu@utsouthwestern.edu; Rhonda.Bassel-Duby@utsouthwestern.edu

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developed to maintain and update the muscle disease information in an interactive format. Here, our intention is not to give a comprehensive list of these neuromuscular diseases but to spotlight various monogenic muscle diseases, such as congenital myopathy, muscular dystrophy, and inflammatory myopathy, and to define the molecular basis of these muscle diseases (Table 2.1). By identifying the underlining genetic mutation causing the disease, gene therapy approaches can be designed to correct the mutation and restore muscle function.

2.1.1 Skeletal Muscle Fiber Types and Adaptation

Skeletal muscle accounts for ~40% of human body mass and plays vital roles in locomotion, physical strength, energy expenditure, and overall metabolism. Each muscle group is comprised of heterogeneous myofibers that differ in their biochemical, physiological, and metabolic parameters. The heterogeneity of muscle fibers is the basis of the flexibility which allows striated skeletal muscle to be used for a variety of tasks, ranging from continuous low-intensity activity (e.g., posture), to repeated submaximal contractions (e.g., locomotion), to fast and strong maximal contractions (jumping, kicking) [4].

Mammalian skeletal muscle comprises different fiber types that differ in contractile properties, metabolic parameters, and expression of distinctive myosin isoforms [4]. Based on distinct myosin heavy-chain isoform expression, myofibers are classified into four major fiber types: one type of slow-twitch fiber (type I) and three types of fast-twitch fibers (types IIa, IIx/d, and IIb). While type I and type IIa fibers exhibit an oxidative metabolism and high endurance, type IIx and IIb fibers are glycolytic and display low endurance. Type I myofibers, also termed slow-twitch fibers, exert a slow contraction owing to the ATPase activity associated with type I myosin. Slow-twitch myofibers are rich in mitochondria, have more capillaries surrounding each fiber, exhibit oxidative metabolism, have a low velocity of shortening, and have a high resistance to fatigue. Type II fibers, termed fast-twitch myofibers, exert quick contractions and fatigue rapidly. The slow oxidative fibers are required for maintenance of posture and tasks involving endurance, whereas fast glycolytic fibers are required for movements involving strength and speed. The four major fiber types are distributed throughout the mammalian musculature, including limb, trunk, and head muscles.

Fiber type is assessed using assays that delineate the differences in ATPase activity that correlate with specific myosin heavy-chain isoforms [5]. The basis of the reaction is the deposition of insoluble salts of inorganic phosphate cleaved from ATP by myofibrillar ATPase(s) followed by substitution of the phosphates with less soluble chromogenic salts (Fig. 2.1a). Immunohistochemistry using monoclonal antibodies that recognize isoform-specific myosin heavy chain is another method used to determine fiber type specificity (Fig. 2.1a).

Myofiber identity is first established during embryonic development by myogenic transcription factors and is later modulated by neural and hormonal factors.

Table 2.1 Genes involved in congenital myopathy and muscular dystrophy

Category	Disease symbol	Disease name	Inheritance	Genes	Protein encoded
Congenital myopathy	NM	Nemaline myopathy	AD, AR	TPM3	Tropomyosin alpha-3
			AR	NEB	Nebulin
			AD, AR	ACTA1	Skeletal muscle alpha actin
			AD	TPM2	Tropomyosin beta-2
			AR	TNNT1	Troponin T slow
			AR	KBTBD13	Kelch repeat and BTB domain containing protein 13
			AR	CFL2	Cofilin 2
			AR	KLHL40	Kelch-like family member 40
			AR	KLHL41	Kelch-like family member 41
			AR	LMOD3	Leiomodin-3
	CNM	Centronuclear myopathy	XL	MTM1	Myotubularin
			AD	DNM2	Dynamin 2
			AR	BIN1	Amphiphysin 2
			AR	RYR1	Skeletal muscle ryanodine receptor
			AR	TTN	Titin
			AR	MTMR14	hJUMPY
			AD	CCDC78	Coiled-coil domain containing protein 78
		Central core myopathy	AD, AR	RYR1	Skeletal muscle ryanodine receptor
			AR	SEPN1	Selenoprotein 1
			AD	ACTA1	Skeletal muscle alpha actin
			AR	TTN	Titin
Muscular dystrophy	DMD	Duchenne muscular dystrophy	XL	DMD	Dystrophin
	BMD	Becker muscular dystrophy	XL	DMD	Dystrophin
	LGMD1A	Limb-girdle muscular Dystrophy type IA	AD	TTID	Myotilin
	LGMD1B	Limb-girdle muscular dystrophy type IB	AD	LMNA	Lamin A/C

(continued)

Table 2.1 (continued)

Category	Disease symbol	Disease name	Inheritance	Genes	Protein encoded
	LGMD1C	Limb-girdle muscular dystrophy type IC	AD	CAV3	Caveolin 3
	LGMD2A	Limb-girdle muscular dystrophy type 2A	AR	CAPN3	Calpain 3
	LGMD2B	Limb-girdle muscular dystrophy type 2B	AR	DYSF	Dysferlin
	LGMD2C	Limb-girdle muscular dystrophy type 2C	AR	SGCG	γ-Sarcoglycan
	LGMD2D	Limb-girdle muscular dystrophy type 2D	AR	SGCA	α-Sarcoglycan
	LGMD2E	Limb-girdle muscular dystrophy type 2E	AR	SGCB	β-Sarcoglycan
	LGMD2F	Limb-girdle muscular dystrophy type 2F	AR	SGCD	δ-Sarcoglycan
	LGMD2G	Limb-girdle muscular dystrophy type 2G	AR	TCAP	Telethonin
	LGMD2H	Limb-girdle muscular dystrophy type 2H	AR	TRIM32	Tripartite motif containing 32
	LGMD2I	Limb-girdle muscular dystrophy type 2I	AR	FKRP	Fukutin-related protein
	LGMD2J	Limb-girdle muscular dystrophy type 2J	AR	TTN	Titin
	LGMD2K	Limb-girdle muscular dystrophy type 2K	AR	POMT1	Protein-*O*-mannosyl transferease 1
	LGMD2L	Limb-girdle muscular dystrophy type 2L	AR	ANO5	Anoctamin 5
	CMD	Congenital muscular dystrophy	AR	POMT1	Protein-*O*-mannosyl transferease 1
			AR	POMT2	Protein-*O*-mannosyl transferease 2
			AR	POMGnT1	Protein-*O*-mannose 1,2-*N*-acetylglucosaminyl transferase
			AR	FKRP	Fukutin-related protein
			AR	LARGE	Glycosyltransferase-like protein LARGE1
			AR	LAMA2	Laminin α2
			AR	ITGA7	Integrin α7
	FSHD	Facioscapulohumeral muscular dystrophy	AD	DUX4	Double homeobox 4
	DM1	Myotonic muscular dystrophy type I	AD	DMPK	Myotonin-protein kinase
	DM2	Myotonic muscular dystrophy type I	AD	CNBP/ZNF9	Zinc finger 9
	EDMD	Emery-Dreifuss muscular dystrophy	XL	EMD	Emerin
			AD	LMNA	Lamin A/C

AD autosomal dominant, *AR* autosomal recessive, *XL* X-linked

Adult skeletal muscle has the ability to adapt and remodel its biochemical, morphological, and physiological states in response to environmental demands [4, 5]. The remodeling process provides an adaptive response that serves to maintain a balance between physiological demands for contractile work and the capacity of skeletal muscle to meet those demands. Many remodeling responses involve activation of intracellular signaling pathways and consequent genetic reprogramming, resulting in alterations of muscle mass, contractile properties, and metabolic states [4, 5]. For example, exercise training can change skeletal muscle by transforming the myofibers to an increased oxidative metabolism and inducing fiber type transitions from type IIb → type IIx/d → type IIa. Upon cessation of exercise training, these myosin heavy-chain isoform transitions and metabolic changes are reversed.

The relative proportion of any fiber type may vary according to species and anatomical site. For example, the diaphragm, a continuously active respiratory muscle, is a fast muscle in a rat and a mouse but a slow muscle in large mammals, such as a cow [6]. In the rat, the diaphragm consists predominantly of type IIx fibers and lacks type IIb fibers that are abundant in leg muscles. In leg muscles, the most studied muscle of the body, slow type 1 fibers are more abundant in the posterior compartment, where the typical slow soleus muscle is also located, in relation with the greater postural role of posterior muscles [6]. Finally, in many species, type II fibers are more numerous in forelimbs than in hind limbs, and, accordingly, in human upper limb muscles are faster than lower limb muscles [7, 8].

2.1.2 Regeneration and Satellite Cells

Adult skeletal muscle has a remarkable ability to regenerate in response to exercise, injury, and disease. In mice, injuries such as cardiotoxin injection cause severe muscle damage and degeneration. However, within 3 weeks muscle can fully regenerate and restore morphology (Fig. 2.1b). Regenerated muscles are marked by the presence of centralized nuclei. Skeletal muscle regeneration relies on a small population of stem cells, known as satellite cells (SCs), which reside beneath the basal lamina of myofibers. They are marked by expression of Pax7, a paired-box transcription factor (Fig. 2.1c) [9, 10]. SCs are normally quiescent but in response to stress or injury become activated to proliferate, differentiate, and fuse into multinucleated myotubes [9, 10]. Activated SCs also undergo asymmetric division, generating progeny that replenish the pool of quiescent SCs (Fig. 2.1d). Abnormalities in SC specification, proliferation, or differentiation result in skeletal muscle dysfunction during aging and can promote muscle disease. Pax7 is a specific marker for quiescent and activated SCs and is downregulated when SCs differentiate into myotubes [11, 12]. Pax7 activates expression of the myogenic regulatory factors Myf5 and MyoD in activated SCs and proliferating myoblasts, which in turn drive the myogenic differentiation program. Genetic ablation experiments demonstrated that Pax7-expressing SCs are essential for adult skeletal muscle regeneration [13–16].

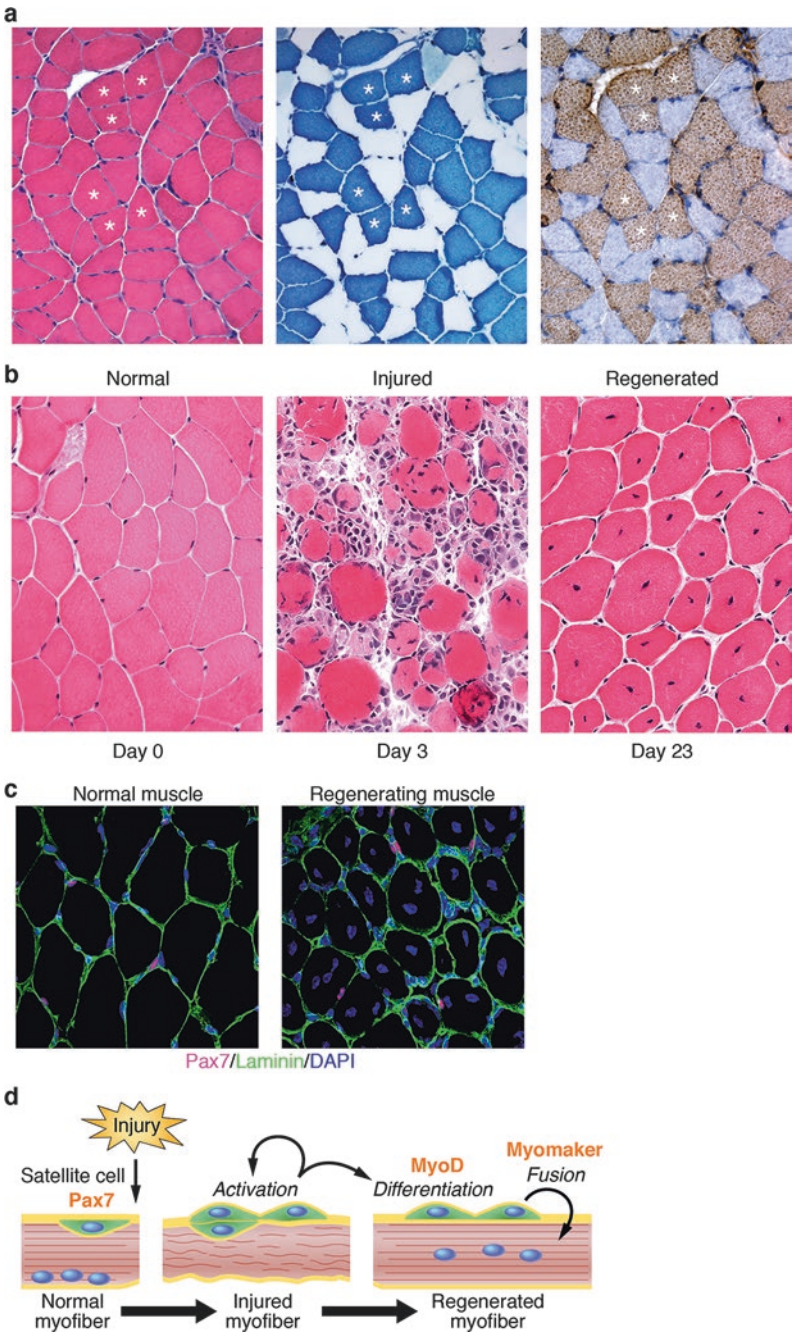

Fig. 2.1 Skeletal muscle fiber type diversity and regeneration. (**a**) Fiber-type analysis of serial transverse sections of mouse soleus muscle by hematoxylin and eosin stain (left panel) shows a checkerboard pattern of fibers, metachromatic dye-ATPase method (middle panel) shows type I

2.1.3 Primary Muscle Disorders

Primary muscle disorders are a diverse group of muscle diseases that involve muscle weakness, atrophy, and myofiber degeneration and regeneration and inflammation. In general, there are three groups of diseases: congenital myopathy, the muscular dystrophy, and inflammatory myopathy (also called myositis). Congenital myopathies are caused by various genetic defects affecting the contractile apparatus of muscles. Symptoms include generalized weakness and hypotonia of variable severity, manifesting from early childhood. Muscular dystrophies are caused by genetic abnormalities primarily affecting the sarcolemmal membrane or its supporting structures, thus leading to pathological degeneration and regeneration of skeletal muscles and progressive loss of skeletal muscle structure and function. Inflammatory myopathy is further classified into polymyositis, dermatomyositis, and sporadic inclusion body myositis, all of which involve inflammation of the muscle. The cause of inflammatory myopathy is unknown. In this chapter, we will focus on congenital myopathies and muscular dystrophies.

2.2 Congenital Myopathies

Congenital myopathies are a heterogeneous group of inherited muscle diseases characterized by early infantile or childhood onset of muscle weakness, hypotonia, and developmental delay, which have a static or slowly progressive course [17]. Classically, they are defined by skeletal muscle dysfunction and a non-dystrophic muscle biopsy with the presence of one or more characteristic histological features [18]. There are three major groups of congenital myopathies: (1) nemaline myopathies, characterized by the presence of electron-dense nemaline bodies or rods within myofibers; (2) centronuclear or myotubular myopathies, marked by the presence of internally located myonuclei; and (3) core myopathies, which have foci devoid of oxidative enzymes in myofibers. Congenital myopathies are caused by various genetic defects affecting the contractile apparatus of muscles. To date, more than 20 genes have been associated with the congenital myopathies, and more genes are expected to be discovered with the advances in molecular diagnostics and next-generation sequencing.

Fig. 2.1 (continued) fibers stained *dark blue* and type IIa stained *light blue*, and immunohistochemistry (right panel) using a monoclonal antibody that recognizes type I myosin heavy chain (brown). Asterisks mark the same type I fibers in each panel. (**b**) Adult skeletal muscle regeneration following cardiotoxin injury. Tibialis anterior muscle is injected with cardiotoxin to induce myofiber damage (day 3). Muscle regenerates and restores morphology within 3 weeks (day 23). Hematoxylin and eosin staining of transverse sections of tibialis muscle is shown. (**c**) Immunostaining for Pax7, a marker for satellite cells in normal and regenerating muscle. Note that muscle nuclei (stained by DAPI) are located in the periphery of normal muscle and are centrally located in regenerating muscle. (**d**) Model for the role of satellite cells in regeneration. Satellite cells are quiescent in normal muscle but become activated upon injury to participate in regeneration

2.2.1 Nemaline Myopathies

Nemaline myopathy (NM) is one of the most common forms of congenital myopathy, affecting 1 in every 50,000 births [19]. NM encompasses a set of genetically heterogeneous diseases defined by the presence of rod-like structures (called nemaline bodies) in skeletal muscle fibers. Nemaline bodies are formed by the abnormal aggregation of proteins within the thin filaments, such as tropomyosin, actin, myotilin, and nebulin. Patients with NMs are associated with myofibril disorganization, reduced contractile force, and mitochondrial dysfunction and clinically present a spectrum of muscle dysfunctions from mild muscle weakness to complete akinesia [19, 20]. Currently, there is no effective treatment for NM patients other than symptomatic treatments.

NM is significantly heterogeneous from a genetic point of view, and its inheritance can be autosomal dominant, sporadic, or autosomal recessive. The two most common causes of NM are recessive mutations in nebulin and de novo dominant mutations in skeletal muscle α-actin [19]. To date, 11 genes encoding proteins of skeletal muscle thin filaments, Kelch domain-associated proteins, and an unconventional myosin have been implicated in NM [20]. These findings lead to the current hypothesis that NM is a thin filament disease.

2.2.1.1 Nebulin

The nebulin (NEB) gene is the most commonly mutated gene in NM, accounting for approximately 50% of genetically diagnosed cases of NM [20]. Mutations in NEB have been known to cause severe, intermediate, mild, and typical forms of NM but most often the typical form [21, 22]. The typical NEM patients with NEB mutations survive to adulthood, express low (but detectable) levels of nebulin, and display a considerably mild phenotype. All mutations hitherto identified in this gene have been recessive [19].

The NEB gene has 183 exons (in humans) that are predicted to encode a protein of maximally 900 kDa [23]. Nebulin is a large sarcomeric protein in skeletal muscle, located along the length of the thin filament, with its C-terminus anchored in the Z-disk and its N-terminus positioned near the thin filament pointed end. The majority of nebulin is composed of 35-residue domains that bind actin with high affinity, which are organized into super-repeats that match the repeat of F-actin, and might determine the minimal length of the thin filament [23].

Several mouse models of NEB deficiency have been established to understand the mechanism underlining NEB-associated NM, including Neb KO, NebΔex55, and a muscle-specific deletion of Neb (Neb-cko) [24–27]. These mice all present many aspects of NM phenotypes including muscle weakness, nemaline rods, and variable trophicity effects. Studies in these mice have revealed the essential functions of NEB in muscle. NEB acts as a molecular ruler to regulate thin filament lengths. It also functions in the regulation of muscle contraction, force development, and calcium homeostasis, as NEB deficiency contributes directly to a loss in muscle contractility due to dysregulation of actin-myosin cross-bridge formation.

In addition, NEB deficiency also leads to fiber type switching toward oxidative types [24, 27]. Finally, the NEB-KO mouse models also highlight the role of nebulin in the assembly and alignment of the Z-disks [24, 27].

2.2.1.2 Kelch Proteins

Out of the 11 NM-related genes, 3 genes (*Klhl40*, *Klhl41*, and *Kbtbd13*) encode proteins that belong to the Kelch family proteins. Kelch proteins are characterized by the presence of a Kelch repeat domain, a BTB/POZ domain involved in protein-protein interaction, and a BACK domain that binds E3 ubiquitin ligases (Fig. 2.2a).

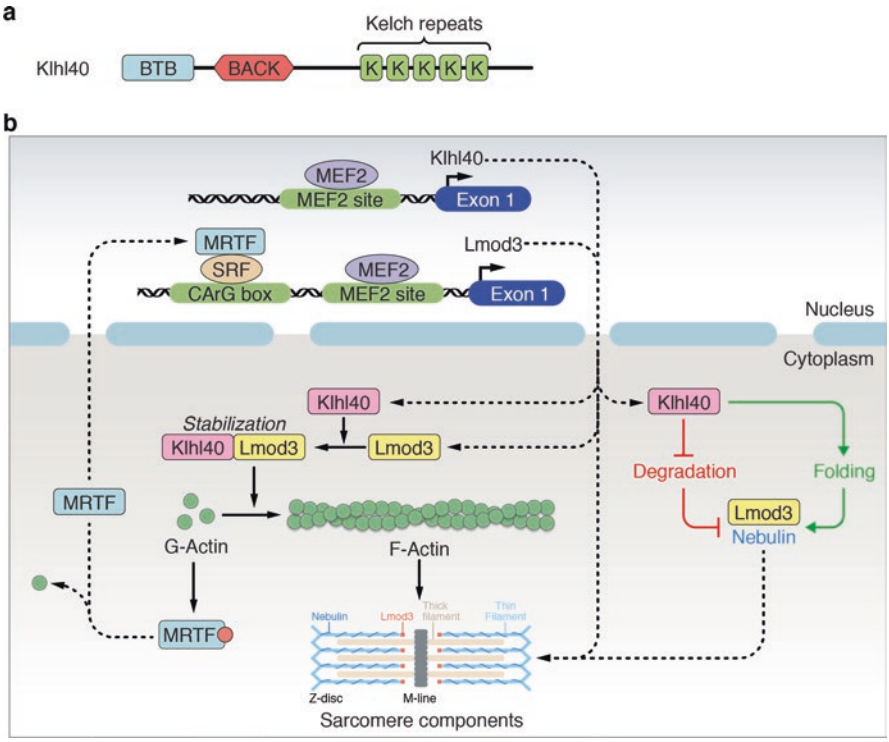

Fig. 2.2 Model for Kelch 40 and Lmod3 regulating actin cycling and sarcomere integrity. (**a**) Domain structures of Kelch 40 protein. KLHL40, like other members of the Kelch protein family, contains a BTB/POZ domain involved in protein-protein interaction, a BACK domain that binds E3 ubiquitin ligases, and a Kelch-repeat domain. (**b**) A model for the role of LMOD3 and KLHL40 in actin cycling and sarcomere integrity. In normal muscle cells, MRTF/SRF and MEF2 regulate LMOD3 expression, and MEF2 regulates KLHL40 expression. KLHL40 functions in the cytoplasm to stabilize LMOD3 and nebulin proteins that are components of sarcomeres. KLHL40 and LMOD3 together promote actin polymerization by converting G-actin to F-actin, allowing normal sarcomeric function. In muscles lacking KLHL40, nebulin and LMOD3 protein levels are reduced, resulting in destabilization of thin filaments, sarcomere dysfunction, and subsequent nemaline myopathy. Similarly, in muscles lacking LMOD3, accumulation of G-actin monomers not only disrupts sarcomeric integrity, but also represses MRTF-A expression, which in turn suppresses SRF-dependent target genes encoding cytoskeletal proteins and components of the contractile apparatus, leading to nemaline myopathy

Many Kelch proteins function as substrate-specific adaptors for Cullin E3 ubiquitin ligase (Cul3), mediating the ubiquitination and, in most cases, degradation of their respective protein substrates [28].

KLHL40 is localized to both the I band and A band in the sarcomere and binds NEB and the thin filament protein, leiomodin 3 (LMOD3) [29, 30]. Unlike other BBK proteins that promote target proteins to degradation, KLHL40 blocks proteasome-mediated LMOD3 and NEB degradation by inhibiting ubiquitination. Loss of KLHL40 in mice leads to a NM-like phenotype comparable to that of patients with NM lacking KLHL40 [30, 31]. Klhl40 KO mice display neonatal lethality, with defects in sarcomere structure and significant muscle weakness [30]. NEB and LMOD3 were reduced in skeletal muscle of both Klhl40$^{-/-}$ mice and KLHL40-deficient patients. This results in thin filament disruption, with subsequent irregularities of the sarcomere Z-disks and, in extreme cases, sarcomere dissolution with the formation of ovoid Z-disks, culminating in a fatal loss of muscle function [30]. These findings provide the first example of a Kelch protein that can mediate protein stabilization rather than degradation and highlight the importance of maintaining the balance between protein synthesis and degradation in skeletal muscle.

Similar to KLHL40, KLHL41 mutations in humans have been associated with NM [32]. KLHL41 shares 52% identity with KLHL40, indicating overlapping functions. Morpholino knockdown of KLHL41 in zebra fish causes NM-like abnormalities with aberrant myofibril formation [32]. The absence of KLHL41 in mice results in severe NM with general sarcomere disarray, accumulation of nemaline bodies, and perinatal death as seen in humans with KLHL41 mutations [33]. KLHL41 also presents the unique stabilizing activity, in which it prevents NEB aggregation through poly-ubiquitination of its BTB domain. Under normal conditions KLHL41 functions as a chaperone, preventing NEB aggregation and degradation [33]. Loss of KLHL41 or reduced poly-ubiquitination of KLHL41 results in loss of KLHL41 activity, NEB aggregation, and NM [33].

KLHL40 and KLHL41 possess distinct functions. While both proteins stabilize NEB, only KLHL40 stabilizes LMOD3. The stabilization of LMOD3 by KLHL40 occurs through a proteasome-mediated pathway, distinct from the stabilization of NEB. In the absence of KLHL40, LMOD3 levels are increased by proteasome inhibition [30]. Although KLHL40 and KLHL41 are very similar in their BTB and BACK domains, the homology is decreased throughout the Kelch repeats, which likely enables them to discriminate between different substrates.

2.2.1.3 Leiomodin-3 (LMOD3)

LMOD3 is another sarcomere protein associated with NM. Frameshift and nonsense mutations in LMOD3 were found to NM in humans [34]. LMOD3 belongs to a family of tropomodulin-related proteins known as leiomodins that comprise three predicted actin-binding domains and a tropomyosin-binding domain [35–38].

Like tropomodulins, LMOD proteins bind to the pointed ends of actin filaments and promote actin polymerization by stabilizing binucleated or trinucleated actin.

Similar to KLHL40, LMOD3 is localized to the A band in both contracted and relaxed muscle. Loss of function of LMOD3 in mice causes lethal NM and severe disruption of skeletal muscle sarcomeric structure and function [29, 39]. Skeletal muscle from Lmod3-KO mice also displays abnormal glycogen accumulation and nemaline rods. Mechanistic studies in mice revealed that LMOD3 promotes actin polymerization and diminishes G-actin levels [29]. The decrease in cytoplasmic G-actin pool triggers activation of serum response factor (SRF) in the nucleus to activate expression of sarcomeric components including LMOD3 itself [29]. By stabilizing LMOD3, KLHL40 can also stimulate the expression of LMOD3 and other sarcomeric components (Fig. 5.2b).

2.2.2 Centronuclear Myopathy

Centronuclear myopathies (CNMs) are a heterogeneous group of congenital myopathies characterized pathologically by the presence of abundant and centrally located nuclei. The clinical presentation of patients is extremely heterogeneous, ranging from severe hypotonia in newborns to a relatively late onset of muscle weakness with extraocular muscle involvement in adolescent and young adults [40, 41]. There are three main forms of CNMs according to the mode of inheritance and clinical presentation: (1) the X-linked recessive form, also named myotubular myopathy, caused by mutations in MTM1 gene; (2) the classical autosomal dominant form caused by mutations in DNM2 gene; and (3) an autosomal recessive form caused by mutations in BIN1 gene. Other genes have also been associated with CNMs, including the RYR1 gene encoding the skeletal muscle ryanodine receptor, the TTN gene encoding titin, the CCDC78 gene encoding coiled-coil domain containing protein 78, the MTMR14 gene encoding myotubularin 14, and the SPEG gene encoding striated muscle preferentially expressed protein kinase [20].

The X-linked recessive myotubularin myopathy (XLMTM) is the most common form of CNMs, affecting approximately 2/100,000 male births per year [41]. The disease is characterized by a severe phenotype in males with marked extraocular, facial, respiratory, and axial muscle weakness at birth. Most affected boys die within the first year of life despite supportive treatment. XLMTM is caused by mutations in the myotubularin (*MTM1*) gene located on the X chromosome. MTM1 encodes a 3′-phosphoinositides phosphatase that is implicated in many cellular processes, including phosphatidylinositol-3-phosphate (PI3P) signaling pathway and membrane trafficking [42]. More than 300 MTM1 mutations have been identified to date, distributed throughout the entire coding sequence [43–45].

The autosomal dominant DNM2-related CNMs usually have much milder phenotypes than XLMTM, with typical onset in adolescence or early adulthood [40, 41]. Patients show predominant proximal weakness with additional distal involvement,

particularly in the lower limbs, and ptosis with external ophthalmoplegia, over a stable or slowly progressive course. Histologically, muscles from DNM2-related CNMs have a radial arrangement of sarcoplasmic strands, as well as significant nuclear centralization and internalization. The disease is caused by mutations in the DNM2 gene, which encodes dynamin 2, a ubiquitously expressed large GTPase protein [46]. Dynamin 2 is one of three members of the dynamin family, which is involved in membrane fission, vesicle trafficking, endocytosis, actin cytoskeleton assembly, and centrosome cohesion [47, 48].

Autosomal recessive CNMs caused by BIN1 mutations are generally very rare. Patients usually present as an intermediate form of disease between XLMTM and DNM2-related CNMs [2, 3]. BIN1 encodes amphiphysin-2, a ubiquitously expressed BAR domain containing protein. Members of the BAR domain proteins are involved in membrane recycling and endocytosis [49]. BIN1 also contains a phosphoinositide-binding domain and is also involved in T-tubule formation [50].

It is intriguing that the three major proteins affected in CNMs, MTM1, dynamin-2, and amphiphysin-2 are all involved in various aspects of membrane trafficking, marking it a pathogenic "master mechanism" for different types of CNMs [41]. Other common defects include aberrant T-tubule formation, abnormalities of triadic assembly, and disturbance of the excitation-contraction machinery. Abnormal autophagy has recently been recognized as another important collateral of defective membrane trafficking in different genetic forms of CNM, suggesting an intriguing link of defective autophagy to primary disorders with overlapping histopathological features [18]. It remains unknown whether other CNM-associated genes such as RYR1 and TTN are also involved in the same pathways.

2.2.3 Core Myopathies

Core myopathies are characterized by foci (cores) devoid of oxidative enzymes in the central area of myofibers. Histologically, the cores are areas of abnormal sarcomeric structures, including Z-line streaming, complete myofibrillary disorganization, and accumulation of Z-band material, and the core regions are devoid of mitochondria [51, 52]. Core myopathies are associated at a varying degree of disease severity, and the pathological features most often progress over time.

Core myopathies are among the most common congenital myopathies and more than half of them can be attributed to RYR1 mutations, both autosomal dominant and recessive forms [20, 53]. Other disease-associated mutations include autosomal recessive mutations in the selenoprotein N1 (*SEPN1*) gene encoding an endoplasmic reticulum glycoprotein [51]. Mutations in the skeletal muscle α-actin 1 (*ACTA1*) and titin (*TTN*) genes can also result in core myopathies [17]. However, the molecular mechanisms of core myopathies are poorly understood.

2.3 Muscular Dystrophies

Muscular dystrophies are characterized by muscle degeneration with cycles of muscle necrosis and regeneration and progressive loss of muscle structure and function. Muscular dystrophies are caused by many different genetic mutations primarily affecting the sarcolemmal membrane or its supporting structures, leading to defects in muscle membrane integrity [54, 55]. Muscle membrane disruption triggers an increase in intracellular calcium, which can both activate proteolysis to exacerbate muscle damage and also stimulate a muscle membrane repair system containing the protein dysferlin [56]. Extensive muscle damage also activates SCs to repair and regenerate necrotic myofibers. As muscle disease advances, SCs are rapidly depleted; thus muscle repair cannot adequately compensate for damage, and muscle is gradually replaced by fibrotic tissue. Life-threatening cardiac and respiratory symptoms can also occur in the most severe dystrophies.

2.3.1 Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD), an X-linked inherited or spontaneous disorder, is the most common type of muscular dystrophy, affecting 1 in every 5000 boys at birth [57]. DMD boys are usually diagnosed within the first few years of life by delayed and abnormal walking ability. Their symptoms progress with age, and between ages 7 and 12, DMD children are wheelchair dependent. DMD patients die prematurely in their 20 s and/or 30 s, due to cardiac complications and/or respiratory issues. Since DMD is an X-linked recessive disorder, female carriers have little to no symptoms. However, it has been recently recognized that although fully ambulatory, adult carrier females may develop clinical manifestations, such as a dilated cardiomyopathy [58, 59].

DMD is caused by mutations in the dystrophin gene (*DMD*), one of the largest human genes comprised of 79 exons, encoding dystrophin, a 427-kDa intracellular protein [60, 61]. The large dystrophin protein has four main functional domains: an actin-binding amino-terminal domain, a central rod domain containing 24 spectrin repeats interrupted by four hinge proteins, a cysteine-rich domain, and a carboxyl-terminus (Fig. 2.3a) [62–64]. More than 7000 mutations in DMD have been identified, all resulting in loss of dystrophin protein expression [65]. Mutations that only partially disrupt the dystrophin gene resulting in an internally truncated and partially functional dystrophin protein cause Becker muscular dystrophy (BMD), a milder phenotype than DMD [66, 67].

Dystrophin is a central component of the dystrophin glycoprotein complex (DGC), which is a multimeric protein complex essential for sarcolemma integrity and stability of muscle cells (Fig. 2.3b) [57, 62]. The DGC complex can be divided into three groups based on their cellular localization: extracellular (α-dystroglycan),

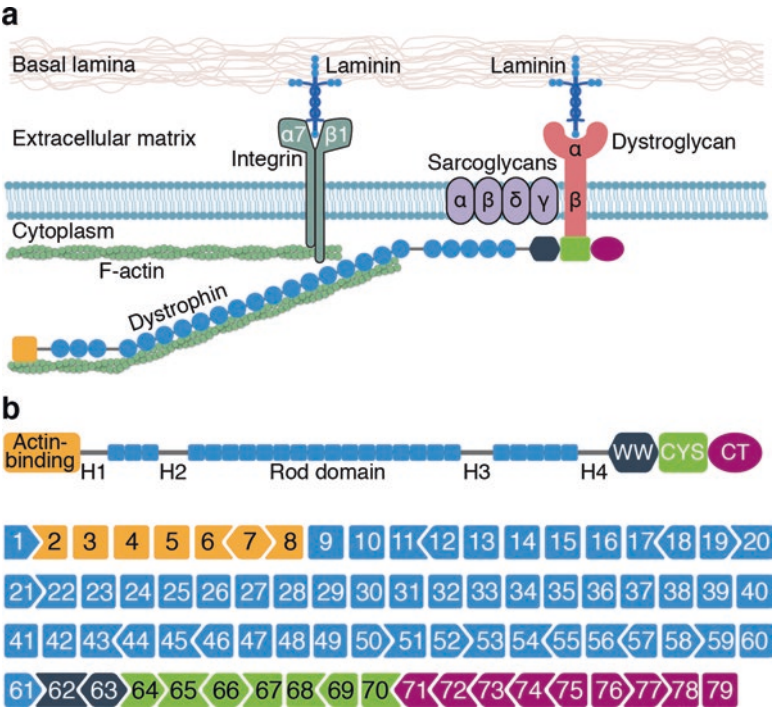

Fig. 2.3 Dystrophin-glycoprotein complex (DGC) complex and domain structure of dystrophin protein. (**a**) The dystrophin glycoprotein complex (DGC) contains sarcoglycans, dystroglycans, dystrophin and other components. Through binding to laminin in the basement membrane on the extracellular site and binding to actin on the cytoplasmic site, DGC provides stability to the sarcolemma during the mechanical changes caused by muscle contraction. Absence of components of the DGC complex can cause muscular dystrophies characterized by sarcolemmal disruptions and muscle degeneration. The α7β1 integrin dimer binds laminin extracellularly and associates intracellularly with actin-binding proteins. (**b**) Domain structure of the dystrophin protein (top panel) and exon arrangement of the dystrophin gene (bottom panel)

transmembrane (β-dystroglycan, sarcoglycans, sarcospan), and cytoplasmic (dystrophin, dystrobrevin, syntrophins, neuronal nitric oxide synthase) [57, 62]. α-Dystroglycan resides on the extracellular surface of the sarcolemma due to its heavy glycosylation and peripheral membrane association [62]. α-Dystroglycan functions as a receptor for the extracellular ligands such as laminin. α-Dystroglycan is tightly associated with β-dystroglycan, a transmembrane protein that also interacts with dystrophin. At the sarcolemma, the sarcoglycan subcomplex is tightly associated with β-dystroglycan. The most prevalent form of the sarcoglycan complex in skeletal muscle is composed of four single-pass transmembrane proteins: α-sarcoglycan, β-sarcoglycan, γ-sarcoglycan, and δ-sarcoglycan [68]. At the cytoplasmic face of the sarcolemma, dystrophin maintains its membrane localization by interacting with β-dystroglycan. Dystrophin also binds filamentous actin through its amino-terminus in the cytoskeleton, thus linking the cytoskeleton to DGC, which in

turn is connected to the basal lamina by interacting with the ECM ligands [56]. Other cytoplasmic components of DGC include α-dystrobrevin, syntrophins, and neuronal nitric oxide synthase (nNOS). The α-dystrobrevin/syntrophin triplet associates with dystrophin. Syntrophin and dystrophin spectrin-like repeat 16 and 17 anchor nNOS to the sarcolemma. The DGC is essential to the integrity of muscle by providing muscle membrane stabilization during contraction.

DMD is associated with mutations that disrupt the reading frame of the dystrophin protein, causing premature stop codons. Mutations in the *DMD* gene cluster into two hot spot regions, within exons 2–20 and exons 45–55 [65]. These mutational hot spot regions are subjected to missense and nonsense substitutions, as well as deletions, insertions, and duplications [69]. Mutations in the first hot spot region exons 2–20 account for ~15% of all exon deletions and ~50% of all exon duplications within the *DMD* gene. Deletion of exons 3–7 are the most frequent. The second DMD hot spot, exons 45–55, accounts for ~70% of all exon deletions and ~15% of all exon duplications [65, 70]. Internal deletion mutations of the dystrophin gene *DMD* that preserve the amino- and carboxy-termini of the protein produce a truncated form of dystrophin and translate into BMD, a milder disease phenotype, in which the severity of symptoms differs depending on the length and structure of the truncated dystrophin. Exon skipping therapies, such as antisense oligonucleotides or CRISPR/Cas9, are conceptually based on using gene therapy to convert the lethal disease of DMD to a clinically milder disease of BMD [57].

2.3.2 Limb-Girdle Muscular Dystrophies

Limb-girdle muscular dystrophies (LGMD) are a heterogeneous group of inherited muscular dystrophies, characterized by progressive weakness of the proximal limb muscles. Other muscles may also be affected, including the heart and respiratory muscles [54, 71]. The phenotypic spectrum is broad, ranging from minimal symptoms to severe, early onset weakness greatly affecting quality of life and life-span [54, 71]. LGMD is further categorized into the autosomal dominant LGMD1 and autosomal recessive LGMD2, based on modes of inheritance [72]. Mutations in more than 50 loci have been reported in LGMD, making accurate diagnosis and genetic counseling a challenge [73]. The underlying genetic changes linked to LGMD1 are missense mutations that occur in structural (desmin, myotilin, lamin A/C), transmembrane (caveolin-3), and signaling (DNAJB6) proteins [73].

2.3.2.1 LGMD Caused by Mutations in Sarcoglycans

The sarcoglycans are single-pass transmembrane proteins that form a tight unit within the DGC [68]. In mammalian skeletal muscle cells, the sarcoglycan complex is composed of α-, β-, γ-, and δ-sarcoglycan. The sarcoglycan proteins have multiple functions in muscle cells and are essential for membrane stability. The sarcoglycan

complex stabilizes the DGC, by enforcing the link between α- and β-dystroglycan and by interacting directly with dystrobrevin. α- and δ-Sarcoglycan also interact with filamin C in the cytoplasm, participating in the mechanoprotection process [62, 68]. The sarcoglycan complex may also regulate cell-cell adhesion via interacting with the integrin complex.

Loss-of-function mutations in the α-, β-, γ-, δ-sarcoglycan genes cause the recessive LGMD types 2D, 2E, 2C, and 2F, respectively [54, 73]. These patients have a presentation similar to the phenotypic range seen in DMD and BMD. The basic pathological features in LGMD-2D, 2E, 2C, and 2F patients are indistinguishable from those found in DMD or BMD muscle. Interestingly, mutations in any single sarcoglycan gene causes the absence of expression of all the other subunits [74]. Absence of dystrophin leads to the loss or reduction of the sarcoglycan subunits from the plasma membrane, but the reverse is not true as sarcoglycan gene mutations do not affect the expression and distribution of dystrophin [55].

2.3.2.2 LGMD Caused by Mutations in Sarcolemmal Repair Complex

LGMD type 2B is caused by loss of function mutations in the dysferlin gene locus [75]. Like other LGMDs, patients with LGMD2B present proximal muscle weakness. A prominent feature of LGMD2B is the presence of inflammatory infiltration in muscle biopsies [75]. Mutations in dysferlin also cause Miyoshi myopathy, a mild form of muscular dystrophy that selectively affects the gastrocnemius muscle but spares other musculature [76]. Surprisingly, identical dysferlin mutations cause both diseases, suggesting that modifiers mediate substantial aspects of the disease pathology [77].

Dysferlin is a 230-kDa membrane-associated protein with a long cytoplasmic domain [78]. Dysferlin is not a component of the DGC, but it forms a muscle repair complex by interacting with other proteins to mediate membrane repair. Dysferlin interacts with caveolin-3, a muscle-specific form of caveolar membranes that participate in membrane trafficking [79, 80]. Dysferlin also interacts with AHNAK (desmoyokin), calpain-3, a calcium-activated protease, and annexins, which are calcium-dependent phospholipid-binding proteins that also participate in vesicle aggregation [81–83].

Damage to the sarcolemma results in an influx of calcium which activates and alters the binding properties of proteins in the membrane repair complex [56, 84]. Annexins bind dysferlin and phospholipids with higher affinity in the presence of calcium, dysferlin binds phospholipids in a calcium-dependent manner, and calpains are activated [56, 84]. These interactions result in the recruitment of internal vesicle structures. Within seconds of activation, membrane lesions are resealed, calcium concentrations are normalized, and the repair complex is deactivated. The deactivation of the complex may be mediated in part by calpain-dependent cleavage of annexins and AHNAK. In addition, mutations in the genes encoding proteins of this sarcolemmal repair complex cause LGMD2B and Miyoshi myopathy (dysferlin), LGMD1C (caveolin), and LGMD2A (calpain-3) [56, 73].

2.3.3 Congenital Muscular Dystrophy

Congenital muscular dystrophy (CMD) is an uncommon group of muscular dystrophies characterized by early onset of muscle weakness and hypotonia within 1 year of age. CMD patients present typical features of dystrophic muscle histology, with an elevated serum creatine kinase (CK), indicating disruption of the sarcolemma [85]. In addition, more than 50% of patients have moderate to severe cognitive impairment and neurological features such as lissencephaly and ocular and retinal defects [55, 86]. The prevalence of CMDs is poorly known, and it is estimated in the range of 1/100,000 individuals [87]. The majority of CMDs are autosomal recessive, and they are presented as several forms, including Walker-Warburg syndrome, muscle-eye-brain disease, Fukuyama-type CMD, Ullrich CMD, and Bethlem myopathy [88]. CMDs are highly heterogeneous with regard to genetic background, but many of the mutated genes encoded enzymes in the protein *O*-mannosylation biosynthetic pathway that is involved in the α-dystroglycan protein in the DGC [89, 90].

Dystroglycan is composed of two subunits produced from a single gene, α-dystroglycan and β-dystroglycan [90]. β-dystroglycan is a transmembrane protein that interacts with dystrophin in the cytoplasm and anchors α-dystroglycan. α-Dystroglycan is a soluble secreted glycoprotein that interacts with both β-dystroglycan and multiple components of the extracellular matrix such as laminin. These extracellular matrix proteins recognize and bind the unusual glycan structures on α-dystroglycan. Thus, proper glycosylation of α-dystroglycan is essential for binding to extracellular matrix components [90, 91]. Multiple studies have clearly demonstrated that it is the *O*-mannosylated glycan structures that serve as binding sites for laminin and presumably other extracellular matrix proteins [89]. Many enzymes in the *O*-mannosylation process have been associated with CMDs, including POMGnT1 (protein O-mannose 1,2-*N*-acetylglucosaminyl transferase), FKRP (fukutin-related protein), POMT1/2 (protein *O*-mannosyltransferase), and LARGE (like acetylglucoseaminyltransferase) [85]. Other forms of CMDs include mutations in the gene encoding the α2 chain of laminin, a major ligand for α-dystroglycan, and in the gene coding integrin α-7, which mediates cell membrane interactions with the extracellular matrix [85, 92, 93]. Therefore, in addition to *O*-mannosylation defects, disruption between the extracellular matrix and the membrane can also cause CMDs.

2.3.4 Facioscapulohumeral Muscular Dystrophy (FSHD)

Facioscapulohumeral muscular dystrophy (FSHD) is the most common autosomal dominant form of muscular dystrophy, affecting approximately 1 in 8,000 individuals worldwide [94]. FSHD is unique from other types of muscular dystrophies, in which muscle weakness is asymmetric and involves the face, shoulder, and upper arm muscles. Trunk and lower extremities often become affected with disease progression in FSHD, but the extraocular, pharyngeal, and cardiac muscles are spared. Unlike

other muscular dystrophies such as DMD and LGMD that show striking evidence of myofiber degeneration accompanied by elevated serum creatine kinase, FSHD muscle shows minimal myopathic changes with evidence of inflammatory infiltrates specific to the perivascular region. FSHD is further divided into two groups, FSHD1 and FSHD2, based on different genetics and epigenetics of the disease [95, 96].

FSHD1 is an autosomal dominant gain-of-function disease, representing about 95% of FSHD patients. Genetically, FSHD1 is linked to a contraction of a macrosatellite repeat called D4Z4 and the consequent misexpression of subtelomeric gene(s) on chromosome 4 [97–99]. Normal individuals carry 11–100 repeat units within the highly condensed D4Z4 macrosatellite elements on the subtelomeric region of chromosome 4q35. Contraction of D4Z4 repeats in FSHD1 (less than 10 repeats) relaxes the chromatin structure and induces the expression of the *DUX4* gene from the distal-most repeat unit. *DUX4* encodes double homeobox 4, a putative transcription factor, which induces apoptosis and inflammation in muscle cells [100, 101]. It is believed that transient over-expression of DUX4 causes toxicity in muscle cells, leading to FSHD1. FSHD2 represents 5% of the FSHD cases, and patients with FSHD2 typically do not harbor a contraction of the D4Z4 repeats [102]. Instead, chromatin relaxation of the D4Z4 locus is caused by heterozygous mutations in the SMCHD1 gene encoding a protein essential for chromatin condensation [102]. Mutated SMCHD1 fails to methylate D4Z4 and to suppress DUX4 expression. FSHD1 and FSHD2 may have an additive effect: patients harboring D4Z4 contraction and SMCHD1 mutations display a more severe clinical phenotype than with either defect alone [95, 96].

2.3.5 Myotonic Dystrophy

Myotonic dystrophies (DMs) are the most common types of muscular dystrophy in adults, affecting 1 in 8,000 individuals [103, 104]. DMs are characterized by progressive muscle degeneration leading to disabling weakness and wasting with myotonia, muscular dystrophy, cardiac conduction defects, posterior iridescent cataracts, and endocrine disorders. DMs are autosomal dominant diseases with two genetically distinct types causing similar nucleotide repeat expansions. Myotonic dystrophy type 1, DM1 (also known as Steinert's disease), is caused by a (CTG) microsatellite repeat expansion in the untranslated 3′ region of DMPK (dystrophia myotonica protein kinase) gene in chromosome 19 [105–107]. This results in the nuclear accumulation of RNA containing the abnormal CUG expansions, forming stable secondary structures detectable as RNA foci [103]. Members of the muscleblind-like (MBNL) protein family, such as MBNL1, are sequestered in ribonuclear foci leading to loss of function and dysregulation of MBNL splicing and transcription targets and microRNA metabolism [108, 109]. Myotonic dystrophy type 2, DM2, is caused by a (CCTG)n expansion in intron 1 of CNBP (also called Znf9) gene in chromosome 3 [110, 111]. Similar to DM1, the accumulation of (CCTG)n repeats causes MBNL1 sequestration and subsequent abnormal splicing

of effector genes. The prevailing paradigm therefore is that both disorders are toxic RNA diseases. However, research indicates several additional pathogenic effects take place with respect to protein translation and turnover [103].

2.3.6 Emery-Dreifuss Muscular Dystrophy (EDMD)

Emery-Dreifuss muscular dystrophy (EDMD) is a unique type of dystrophy caused by mutations in genes encoding nuclear proteins. Patients with EDMD are characterized clinically by humero-peroneal muscle atrophy and weakness, multi-joint contractures, spine rigidity, and cardiac insufficiency with conduction defects [112]. Cardiac abnormality such as atrioventricular block is frequent in EDMD patients. EDMD was first described as an X-linked muscular dystrophy clinically distinct from DMD or BMD, caused by mutations in Emerin gene on chromosome X [113, 114]. In the 1990s, it was determined that EDMD may also be caused by autosomal dominant mutations in the gene encoding lamins A and C (LMNA) on human chromosome 1 [115]. To date, there are at least six types of EDMD, five of which have been associated with mutations in genes encoding nuclear proteins [112].

Emerin is a 34-kDa protein that embeds in the inner nuclear membrane. Emerin binds to barrier-to-autointegration factor (BAF), a small peptide that oligomerizes and directly binds to DNA [116]. Lamins polymerize to form a protein meshwork known as the nuclear lamina under the inner nuclear membrane. Lamin and emerin directly interact at the inner nuclear membrane [117]. Lamin and emerin, together with other nuclear proteins, maintain nuclear morphology, regulate chromatin organization and gene transcription, link the cytoskeleton to the nuclear skeleton, and serve as a scaffold for other nuclear proteins involved in gene regulation and DNA replication [118, 119]. Emerin and lamin proteins are expressed in all tissues but have muscle-specific phenotypes when mutated. It is postulated that muscle nuclei are susceptible to damage from mechanical forces from contraction, because they are joined to the cytoplasm actin cytoskeleton via the interaction of the nuclear membrane complex with the intermediate filament network [118, 119]. Through this interaction network, contractile stress can pass from the sarcolemma through the cytoskeleton to the nucleus. In EDMD patients, this force may be enough to disrupt nuclear structure and lead to the subsequent changes in gene expression and death of dystrophic muscle fibers [118].

2.4 Concluding Remarks

Skeletal muscle is highly specialized and designed to support body movement, maintain posture, and provide body strength. Skeletal muscle diseases are debilitating illnesses that impair skeletal muscle function and, in many cases, shorten life expectancy. With the entire human genome sequenced, it is possible to identify the

gene responsible for causing skeletal muscle disease, especially for monogenic diseases. Skeletal muscle diseases generated by multiple genetic mutations still remain a challenge but are being actively pursued. Additionally, the relationship between epigenetic mutations and skeletal muscle diseases is just being realized. We appreciate that identification of the gene responsible for skeletal muscle disease is not a guarantee of a cure for the disease; nevertheless it does provide a major step in allowing us to formulate a genetic therapy approach to ameliorate the disease and restore muscle function.

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Chapter 3
Animal Models for Muscle Disease and Muscle Gene Therapy

Stephanie M. Shrader, Roberta Wrighten, and Bruce F. Smith

Abstract It is currently estimated that roughly 50,000 Americans are affected by some form of muscular dystrophy and many others by the various forms of myopathies. Animal models are critical for our understanding of the numerous muscular diseases that affect people and for the development of targeted gene therapeutics to treat such diseases. Our current understanding of the pathophysiology of these diseases would not be possible without the aid of animal modeling. Multiple animal models have been described, including mice, cats, dogs pigs, etc.; however, the discussion in this chapter will primarily focus on mice and dogs because these two animal models have been more rigorously researched and described. The overall objectives of this chapter are to review the available animal models and their limitations, disease-specific mutations, clinical disease manifestations, and recent advances in associated therapeutic modalities for various muscular disorders, with a focus on dystrophinopathies and limb-girdle muscular dystrophies.

Keywords Muscular dystrophy · Duchenne · Myopathy · Dystrophinopathy

S. M. Shrader (✉)
Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, AL, USA
e-mail: sms0059@auburn.edu

R. Wrighten
Opelika, AL, USA
e-mail: robbywrighten@gmail.com

B. F. Smith
Department of Pathobiology, Scott-Ritchey Research Center, College of Veterinary Medicine, Auburn University, Auburn, AL, USA
e-mail: smithbf@auburn.edu

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3.1 Introduction

There are various forms of genetic muscular diseases which have been identified in humans, including muscular dystrophies, congenital myopathies, and metabolic muscular disorders. Importantly, similar diseases have been discovered (or created) in other animals, allowing for the development of animal models that can aid in the understanding of muscular disease pathogenesis and generation of novel therapeutics to treat such diseases. One of the most well-known and well-studied forms of the muscular dystrophies is Duchenne muscular dystrophy (DMD), which will be discussed at length in this chapter. Much of our understanding of DMD has come from the study of multiple animal models which have been identified in a variety of species. Advances in the understanding of other muscular diseases have also been the result of animal disease modeling. This chapter shall endeavor to review the benefits and limitations of the various animal models as they pertain to the understanding and treatment of muscular diseases in humans.

The two most commonly used animals for human disease modeling are the mouse and dog. Although this chapter shall focus on these two animal models, it is important to note that other animal models can provide useful information as well. Rodent models (particularly mice) have been extensively used in disease research for multiple reasons. They have relatively fast reproduction rates with a gestational period ranging from 18 to 22 days. As a comparison, cats have a gestational period of 63–65 days, and dogs have a gestational period ranging from 58 to 65 days. Mice are also favored as animal models due to their small size, relative ease of handling, availability, and lower costs of colony maintenance. Additionally, mice and humans have remarkable genomic similarities. Although the mouse genome is about 14% smaller than the human genome, both contain about 30,000 protein-coding genes and have over 90% corresponding regions of conserved synteny [1].

Because of these advantages, many murine models have either been discovered (i.e., they develop spontaneous disease that parallels a known disease in humans) or created (utilizing genetic manipulation or mutagenic agents). Using homologous recombination in embryonic stem cells, researchers are able to modify the mouse genome at a specific locus. Based on this gene targeting technology, various strains of mice have been developed using knock-in, knockout, and conditional gene modification strategies. Embryonic stem cell technologies are not currently available for larger animal models such as cats and dogs; therefore, these animal models are spontaneous only. Discovery of these animal models typically relies on astute observations and disease recognition by veterinarians in private practice, specialty clinics, or academic institutions. Following discovery of an animal with a spontaneous inherited disease of interest, additional study of the disease requires the establishment of an appropriate breeding program and study colony.

3.2 Dystrophinopathies

The dystrophin gene, the largest gene currently known in nature, measures 2.4 megabases at locus Xp21. It was discovered in 1986 as the gene that causes DMD [2]. Roughly a year later, Louis Kunkel and his colleagues described the associated protein product [3]. The dystrophin protein is large (427 kD), located in the cytosol subjacent to the cellular membrane, and functions to link cytoskeletal F-actin and transmembrane beta-dystroglycan to form the dystrophin-associated protein complex (DAPC) [4, 5]. Appropriate functionality of this complex is vital because it mediates signaling between the intracellular cytoskeleton and the extracellular matrix. As such, its role is to protect myocytes and myofibers from injury associated with normal contractile forces.

Mutations in the dystrophin gene are responsible for what are known as the dystrophinopathies, which result in a spectrum of related clinical diseases which include DMD, the less clinically severe Becker muscular dystrophy (BMD), and X-linked dilated cardiomyopathy (XLDCM) [6]. Since DMD has a more severe clinical manifestation and there is a greater interest in the development of animal models to study it, the remainder of this section will focus primarily on DMD. DMD is an X-linked recessive disorder that results from various mutations in the dystrophin gene (to be discussed in depth later). Due to the role that dystrophin plays in muscular stabilization and contractility, patients eventually develop degenerative changes in skeletal and cardiac muscle. Boys with DMD are typically diagnosed prior to 5 years of age and generally become nonambulatory and wheelchair-dependent by 10–12 years of age. Historically, death occurs in the second decade of life, attributed primarily to pulmonary infections and respiratory failure [4]. In more recent years, the treatment of secondary respiratory disease has improved, resulting in both prolongation of life and in the unmasking of cardiac disease. Almost all patients with DMD that survive into their 30s will be diagnosed with cardiomyopathy. Electrocardiographic findings include persistent sinus tachycardia, an increased R-S ratio, deep Q waves, various conduction abnormalities, and arrhythmias. Over time, myocardial damage can progress to myocardial fibrosis and eventually to dilated cardiomyopathy. Because early recognition is the key, the recommended cardiac screening protocol for boys with DMD now includes an ECG and transthoracic echocardiography every 2 years until the age of 10 and then on a yearly basis [7]. Cardiac magnetic resonance imaging (MRI) is also starting to gain favor for the diagnosis of early myocardial damage and remodeling [8].

Although various types of dystrophin mutations have been documented, most of the mutations in people are deletions. These are thought to occur during meiosis, resulting from the malalignment of exons containing highly repetitive sequences. The mutation can either disrupt the reading frame or cause premature termination of translation. The end result is a loss of dystrophin functionality, an altered DAPC, and subsequent muscular damage. Often, DMD patients lack detectable skeletal muscle dystrophin expression. Point mutations that result in stop codons can cause a similar outcome [6]. Milder disease states, such as occurs with BMD, can result if

the mutation maintains the reading frame, allowing for preservation of some levels of a truncated dystrophin protein.

Over the last 30 years, various animal models for DMD have been discovered or developed. Although the mouse and dog are most popular, dystrophin deficiency has also been studied in cats, pigs, zebrafish, and nematodes. In cats, deficiency results in hypertrophic feline muscular dystrophy. It is associated with periods of muscular degeneration and regeneration, but lacks the characteristic debilitating fibrosis, which occurs in people with DMD [9]. Similar to what is seen in people, dystrophin-deficient pigs develop skeletal muscle degeneration, regeneration, inflammation, fibrosis, and impaired metabolic activity [10]. However, their larger size typically makes large-scale studies cost prohibitive. Nonmammalian models, such as the zebrafish and the nematode *Caenorhabditis elegans*, express a dystrophin ortholog, making them useful for DMD-related gene analysis and drug discovery studies [11].

The most well-known animal model of DMD is the *mdx* mouse. Similar to what occurs in humans, the *mdx* mutation of dystrophin is recessive and was first described in 1984 in mice from a C57BL/10ScSn background. It results from a T to C substitution at position 3185, creating a stop codon in exon 23 [12]. Homozygous females and hemizygous males are useful for the study of DMD because they are cheaper to house than larger animal models and can survive for up to 2 years (similar to that of the parental strain). The ability of affected male mice to reach sexual maturity is important because it allows for breeding of affected males to carrier and affected females, resulting in a greater number of affected offspring per litter. Although they have a fairly normal life-span, the *mdx* mouse does develop a disease phenotype similar to (although less severe than) that which occurs in humans, including stunted growth, muscle atrophy, muscular weakness, and compensatory hypertrophy [13] which can be exacerbated with eccentric contraction (e.g., walking on a downhill treadmill) [14]. Nevertheless, aged *mdx* mice show a more severe patient-like phenotype. Cardiac dysfunction has also been reported as early as 9–10 months of age in some mice [4]. Characteristic dilated cardiomyopathy can be detected in female *mdx* mice that are $\geq$ 21 months old [15].

Histologically, *mdx* mice develop pronounced skeletal myofiber degeneration, necrosis, inflammatory cell infiltrates, and foci of regeneration, but do not frequently develop fibro-fatty replacement. The exception, however, is in the diaphragm, which can develop severe fibrosis [16]. These mice also develop increased numbers of satellite cells, which when normalized to myonuclear number, appear to be stable. Although the mean myofiber diameter appears to be unaltered in *mdx* mice, the increased number of myonuclei per fiber and extensive fiber branching results in muscular hypertrophy. Hypernucleation in *mdx* myofibers results from abundant central nucleation, a characteristic feature of the disease. It is not currently clear whether these centralized nuclei are contributors to the myopathic process or resultant from it [17, 18].

Another interesting feature in DMD patients and in murine and canine models is the presence of "revertant fibers." These skeletal and cardiac myofibers are named as such because although there is an absence of dystrophin, with time, they may

develop dystrophin expression. The presence of revertant fibers does not alter the disease phenotype very likely because of their low abundance. It is thought that the acquisition of dystrophin expression in dystrophin-deficient myofibers is due to epigenetic alterations that involve splicing of the dystrophin mRNA. The degree of reversion, muscles involved, and age at which it is noted vary between *mdx* strains. For example, *mdx* and *mdx*^{2cv} strains (which have point mutations in exon 23 and intron 42, respectively) have roughly 10x more revertant fibers than *mdx*^{4cv} and *mdx*^{5cv} mice (which have point mutations in exons 53 and 10, respectively) [19].

Because the disease phenotype of the *mdx* mouse is less severe than what is observed in people with DMD, additional mouse models have been created, including mice deficient in both dystrophin and utrophin, a double knockout (dko) model. Utrophin is a paralog of dystrophin that is found at the neuromuscular junction and can redistribute to the sarcolemma. Because utrophin and dystrophin have complementary roles in muscle function and development, it was hypothesized that utrophin could compensate for the lack of dystrophin in DMD. In confirmation of this notion, dko mice develop a disease phenotype that resembles what is seen in DMD patients (i.e., progressive muscular dystrophy, premature death, and cardiomyopathy) [20, 21]. Because DMD patients often develop osteopenia, fractures, and scoliosis, the dko mouse model has also proven useful in the study of DMD-associated premature musculoskeletal aging. In addition to the previously described muscular changes, they also develop degenerative changes in bone, articular cartilage, and intervertebral discs [22].

The *mdx* mouse has more recently been crossed to multiple genetic backgrounds, including the albino, BALB/c, C3H, C57BL/6, C57BL/10, DBA/2 and FVB strains, and various immune-deficient strains (including nude and SCID mice). The resultant phenotypes vary significantly but offer opportunities to study different disease aspects. For example, the DBA/2-*mdx* mice develop pronounced muscular weakness, decreased muscle weight, more fibrosis, and less regeneration when compared to other *mdx* crosses [23]. Crosses with immune-deficient strains may prove useful for transplantation studies evaluating the effect of donor cells on skeletal muscle regeneration.

Using a C57BL/6 background strain, four chemical variant (cv) *mdx* strains have been created via *N*-ethyl-*N*-nitrosourea (ENU) chemical mutagenesis (*mdx*^{2cv}, *mdx*^{3cv}, *mdx*^{4cv}, and *mdx*^{5cv}). Although each of these model strains results from a different point mutation, the outward phenotypes are similar to that of the *mdx* mouse [24, 25]. Even though the cv strains do not develop a severe disease phenotype, each is useful for the study of specific aspects of DMD pathogenesis. For example, the *mdx*^{3cv} mice express ~5% of a nearly full-length dystrophin protein and have increased neonatal mortality [25]. When compared to the *mdx* or *mdx*^{2cv} models, the *mdx*^{4cv} and *mdx*^{5cv} strains have a tenfold reduction in revertant fibers [19].

Although uncommon, spontaneous dystrophin deficiency has been identified in domestic shorthair cats. In cats, deficiency results in hypertrophic feline muscular dystrophy, a disease which is characterized by substantial glossal and diaphragmatic hypertrophy with resultant esophageal occlusion. Histopathologic findings typically include fiber size variation, fiber hypertrophy, foci of mineralization, fiber

splitting, nuclear centralization, and minimal endomysial fibrosis [9, 26, 27]. Although clinical signs of heart disease develop infrequently, gross, histopathologic, and imaging studies have revealed that affected cats do develop myocardial hypertrophy [28].

Spontaneous dystrophin deficiency is much more commonly diagnosed in dogs than in cats, likely owing to the severe disease phenotype that can occur in this species. Table 3.1 lists known affected breeds (both spontaneous and experimentally derived) and their associated mutations (if known).

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

Table 3.1 Dystrophin-deficient dog breeds and their associated mutations

Breed	Mutation	Comments
Golden retriever	Point mutation in intron 6 [29]	Original model, some revertant fibers
German shorthaired pointer	Deletion of entire dystrophin gene [30]	
Rottweiler	Point mutation in exon 52 [31]	
Labrador retriever	Insertion in intron 19 [32]	
Alaskan malamute	Undetermined [33]	Typical clinical signs, elevated CK, and abnormal EMG
Australian labradoodle	Nonsense mutation in exon 21 [34]	
Old English sheepdog	Undetermined [35]	
Grand Basset Griffon Vendéen	Undetermined	
Norfolk terrier	Undetermined [36]	
Tibetan terrier	Exon 8–29 deletion [37]	
Japanese Spitz	Inversion disrupts the dystrophin and retinitis pigmentosa GTPase regulator gene [38, 39]	
Cavalier King Charles spaniel	Point mutation in intron 50 [40]	
Beagle	Point mutation in intron 6 accepter splice site [41, 42]	CXMD and CXMDJ models
Pembroke Welsh corgi	Repetitive element-1 [LINE-1] insertion in intron 13 [43]	Outbred to beagle
Cocker spaniel	Deletion in exon 65 [37]	

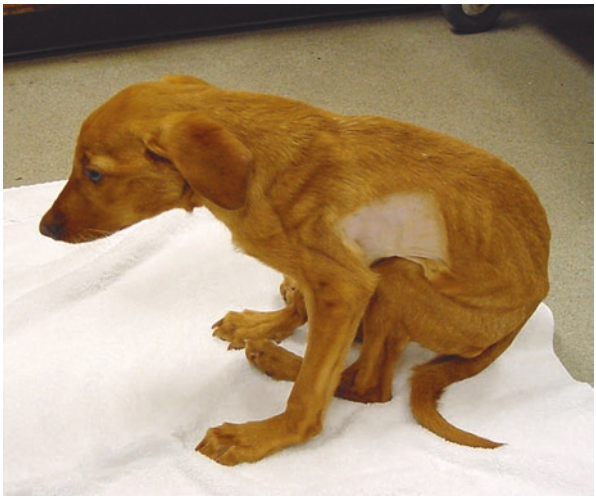

Fig. 3.1 A photograph of an affected male yellow Labrador retriever dog at 5 months of age. This dog shows severe muscle loss and fibrosis, kyphosis, weakness, and hyperextension of the carpus and tarsus. This represents the extreme of this phenotype

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. 3.1). 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 others can survive into adulthood. However, the "longer-lived" affected dogs show obvious 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 are inability to eat, recumbency, respiratory disease secondary to compromised respiration, and heart failure.

The golden retriever was the first canine model of DMD (named GRMD), the first model in which the mutation was identified, and has subsequently been the most extensively studied. GRMD is caused by a point mutation in the splice acceptor site of intron 6, resulting in elimination of exon 7 and a premature stop codon in exon 8 [29]. As is the case in humans with DMD, phenotypic variability occurs in GRMD. Although severity and age of onset can differ, affected dogs completely lack the dystrophin protein and develop both skeletal and cardiac abnormalities. Common clinical signs include decreased life-span, stunted growth, a plantigrade stance, ptyalism, and lordosis. Histologically, affected muscles have foci of degeneration, regeneration, mineralization, and fibro-fatty infiltration. Revertant fibers are also occasionally seen in this model, owing to alternative splicing out of exons 3–9 or 5–12 [44]. As is the case with the other canine models, euthanasia is often opted for due to an inability to eat, prolonged recumbency, and secondary respiratory and/or heart failure.

Because the GRMD model has been most rigorously studied, most preclinical studies in dystrophin-deficient dogs have been completed in this model. Earlier

interventions focused on plasmid- and vector-based gene therapies. For example, Howell and colleagues successfully used adenovirus-mediated dystrophin minigene transfer in GRMD dogs to accomplish high-level dystrophin expression in affected skeletal muscles [45]. Various cell-based therapies have also been pursued over the last 25 years, with myoblast transplantation studies leading the way. Although it sounded promising, significant implantation of myoblasts was not able to be achieved in the GRMD model [46, 47]. During roughly the same time period, myoblast implantation clinical trials were conducted in DMD patients; the results were unimpressive. The treated patients lacked functional gains, even though some donor cells could be detected in skeletal muscles [48–50]. More recently, pharmacologic interventions for the treatment of DMD have been explored using the GRMD model. One study showed that chronic infusion of a membrane sealant (a poloxamer) resulted in effective membrane stabilization with reduced myocardial fibrosis, decreased cardiac troponin I and brain natriuretic peptide elevations, and a lack of left ventricular remodeling [51]. Although the GRMD model has thus far been the focus of DMD-associated canine model research, further investigation of other dog breeds with dystrophin deficiencies may also prove useful in the study of DMD and the development of therapeutic strategies.

Animal husbandry in colonies of DMD dogs can be challenging. Dystrophic puppies have a higher neonatal mortality rate than normal puppies. Enhanced survival of 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, 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.

3.3 Limb-Girdle Muscular Dystrophies

Limb-girdle muscular dystrophy (LGMD, also known as Erb's muscular dystrophy) is a broad term that encompasses a group of rare diseases that result in characteristic wasting and weakness in the proximal limb muscles (especially muscles of the upper

Table 3.2 Animal models of limb-girdle muscular dystrophies

Type	Species	GENE	Protein	Comments
LGMD1A	Mouse	*MYOT*	Myotilin	Transgenic mice express the myotilin T57I mutation
LGMD1B	Mouse	*LMNA*	Lamin A	Most severe phenotype seen with $Lmna^{GT-/-}$
LGMD1C	Mouse	*CAV3*	Caveolin 3	Cav-3 P104L Tg and Cav-3 (−/−) null mice
LGMD2A	Mouse	*CAPN3*	Calpain 3	C3KO mice have reduced muscle mass and histologic evidence of muscle damage
LGMD2B	Mouse	*DYSF*	Dysferlin	Spontaneous disease in SJL/J and A/J strains
LGMD2C	Mouse	*SGCC*	γ-sarcoglycan	Cardiomyopathy and death by 20 weeks of age
LGMD2D	Mouse	*SGCD*	α-sarcoglycan	Progressive muscular dystrophy
LGMD2E	Mouse	*SGCE*	β-sarcoglycan	Skeletal muscle endomysial fibrosis
LGMD2F	Mouse	*SGCF*	δ-sarcoglycan	Skeletal and cardiac muscle involvement
	Hamster			Bio14.6, TO-2, J2N-k, and UMX7.1
LGMD2C-F	Dog	Unknown		Clinical myopathy and altered sarcoglycan expression; exact mutation(s) unknown
LGMD2H	Mouse	*TRIM32*	Trim-32	T32KO mice have myogenic and neurogenic disease components
LGMD2I	Mouse	*FKRP*	Fukutin-related protein	Multiple models that develop varying neural, ocular, and muscular defects

arms, shoulders, thighs, and pelvic girdle). Clinical signs, severity, and rate of progression can vary dramatically between individual patients. Most cases of LGMD are inherited in an autosomal recessive pattern (subclassified as LGMD2). Less commonly, LGMD may be inherited in an autosomal dominant pattern (subclassified as LGMD1). LGMD1 and LGMD2 can be further characterized based on the affected gene and locus (e.g., LGMD1C is resultant from mutations in the *CAV3* [caveolin-3] gene). A variety of transgenic mice have been developed to study the different forms of LGMD [52]. Research efforts in murine animal models have resulted in the development of multiple therapeutic interventions which have entered the clinical trial stage. Specific subtypes of LGMD, relevant animal models, and information on drug discovery/development are reviewed in the following subsections. Current animal models of limb-girdle muscular dystrophy are shown below (Table 3.2).

3.3.1 Myotilinopathies

Myotilin is a structural Z-line protein that plays a critical role in sarcomeric assembly and structural support of contracting myocytes. Myotilinopathies result from mutations in the highly conserved myotilin gene (*MYOT*); the consequent spectrum of diseases includes LGMD1A, myofibrillar myopathy, spheroid body myopathy,

and distal myopathy. LGMD1A is the best characterized and will therefore be the focus of this discussion [53].

LGMD1A has an autosomal dominant pattern of inheritance, and its prevalence is less than 1/1,000,000. It is characterized by adult-onset limb-girdle weakness and dysarthric speech patterns. Histopathologic findings in affected muscles include fiber size variation, fiber splitting, vacuolar change, and foci of Z-line streaming [53, 54]. The disease is so rare that it has only been reported in three families (German, Argentinean, and Turkish ancestries) and in one person of Japanese ancestry [55–58]. In 2006, a transgenic mouse model was generated to express mutant myotilin under the control of the human skeletal actin promoter. The mice develop similar muscular weakness and histopathologic changes in myofibers [59]. Garvey and colleagues subsequently produced a second LGMD1A mouse model by overexpressing myotilin. To do this, they crossed wild-type and mutant transgenic mice. They found that when compared to single-transgenic mutant mice, the double-transgenic mice that overexpressed myotilin had similar, but more severe, skeletal muscle pathology. They concluded that therapeutic modalities designed to lower myotilin levels in LGMD1A patients might be effective in ameliorating the clinical signs [60]. More recently, RNA interference (RNAi) technologies have been utilized to address LGMD1A. To do this, researchers developed adeno-associated viral vectors (AAV) with microRNAs targeting mutant myotilin. The result was a significant reduction in mutant myotilin mRNA and soluble protein expression in the muscles of TgT57I mice. RNAi-mediated gene silencing also resulted in improvement of clinical signs and less severe microscopic myofiber alterations [61]. Thus far, the myotilin overexpression mouse model proves to be invaluable for the study of LGMD1A, but further investigations are necessary to better characterize the disease pathogenesis and to develop therapeutic interventions.

3.3.2 Laminopathies

As the name suggests, lamins are the major components of the nuclear lamina. They are necessary for appropriate nuclear architecture, help anchor nuclear proteins, and aid in cellular signaling processes. Lamins A and C are the major alternative splicing variants of the LMNA gene. In 1999, studies of familial genetic linkage in people (of French pedigree) with autosomal dominant Emery-Dreifuss muscular dystrophy (AD-EDMD) found a strong positive LOD score at an 8-cM locus on chromosome 1q21–1q23. All affected subjects were shown to have mutations in *LMNA*, which is located within this locus [62]. Mutations have also been found to be responsible for two other diseases which clinically resemble AD-EDMD: limb-girdle muscular dystrophy with cardiac conduction disturbances (LGMD1B) and dilated cardiomyopathy with conduction defects (DCM-CD) [63, 64].

In order to study the human diseases associated with *LMNA* mutations, mouse models have been developed. Depending on the deletion alleles, mice with *LMNA* mutations have postnatal lethality at either 16–18 days or 4–8 weeks. Typically, these

mice have defects in both cardiac and skeletal muscles, similar to what is observed in people with LGMD1B. Homozygous knockout mice develop gait abnormalities, splayed hind limbs, and decreased strength. Heterozygous knockout mice can develop atrial and ventricular arrhythmias, atrioventricular conduction anomalies, and dilated cardiomyopathy [65, 66]. More recently, a novel *LMNA* germline knockout mouse ($Lmna^{GT-/-}$) has been developed using gene trap technology. This new mouse model is important because it produces a severe mutant phenotype which includes growth retardation, cardiac anomalies (excluding dilated cardiomyopathy), abnormal myocyte hypertrophy, and decreased subcutaneous adiposity [67]. Although none of the mouse models perfectly recapitulate LGMD1B, each one allows us to better understand the disease and potentially develop therapeutic interventions.

3.3.3 Caveolinopathies

Caveolin-3 gene (*CAV3*) mutations can result in a broad spectrum of clinical syndromes (caveolinopathies) and phenotypes, which include limb-girdle muscular dystrophy type 1 C (LGMD1C), rippling muscle disease, distal myopathy, long QT syndrome 9, etc. [68]. In general, caveolins are membrane proteins that aid in the formation and maintenance of caveolae, plasma membrane invaginations that play an integral role in cellular signal transduction and vesicular transport. Although caveolins are found in many different cell types, Caveolin-3 is the only caveolin found in striated muscle (i.e., skeletal muscles and the heart).

Because we are focusing on limb-girdle muscular dystrophies in this section, we'll concentrate on animal models used for the study of LGMD1C. To date, only mouse models are utilized, and it wasn't until recently that the first model of LGMD1C was created by expressing the CAV3 P104L mutant in murine skeletal muscle tissue as a transgene. Although the transgenic mice have a myopathic phenotype that resembles human disease, the underlying muscle damage is more severe. Histologically, muscular changes are characterized by fiber size variations, myofiber atrophy, centralized nuclei, and increased amounts of endomysial connective tissue [69]. Caveolin-3 null mice, which lack expression of caveolin-3 in skeletal muscles, have also been developed. These mice do not have overt clinical signs of disease; however, they do develop microscopic changes in the skeletal muscles which include areas of myofiber necrosis and variation in fiber size. Similar to what occurs in people with LGMD1C, Cav-3 null mice also develop T-tubule system abnormalities. Cardiac abnormalities vary in this mouse model—one study reports a lack of gross and histopathologic cardiac findings, while another reports on the presence of a progressive cardiomyopathy, characterized by cardiac hypertrophy, dilation, and reduced fractional shortening [70, 71]. It has also been noted that Cav-3 null mice have normal expression levels of dystrophin and the DAPC, but that these components have abnormal localization within the cholesterol-sphingolipid rafts/caveolae [71]. Although further characterization of these mouse models may be necessary, they are potentially useful for the understanding of LGMD1C and other caveolinopathies.

3.3.4 Calpainopathies

Calpain 3 functions as a protease to cleave various cytoskeletal and myofibrillar proteins. It plays an important role in a number of processes including muscle remodeling, myocyte differentiation, sarcomere formation, cytoskeletal rearrangements, and apoptosis [72, 73]. In humans, there are three calpainopathy phenotypes that comprise LGMD2A, which vary depending on the distribution of muscle weakness and age of onset. They include pelvifemoral limb-girdle muscular dystrophy (Leyden-Möbius LGMD), scapulohumeral LGMD (Erb LGMD), and hyperCKemia. Histologically, patients with mild or preclinical LGMD2A have minimal muscular changes, with the exception of small foci of myonecrosis. In these patients, the diaphragm and the soleus muscles are the most severely affected.

Calpain 3 knockout (C3KO) mice have been used to study LGMD2A. They are typically smaller than age-matched wild-type mice, but they are viable and fertile. Grossly, they have reduced muscle mass that corresponds to a reduction in both fast- and slow-type myofibers. Atrophic myofibers contain small foci of necrosis with inflammatory cell infiltrates and nuclear centralization that progresses with age. Electron microscopy has also demonstrated that affected myocytes lack normal sarcomeric organization [74, 75]. Thus far, the C3KO mouse model has proven to be invaluable for the understanding of LGMD2A pathophysiology.

3.3.5 Dysferlinopathies

The protein dysferlin plays an important role in the process of membrane repair, intracellular vesicular transport, and in myocyte T-tubule development. Limb-girdle muscular dystrophy 2B (LGMD2B), Miyoshi myopathy (MM), and distal anterior compartment myopathy result from recessively inherited *DYSF* mutations. Both are associated with muscular weakness and atrophy, slow disease progression, and symmetric involvement of the limb-girdle muscles.

Two mouse strains, SJL/J and A/J, have a long history as models for various non-neoplastic and neoplastic processes. They are also spontaneous models for LGMD2B. SJL mice have a splice site mutation that results in removal of the *DYSF* C2E domain. The A/J mouse model has an ETn retrotransposon insertion near the 5’ end of the *DYSF* gene [76]. Although skeletal muscle lesion distribution differs between the two mouse models, histopathologic findings include myofiber degeneration and necrosis, myofiber size variation, myofiber atrophy, inflammation (predominantly macrophages), centronuclear fibers, and fibro-fatty infiltration [76, 77]. Because these models have clinical heterogeneity and a general lack of muscular weakness, they are generally only employed for the study of therapeutic interventions via histopathologic evaluation [78].

3.3.6 Sarcoglycanopathies

Sarcoglycanopathies are caused by mutations in any of the four sarcoglycan genes (α, β, γ, or δ). The resulting diseases are categorized as LGMD2D (α-sarcoglycanopathy), LGMD2E (β-sarcoglycanopathy), LGMD2C (γ-sarcoglycanopathy), and LGMD2F (δ-sarcoglycanopathy). The sarcoglycans form a sarcolemmal complex that interacts with the DAPC; this interaction functions to stabilize the plasma membrane cytoskeleton. Although the mutations vary, all of the sarcoglycanopathies result in muscular dystrophy that typically manifests in childhood.

The predominant animal model for LGMD2D is the *Sgca*-null mouse. Although these mice do not develop overt clinical signs of a myopathy, they do develop muscular dystrophy characterized by myonecrosis that progresses with age, a hallmark of human LGMD2D. Other findings in this model include loss of sarcolemmal integrity, elevated serum levels of muscle enzymes, and alterations in absolute contractile force [79].

LGMD2E is interesting because affected patients develop cardiomyopathy in addition to skeletal muscle disease. Patients are usually asymptomatic until late childhood; clinical signs of cardiac involvement typically parallel the development of skeletal myopathy [80]. Similar to the human pathology, the *Sgcb*-null mouse develops significant endomysial fibrosis in skeletal muscles. Recently, β-sarcoglycan gene transfer has shown to be a promising therapeutic intervention by decreasing fibrosis and restoring muscular force in LGMD2E mice [81].

Similar to LGMD2E, LGMD2C usually appears in childhood (around 6–8 years of age). As is typical of the muscular dystrophies, clinical severity can vary, and some patients may be wheelchair bound by 12–16 years of age. LGMD2C can be difficult to distinguish from Duchenne muscular dystrophy because symptomology often overlaps and includes hypertrophy of the calves, macroglossa, cardiomyopathy, and respiratory disease [82, 83]. γ-Sarcoglycan-deficient mice have been produced by homologous recombination to aid in the study of LGMD2C. These mice develop muscular dystrophy in early life characterized by myocyte membrane defects, myocyte degeneration, and abundant apoptotic myonuclei. Typically by the time they are 20 weeks old, they develop cardiomyopathy (both dilated and hypertrophic forms occur) and premature death [84].

It wasn't until 1996 that a single nucleotide deletion in the δ-sarcoglycan gene was proven to be the cause of LGMD2F in people. Clinical signs typically occur in childhood and include wasting of the proximal muscles in the upper and lower extremities, decreased muscle strength, calf hypertrophy, and toe walking [85, 86]. Both hamster and mouse models have been used for the study of LGMD2F (δ-sarcoglycanopathy). An inbred line of Syrian hamsters (Bio14.6) develop a spontaneous myopathy with both skeletal and cardiac muscle involvement. Not only do they have reduced δ-sarcoglycan expression, but they also have a secondary reduction of the other three sarcoglycan proteins. Changes in the heart consist of myocardial hypertrophy and myofiber necrosis; changes in the skeletal muscles are characterized by myocyte degeneration and necrosis [87]. Although people with LGMD2F don't typically

develop cardiomyopathy, the hamster model has been useful for the study of skeletal muscle changes associated with LGMD2F. In 1998, researchers showed long-term expression of delta-sarcoglycan and rescue of the sarcoglycan complex in the Bio14.6 hamster using a recombinant *SGCD* adenovirus [88]. Histologically, treated hamsters lacked histologic evidence of muscular dystrophy and had restored plasma membrane integrity [88]. Although the Bio14.6 hamster is the most commonly used hamster model, other hamster models, including TO-2, J2N-k, and UMX7.1, have also been used in the study of LGMD2F [89–91]. Recently, several groups showed excellent rescue of LGMF2F in various hamster models using AAV-mediated gene therapy.

Two transgenic mouse models (one on a C57BL6 background and the other on a 129SvJ/129SvEms- $+^{Ter}$/J background), both of which develop skeletal and cardiac abnormalities, have also been utilized to better understand the pathophysiology of LGMD2F. These *Sgcd*$^{-/-}$ mice on a 129SvJ/129SvEms- $+^{Ter}$/J background develop a more severe phenotype which includes premature death (50% survival at 28 weeks), myocyte degeneration, regeneration, and fibrosis and cardiac changes that are evident by 12 weeks of age [84, 92]. In addition to the AAV vector therapies which were previously mentioned, myosphere-derived progenitor cells (MDPCs) have been shown to enhance neoangiogensis and restore δ-sarcoglycan expression in the vasculature of *Sgcd*$^{-/-}$ mice [93]. Thus far, as with AAV gene transfer, these therapeutic strategies are promising, but require further development to be acceptable to the human immune system.

Although extremely rare, sarcoglycan deficiencies are occasionally reported in dogs, including a Boston terrier, cocker spaniel, Chihuahua, and most recently, a Doberman pinscher. In all cases, clinical signs have included failure to thrive, loss of body condition, and exercise intolerance. Affected dogs also had markedly elevated serum creatine kinase, electromyographic abnormalities, and histologically evident skeletal muscle damage (i.e., varying fiber types, degeneration, necrosis, regeneration, and mineralization) [94, 95]. Although the specific mutations have not been identified, dogs represent the only intermediate animal model to date and therefore present a novel opportunity to study potential LGMD2F therapeutic interventions.

3.3.7 TRIM32 Mutations

Limb-girdle muscular dystrophy type 2H (LGMD2H) is an autosomal recessive myopathy characterized by proximal muscle weakness and facial muscle wasting. It is caused by mutations in the gene encoding tripartite motif-containing protein-32 (TRIM32), an E3 ubiquitin ligase that functions in the maintenance and degradation of myofibrils during remodeling. Four known *TRIM32* mutations are linked to LGMD2H. A fifth *TRIM32* mutation is associated with a disparate, multisystemic oligogenic disorder known as Bardet-Biedl syndrome type 11. To date, LGMD2H has been reported in Hutterite and non-Hutterite European populations. Affected patients exhibit slowly progressive proximal muscle weakness, muscular wasting,

and respiratory weakness that typically manifests in middle age. They also have abnormal electromyograms and histologic evidence of skeletal muscle damage (including rounded muscle fibers, centralized nuclei, and vacuolar change). Some of the patients also have a neurogenic component to the disease, characterized by paresthesia, paresis, and hypoactive tendon reflexes [96, 97].

TRIM32 knockout (T32KO) mice have been developed in order to better understand the pathophysiology of LGMD2H. Clinically, the mice develop skeletal muscle weakness. Histologically, skeletal myofibers have foci of fiber splitting, ring fibers, angulated fibers, variation in fiber size, and internal nuclei. Interestingly, neural tissue from T32KO mice has fewer neurofilaments and smaller myelinated motor axons. Because T32KO mice develop a disease with both myogenic and neurogenic components (similar to the human disease spectrum), they are a promising tool for future LGMD2H research and therapeutic developments.

3.3.8 Fukutin-Related Protein Mutations

Fukutin-related protein (FKRP) is present in various tissue types but is particularly abundant in skeletal muscles, the heart, and the brain. FKRP is found in the Golgi apparatus and functions to glycosylate α-dystroglycan (part of the DAPC). *FKRP* mutations are associated with Walker-Warburg syndrome (associated with skeletal muscle, neural, and ocular abnormalities), congenital muscular dystrophy type 1C (associated with skeletal muscle weakness, brain abnormalities, and intellectual disability), and LGMD2I. LGMD2I is characterized by proximal limb-girdle weakness, winging of the scapulae, abdominal muscle weakness, waddling gait, calf hypertrophy, cardiomyopathy, and respiratory difficulties [98, 99].

To better understand the pathogenesis of LGMD2I, multiple mouse models have been developed. The FKRP-Neo^{Tyr307Asn} mouse has a missense mutation and a neomycin cassette. Homozygous mice with this mutation die perinatally and have decreased levels of FKRP transcripts. They also have a reduction in the laminin-binding epitope of α-dystroglycan in skeletal, ocular, and neural tissues. Histologically, skeletal muscles have foci of edema and α-dystroglycan hypoglycosylation. A similar model has the FKRP^{Tyr307Asn} mutation but lacks the neomycin cassette; as a result, it lacks phenotypic evidence of disease [100]. Since these first knock-in models were produced, additional mouse models for LGMD2I have been developed to better understand disease pathogenesis and circumvent the issue of early lethality. For example, RNA interference and AAV technology have been utilized to knock down *FKRP* expression via postnatal gene delivery. At 10 months postinjection, *FKRP* expression was reduced by roughly 50% using a single shRNA and by 75% using a dual shRNA cassette [101]. More recently, researchers have developed homozygous and compound heterozygous murine models with human mutations in the murine *FKRP* gene. The P448Lneo+ mutant mouse develops a severe dystrophic phenotype, while the E310delneo+ mutant mouse develops embryonic lethality. Interestingly,

P448Lneo+/E310delneo+ compound heterozygotes develop neural defects and severe muscular dystrophy [102]. Although phenotypically different, each of these mouse models offers insight into ocular and brain development, the manifestation of muscular dystrophy, and future drug discovery.

3.4 Summary and Future Direction

Although this chapter focused on animal models and their usage in the study of human dystrophinopathies and limb-girdle muscular dystrophies, there are certainly other human muscular diseases for which animal models are being utilized. Muscle disease research is likely to continue to grow for the foreseeable future. Technologies have advanced to the point that we can now generate a murine model for almost any known human mutation. Although more traditional knock-in and knockout models will continue to be of benefit, newer technologies such as RNAi, CRISPR/Cas9-mediated gene editing, and embryonic stem cell microinjection techniques will lead the way in murine model development. It will also continue to be advantageous to investigate intermediate animal models. Even though identifying spontaneous mutations and establishing a colony can be time-consuming and costly, these larger models (such as dogs) often show more similarities with the investigated human disease than do their murine counterparts. Additionally, the intermediate models typically have appropriate immune function, body mass that is closer to that of humans, and a history of outbreeding, which makes them a more realistic model for preclinical studies.

In recent years, pharmaceutical companies and contract research organizations have come under scrutiny because of promising preclinical results in animal models and subsequent clinical trial failure in people. Animal models for therapeutic development have been increasingly criticized for the perceived inability to predict drug efficacy, safety, and toxicity in human patients. This criticism is generally unfounded. Animal models are the basis for many medical discoveries and the advancement in the understanding of disease. One must remember that without appropriate interpretation, the predictive value of any given model system is useless. Moving forward, these potential pitfalls of animal model research can be ameliorated by the appropriate design and selection of animal models, a better understanding of the model that is being utilized, and a greater knowledge of the intervention that is being investigated.

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Chapter 4
Muscle Stem Cell Biology and Implications in Gene Therapy

Terence Partridge

Abstract As the most abundant tissue in the body, skeletal muscle is a challenging target for the would-be gene therapist, particularly in the context of a severe disease such as Duchenne muscular dystrophy (DMD), where the bulk of tissue is simultaneously being destroyed and regenerated. Such instability poses the problem of maintaining any potential therapeutic expression construct or genetically corrected nuclear information but offers the compensation of the prospect of using the repair mechanism itself as a vector for genetic material. The best-attested myogenic stem cell is the skeletal muscle satellite cell, a Pax7^{+ve} cell, sandwiched between the muscle fibre plasmalemma and the overlying basement membrane. However other cell types capable of myogenesis have been identified, lying outside the muscle basement membrane and, in the dormant state, not expressing Pax7; their place in muscle development and maintenance has yet to be definitively established. Two major unresolved problems for the strategy of direct intramuscular transplantation of muscle precursor cells are the massive necrotic loss of such cells when grafted into muscle and their poor migration within the recipient muscles. The main alternative approach involves grafting cells derived from pericytes or CD133-expressing cells isolated from muscle, which are shown to be distributable via the blood to widespread muscles where they extravasate, adopt a myogenic phenotype and repair diseased and damaged muscle fibres. This approach is subject to the problems that the cells are incompletely characterized, and their property of being distributable via the vasculature has not been widely reproduced.

Keywords Satellite cell · Muscle precursor cell · Stem cell · Myoblast transplantation · Myogenesis

T. Partridge (✉)
Children's National Medical Center, Washington, DC, USA
e-mail: TPartridge@childrensnational.org

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4.1 What Is a 'Stem Cell'?

Perhaps because the stem cell concept has entered biological science via two separate routes, it has acquired at least two different *personae* [1]. The paradigm arose originally from observations of the hierarchy of increasing cellular specialization, or restriction of potential function, that occurs during the generation of each specific tissue in the course of development from the fertilized ovum to the adult individual. Subsequently, it was discovered that grafts of cells from the bone marrow [2, 3] or epithelium [4, 5] could fully reconstitute these tissues, implying that some cells conserve remnants of the terminal stages of this developmental hierarchy. This notion of adult stem cells has become further broadened to cover most situations where there is evidence of cell proliferation within a distinguishable precursor cell population, as a component of tissue maintenance during adult life. In some cases this reaches the point where the term loses much of its original meaning. Indeed the concept behind the term 'stem cell' as a discrete cellular property begins to fail under detailed scrutiny, and we are left with an operational definition, a cell that is able to reconstitute a tissue or an element of a tissue within the microenvironment of that tissue [6, 7]. One uniting feature of these adult stem cell systems is the notion of perpetuation of 'stemness' within a subsection of the population by virtue of asymmetric cell division that endows the daughter cells with two different fate predilections. One perpetuates the stem cell properties. The second is, together with its progeny, committed to maintaining the function of the specific tissue. This overall binary outcome property is increasingly being recognized as an emergent phenomenon of a complex of interactions within the tissue as a whole, involving the participation of other cells and interstitial connective tissue together with cytokines of local and remote origins. This broad functional entity is commonly encapsulated within the term 'stem-cell niche'. It should be noted that the commercialization of the 'stem cell' has further loosened, some would say corrupted, this definition, and in many cases, what is sold on the international market as a 'stem cell' is not specifically associated with any identified differentiated tissue.

4.2 What Is a 'Muscle Stem Cell'?

From the early 1960s until recent years, the skeletal muscle stem cell was identified, with some confidence as the satellite cell, first identified by Mauro [8] on extrafusal muscle fibres and Katz [9] on intrafusal fibres, a confidence founded, in part, on lack of firm contradictory evidence. This, in turn, is attributable largely to the technical difficulty of experimentally linking the electron-microscopic characterization of the satellite cell, as a small quiescent cell sandwiched between the plasmalemma of the muscle fibre and its basement membrane, to myogenic functional qualities that involve activation, proliferation and migration [10]. Confirmatory evidence of a central myogenic role of the satellite cell began to arrive with development of

antigenic markers and, more recently, of reporters of genes that characterize satellite cell determination and function. The most influential of these is Pax7 [11], which is as close as we get to a 'gold standard' for identification of this cell type. Intriguingly, in the mouse, absence of PAX7 has little or no impact on prenatal myogenesis; it is required for postnatal myogenic function [12, 13]. However, discoveries of new myogenic stem cell markers, far from solidifying the status of the satellite cell, have led to a picture of increasing complexity, with the regular unveilings of new myogenic stem cell candidates. These cells are generally distinguished from satellite cells by lack of Pax7 expression in their quiescent state and their position outside the muscle fibre basement membrane (Fig. 4.1). But where their course to myogenic differentiation has been fully investigated, they are seen to pass through a satellite cell-like phase where they do express Pax7 [14–18].

Use of Pax7 as a driver of CreERT constructs designed to ablate Pax7-expressing cells conditionally upon treatment with tamoxifen has shown that such cells are essential for regeneration of limb muscles [19–21]. While these experiments fall short of showing that Pax7^{+ve} cells are the only participants in regeneration, the fact

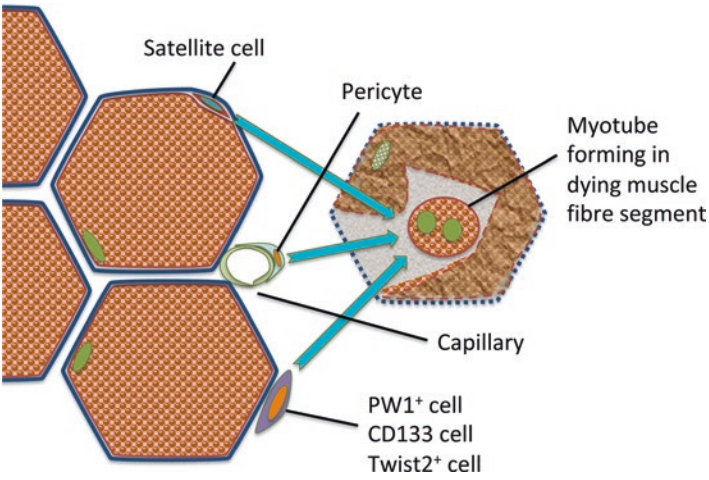

Fig. 4.1 Skeletal muscle shows very swift and competent regeneration when damaged by either externally administered injury or as a result of endogenous defects in genes encoding functionally important proteins such as dystrophin in Duchenne muscular dystrophy. The best characterized source of reparative cells is the satellite cell, defined by its position between the muscle fibre plasmalemmal surface and the overlying basement membrane and by its expression of the Pax7 gene in the quiescent state. However, other sources of myogenic precursor have also been identified that are located outside the muscle fibre basement membrane and that do not express Pax7 except when activated into the myogenic pathway. Of these, the pericyte is best characterized by its location around the microvessels. This shows very similar functional properties to the CD133^{+ve} cells, in that both are reported to be deliverable to muscle by intravascular injection. PWI^{+ve} cells lying in the interstitium have also been demonstrated to exhibit myogenic capacity as have the more recently described Twist2^{+ve} cells which are shown to contribute preferentially to the regeneration of the fast type IIb/X fibre type. The normal functions of these various Pax7^{-ve} classes of cells have yet to be fully elucidated

that ablation of Pax7^{+ve} cells extinguishes any significant regeneration brings into question the normal roles of the various Pax7-negative cells that have been shown to display myogenic function. It suggests that, at the least, they require the company of Pax7^{+ve} cells to participate in effective myogenesis in vivo.

Even within the conventional myogenic satellite cell category, attempts at comprehensive molecular characterization run into the problem that, for much of the head musculature, the myogenic participants pass through different developmental pathways from that of the main limb and trunk muscles [20]. A recent report that the postnatal satellite cells of the posterior region of the body of the mouse are formed by a distinct migration of myogenic precursors, temporally separate from those that formed the very same fibres on which these satellite cells come to reside [22], adds further to the conundrum of constructing a coherent and unifying picture of the myogenic stem cell.

This raises a more general question of myogenic stem cell taxonomy; can we define a myogenic stem cell as a property intrinsic to the cell itself, as opposed to being an emergent property of a responsive cell within a facilitative environment (or niche)? The second view is supported by the contrast between results of heterotopic grafting of suspensions of myogenic cells between developmentally distinct muscles and those obtained when pieces of muscle are heterotopically grafted. Extraocular muscle-derived satellite cells sustain robust muscle regeneration when grafted into damaged limb muscles [23] but lose their characteristic extraocular pattern of gene expression [20], whereas regenerates from strips of jaw muscle grafted into the anterior tibial site retain expression of the myosins that typify this muscle in its original site [24]. This difference would argue for a strong influence of the local tissue environment on the differentiation pathway followed by the satellite cells, a factor that must be taken into account in any attempt to make use of these cells as vectors or to persuade them to behave in a specified way.

A uniting theme might be that the body is permeated by versatile cells whose options include the ability to differentiate into muscle under the influence of appropriate signals. Within such a scheme, the satellite cells would be viewed as those that have followed one of a particular range of definitive pathways that put them into the condition that has come to be regarded as the classical satellite cell, expressing Pax7, and positioned beneath the muscle fibre basement membrane.

4.3 Practical Utility of Muscle Stem Cells

While developmental biologists have been preoccupied with the lineage relationships of the various myogenic cell categories, the gene therapy community has taken a more utilitarian point of view that is more concerned with their practical value; i.e. the ease with any given myogenic stem cell can be obtained, amplified in number, efficiently delivered into the cellular ecosystem of skeletal muscle and, once at such a site, the frequency and efficiency with which they undertake myogenesis. Of these, the question of delivery is a strong priority for all stem cell therapies but

especially so in the case of skeletal muscle, where the ideal would consist of dissemination of myogenic stem cells throughout the large mass of this tissue spread throughout the body. Thus far, direct intramuscular injection has yet to achieve the modest aim of dispersion over more than a few millimetres from the injection site. In response, there has been abiding interest in the idea of delivery to muscle via the vasculature, with the hope of accessing the entire musculature via this all-pervasive highway. It should be noted, however, that it would require the myogenic cells to negotiate the barriers of the microvascular endothelium and basement membranes as well as the muscle fibre basement membrane to attain contact with its target tissue.

Muscle stem cells, in the form of tissue cultured myogenic cells, were first explored by would-be therapists for their dual function as vectors to carry normal dystrophin genes into genetically dystrophic muscle fibres and, by direct participation, to boost the repair of those muscle fibres [25–44]. This approach, inspired largely by analogy with the haematopoietic model, originally addressed the idea of using myogenic precursors from normal donors but subsequently acquired the additional notion of using autologous myogenic cells in which the genetic defect is in some way repaired, modified or supplemented by a functional copy of the gene of interest [45, 46]. This second option was motivated primarily as a means of minimizing the problem of immune rejection of allogeneic donor cells. But, increased understanding on the sophistication of muscle stem cell biology and parallel advances in the development of techniques for tightly targeted genetic manipulation has shifted emphasis progressively towards the use of stem cells as vectors for carrying therapeutic genetic modifications that can be made efficiently ex vivo into regenerating muscles. Such protocols of ex vivo genetic correction, in combination with the transplantation of the corrected cells, are commonly viewed as intermediates on the road to direct genetic modification, in situ, of muscle cells or their stem cell progenitors in vivo, pending resolution of uncertainties about safety and efficiency of direct in vivo application of the techniques for genetic correction. At present, neither the direct in vivo approach nor the use of stem cell intermediaries has achieved standards of efficiency or well-validated safety that would commend them above adeno-associated virus (AAV)-mediated transfer of minimized dystrophin genes into fully differentiated muscle fibres.

4.4 Stem Cells as Vectors of Genetic Modification

Our therapeutic stem-cell aspirations are modelled on the archetypes of bone marrow/haematopoietic and the epidermal keratinocyte systems. Both possess advantages over most other tissues. They exhibit high cellular turnover sustained by what might be considered to be professional stem cells, so as to maintain a dynamic homeostatic status. This conspicuous maintenance activity underlies their discovery as the first adult stem cell systems and early exploitation for therapeutic replacement of the tissues in question. This pinpoints the major impediment to application

of stem cell transplantation therapies to other candidate targets, none of which have anything approaching the extent of homeostatic cellular turnover of the epithelial and haematopoietic systems.

For skeletal muscle, the fact that it regenerates rapidly and nearly faultlessly after traumatic injury offers some hope of beneficial stem cell intervention, but the question of effective and efficient delivery of myogenic cells into mature skeletal muscle remains a major bottleneck. An unexpected obstacle was, and is still, the rapid massive death of the transplanted cells [47–51], for reasons that have yet to be determined and for which no effective remedy has been discovered. Certainly, this loss increases disproportionately with the number of cells injected per site [52], but where the data permits calculation, even with the use of multiple site injections, the yield of muscle per injected stem cell does not rise far above parity [53]; i.e. the amounts of muscle produced are less than what would have arisen from direct conversion of each putative muscle stem cell into a myonucleus [54]. This compares poorly with the massive expansions seen with the classical stem cell systems underlying haematopoiesis from bone marrow grafts or skin replacement with keratinocyte precursors, again raising the question of general applicability, between different tissues, of the term stem cell. A significant expansion of grafted myogenic stem cells was seen only when they were grafted while still attached to muscle fibres [55, 56] or with a minimum of experimental manipulation [37, 57].

A second obstacle to the practical use of muscle stem cells is that of delivery to the large mass of skeletal muscle that we would wish to modify. For intramuscular injections, migration of myogenic cells is limited to a millimetre or so from the needle track [58, 59] and has only been extended to a maximum of 7 mm by extensive mechanical damage to the muscle neighbouring the graft site [60]. It is true that the syncytial nature of skeletal muscle permits diffusion of the gene product within the muscle fibres. Indeed this is held to account for the near-asymptomatic condition of many DMD carriers [61], but movement of dystrophin along the long axis of the muscle fibre, as detected by immunostaining, is limited in extent to some tens of microns from the nearest competent myonucleus [54, 62, 63]. Thus efficient widespread expression of this protein would be heavily dependent on efficient dispersion of the graft-derived myonuclei within the recipient muscle.

In response to the limited dispersion achievable from local intramuscular of myogenic cells, a number of attempts have been made to deliver myogenic stem cells via the blood circulatory system. Claims of impressive success are intermixed with less encouraging reports of this approach, with puzzling variability between the model systems employed and between investigators. The two strongest contenders for efficient vascular delivery of myogenic cells into muscle are cells associated with the microvascular or haematopoietic systems. One, originally termed the mesoangioblast, is now identified as a derivative of the pericytes [15, 64–66] that surround the arterial end of the microvasculature. This was first demonstrated to participate effectively in regeneration and reduce pathology in the α-sarcoglycan-null, dystrophic mouse [67] and subsequently in the GRMD dystrophic dog model of DMD [68] but was reported to be unsuccessful in the mdx

mouse [69]. A recent clinical trial of such pericyte-derived cells in Duchenne boys has reported trace levels of engraftment and, in one case, of dystrophin production [70]. Successful systemic delivery has also been reported for a cell identified by expression of CD133. Its relationship to the pericyte is uncertain, but it was shown to engraft successfully to widespread muscles in dystrophic dog [68, 71] and mouse [16] but again with contrary reports from some other laboratories [72]. Less encouraging results have also been reported of attempts at intravascular delivery of myogenic cells into non-human primates, with little or no integration into regenerating myofibres [73]. Further intricacy is added to the story by the report of a muscle-derived stem cell that, on intravenous delivery to dystrophic dogs, greatly ameliorates the clinical phenotype without producing commensurate amounts of dystrophin [74], a finding that has prompted an investigation of potentially beneficial effects of these 'MuStem' cells other than by restoration of dystrophin [75, 76].

Quite apart from the exploitation of endogenous myogenic cells, considerable excitement has been generated by the development of myogenic cells from pluripotent stem cells. Initially these were mainly of embryonic origin but with a progressive switch to induced pluripotent stem cells (iPSC). The latter have the twin virtues of avoiding the ethical constraints placed upon cells of embryonic origin and of being readily derivable from a variety of differentiated tissue cells from individuals carrying genetic defects of interest. The conversion of pluripotent cells to a myogenic phenotype has been accomplished by a number of means, most commonly by introduction of Pax7 [77] expression plasmids but also by serial exposure to a series of cytokines designed to simulate the normal in vivo developmental process [78]. This latter approach has great appeal as a practical route to the transplantation of genetically corrected autologous stem cells but does still require a radical improvement in the efficacy of myogenic cell transplantation.

A gene therapist's ideal would be to directly target resident satellite cells, in situ, with the therapeutic gene of interest and make use of its regenerative function to maintain and introduce the genetic modification into the muscle during subsequent repair. Such a strategy would require efficient transduction of the satellite cells which, at around 2% of fibre-associated nuclei in the mouse [79], constitute a rare target. In addition, to perpetuate the modification during proliferation, sufficient stability of expression would be required. AAV vectors have been found to integrate only rarely into the genome [80], but where AAVs have been used to deliver components of CRISPR/Cas9, there is some evidence of genetically transmitted modification of satellite cells, presumably reflecting the 'hit-and-run' nature of the mechanism [81]. Lentiviral vectors do integrate but are conventionally thought, like AAV vectors [82], to be unable to package a complete dystrophin expression construct. Recently, this view has been contradicted [83]. But, for direct use of such a vector for in vivo delivery, the questions of efficiency, accuracy of targeting and accompaniment by unforeseen side effects remain open [84].

Recent rapid developments of targeted CRISPR/Cas9-induced modification of the genome have triggered a new impetus to the use of stem cell vectors to effect rescue of the mutated DMD locus in the mdx mouse, by restoration of open reading

frame [81, 85–88]. This technology promises the dream scenario of engineering specific and tightly targeted rectification of genetic defects and faces us with the dilemma of whether to make such genetic alterations directly in vivo, with the attendant risks of off target or other unforeseen side effects, or to make the corrections, ex vivo, in stem or precursor cells, where, for muscle, ineffectual grafting remains the main barrier to success.

4.5 Stem Cell Function as a Target of Genetic Therapies

Implicit in the notion of using myogenic cells as vectors is the fact that they are able to form new muscle or to contribute to repair of existing muscle. The balance of interests for diseases such as Duchenne muscular dystrophy, where muscle repair falls into cumulative deficit, veers towards consideration of the tissue repair function of the stem cell itself as an important therapeutic target. The resulting interest in the prospect of combatting the loss of regenerative potency has been pursued by a number of means, including the expression of genes that may directly enhance satellite cell activity or may modify the environment to one that favours myogenic activity. Both IGF-1 overexpression [89] and inactivation of myostatin [90] have been shown to boost muscle size and resilience in mdx mice, but it has not been ascertained whether this is attributable to increased satellite cell activity or to increases in myonuclear domain. Attempts have also been made to enhance myogenic stem cell function by favourably modifying the local environment [91, 92] or by limiting the deposition of scar tissue [93–99]. The inflammatory pathways involving TGFβ or TNFα have also become favoured targets but with mixed results [100], while others have turned their attention to the systemic signalling systems that impinge on fibrogenic [101, 102] versus myogenic activities [85]. This is a research area where the mdx mouse is a far from ideal model, because it does not mimic the atrophic processes that characterize DMD [79, 103]. On the contrary, the mdx mouse shows no sign of muscle atrophy throughout the majority of its life; indeed its muscles are hypertrophic containing larger than normal numbers of muscle fibre nuclei and of satellite cells than the normal mouse [79]. Against such a hypertrophic background, it is difficult to identify beneficial levels of enhancement of regeneration induced by a test therapy or to determine the mechanisms whereby any hypertrophic effect is achieved. Balanced against this disadvantage is the time and cost of performing equivalent experiments in animals such as the GRMD dog, where the pattern of muscle loss more closely resembles that in DMD [104]. Recent reports of a marked atrophy when the mdx mutation is bred onto the DBA/2J background may provide a more useful model for testing methods of boosting myogenic stem cell function, but its value for translational purposes will depend on the extent to which its regenerative failure [105, 106] is shown to mechanistically parallel that seen in DMD.

4.6 Hopes, Aspirations and Hurdles

Our current position in gene therapy for genetic diseases of muscle lies at a point of balance between two alternative paths. On one side lies the application of vector biology and gene engineering to direct delivery of therapeutic genetic constructs directly into muscle in vivo. The alternative course of action is to profit from the higher levels of safety and efficiency of in vitro modification of the stem cells that would then be used to carry the corrections into the muscle fibres. Current literature does not provide us with sufficiently precise and reliable information to make this choice definitively. The dilemma is evident in its most acute form in Duchenne muscular dystrophy, where AAV vectors are currently seen as the most promising means of direct in vivo delivery into muscle. This virtue is counterbalanced by two big problems. First, AAV vectors are unable to carry a construct large enough to express the full-length dystrophin protein, necessitating the use of truncated versions of the gene. Second, rapid appearance of AAV-neutralizing antibodies gives no assurance of repeat delivery of AAVs. These issues, in combination, present a major hurdle. If the effect of the genetic intervention with a suboptimal truncated mini-dystrophin is not adequate to preserve muscle fibre integrity, then the resulting chronic loss of muscle fibres will lead to loss of the therapeutic construct that cannot be replenished, assuredly, by further administrations of the same AAV vector. This effect has been observed in mdx mouse, where an exon-skipping construct that elicits production of a truncated, partially functional dystrophin protein, that gave suboptimal protection of myofibres, was gradually lost during the following months [107]. One obvious response, development of a fully functional therapeutic construct, is an area where much progress has been made in the design of mini- or micro-dystrophin expression constructs [108–110] that perform well in dystrophic mice and dogs. But their use in man still carries a substantial element of the unknown, whose resolution would entail a thorough understanding of the relative functional properties of the various truncated dystrophin isoforms. Indeed, resolution of the immune neutralization of AAV by blockade or evasion of the immune response [111–113] would, at a stroke, give us an effective route to gene therapy for many genetic conditions of both muscle and other tissues.

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Chapter 5
Pluripotent Stem Cells for Gene Therapy of Hereditary Muscle Disorders

Thierry VandenDriessche, Yoke Chin Chai, Dimitri Boon, and Marinee K. Chuah

Abstract Stem cells and their myogenic derivatives offer unprecedented opportunities to treat degenerative muscular disorders by autologous or allogeneic cell-based therapy. This could be attributed to their self-renewal properties, their myogenic differentiation potential and their capacity to enhance muscle regeneration. In particular, different types of adult stem cells that participate in muscle regeneration have been explored for cell-based therapies of degenerative muscle disorders. Nevertheless, these adult stem cells cannot be expanded indefinitely due to cell exhaustion. To overcome this limitation, bona fide pluripotent stem cells could be used instead, such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells. They could be induced to differentiate into myogenic cells that contribute to muscle regeneration upon transplantation. Most importantly, patient-derived adult stem cells, ES and iPS cells, have been engineered by gene therapy, primarily using integrating vectors (with γ-retroviral, lentiviral or transposons). This allowed sustained expression of the therapeutic gene in the stem cells and their differentiated progeny. More recently, gene editing strategies have been explored (using either ZFNs, TALENs or CRISPR/Cas9) enabling site-specific gene correction. Proof-of-concept studies demonstrate the potential of gene-engineered adult or pluripotent stem cells for muscle regeneration in preclinical disease models, including Duchenne muscular dystrophy. Nevertheless, the overall efficacy of functional integration of gene-corrected myogenic cells into the degenerating muscle would need to be increased. In this review, we discuss some of the challenges that need to

T. VandenDriessche (✉) · M. K. Chuah (✉)
Department of Gene Therapy & Regenerative Medicine, Faculty of Medicine and Pharmacy, Vrije Universiteit Brussel (VUB), Brussels, Belgium

Center for Molecular & Vascular Biology, Department of Cardiovascular Sciences, University of Leuven, Leuven, Belgium
e-mail: thierry.vandendriessche@vub.be; marinee.chuah@vub.be

Y. C. Chai · D. Boon
Department of Gene Therapy & Regenerative Medicine, Faculty of Medicine and Pharmacy, Vrije Universiteit Brussel (VUB), Brussels, Belgium
e-mail: yoke.chin.chai@vub.be; dimitri.boon@vub.be

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be addressed in order to harness the full potential of gene-engineered patient-specific pluripotent stem cells for regenerative medicine.

Keywords Muscle stem cells · iPS · Gene therapy · Gene editing · Myogenic Muscular dystrophy · Duchenne

5.1 Introduction

The regenerative capacity of skeletal muscle is essential for tissue repair and can be attributed to the presence of resident adult stem/progenitor cells in the muscle tissue [1, 2]. Exhaustion and dysfunction of muscle stem/progenitor cells have been reported in several muscular dystrophies, which may contribute to the muscle degeneration [3–9]. Consequently, adult stem/progenitor cells are attractive to achieve muscle repair and regeneration in the context of degenerative muscle disorders, including muscular dystrophy [10]. Unfortunately, these adult stem cells have a limited regeneration capacity after in vitro expansion, mostly caused by dedifferentiation. To overcome this limitation, embryonic stem (ES) cells [11, 12] and induced pluripotent stem (iPS) cells [13, 14] have been explored by virtue of their unlimited self-renewal ability. Moreover, they can be coaxed to differentiate into myogenic cells capable of promoting muscle regeneration. We hereby review the state-of-the-art technologies and discuss the challenges that need to be addressed to develop a gene and cell therapy strategy for muscular dystrophy based upon pluripotent stem cells. We will focus specifically on the use of genetically modified myogenic cells derived from patient-specific iPS cells.

5.2 Myogenic Adult Stem/Progenitor Cells for Muscle Regeneration

Different types of myogenic adult stem/progenitor cells have been shown to engraft and differentiate into muscle fibres. Only the most salient features of some quintessential myogenic adult stem/progenitor cells are highlighted here. A detailed description of their properties falls outside the scope of this review and has been described elsewhere [10, 15].

In healthy individuals, adult skeletal muscle is capable of regeneration upon exercise, injury or disease, mainly due to the presence of resident adult stem/progenitor cells, particularly satellite cells (SCs) that act as the primary drivers of this regenerative capacity. In muscular dystrophy, the skeletal muscles typically undergo repeated waves of contraction-induced fibre damage followed by constant attempts in repairing fibre degradation by the resident SC pool [16]. Eventually, this high tissue turnover leads to exhaustion of these resident stem cell populations [8]. Consequently, patients progressively lose muscle regeneration capacity due to diminishing SCs. Consequently, restoring tissue regeneration by replenishing the

pool of functional muscle stem/progenitor cells represents a promising therapeutic modality for muscular dystrophy. The potential of SC-derived myoblasts for muscle regeneration in muscular dystrophy is supported by preclinical studies in dystrophic *mdx* mouse models [17, 18]. Consequently, several clinical trials had been initiated based on intramuscular transplantation of allogeneic SC-derived myogenic progenitors or myoblasts. Though dystrophin expression was apparent, it was mainly confined to the proximity of the injection site, accounting for the low therapeutic efficacy [19]. Several confounding variables may have contributed to this outcome, including limited cell engraftment, survival, migration and/or differentiation. This may have been compounded by possible immune rejection impeding long-term engraftment. Nevertheless, intramuscular injection of myoblasts may still have merit, particularly for the treatment of specific types of muscular dystrophy that manifest themselves predominantly in specific muscle groups. For instance, transplantation of autologous myoblasts in the pharyngeal muscles yielded promising results in patients suffering from oculopharyngeal muscular dystrophy, showing improvement in both swallowing and quality of life [20, 21].

Alternative adult myogenic stem/progenitor cells have been explored to circumvent the intrinsic limitations of these SC-derived myoblasts [10]. In particular, preclinical studies have shown that $CD133^+$ stem/progenitor cells have the ability to contribute to SC formation and muscle regeneration, paving the way to clinical studies in Duchenne muscular dystrophy (DMD) patients [22–24]. Alternatively, $PW1^+$-expressing interstitial cells contribute to the SC pool and are capable of generating new muscle fibre [25]. Improved therapeutic outcomes have also been reported by using so-called muscle-derived stem cells (MDSC) in dystrophic dogs via systemic delivery [26]. Additionally, skeletal muscle aldehyde dehydrogenase-positive ($ALDH^+$) cells have also been identified in endomysial space of human skeletal muscle with high myogenic capacities [27], capable of forming multinucleated myotubes. Interestingly, the $ALDH^+CD34^-$ cells were highly proliferative in vivo and contributed to muscle formation in immunodeficient mice. Skeletal muscle pericytes are equivalent to mesoangioblasts and represent another myogenic adult stem cell population that is normally associated with capillaries [28–30]. These mesoangioblast-like pericytes can promote muscle regeneration in dystrophic mice and dogs upon intraarterial transplantation [31–36]. These encouraging findings have led to a first-in-human phase I/II trial based upon intraarterial delivery of allogeneic mesoangioblasts in five subjects with DMD (EudraCT no. 2011-000176-33). Escalating doses of donor-derived HLA-matched mesoangioblasts (in the order of 10^9 cells) were transplanted intraarterially in DMD patients under immunosuppressive therapy. Donor DNA was detected in muscle biopsies of most of patients, albeit at low levels. Donor-derived dystrophin was detected in only one of the subjects. Overall, no functional improvements were observed, indicating that a much higher dose of cells and/or younger subjects with less advanced disease may be required to reach clinical efficacy [37]. The study was considered relatively safe although one subject developed a thalamic stroke with no clinical consequences and whose correlation with mesoangioblast infusion remained unclear.

5.3 ES and iPS Cells for Muscle Regeneration

ES cells are pluripotent stem cells that can be isolated from the inner cell mass of blastocysts during the early stages of embryonic development [11, 12, 38]. In contrast, iPS cells are pluripotent stem cells that are derived from normal somatic cells by expressing reprogramming factors (i.e. Oct4, Klf4, Sox2 and c-Myc) [39]. It is particularly encouraging that the ES- or iPS-derived myogenic cells show similar myogenic potential in vitro and regenerative capability in vivo [40]. This further supports the use of iPS cell-derived myogenic cells as an attractive alternative to ES cell derivatives for future clinical applications. iPS cells were originally generated through retroviral vector-mediated integration of the reprogramming cassettes encoding oncogenic factors. Hence, this original approach poses an intrinsic tumorigenic risk [41]. Recently, nonintegrating vectors, protein transduction or 'transgene-free' reprogramming method has been developed potentially as safer reprogramming alternatives [42, 43].

The main advantage of patient-specific iPS cells and their myogenic progeny is that immune rejection could in principle be avoided enabling stable autologous transplantation. In contrast, since ES cells are not patient-specific, autologous transplantation of ES-derived myogenic cells is not possible raising potential immune concerns. This is compounded by the increase in immunogenicity during differentiation of ES cells [44, 45] and possibly expression of major histocompatibility complex (MHC) class I molecules. However, even syngeneic mouse iPS cells can be rejected following transplantation in vivo into mice. This indicates that even autologous iPS cells and their myogenic derivatives can potentially be recognised by the immune system [46]. It is encouraging however that human iPS cell-derived mesoangioblast-like cells exhibit a reduced risk of evoking inadvertent immune responses, potentially mediated through the suppression of T-cell proliferation via the IDO- and PGE-2-dependent pathways [47]. Another advantage of iPS cells is that they circumvent the ethical concerns associated with the use of ES cells since they can be derived from virtually any somatic cells and consequently obviate the need for human embryos altogether. Moreover, the generation of iPS cells requires only a limited number of patient-derived cells, which could be obtained from minimally invasive skin biopsies, urine or blood [48, 49]. This avoids the need for relatively invasive procedures required to harvest sufficient cells, as in the case of adult stem/progenitor cells.

ES and iPS cells overcome the limitations of conventional adult muscle stem/progenitor cells by virtue of their indefinite in vitro expansion capability without compromising their 'stemness'. This attribute of ES and iPS cells makes them ideally suited for stem cell-based therapies of skeletal muscle disorders, since they can be expanded to large numbers, prior to inducing their differentiation into transplantable myogenic cells. Hence, safe and efficient myogenic differentiation protocols need to be established in order to produce sufficient myogenic progenitors of high regenerative capacity. Moreover, to ensure that ES/iPS cell-derived myogenic progenitor/stem cells function normally in vivo after transplantation, they would need

to optimally integrate with the skeletal myofibres and, ideally, within the satellite cell niche [50–52]. This niche will be important for controlling the self-renewal and differentiation of the myogenic ES/iPS cell progeny. Ideally, these myogenic progenitors should enable body-wide restoration of muscle function after systemic delivery. This would obviate the limitations associated with intramuscular cell delivery which is not a viable option to treat systemic muscle disorders due to the high number of required injections.

Myogenic differentiation of ES and iPS cells essentially attempts to replicate the normal myogenic differentiation in a developing embryo. Consequently, many of the factors known to play a role in myogenic differentiation during development have been explored to coax myogenic differentiation of ES and iPS cells. The various myogenic differentiation approaches of ES and iPS cells have been discussed in detail elsewhere [2] and will be summarised here. Myogenic differentiation of ES and iPS cells can be accomplished by specific coculture conditions, growth factor combinations, small molecules and even genetic engineering with genes encoding myogenic differentiation factors (including MyoD, Pax3 or Pax7). The desired differentiated myogenic cells could subsequently be enriched based on the expression of specific cell surface markers. Some of the potentially most attractive differentiation protocols that may facilitate ultimate clinical translation rely on serum-free, chemically defined conditions that do not require any genetic modification of the ES or iPS cells [53]. The efficacy of myogenic differentiation may also vary depending on the tissue of origin of the somatic cell from which the iPS was derived [54–57]. Nevertheless, robust skeletal myogenic differentiation protocols may potentially over-ride the effects of this ‘epigenetic memory’ on this differentiation bias [9].

Regardless of the differentiation method used, it is critically important to develop standardised protocols to obtain pure myogenic cell populations. Inadvertent transplantation of any undifferentiated ES or iPS cells should be prevented as this poses a significant risk of teratomagenesis. The incorporation of genetic safety switches (e.g. based on HSV-TK or iCASP9) that are specifically designed to eliminate any residual undifferentiated pluripotent stem cell may therefore be required to further enhance the overall safety of ES- or iPS-based strategies for muscle repair [58, 59]. It is particularly encouraging that transplantation of these ES- and iPS-derived myogenic cells in preclinical models showed engraftment, myogenic differentiation and expression of therapeutic transgenes, like dystrophin. Though functional benefits were reported in some of the transplanted mice, the overall therapeutic effects were limited since myogenic cells were typically injected intramuscularly instead of systemically. This is compounded by the clearance of the ES- or iPS-derived myogenic cells by the reticuloendothelial system. Moreover, robust body-wide phenotypic correction of muscular dystrophy in preclinical models has not yet been realised after transplantation of human ES- or iPS-based myogenic cells. However, xenogeneic models may not necessarily replicate all of the key features of human myogenic cell transplantation in a clinical setting. This is compounded by the fact that the interaction of the transplanted cells with the host micro-environment may result in species-specific differences in survival, migration and differentiation of iPS-derived myogenic cells. Once the overall robustness can be increased, it will be

possible to contemplate efficacy and safety studies in large animal disease models prior to human trials. Alternatively, synthetic scaffolds and decellularised devices from large animal models could ultimately be used to optimise the maturation, differentiation and engraftment of ES/iPS-derived myogenic cells towards possible clinical applications. Biomimetic 3D in vitro models of human skeletal muscle are currently being generated in an attempt to mimic the in vivo environment [60–62].

5.4 Genetic Modification of Pluripotent Stem Cells

The field of gene therapy is gaining momentum due, in part, to the development of improved gene transfer and gene editing technologies and a better understanding of its in vivo consequences. Consequently, this will greatly benefit the development of autologous patient-specific iPS-based cell therapies. This 'personalised medicine' approach requires multiple steps (Fig. 5.1). Patient-specific somatic cells are first harvested to generate iPS cells. Subsequently, these patient-specific iPS cells are induced to differentiate into myogenic cells prior to transplantation into the same patient. To treat a genetic disease with iPS-derived myogenic cells, it is imperative to correct the underlying genetic defect prior to transplantation of the autologous genetically engineered myogenic cells into the patient. Ex vivo genetic correction of the genetic defects can be performed at several different stages: (1) before cellular reprogramming, (2) directly on the iPS cells themselves or (3) at the stage of the iPS-derived committed myogenic cells, just prior to transplantation. The genetic modification could be accomplished either by introducing a functional copy of the therapeutic gene de novo into the desired target cells (i.e. 'gene addition') or by in situ targeted correction of the defective gene itself (i.e. 'genome editing') in the iPS cells or their myogenic derivatives. Since a large number of myogenic cells are needed for transplantation, stable expression of the therapeutic gene typically requires an integrating vector platform. In particular, retroviral or lentiviral vectors or transposons have been used to stably express a therapeutic gene in iPS or their myogenic progeny. Alternatively, stably persisting nonintegrating episomes such as human artificial chromosomes (HACs) encoding the gene of interest have been used. The main advantage of 'gene addition' is that it can be used broadly in most patients irrespective of the underlying mutation in the defective gene.

For specific and targeted gene correction by genome editing, the use of engineered designer nucleases is required that are specifically designed to induce a double-strand DNA break (DSB) at any desired locus. This can then result in 'gene repair' by non-homologous end joining by exploiting the cellular DNA repair machinery. Alternatively, the presence of a DSB at the desired target locus can dramatically enhance homology-directed gene repair up to 10^4 to 10^5-fold if a homologous donor DNA is provided. The most commonly used designer nucleases are zinc finger nucleases (ZFN), transcription activator-like effector nuclease (TALENs) or homing endonucleases. In the case of ZFNs, TALENs or homing endonucleases, the designer nucleases are specifically engineered de novo in function of the target

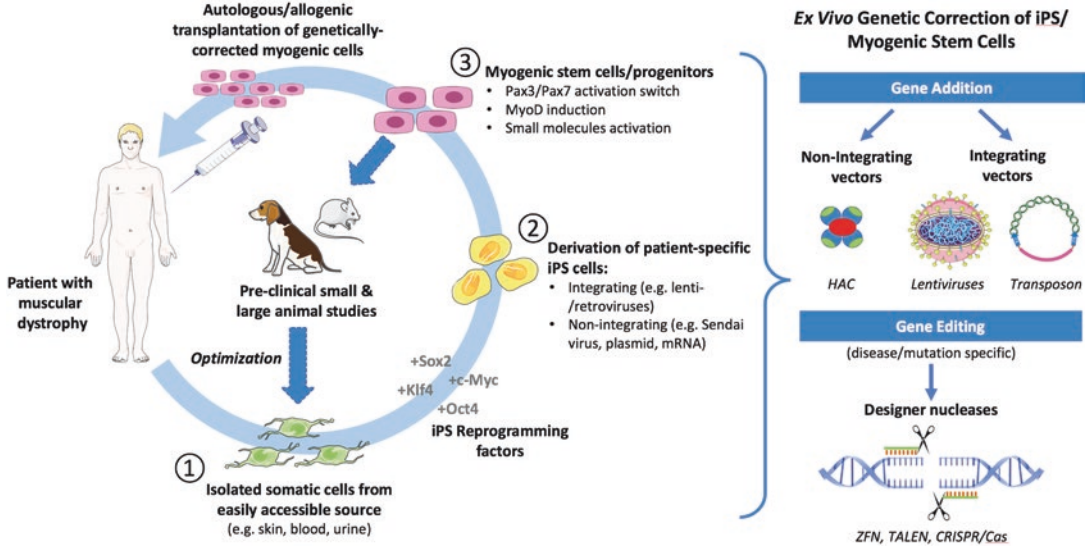

Fig. 5.1 Autologous iPS-based therapies for hereditary muscle disorders. Patient-specific somatic cells are first isolated from easily accessible tissues or body fluids (skin, urine, blood) of patients with muscular dystrophy. These somatic cells are then reprogrammed into iPS de novo expression of reprogramming factors. The patient-specific iPS cells are subsequently induced to differentiate into myogenic stem cells/progenitors that can be expanded to large numbers. Coaxed myogenic differentiation can be achieved using coculture, growth factors, small molecules or forced expression of myogenic factors Pax3/Pax7 or MyoD. Ex vivo genetic correction of the genetic defects can be performed at several different stages: (1) before cellular reprogramming, (2) directly on the iPS cells themselves or (3) at the stage of the iPS-derived committed myogenic cells, just prior to transplantation. Several methods of gene correction are currently under development which involve either the addition of a new functional copy of the disease-causing gene or mutation-specific gene editing. Several examples of each of these methods have been tested in patient-derived iPS cells. Prior to clinical applications, preclinical animal non-disease or disease models will be carried out to assess the safety and regenerative potential of the corrected cells in reversing the dystrophic phenotype upon systemic delivery. *CRISPR/Cas9* clustered regularly interspaced short palindromic repeat/associated protein-9 nuclease, *HAC* human artificial chromosome, *iPS cells* induced pluripotent stem cells, *Klf4* Kruppel-like factor 4, *MyoD*, myogenic differentiation 1, *Oct4* octamer-binding transcription factor 4, *Pax3/7* paired box 3/7, *Sox2* sex-determining region Y-box 2, *TALEN* transcription activator-like effector nuclease, *ZFN* zinc finger nuclease. Figure was made using Servier Medical Art (http://www.servier.com)

DNA that needs to be cleaved. More recently, clustered regularly interspaced short palindromic repeat (CRISPR/Cas9) platforms have been developed. In this case, a single Cas9 protein is required that is guided to its cognate DNA target by virtue of a specific complementary guide RNA. Typically, gene editing is specifically tailored towards correcting a specific mutation and thus restricted to specific patient subsets. Nevertheless, by carefully designing the targeting strategy, it may be possible to broaden the scope of the gene editing making it mutation-independent (e.g. by targeted integration of an entire cDNA into the desired target locus).

5.4.1 Gene Addition

One of the first proof-of-concept studies based on gene addition in iPS cells was aimed at correcting limb-girdle muscular dystrophy 2D (LGMD2D), a neuromuscular disease due to a mutation in the α-sarcoglycan gene. To this end, patient-specific iPS cells were first generated from patients suffering from LGMD2D. These LGMD2D-iPS cells were then induced to differentiate into mesoangioblast-like myogenic cells (designated as HIDEMs or *h*uman *i*PS-*d*erived *m*esoangioblast-like cells). These LGMD2D-HIDEMs were then transduced with a lentiviral vector encoding the therapeutic human α-sarcoglycan gene under the control of a muscle-specific promoter. The LGMD2D-HIDEMs were also transduced with another lentiviral vector containing an inducible MyoD expression cassette to induce myogenic differentiation. The genetically corrected LGMD2D-HIDEMs were then transplanted into immunodeficient α-sarcoglycan-null mice (*Sgca-null/scid/beige*) by intramuscular or intraarterial injection. The genetically engineered myogenic cells were able to engraft and produced α-sarcoglycan-positive muscle fibres 1 month post transplantation [9]. The expression of the α-sarcoglycan protein prompted the reconstitution of the dystrophin-associated protein complex in the myofibres of the recipient mice. Similarly, transplantation of wild-type mouse iPS cell-derived progenitors into *Sgca-null/scid/beige* mice resulted in relatively robust engraftment in the recipient muscle and reestablishment of functional pericytes, consistent with amelioration of the dystrophic phenotype.

In a separate study, patient-specific iPS cells were generated from patients suffering from DMD. These DMD-iPS cells were then transfected with a HAC containing the entire 2.4 Mb dystrophin genetic locus (DYS-HAC) that was stably maintained as a nonintegrated episome, even upon successive cell divisions [9, 63]. This type of genetic modification circumvents safety concerns associated with randomly integrating vectors. Unfortunately, microcell fusion typically results in relatively low transfection efficiencies. Nevertheless, DMD-iPS clones could be obtained that contained the full-length dystrophin gene. These genetically engineered DMD-iPS cells were subsequently induced to differentiate into HIDEMs, as described above.

As an alternative to the DYS-HAC system, we recently explored the use of piggyBac transposons that encoded the full-length dystrophin cDNA instead. We initially demonstrated that the full-length dystrophin cDNA could be stably expressed

in primary mesoangioblasts derived from dystrophic dogs following transposition with the piggyBac transposon system using a hyperactive PB transposase [64]. Subsequently, we demonstrated that HIDEMs derived from DMD patients could be engineered with this piggyBac transposon to stably express the full-length dystrophin protein. Xenogeneic transplantation into immunodeficient mdx/SCID mice resulted in detectable dystrophin expression in vivo (Loperfido et al., in revision). Since DMD can be caused by a spectrum of mutations that essentially encompass the entire dystrophin gene, delivery of the entire dystrophin cDNA ensures a mutation-independent approach. This underscores the potential of the piggyBac transposon system to deliver and stably express large therapeutic genes in iPS-derived myogenic cells. This non-viral vector approach is more efficient than HAC-microcell fusion and overcomes the limitations imposed by the intrinsic packaging constraints of retroviral or lentiviral vectors. However, the safety consequences of random genomic transposon integrations would still need to be addressed in this system.

Another transposon system, derived from Sleeping Beauty, has been used to effectively deliver micro-utrophin (μUTRN) in mouse iPS cells derived from dystrophin/utrophin null mouse [65]. Utrophin is a protein closely resembling dystrophin, whose overexpression could effectively reverse the dystrophic phenotype in mdx mice [66]. The myogenic differentiation of the transposon-modified mouse iPS cells was induced with Pax3, and the differentiated myogenic population was enriched by sorting for PDGF-αR$^+$/Flk1$^-$ expression. The genetically corrected myogenic cells were transplanted in dystrophin/utrophin null mice and contributed to muscle regeneration, consistent with an improvement in contractility. By adopting a similar piggyBac transposon-based gene transfer approach, Tanaka et al. reported on the correction of human iPS cells derived from patients with Miyoshi myopathy (MM) with a full-length dysferlin (DYSF) transgene [67]. In this study, expression of the DYSF protein was detected on the corrected cells in vitro, which in turn reversed the MM phenotype.

5.4.2 Gene Editing

Genome editing using TALENs, ZFNs, homing endonucleases and CRISPR/Cas9 represents a powerful approach for the correction of various disease mutations in iPS cells. Indeed, this approach has been shown to be effective in deriving genetically edited iPS cells for the treatment of several diseases such as ß-thalassemia [68], α1-antitrypsin deficiency [69], epidermolysis bullosa [70] and muscular dystrophies [71].

In the case of DMD, Gersbach and colleagues provided proof of concept demonstrating correction by targeting exon 51 of the dystrophin gene using TALENs specifically designed to target this locus [72]. Consequently, dystrophin expression was restored in the DMD skeletal myoblasts and in the dermal fibroblasts that were coaxed to undergo myogenic differentiation upon induction of MyoD expression. It

is somewhat reassuring that this TALEN-mediated gene editing appeared to be highly specific for the targeted locus as no off-target effects were detected by exome sequencing of any of the in silico predicted target sites. Alternatively, designer ZFNs have been used to permanently remove the essential splicing sequences in exon 51 of the dystrophin gene, resulting in dystrophin transcripts without exon 51 [73]. Interestingly, ZFN-edited DMD myoblasts containing this deleted exon 51 gave rise to concomitant restoration of dystrophin protein expression. Upon transplantation into immunodeficient mice, the ZFN-edited DMD myoblasts contributed to in vivo expression of the human dystrophin. These gene editing approaches permanently and irreversibly restore the dystrophin reading frame and protein production. This is in contrast to conventional oligonucleotide-based exon skipping strategies that yield only transient therapeutic effects and require repeated oligonucleotide administration.

In order to restore the dystrophin reading frame in DMD myoblasts, recently Ousterout et al. developed a multiplex CRISPR/Cas9-based gene editing system capable of targeting the exons 45–55 mutational hotspot [74]. Encouragingly, this multiplex designer nuclease system allowed for the correction of >60% of DMD patient mutations with a single genome-editing strategy; however the technique produced a shorter version of the human dystrophin. Unfortunately, all of these proof-of-concept studies relied on DMD myoblasts which are not ideal candidates for cell therapy in DMD patients due to their limited proliferation and self-renewal potential and their inefficient extravasation capacity as outlined above. However, a recent study by Li et al. suggests that gene editing can also be achieved using DMD patient-specific iPS-derived myogenic cells instead of myoblasts. The authors showed that TALEN- and CRISPR/Cas9-based genome editing were able to restore the expression of full-length human dystrophin [75].

Though the overall efficiency of gene editing is currently not as high as with more conventional gene addition strategies, incremental changes in technology may eventually bridge this gap. It will be important to conduct the necessary in vivo studies with gene-edited iPS-derived myogenic cells to establish safety and efficacy in the appropriate preclinical models. Finally, comprehensive genome-wide analysis of off-target effects would be required to formally rule out any off-target effects.

5.5 Concluding Remarks and Future Perspectives

Preclinical proof-of-concept studies highlight the potential of myogenic adult stem/progenitor cells, ES and iPS cells, to treat degenerative muscle disorders by exploiting their myogenic and self-renewing potential. Recent clinical trials indicate that large numbers of cells are needed to treat dystrophic patients, the amount of cells and their delivery depending primarily on the type of adult stem/progenitor cells, the type of dystrophic disorder that is targeted [21] and possibly also the age of the patient and the extent of muscle function deterioration. In contrast to adult stem/progenitor cells, ES and iPS cells can be expanded

indefinitely, potentially enabling body-wide regeneration of the deteriorating skeletal muscles.

The advances in gene therapy will likely benefit the field of regenerative medicine to treat muscle disorders by enabling the use of gene-corrected autologous cells for muscle repair. For ex vivo correction of patient-/disease-specific iPS cells, different viral and non-viral vectors have been explored that enable stable genomic integration and sustained expression of the therapeutic transgene. Achieving expression of either truncated or full-length dystrophin proteins after gene therapy enables phenotypic correction, irrespective of the patient's underlying mutation. Consequently, these types of 'gene addition' approaches will be amenable to treat a larger number of patients. Whereas random genomic integration raises possible concerns associated with insertional oncogenesis, this risk could be reduced by keeping the number of vector copies per cell to a minimum (typically <2 integrations) and by optimising the vector design. Furthermore, it is particularly reassuring that these concerns could be overcome by developing efficient site-specific integration in safe-harbour loci or by gene editing of the defective allele. However, even with these emerging technologies, 'off-target' effects cannot be excluded and would still need to be addressed.

Despite these advances, clinical translation of iPS cells for autologous gene and cell therapy of genetic diseases remains challenging as there are several hurdles that would need to be overcome including (1) the need for efficient long-term engraftment and robust functional reconstitution and contribution to host myofibres; (2) possible genome instability of the transplanted iPS cells and their derivatives; (3) tumorigenic risk due to residual undifferentiated iPS cells and/or re-expression of oncogenic reprogramming factors; and (4) loss of self-renewing and regenerative potential due to prolonged in vitro cell expansion. The epigenetic memory of the cells of origin may also have a lasting impact on cellular behaviour in vivo and would need to be considered. It is also important to efficiently correct not only the skeletal muscle dysfunction but also the other afflicted tissues and organs, such as the heart, the diaphragm and the intercostal muscles. This is compounded by the fact that DMD patients typically die from cardiorespiratory failure.

Further studies are needed to optimise large-scale manufacturing of iPS cells and their myogenic derivatives under GMP/GLP conditions. Based on safety considerations, genome-integration-free protocols would be preferred either to generate iPS cells and/or to coax their myogenic differentiation. The development of chemically defined methods enabling efficient myogenic differentiation is therefore important while ensuring that the myogenic progenitors retain their ability to efficiently migrate and engraft into muscles upon systemic or loco-regional delivery.

In conclusion, the generation and genetic correction of pluripotent stem cells and their myogenic derivatives foster the development of new therapeutic approaches to treat degenerative muscle disorders, like Duchenne. While it is encouraging that the 'first-in-man' iPS-based therapies have been initiated to treat age-related macular degeneration [76–78], the need for widespread distribution to replace missing skeletal muscle gene products to multiple muscles of the limbs and diaphragm, plus cardiomyocytes in Duchenne and other muscular dystrophies, provides greater

challenges than singularly targeting the macula of the retina. Nevertheless, the opportunity to obtain valuable insights into the efficacy and safety of iPS-based regenerative medicine approaches cannot be underestimated.

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Chapter 6
MicroRNAs (miRs) in Muscle Gene Therapy

Alessio Rotini, Giorgia Giacomazzi, Ester Sara Di Filippo, and Maurilio Sampaolesi

Abstract Despite recent advances in scientific knowledge and several clinical trials, muscle gene and cell therapies remain a major challenge. As a matter of fact, novel technologies are being developed for targeting muscle tissues including CRISPR, TALEN, and iPS technologies indicating that gene-based therapies still hold significant promises. Recent findings from our laboratory and others unveiled that microRNAs (miRs), small nonprotein-coding RNAs, are able to posttranscriptionally regulate many genes and exert pleiotropic effects in the muscle. Deleterious changes in miR expression play an important role in muscle diseases. In this regard, miRs are possible therapeutic targets, and miR-based gene therapy for smooth, skeletal, and cardiac muscles is an extremely interesting field for harnessing the com-

A. Rotini · E. S. Di Filippo
Translational Cardiomyology, Stem Cell Research Institute, Stem Cell Biology and Embryology Unit, Department of Development and Regeneration, KU Leuven, Leuven, Belgium

Interuniversity Institute of Myology, Chieti, Italy

Department of Neuroscience, Imaging and Clinical Sciences, University "G. d'Annunzio" Chieti-Pescara, Chieti, Italy
e-mail: alessio.rotini@inserm.fr; es.difilippo@unich.it

G. Giacomazzi
Translational Cardiomyology, Stem Cell Research Institute, Stem Cell Biology and Embryology Unit, Department of Development and Regeneration, KU Leuven, Leuven, Belgium

Interuniversity Institute of Myology, Chieti, Italy
e-mail: Giorgia.Giacomazzi@ieo.it

M. Sampaolesi (✉)
Translational Cardiomyology, Stem Cell Research Institute, Stem Cell Biology and Embryology Unit, Department of Development and Regeneration, KU Leuven, Leuven, Belgium

Interuniversity Institute of Myology, Chieti, Italy

Human Anatomy Unit, Department of Public Health, Experimental and Forensic Medicine, University of Pavia, Pavia, Italy
e-mail: maurilio.sampaolesi@kuleuven.be

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plexity of miR-based therapeutic approaches. In this chapter, we will focus on miR-driven regulation of myogenic routes in homeostatic and challenging states. We will also survey the intriguing perspective of miR biological transfers, including the delivery of functional miRs via exosomes that unlike other vectors are cell-free natural systems for ferrying RNAs between cells. Finally, we will review the recent literature on key miR targets to treat skeletal, cardiac, and smooth muscle diseases and novel valuable clinical tools for more effective treatment strategies in muscle degeneration.

Keywords Cardiomyopathies · Muscular dystrophies · MiR-based therapies MiRs · Smooth muscle cells · Exosome

6.1 Introduction: miR Biogenesis and Delivery

MiRs are small endogenous noncoding RNAs that direct the posttranscriptional regulation of gene expression [1]. Approximately one-third of human genes are regulated by miRs, and currently 3196 murine (as mmu-miRs) and 4552 human (as has-miRs) mature miR entries have been reported in the miRbase sequence repository (mirbase.org; March 2017). MiRs are 21 ± 2 nucleotides in length and are present in the genome as independent transcriptional units or intergenic clusters. The deregulation of miRs is observed in many diseases such as cancer, neurologic diseases, metabolic disorders, as well as cardiovascular and skeletal muscle diseases [2].

MiR-encoding genes are transcribed by RNA polymerase II into principal miRs (pri-miRs) as hairpin structures. RNA polymerase III has the ability to generate pri-miRs although this is limited to the miR cluster of the human chromosome 19 among repetitive Alu sequences. RNase III Drosha, a double-stranded specific endonuclease, processes the pri-miRs into ≈70-nt stem-loop precursor molecules (pre-miRs), which are further shuttled into the cytoplasm by the nuclear export protein exportin-5. The final 22-nt miR/miR* duplex is produced in the cytoplasm by the endoribonuclease Dicer. Argonaute proteins (AGOs) bind miRs into the RNA-induced silencing complex (RISC), and the weaker strand (miR* or passenger) is then degraded. After the integration of the RISC complex, miRs are able to target specific mRNAs. However, the modus operandi of miR processing machinery is still largely unknown [3, 4]. The principal RISC components interact with the proteins responsible for RNA remodeling and for the generation of processing bodies (P-bodies), or glycine-tryptophan bodies (GW-bodies), which account for mRNA decapping, deadenylation, and degradation. It has been reported that circulating high-density lipoprotein (HDL) particles [5], exosomes [6], and liposomes [7] transfer endogenous miRs to host cells for functional mRNA targeting in a non-cell autonomous manner. It has further been described that long noncoding and alternative polyadenylation RNAs constitute posttranscriptional controls on miR activity [4].

Synthetic liposomes and viral vectors are extensively used for many applications, but both strategies present major limitations, including immune stimulatory properties, which restrict gene delivery applications [8]. Generally, viral-based systems exploit the use of retroviruses, lentiviruses, and adenoviruses or adeno-

associated viruses (AAV) [9]. The advantage of this delivery system is the high efficiency and the stable expression of miRs or antagomiRs. For example, in mouse embryonic fibroblasts, retrovirus-based delivery of miR-138 was adopted to improve the efficiency of iPS cell generation [10]. In other study, the use of lentivirus-based approach guaranteed the long-term stable expression of miR-143 (2500-fold) in corneal epithelial progenitor cells [11]. Also, AAV vectors are suitable in transferring miRs [12], and miR-590 and miR-199a were delivered into neonatal mouse heart by a rAAV9 vector in order to improve cardiac regeneration [13]. Nevertheless, immunogenicity and random integration hamper the use of viral vectors for miR gene therapy approaches. Non-viral systems are less toxic and less immunogenic and have no limitation of the size of the transferred DNA. However, the low efficiency of miR delivery of non-viral systems compared to the viral systems has limited their use in therapeutic applications. Gene gun, electroporation, hydrodynamic, ultrasound, laser-based energy, and inorganic carriers have been explored to improve the efficiency of miR delivery. However, damages of cell integrity and apoptosis are frequently observed in all these procedures [14].

Recently, exosomes have emerged to be important mediators of cell–cell communication and cross talking. Exosomes are extracellular small vesicles (40–100 nm diameter) of endocytic origin secreted by several cell types and transporting macromolecules including lipids, proteins, mRNA, and miRs [15]. Different from other delivery vectors, exosomes are a unique cell-free natural system acting as signaling shuttles for short- and long-range communications between cells. Exosomal membranes can protect and promote intracellular release of cargo molecules [15]. For these reasons, exosomes are now considered as an excellent delivery system for gene therapy.

An ideal miR delivery system for regenerative medicine should be able to target specific tissues or organs with low cytotoxicity and high efficiency. In this regard, scaffold-based miR delivery systems represent an innovative approach to optimize the miR distribution to target tissues, minimize miR degradation, and mitigate immune responses [16]. Successful miR therapies can be achieved through the overexpression (miR-mimetics) or the inhibition (antagomiRs) of miRs or even through a combination of both miR-mimetics and antagomiRs [17]. An overview of the miRs employed in gene therapy protocols for skeletal, cardiac, and smooth muscle tissues is shown in Fig. 6.1.

6.2 MiRs and Skeletal Myogenesis

The skeletal muscle tissue of vertebrates originates through a complex and multistage process called myogenesis where numerous genes are cooperatively involved in the regulation of each stage. Numerous miRs can be highly and specifically enriched at different phases of embryonic and adult myogenesis. The miRs that are specifically expressed in skeletal muscle are referred as myomiRs, which include miR-1, miR-133 a/b, and miR-206 [18–20]. The myogenic transcription factors

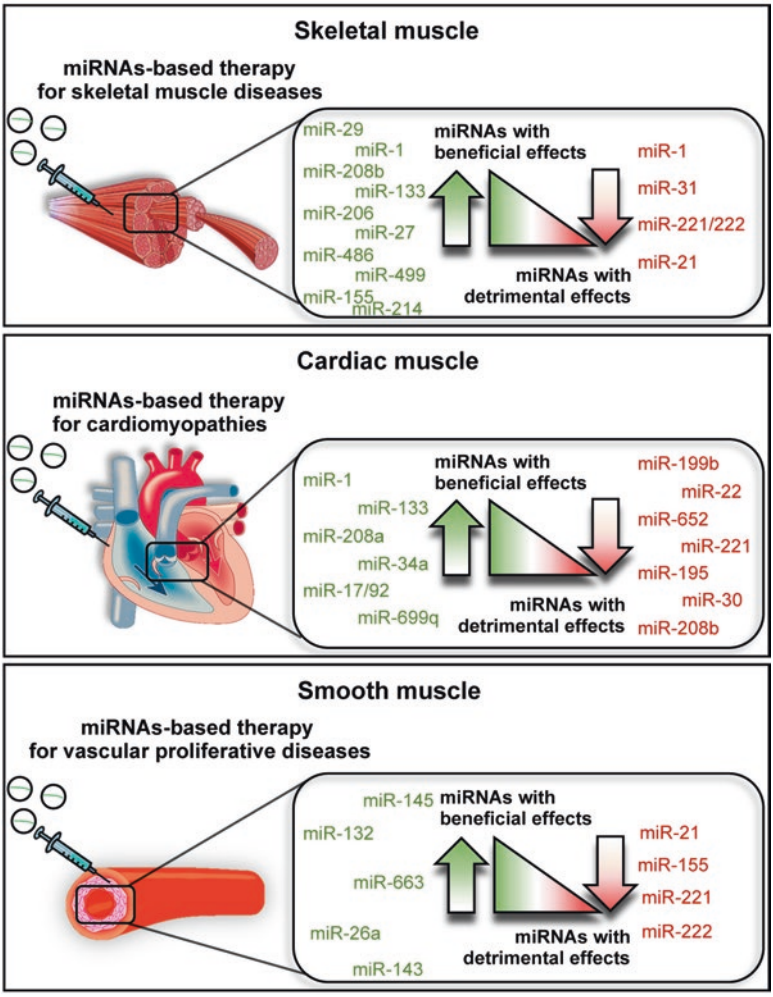

Fig. 6.1 Overview of the miR-based gene therapies for muscle diseases. MiR-based gene therapy holds great potential for treating disorders that affect skeletal, cardiac, and smooth muscles. Green marks miRs that exert beneficial effect when upregulated. Red depicts miRs that exert detrimental effects when upregulated

MyoD, MEF2, and SRF directly regulate the expression of miR-1 and miR-133a in skeletal muscle, whereas the expression of miR-206 is controlled by MyoD and MyoG. In addition to muscle-specific miRs, numerous non-muscle-specific miRs, referred here as non-myomiRs, are also important in the regulation of myogenesis. It has been demonstrated that these non-myomiRs modulate muscle proliferation and differentiation through different mechanisms via repression of specific target genes [21–24]. At the onset of myogenesis, miR-27b induces migration and early differentiation of myoblasts by targeting the Pax3 protein [22]. MiR-26a [21] and

miR-214 [23, 24] also promote myogenesis by targeting enhancer of zeste homolog 2, a known inhibitor of myogenesis. It is noticed that the timing of expression of miR-26a and miR-214 differs upon myogenesis. Once muscle differentiation begins, miR-214 is upregulated via MyoD/MyoG, which promote $P21^{Cip1}$ and MyoG expression, while miR-26a increases during the later course of myogenesis. Another crucial step in muscle differentiation is the inhibition of homeobox-protein A11 (HOX11) by miR-181 [25]. Lower expression of HOX11 leads to an increase in MyoD, a target of HOX11, and therefore to the myogenic differentiation of cells. This is consistent with the finding that inhibition of miR-181 decreased differentiation of C2C12 myoblasts [25]. Similarly, miR-148a has been found crucial for skeletal myogenesis as its main target, ROCK1, prevents myoblast fusion and antagomiR-based experiments results in myogenic differentiation impairment [25].

6.3 MiR-Based Therapy for Skeletal Muscle Diseases

Myopathies are characterized by muscle weakness and the loss/wasting of muscle tissue. Among these diseases, muscular dystrophies (such as Duchenne muscular dystrophy (DMD), Becker muscular dystrophy, facioscapulohumeral muscular dystrophy, and limb-girdle muscular dystrophy type 2A and type 2B) have emerged as potential targets of miR-based therapies. Indeed, it has been demonstrated that manipulation of miRs can ameliorate the phenotype of these diseases in combination with changes in the expression levels of specific miRs [26–28]. The loss/wasting of muscle tissue is commonly associated with the depletion of satellite cells (SCs), which represents the reservoir of resident muscle stem cells in adulthood. After an injury, SCs receive external cues and, as a consequence, activate, proliferate, and give rise to new myofibers [29]. This fine-tuned process, which replaces damaged myofibers with newly formed myofibers, is called skeletal muscle regeneration. In dystrophic muscle, cycles of regeneration/degeneration continuously occur causing SCs to exit their quiescent state (SC activation), start to proliferate, differentiate, and later reenter the quiescent state [29] in order to replenish the SC pool. Crist et al. in 2012 showed that miR-31 maintains SCs in quiescent state by downregulating Myf5, [30] a protein with a key role in activating the myogenic program during the development of skeletal muscle. In quiescent SCs, Myf5 is transcribed but not translated because of the presence of miR-31 in the messenger ribonucleoprotein (mRNP) granules that directly target Myf5 mRNAs. Once SCs are activated, mRNP granules are dissolved including miR-31, releasing Myf5 mRNAs and allowing the translation and protein expression [30]. Manipulation of miR-31 levels affects SC differentiation ex vivo and muscle regeneration in vivo and might provide a feasible treatment aimed at enhancing the muscle regeneration in DMD. Sequence analysis of myostatin (Mstn) 3′ UTR showed a single highly conserved miR-27a/b-binding site, and increased expression of miR-27a/b was correlated with decreased expression of Mstn. Moreover, the authors also showed that Mstn gene expression was regulated by miR-27a/b in vitro and in vivo [31].

Treatment with miR-27a/b-specific antagomiRs resulted in increased Mstn expression, reduced myoblast proliferation, impaired SCs activation, and induced skeletal muscle atrophy that was rescued upon either blockade of, or complete absence of, Mstn [31]. Another study showed that the transcriptional factor MyoD was further regulated by miR-221/222. The miR-221/222-myoD-myomiRs regulatory pathway was confirmed by overexpressing or knocking down the miRs in C2C12 cells, resulting in direct modulation of MyoD expression [32].

After proliferation, myoblasts start differentiation and fusion giving rise to new functional myofibers, and several miRs, including myomiRs, are involved in the process. It has been shown that miR-1 and miR-206 repress HDAC4, which is an inhibitor of MEF2, a myocyte enhancer factor. Thus, miR-1/miR-206 release MEF2 in order to promote muscle differentiation [33, 34]. Moreover, miR-1/miR-206 are able to repress Pax7 and Pax3 [35], quiescence markers of SCs. Therefore, the miR-1/miR-206 inhibiting action on Pax7 leads to muscle differentiation. Moreover, miR-206 promotes myoblast differentiation by repressing other targets, including Pola-1, the largest DNA polymerase subunit, thereby stopping the proliferation machinery favoring the differentiation process [19]. An additional target of miR-206 is Cx43, a protein involved in the complete maturation of muscle skeletal fibers [36]. Other studies have demonstrated that miR-133a/b suppresses myoblast proliferation and promotes differentiation by regulating MAPK (mitogen-activated protein kinase) through direct downregulation of its transducers, FGFR1 (fibroblast growth factor receptor 1) and PP2AC (protein phosphatase 2A catalytic subunit) [37]. Recently, Puri et al. showed that HDAC inhibition induces two components of the myogenic transcriptional machinery, MyoD and Baf60C, and upregulates myomiRs. MyomiRs, in turn, target the BAF60A and BAF60B subunits of SWI/SNF complex, ultimately directing promyogenic differentiation and suppressing adipogenic differentiation of fibro/adipogenic progenitors (FAPs) [38]. Authors showed direct evidence of induction of miR-206 and BAF60C and subsequent reduction of BAF60A and BAF60B in FAPs isolated from *mdx* mice treated with the HDAC inhibitor trichostatin A (TSA) [39]. Moreover, in SCs isolated from *mdx* mice injected with TSA, the overexpression of miR-1, miR-133, and miR-206 enhanced their myogenic differentiation and rescued their phenotype when compared to wild-type mice [38]. MiR-206 has been widely described as posttranscriptional inhibitor of utrophin and follistatin [40, 41]. However, more recently Amirouche and coworkers [42] showed that miR-206 can activate two distinct pathways causing alternatively repression or activation of utrophin A gene. In this view, miR-206-induced utrophin may replace dystrophin in dystrophic skeletal muscles and may limit disease progression [43]. In addition, Nakasa et al. [44] showed that the muscle injected with miR-206 in a rat model of skeletal muscle injury resulted in enhanced muscle regeneration and reduced muscle fibrosis. Finally, genetic deletion of miR-206 delayed muscle regeneration in cardiotoxin-injured mice, and the loss of miR-206 accelerated and exacerbated the dystrophic phenotype in miR-206–KO *mdx* mice [26]. Taken together these studies highlight the pivotal role in muscle regeneration of miR-206 (Fig. 6.2), which represents a preferential target for the treatment of skeletal muscle damage.

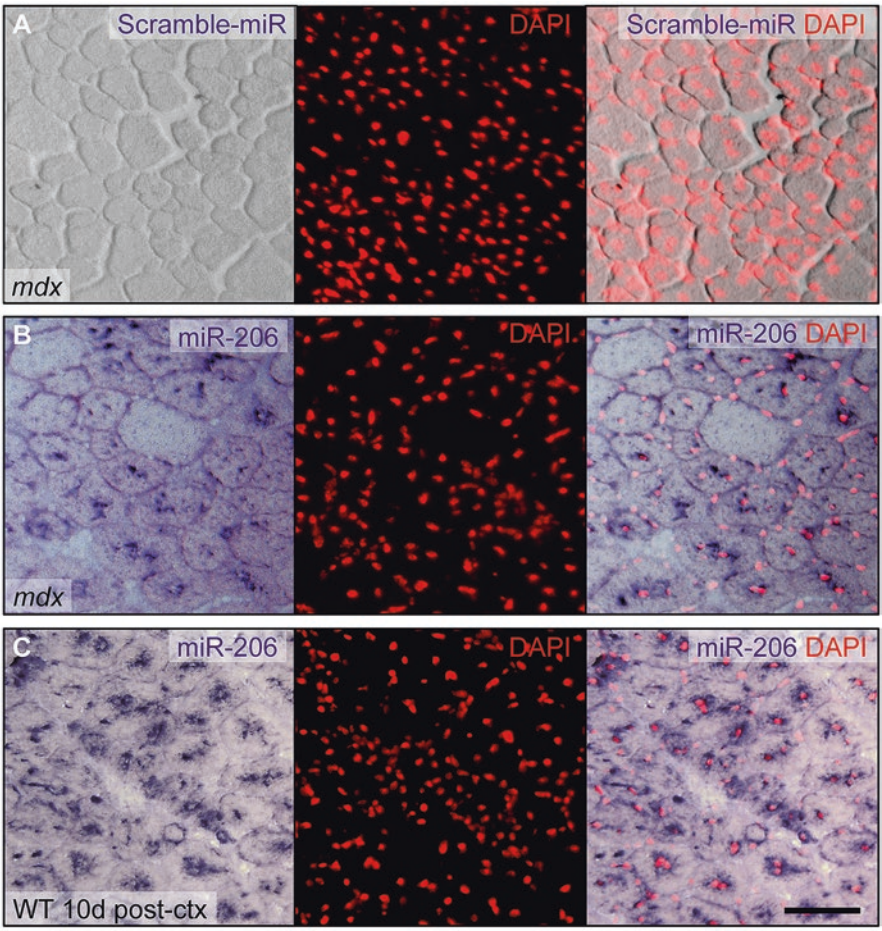

Fig. 6.2 In situ hybridization showing the expression of miR-206 in regenerating muscles from *mdx* and cardiotoxin-injured mice. Muscle sections from *mdx* dystrophic mice were hybridized with the scramble-miR, LNA™ probe as negative control (**a**), *or* using LNA™ probe for miR-206 detection (**b**). Muscle sections from cardiotoxin-injured mice were hybridized as in **b** (**c**). Nuclei were counterstained with DAPI (in red). Note that the miR-206 signal (dark purple) is present in regenerating centronucleated fibers but not in large mature fibers. Bar = 100 μm

Recent studies suggest that miR-29 was repressed by NF-kappaB through the chromatin remodelers YY-1 [45] and Rybp [46]. During myogenesis, the downregulation of NF-kappaB and YY1 causes derepression of miR-29 leading to an acceleration of differentiation in a loop-forward mechanism. Further, miR-486 is reduced in the muscles of dystrophin-deficient mice (Dmdmdx-5Cv mice) and DMD patients. The muscle-specific miR-486 overexpression in Dmdmdx-5Cv mice resulted in reduced serum creatine kinase levels, improved sarcolemmal integrity, increased myofiber size, and improved muscle physiology and performance [27].

Muscle tissues represent a mixture of two fiber types, slow- and fast-twitch fibers. Fast-twitch muscles have been shown to have quick and short contraction time, while slow-twitch have a longer contraction time (about five times longer) [47]. To date, different transgenic mice provide evidence that slow fibers are less susceptible to injury than fast-twitch muscles [48]. Indeed, it has been shown that the higher amount of slow fibers in *mdx* mice, induced by a PGC-1α transgene [49], by Wnt7a treatment [50], or by activation of calcineurin [51], displayed improvements in the regression of the disease. In this context, miR-208b and miR-499 have been shown to be involved in muscle fiber-type regulation [52]. Double knockout of both miR-208b and miR-499 (dKO) in mouse soleus displayed loss of slow myofibers. Conversely, overexpression of miR-499 in EDL, soleus and TA muscles (miR-499 Tg mice) induced a complete conversion of all fast myofibers to slow type I myofibers [52]. These miRs exerted their effect by targeting repressors of slow muscle genes Sox6, Purβ, and Sp3 [52]. Moreover, miR-208b and miR-499 targeted HP-1β, a corepressor of MEF2 involved in activating slow fiber gene expression program [53]. Taken together, these results suggest that miR-499 and miR-208b are potential therapeutic agents for MDs by inducing the conversion from fast-twitch fibers to the slow-twitch fibers that are more resistant to contraction-induced damage.

Another important feature in myopathies is the detrimental accumulation of fibrotic tissue. Several approaches (pharmaceutical, nutritional, exercise-based) are tested to reduce/control fibrotic tissue accumulation [54]. Many miRs have also emerged as novel-antifibrotic molecules. For example, miR-29 can reduce fibrosis in *mdx* mice by repressing the expression of collagen (Col1a1) and elastin (Eln), both responsible for fibrotic tissue accumulation [55–57]. In 2014, Meadows et al. used adeno-associated viral vector (AAV) to deliver miR-29 into muscles of *mdx*/*utrn*± mice and demonstrated a decline of muscle fibrotic tissue [58]. MiR-29 is also a direct target of HDAC2 via dystrophin/nNOS pathway [59] and an ideal candidate for reducing muscle fibrosis in future clinical trials. Indeed MRG-201, a synthetic miR mimic (promiR) to miR-29b, has been developed by miRagen Therapeutics, Inc. and is in an ongoing phase I clinical trial study (https://clinicaltrials.gov/ct2/show/NCT02603224?term=MRG-201&rank=1). Additionally, miR-21 has emerged as an important regulator of fibrotic tissue deposition in the dystrophic muscle and has been found upregulated in different primary muscular disorders [28]. Ardite et al. demonstrated that extracellular plasminogen activator inhibitor-1 (PAI-1)/urokinase-type plasminogen activator regulates miR-21 that controls age-associated muscle fibrosis in *mdx* mice [28]. To date, the PAI-1-miR-21 fibrogenic pathway is well recognized as a target to treat fibrosis and MDs [28].

In 2011, Cacchiarelli et al. highlighted that miR-31a selectively represses dystrophin expression. Specifically, miR-31 targets directly the 3′ untranslated region (UTR) of the dystrophin mRNA. In combination with an exon-skipping therapy, the inhibition of miR-31 has been shown to enhance the dystrophin gene expression in a trial with DMD myoblasts of patients with exon 48–50 deletion. Moreover, local injection of miR-31 inhibitors strongly improved dystrophin translation in *mdx* mice treated with 48–51 exon-skipping approaches [59].

The oxidative stress, through the release of reactive oxygen species (ROS), plays an important role in the progression of DMD and has been widely reviewed in literature in human [60], *mdx* mouse [61], and both human and mouse [62–64]. Moreover, dystrophin-deficient myofibers seem to be more exposed to oxidative stress, as previously reported [65]. Expressions of the enzymes associated with antioxidant defense are increased in DMD patients, as well as in *mdx* mice [61, 65, 66], including reduced glutathione (GSH), the most important free radical scavenger [55]. GSH is formed from oxidized glutathione (GSSG) by glutathione reductase enzyme, using NADPH as an electron donor produced by glucose-6-phosphate dehydrogenase (G6PD) enzyme, a direct target of miR-1. Since miR-1 is downregulated in DMD patients [67], the excess of G6PD determines a dysregulation of GSH/GSSG ratio, thus making the dystrophic muscle fibers more susceptible to oxidative damage [55].

A general inflammatory condition is present in various disorders of the skeletal muscle [68], and miR-155 plays a critical role in the regulation of inflammation that affects both innate and adaptive immunity [69]. MiR-155 regulates the balance between pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages during skeletal muscle regeneration. Mechanistically, it has been found that miR-155 suppresses SOCS1, a negative regulator of the JAK-STAT signaling pathway, during the initial inflammatory response upon muscle injury. Thus, miR-155 plays an important role in DMD physiopathology [70] and provides a novel miRNA target for improving muscle regeneration in degenerative muscle diseases [71].

Recently, vasculature-targeted strategies for DMD, with a major focus on increasing blood flow in existing blood vessels, have emerged [72]. It has been demonstrated that a reduced formation of blood vessels in DMD muscles commonly occurs, and a therapeutic approach to augment angiogenesis by using vascular endothelial growth factor (VEGF)-based strategies has been developed. The identification of miRs, including miR-126, miR-378, miR-296, and miR-17-92 cluster, that regulate angiogenesis has opened a new avenue for therapeutics not only of vascular diseases but also of diseases whereas revascularization is crucial [67]. Mimics of pro-angiomiRs, or antagomiRs of anti-angiomiRs, can be used to elevate angiogenesis in the pathological setting of insufficient angiogenesis, such as myocardial infarction (MI), ischemia, and muscular disorders [73].

6.4 MiRs and Cardiac Myogenesis

As the first fully functioning organ of the body, the heart is guided throughout development by a rather complex organization system made up of genetic pathways, epigenetic players, and posttranscriptional network systems. In vertebrates in general, and more in particular in humans, the underlying mechanisms sustaining the cardiac muscle formation throughout development are very much conserved in the later stages of maturation. MiRs play crucial role in these processes.

Alongside the aforementioned miR-1 and miR-133, two other members of the myomiR family, miR-208a and miR-499, must be added as main actors in many aspects of cardiac development and homeostasis [4]. During cardiac maturation, miRs must be finely regulated; the balance of miR-1 is, for instance, required as its excess leads to a reduction of cardiac progenitor cells, but on the other side, its depletion is embryonically lethal [74]. Similarly, members of the miR-133a family, which are transcribed together with miR1, require a stable level of expression as they are crucial for the growth of progenitor cells. However, when miR-133a is overexpressed, cardiomyogenesis is reportedly hampered [75]. Both miRs are reported as direct regulators of muscle differentiation, as transduction of murine pluripotent stem cells with lentiviral expressed miR-1 and miR-133a increased their cardiomyogenic differentiation [76].

MiR-208a is generally considered the cardiac-specific miR. MiR-208a is embedded in one intron of Myh6. Myh6 encodes α-myosin heavy chain (α-MyHC), the most abundant MyHC (~90%) in the heart. MiR-208a is generally known as the cardiac-specific miR. Research has unraveled the interactions of miR-208a with miR-208b and miR-499, and a lot of interest has grown over this triplet of miRs as promising therapeutic targets. Different from miR208a, miR208b and miR499 are inserted in introns of Myh7 and Myh7b, both encoding for β-MyHC, which constitutes the primary MyHC form during embryonic development. In stress conditions, miR-208 is responsible for removing the transcriptional block on Myh7/7b loci, allowing a switch between MyHC forms. Interestingly, in animal models as well as humans presented with hypertrophic adverse remodeling, high levels of miR-208b and miR-499 were observed, suggesting the pivotal role of the switch between the two miR-208 forms as a marker of certain cardiac-associated diseases and more compellingly pointing at the therapeutic strategy of the trio [77, 78]. For instance, miRagen Therapeutics established MGN-9103, already tested in preclinical studies, which targets miR-208 with clear implications for the treatment of chronic heart failure [79].

The miR-138 and the miR-128 families are evolutionary conserved and provide important cues that guide correct structural formation of the cardiac muscle. More specifically, members of the miR-138 family contribute to establish and sustain a specific gene program in ventricle maturation, while the miR-218 family was found crucial for the formation of the heart tube [77, 80]. Additionally, the miR-143 family has been implicated in the structural regulation of the heart by controlling the distribution of the cytoskeleton in the cells [81].

6.5 MiR-Based Therapy for Cardiac and Smooth Muscle Diseases

It was generally believed that postnatal cardiac growth in mammals was driven by hypertrophic modification of mature cardiomyocytes. However, evidence in mice has shown that mammals' hearts hold a congenital capacity of self-regeneration,

albeit local cardiomyocyte turnover is not sufficient to fully heal the cardiac muscle after injury [82–84]. Several works have shown the role of miRs as direct contributors to cardiomyocyte maturation and renewal [85–88]. MiR-based therapeutic strategies typically consist in either protecting endogenous cardiac progenitors and/or cardiomyocytes to ameliorate the injury-associated features or contributing to enhance differentiation potential of stem cells and progenitors. However, in very recent times, the promise of cell-free alternatives, including exosomes, for stem cell-mediated therapy has emerged [89].

MiR-1 and miR-133 seem to play a pivotal role, alongside others, in directly regulating cardiomyocyte functions. Notably, the lack of miR-133a-1/miR-133a-2 in double-knockout mice has resulted in an increase in the growth rate of cardiomyocytes [75] suggesting an important role for the miR-133 family in maintaining the cardiac phenotype of cardiomyocytes. Similarly, members of the miR-1 family negatively regulate ventricular cardiomyocyte proliferation through targeting of the Hand2 mRNA [74]. The miR-15 and miR-29 families have been implicated as part of regulatory mechanisms in cardiomyocytes promoting fetal to adulthood switch. Patients who underwent ischemic injury show dramatic upregulated levels of miR-195, a member of miR-15 family. Knockdown of miR-195 in neonatal mice with LNA-antagomiRs resulted in increased number of proliferating cardiomyocytes and have thus been further investigated, providing promise in protecting against cardiac ischemic injury [4, 86]. Moreover, upregulation of miR-15 and miR-195a executed a postnatal cell cycle arrest during the process of heart regeneration after myocardial infarction. In this regard, miRagen Therapeutics developed anti-miRs (MGN-1374) against both miR-15 and miR-195a able to induce post-myocardial infarction remodeling [90]. Such remodeling enhanced heart function and induced cardiomyocyte proliferation in mice and pigs. MiR-29 family has more controversial features since miR-29a overexpression has been associated with a decrease in cardiomyocyte proliferation by some authors [91], promoting progenitors' proliferation by others [91, 92]. More recent work has endorsed the idea that miRs can induce the proliferation of resident cardiomyocytes. When injected intraperitoneally with AAV9 vectors expressing miR-590 family or miR-199a, neonatal mice displayed an increased number of mitotic cardiomyocytes. Moreover, this occurs also when AAV9 vectors were injected in adult mice following descending coronary artery ligation [13]. Furthermore in vivo inhibition of miR-199b via antagomiR in a mouse model of heart failure ameliorated the function of the cardiac muscle, reduced fibrosis, and reversed hypertrophy [93]. Hypertrophic remodeling associated with cardiomyopathy was partially halted in mice treated with administration of LNA antimiR-652. These mice displayed also a significant reduction in fibrosis, less apoptosis, and preserved angiogenesis [94]. Further reduction and modulation of cardiac hypertrophy were achieved via angiotensin II silencing by upregulation of miR-34a, whereas its inhibition aggravated the phenotype [95]. Interestingly, this study underlined the role of miR-34a in suppressing cardiomyocyte autophagy via ATG9A silencing. Similarly, other studies have shown evidence of the role of miRs in directly regulating autophagy. Administration of miR-145 in rat models of myocardial infarction improved cardiac function and accelerated cardiomyocyte autophagy

by targeting fibroblast growth factor receptor substrate 2 [96], while overexpression of miR-221 resulted in a marked reduction of autophagic flux, exacerbating the cardiac hypertrophic remodeling [97]. MiR-22 has been also implicated as a strong inhibitor of cardiac autophagy and pharmacological blocking of miR-22 in a post-myocardial infarction (post-MI) model in older mice activated cardiac autophagy, prevented postinfarction remodeling, and improved cardiac function [98]. In other studies, overexpression of the miR-17/92 cluster in mice was shown to have a protecting effect after ischemic injury, promoting proliferation of postnatal cardiomyocytes. Similar results were achieved via intracardiac delivery of miR-199a and miR-590 using an AAV vector in neonatal mice [13, 99]. Additionally, recent work from Shen and colleagues identified miR-30 as a novel regulator of cardiac plasticity post-MI, showing how silencing of miR-30 resulted in cardioprotective features in mice [100]. A further interesting possibility lies in the modulation of miRs for myogenic fate switching purposes. To this end, it is reported that cardiac pericytes from dystrophic mice can differentiate into skeletal pericytes, and this aberrant feature has been attributed to miR-669a/q, a direct regulator of MyoD. Notably, transduction of the neonatal murine myocardium with a miR-699a-expressing AAV-9 vector resulted in partial remission of cardiomyopathies in these mice [101, 102].

MiRs are acquiring growing interest as co-players in stem cell therapy. Stem cells hold the intrinsic ability of enhanced self-renewal and virtually can be differentiated in several lineages and cell types. Different populations of adult stem cells have allured researchers to investigate their cardiac regeneration potential [89]. In more recent times, pluripotent stem cells, ESC and iPSC, have come into the spotlight as therapeutics for cardiac pathologies. From early evidence [103] to much more recent work [104, 105], it is clear that ESC cells can successfully differentiate into cardiac precursors and even contribute to the regeneration of the heart post MI in large animal models. The cardiogenic potential of iPSC has been scrutinized. These studies suggest that iPSC cells can also contribute to regeneration in an injured heart when they are differentiated to mesodermal progenitors [106, 107].

Studies on ESC differentiation toward cardiomyocytes have once more unraveled the central although somewhat divergent role of miR-1 and miR-133. Overexpression of these miRs enhanced the mesodermal profile in early embryo body formation. MiR-1 levels were necessary to sustain further differentiation at later developmental stage, while miR-133 had the opposite effect [76, 108]. Lentiviral mediated expression of miR-1 in ESC cells resulted in increased cardiac repair of these cells in infarcted mice. Similarly, treatment with the already discussed miR-499 family enhanced differentiation potential of cardiac stem cells in vitro and lead to partial restoration of infarcted areas in vivo [109]. Interestingly, these three miRs, together with miR-208a, have shown to be rather efficient at converting cardiac fibroblasts to cells with cardiomyocytes-like properties. This so called direct reprogramming was induced by mirR-1 alone but further increased when four miRs were expressed in combination, additionally boosting maturation [110]. To this regard, other studies conducted on iPSC differentiation have demonstrated that members of the miR-290 cluster (miR-291-3p, miR-294, and miR-295)

enhance murine iPSC reprogramming and, later, further influence cardiomyogenic lineage maturation [111].

MiRs therapeutic research targeting smooth muscle cells has been mainly focused on atherosclerosis, restenosis, and abdominal aortic aneurysm (AAA) formation. Burdensome problems and hallmarks of such diseases are the accumulation of smooth muscle and inflammatory cells in the intima, deposition of fibrotic tissues, and abnormal phenotypic switch of vascular smooth muscle cells (VSMC) from contractile, differentiated cells to a proliferative, dedifferentiated phenotype [112]. Therefore, understanding the molecular mechanisms of VSMC proliferation and remodeling may offer novel insights into disease pathogenesis leading to targeted therapies. MiR-132 has been described as a regulator of VSMC, as data show that miR-132 mimic administered to the rat carotid artery after catheter injury reduced VSMC migration and proliferation in the neointima [113]. One of the most abundant miRs found in the smooth muscle of the vasculature wall is miR-145 [114]. Recent work highlighted a role of miR-145 in the phenotypic modulation of VSMC during pathogenesis. The authors noted that delivery of miR-145 in balloon-injured rat carotid artery successfully promoted differentiation of VSMC and inhibited neointimal growth [115]. Moreover, downregulation of miR-221 and miR-222, found upregulated in the vascular walls with neointimal lesion, significantly decreases VSMC proliferation and subsequent neointimal lesion formation in the rat carotid arteries after angioplasty [116]. Similarly, knockdown of miR-21 decreased neointima formation in rat carotid artery after angioplasty. Inhibition of miR-21 has been reported to decrease proliferation of cultured VSMCs through an apoptotic mechanism [117]. Pharmacological knockdown of miR-21 in human veins resulted in a significant reduction in neointimal formation [118]. Torella et al. demonstrated that adenoviral-mediated overexpression of miR-133a reduced neointimal formation and antagomiR to miR-133a exacerbated neointimal formation following balloon injury [119]. More recently, it was discovered that miR-33 is a novel regulator of smooth muscle cell proliferation in response to arterial stress. MiR-33 acts through the TGFβ-Smad signaling pathway and perivascular injections of agomiR-33 attenuated neointimal hyperplasia in grafted veins [120]. In addition, miR-26a has been found to positively regulate VSMC survival after AAA [121]. The therapeutic potential of miR-33 has been further explored by Regulus Therapeutics that developed an anti-miR-33 a/b for the treatment of atherosclerosis. MiR-33b and miR-33a are encoded in the introns of the transcription factor loci SREBP1 and SREBP2, respectively, and are involved in the regulation of cholesterol and fatty acid homeostasis. Treating African green monkeys subcutaneously with 2′-fluoro-methoxyethyl-phosphorothioate modified antisense-miR-33 oligonucleotides (anti-miR-33) resulted in a decrease in very-low-density lipoprotein and an increase in HDL [122].

Finally, Zhang et al. [123] demonstrated that miR-155 is significantly upregulated in atherosclerotic plaque, functioning to accelerate the proliferation and migration of VSMCs by targeting eNOS. Exogenous overexpression of miR-155 in human aortic SMCs confirmed the detrimental effects [123]. Thus, miR-155 represents a sensitive target to reduce proliferation and migration of SMCs in human vascular diseases, including restenosis and atherosclerosis.

6.6 Conclusion and Remarks

In conclusion, investigation into miR biology has clearly enriched our current understanding on the myogenic processes for cardiac, skeletal, and smooth muscle tissues. There is a large consensus on the involvement of specific miRs in the individual stages of muscle development that affect muscle metabolism, cell proliferation, differentiation, and regeneration. Furthermore, miRs are dysregulated in several muscle pathologies where tissue homeostasis is lost. Those studies established the biological foundation for the use of miR knowledge in disease diagnosis and treatment. For example, since miRs are very stable in most body fluids, they can be used as biomarkers for medical purposes. On the other side, the expression of specific miRs, including myomiRs and angiomiRs, can be modulated to achieve therapeutic benefits. Nevertheless, the lack of gene specificity of miRs and the pleiotropic effects are the major concerns that limit their translational uses. In this context, miR mimics, alone or in combination with antagomiRs, shuttled by exosomes could provide muscle disease-tailored solutions for successful miR-based gene therapies.

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Chapter 7
Immune System Regulation of Muscle Injury and Disease

Jenna M. Kastenschmidt, Ali H. Mannaa, Karissa J. Muñoz, and S. Armando Villalta

Abstract Muscular dystrophy and inflammatory myopathy are muscle diseases that despite their etiological differences share many pathological features, including muscle degeneration, loss of function, and chronic inflammation. Immunological processes induced by muscle injury contribute to the pathology of various muscular dystrophies, whereas autoimmune responses specific for yet undefined muscle antigens are suspected to be the cause of some idiopathic inflammatory myopathies. This chapter discusses the role of the immune system in eliciting immunity and regulating inflammatory responses during acute injury and muscle degenerative diseases. Duchenne muscular dystrophy (DMD) is the most prevalent form of muscular dystrophy. Using DMD as an example, we discuss the role of immune system in the pathogenesis of muscle disease. In addition to the role of innate immunity, we review the literature supporting the elicitation of antigen-specific, adaptive immune responses in DMD, including those specific for dystrophin. We discuss the clinical implications of these adaptive immune responses and their potential in limiting the efficacy of dystrophin gene therapy. Last, we highlight therapeutic approaches that may be used to inhibit degenerative muscle inflammation and to tolerize DMD patients to the protein product of dystrophin gene therapy.

Keywords Muscle immunology · Inflammation · Muscular dystrophy · Gene therapy · Muscle regeneration · Macrophages · T cells · Regulatory T cells Dystrophin immunity · Inflammatory myopathy · Acute muscle injury

J. M. Kastenschmidt · S. A. Villalta (✉)
Department of Physiology and Biophysics, University of California Irvine, Irvine, CA, USA

Institute for Immunology, University of California Irvine, Irvine, CA, USA
e-mail: jkastens@uci.edu; armando.villalta@uci.edu

A. H. Mannaa · K. J. Muñoz
Institute for Immunology, University of California Irvine, Irvine, CA, USA
e-mail: amannaa@uci.edu; kjmunoz@uci.edu

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7.1 Introduction

Skeletal muscle is a highly resilient tissue that maintains homeostasis despite dynamic changes in activity, mechanical load, and stress. Nonetheless, acute trauma or genetic and environmental factors that exceed this homeostatic threshold of resilience cause muscle injury and loss of function. Although the etiology of muscle injury differs between acute and chronic disease, a common feature linking these ailments is the activation of the immune system and inflammatory cascades leading to immune cell infiltration of muscle. Acute injuries that stem from extreme exercise, trauma, or the reloading of muscle following long periods of inactivity induce resolvable inflammation that is required for complete muscle regeneration [1–3]. At the other end of the spectrum, the muscle degeneration observed in muscle disorders elicits chronic inflammation that impairs muscle regeneration [4]. Thus, in a simplified view, one may predict that the severity and chronicity of muscle injury dictates the role immune system plays in muscle injury and repair. While the coordinated recruitment and resolution of inflammation are necessary for regeneration during acute injury, the chronic inflammation caused by asynchronous injury impairs regeneration and exacerbates muscle degeneration. In the sections below, we compare and contrast the role of the immune system in acute injury and chronic muscle disease, providing a basis for discussion on potential therapeutics aimed at suppressing degenerative inflammation. Additionally, we discuss the potential role of the immune system as a barrier limiting the efficacy of dystrophin gene therapy and potential therapeutic strategies aimed at tolerizing patients to dystrophin.

7.2 Immune System Contributions to Muscle Regeneration Following Acute Injury

Muscle injury activates multiple immune system pathways that cooperatively regulate the spatiotemporal dynamics of immune cell subpopulations (e.g., macrophages and T cells) recruited to the injured muscle (Fig. 7.1a). These events are characterized by an early innate immune response, running in parallel with T cell responses that together enhance the regeneration of damaged myofibers by promoting satellite cell proliferation and/or differentiation. Here we describe the carefully controlled series of immunological events that ensure the effective and complete recovery of acutely injured muscle.

Muscle fiber necrosis results in the release of damage-associated molecular patterns (DAMPs) that activate innate immunity [5]. Ly6G^{+} neutrophils are the first immune cells to infiltrate the site of injury [6], causing secondary damage by secreting free radicals [7] and pro-inflammatory cytokines, the latter of which promotes the homing of bone marrow-derived monocytes [8–10]. A recent study revealed that CD18 (integrin beta 2) is required for the extravasation of neutrophils into the acutely injured muscle, and mice deficient in CD18 exhibited enhanced signs of

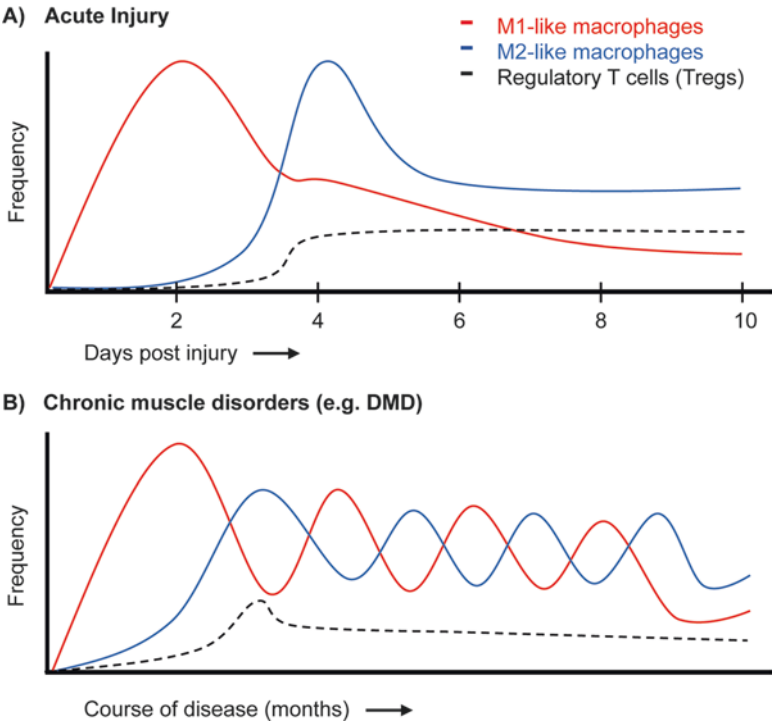

Fig. 7.1 Frequencies of immune cell populations in acutely injured and diseased muscle. Postulated macrophage and Treg frequencies during (**a**) acute muscle injury and (**b**) Duchenne muscular dystrophy (DMD). Red, blue, and dashed black lines represent frequencies of M1-like macrophages, M2-like macrophages, and Tregs, respectively

muscle repair, suggesting that neutrophils have a detrimental role in the repair process [8]. Following the neutrophil response, $CD11b^{+}Ly6C^{hi}CX3CR1^{lo}$ monocytes enter the damaged tissue and differentiate into pro-inflammatory M1-like macrophages [11]. M1 macrophages secrete the inflammatory cytokines, interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNFα) and phagocytose necrotic debris, suggesting that they participate in the repair process by clearing dead muscle [11]. A requirement for M1-like macrophages in muscle repair is further supported by in vitro studies showing that they promote myoblast proliferation while having no effect on differentiation [11]. Although previous studies have shown that M1-like macrophages promote the cytolysis of muscle cells in vitro, it is not clear whether they promote myofiber injury in vivo during acute injury [12].

$CD11b^{+}Ly6C^{lo}F4/80^{hi}$, anti-inflammatory M2 macrophages infiltrate injured muscle as M1 macrophages begin to resolve [11]. Although M2-like macrophages can be further divided into M2a, M2b, and M2c subpopulations (see [13] for a review), in this chapter we will collectively refer to them as M2 macrophages. The transition from M1 to M2 macrophages is necessary for the proper and efficient regeneration of injured muscle. Although the mechanisms that regulate this process

are not completely understood, recent studies highlighted regulatory T cells (Tregs) and IL-10 as having an important role in this transition; the ablation of either impeded on the M1 to M2 transition during acute injury [14, 15]. The importance of this transition is demonstrated by an increase in myofiber injury and altered regeneration in this setting [14, 15].

The central role of M2-like macrophages in muscle repair is supported by several studies showing that depletion or the inability to induce M2 activation impedes muscle regeneration [16, 17]. The depletion of F4/80^{+} macrophages during the time when M2-like macrophages infiltrate muscle impairs regeneration [16]. Moreover, the deficiency of AMPKα1 in macrophages, which prevented M2 activation, also resulted in an impairment in muscle regeneration following cardiotoxin-induced muscle injury [17]. Recent studies also showed that the intramuscular injection of CD11b^{+}Ly6C^{lo}F4/80^{hi} macrophages into mouse skeletal muscle increased the regeneration and functional recovery of skeletal muscle in an ischemia-reperfusion model of injury [18].

Although the role of M2-like macrophages in muscle regeneration is well accepted, we are only recently beginning to uncover the molecular basis of M2 macrophage-mediated regeneration. Recent studies are showing that M2-like macrophages promote regeneration, in part, by secreting factors that regulate inflammation and regeneration. For example, they secrete anti-inflammatory cytokines like transforming growth factor beta (TGF-β) and IL-10 that modulate the inflammatory response to establish an environment favorable for muscle regeneration [19]. Reparative macrophages also express soluble factors such as growth differentiation factor 3 (GDF3) [20] and insulin-like growth factor 1 (IGF-1) that promote regeneration by enhancing satellite cell fusion and differentiation [21–23].

The recruitment of macrophage populations to damaged muscle is an additional layer of regulation controlling the macrophage-specific contributions to muscle regeneration. A recent study by Brigitte and colleagues demonstrated that following acute injury, muscle resident macrophages (CD11b^{+}F4/80^{+}CD11c^{-}Ly6C^{-}CX3CR1^{-}) orchestrate early immune responses through the production of chemoattractants that recruit neutrophils and monocytes [24]. The CCL2:CCR2 chemotactic axis is required for macrophage recruitment to injured muscle and preferentially recruits inflammatory monocytes that are Ly6C^{hi}CCR2^{hi} [6, 23]. In contrast, the chemokine receptor CX3CR1 is not required for macrophage recruitment to injured muscle, but is important for regulating macrophage phagocytosis [25]. Macrophages also promote chemotaxis of immune cells through their intrinsic production of chemokines. For example, macrophages expressing CCL5 (RANTES) recruit CD8+ T cells following cardiotoxin-induced injury [26] and other immune cells including eosinophils [27]. Recent studies showed that eosinophils are required for efficient muscle regeneration and their accumulation in injured muscle paralleled an increase in CCL5 [28]. Given that macrophages are known to express CCL5, a potent eosinophil chemoattractant, these studies collectively suggest that macrophages recruit eosinophils, and both cooperatively promote regeneration.

Although myeloid cells dominate the immune cell infiltrate following injury, lymphocytes are also recruited and regulate muscle regeneration. Recent investigations

showed that following cardiotoxin injury, forkhead box P3 (FoxP3)$^{+}$CD25^{+}CD4^{+} Tregs were recruited to skeletal muscle at the time when the M1 to M2 macrophage transition occurs [14, 29]. The functional importance of Tregs in acute muscle injury was shown by loss- or gain-of-function experiments where muscle regeneration was hindered or enhanced, respectively [14]. Although muscle Tregs in acutely injured muscle expressed high levels of amphiregulin (Areg), and intramuscular and intraperitoneal injections of Areg in injured mice enhanced a transcriptional signature associated with muscle regeneration, it remains to be defined whether Treg-derived amphiregulin directly promotes muscle regeneration in vivo [14]. Burzyn and colleagues showed that Areg treatment enhanced satellite cell proliferation and differentiation, further supporting a regenerative function for Areg in acutely injured muscle. Further corroborating these findings, Castiglioni and colleagues independently demonstrated that muscle Tregs promoted regeneration by increasing satellite cell proliferation but inhibited differentiation [29]. In their studies, Castiglioni et al. co-cultured Tregs with satellite cells, in contrast to the Burzyn et al. study where satellite cells were treated with recombinant *Are*g [14, 29]. Thus, the contradicting results on satellite cell differentiation in these two studies may be attributed to differences in the experimental assays used. Collectively, these studies highlight a critical role for the immune system in muscle regeneration following acute injury. As we discuss next, similar immunoregenerative mechanisms operate in chronic muscle disorders, the dysregulation of which contributes to the pathogenesis of these diseases.

7.3 The Role of the Immune System in Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is a devastating childhood disease attributed to dystrophin gene mutations [30, 31] that arise in approximately 1:5000 males [32, 33]. Clinical symptoms arise shortly after 3–5 years of age and progressively worsen, leading to loss of ambulation by adolescence and death by the second to third decade of life. The dystrophin protein mechanically stabilizes and protects the sarcolemma from longitudinal and radial forces generated during muscle contraction. In the absence of dystrophin, as seen in DMD patients, the sarcolemma is rendered susceptible to contraction-induced injury [34], which subsequently triggers a chronic muscle inflammatory response that contributes to the development and progression of DMD [35].

7.3.1 Immunology in the Pathogenesis of Muscular Dystrophy

The critical function of inflammatory immune cells in muscle regeneration during acute injury is dysregulated in DMD, consequently promoting muscle degeneration. Muscle injury during DMD induces an oscillating pattern of chronic muscle

inflammation that mirrors the asynchronous and cyclic nature of injury (Fig. 7.1b). This dysregulated inflammatory response subsequently promotes fibrosis and failure of regeneration [36]. The increased immune cell numbers and expression of genes related to immune cell function in DMD muscle further support the immune system's contribution to the pathogenesis of muscular dystrophy [37–40]. Moreover, the immunosuppressive activity of glucocorticoids [41], and their known role in promoting muscle atrophy [42], suggests that the incremental delay in disease progression is mediated by inhibition of the immune system. This argument is supported by studies performed in the mdx mouse model of DMD showing that glucocorticoid treatment reduces the expression of adhesion molecules required for immune cell extravasation and the number of immune cells present in dystrophic muscle [43]. More specific methods of immune cell ablation in mdx mice have demonstrated that the depletion of eosinophils, macrophages, or T cells in muscle causes a 60–80% decrease in muscle pathology [44–46]. These early studies aided in establishing inflammation as a secondary disease process contributing to the severity of muscular dystrophy and have provided the basis for research aimed at uncovering the cellular and molecular basis of immune-mediated pathology in DMD.

In addition to promoting muscle injury, the immune system is also critical in mediating muscle regeneration during muscular dystrophy [47–51]. This dichotomy can be partly explained by a division in functional facets of the immune system, in which distinct immune cell subsets are responsible for causing injury or promoting regeneration (Fig. 7.2). This process is exemplified by the accumulation of macrophages with distinct polarized states of M1- or M2-like activation that are known to promote either injury or repair, respectively. M1 and M2 macrophages reflect polar extremes of activation. However, macrophages present in dystrophic muscle likely exist as a broad continuum in which cells transition from one state of activation to another according to changes in the pro- and anti-inflammatory environment [52, 53]. Pro-inflammatory cytokines such as IFNγ and TNFα promote the classical activation of M1 macrophage that induces myofiber injury through an iNOS-dependent mechanism [54]. In contrast, cytokines such as IL-4, IL-13, and IL-10 induce M2 activation of macrophages that antagonize the action of M1-like macrophages via arginase-dependent mechanisms [54]. Recent studies have begun to define the cell types that orchestrate changes in the inflamed environment of dystrophic muscle. Below, we discuss the role of Tregs as critical regulators of muscle inflammation and regeneration, focusing on their capacity to shift the balance between M1 and M2 macrophages in favor of M2 activation.

Treg development and function is highly dependent on FoxP3, a transcription factor critical for the specification of this suppressive $CD4^+$ T cell lineage. The function and viability of $CD4^+FoxP3^+$ Tregs are highly dependent on IL-2, a cytokine expressed primarily by conventional ($CD4^+FoxP3^-$) T cells [55] and to a lesser extent by naive $CD8^+$ T cells [56] and dendritic cells [57]. IL-2 signaling through the IL-2 receptor induces the expression of FoxP3 in a STAT-5-dependent manner [58], thus, enforcing Treg lineage specification. Unlike conventional T cells, Tregs constitutively express CD25 (the IL-2Rα chain), corroborating the importance of IL-2

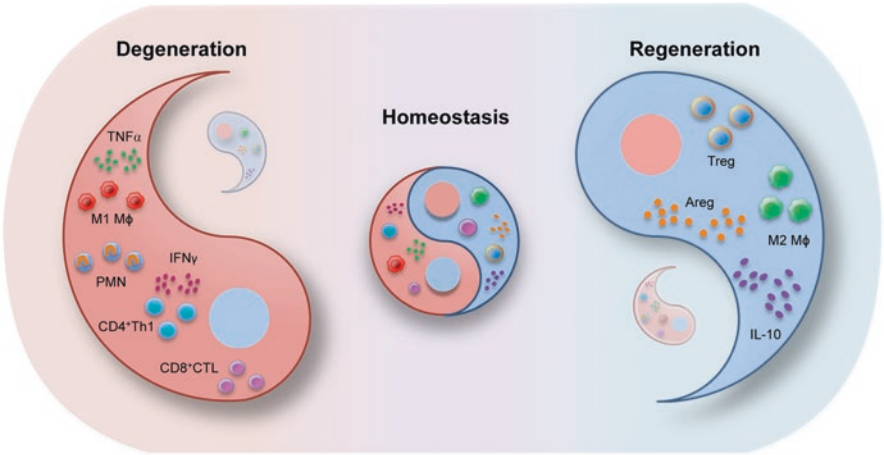

Fig. 7.2 The Yin-Yang function of degenerative and regenerative immune cells in muscular dystrophy. In healthy muscle degenerative and regenerative immune cell populations are balanced, maintaining homeostasis (center). During muscular dystrophy, shifts in this balance either promote degeneration (left) or regeneration (right). A degenerative type 1 inflammatory response is characterized by increased expression of pro-inflammatory cytokines such as IFNγ and TNFα and the presence of polymorphonuclear neutrophils (PMN), M1-like macrophages (M1 Mϕ), $CD4^+$ Th1 cells, and $CD8^+$ cytotoxic T lymphocytes (CTL). Regenerative responses are characterized by the presence of Tregs and M2-like macrophages (M2 Mϕ) that express amphiregulin (Areg) and IL-10. Eosinophils are not depicted because they are suspected to play a role in degeneration and regeneration

signaling for Treg function and/or viability. Accordingly, defects in IL-2/IL-2 receptor signaling have been linked with several inflammatory and autoimmune disorders due to a developmental defect in Tregs [59].

As discussed above, Tregs are elevated in regenerating muscle and promote muscle regeneration by regulating macrophage activation and enhancing myogenesis through Areg-dependent mechanisms. Comparably, studies in the mdx mouse and human indicate that muscle Tregs are also increased in chronic muscle disorders [60–63]. Tregs in mdx muscle were elevated in number and expressed high levels of IL-10. They inhibited M1 macrophage activation and reduced myofiber injury [60]. Similarly, Tregs and IL-10 were elevated in human dystrophic muscle [60], supporting the view that Tregs suppression of muscle inflammation is mediated by IL-10. Additionally, in vitro studies have shown that human Tregs express IL-10 and induce M2 macrophages [64]. Although the Treg-specific role of IL-10 in suppressing muscle inflammation has not been tested, its importance is supported by studies showing that IL-10 deficiency in mdx mice exacerbates dystrophinopathy [65, 66]. We note, however, that the mechanism of Treg-mediated suppression of immunity in other tissues is multifaceted, involving the inhibition of antigen-presenting cells (i.e., dendritic cells), cytolysis of conventional T cells, and the production of anti-inflammatory cytokines, like IL-10 and IL-35 [67, 68]. Moreover, recent studies indicate that tissue Tregs also harness specialized functions adapted to the environment they reside in.

Thus, in addition to suppressive activity, Tregs in dystrophic muscle are likely involved in the promotion of regeneration through Areg, and/or other growth factors, as seen during acute injury [14].

7.3.2 *The Immune System as a Barrier Limiting the Efficacy of Muscle Gene Therapy*

Despite the common view that inflammation is a generalized innate response to muscle injury, several lines of evidence indicate that antigen-specific responses are also operating in dystrophic muscle [49, 50]. Adaptive (antigen-specific) immune responses are mediated by B lymphocytes (B cells) that directly bind antigen; and T lymphocytes (T cells) bind cognate antigen presented on major histocompatibility complex (MHC) molecules [69]. Previous studies have shown that $B220^{+}$ B cells, germinal center reactions, and IFNγ-expressing effector T cells were expanded in lymph node or muscle of mdx mice [60, 70, 71]. Due to the required activation of the antigen-specific receptors for B and T cell expansion [72, 73], one may postulate that an unidentified antigen/s is responsible for the activation of adaptive immune cells in dystrophic muscle. This hypothesis is further supported by multiple studies suggesting the clonal expansion of antigen-specific T cells in mdx mice and human DMD patients [14, 63, 74].

The activation of T cells requires engagement of the T cell receptor (TCR) by cognate antigen loaded on MHC, which causes the clonal expansion of antigen-specific T cells that promote immunity [73]. Previous reports showed that relative to other Vβ T cell populations, $V\beta 8.1/8.2^{+}$ T cell frequencies were increased in mdx muscle, and a conserved amino acid motif, RVSG, was observed in the TCR's third complementary determining region (CDR3) in multiple DMD patients [63, 74]. Burzyn and colleagues provided additional evidence for clonal expansion by examining the TCR repertoire of Tregs and found an enrichment of several TCR rearrangements in mdx muscle [14]. Collectively, these studies indicate that the recognition of uncharacterized muscle antigens in mdx mice and DMD patients elicit adaptive immunity during muscular dystrophy. Although the antigens driving adaptive immunity are unknown, studies have shown that some of the specificity of B and T cells is directed toward dystrophin [75–77].

Recent studies revealing the development of dystrophin-specific immune responses raise a concern about the potential lack of immunological tolerance to the protein product of exogenously introduced dystrophin transgenes [76, 77]. This challenge was highlighted in a recent gene therapy trial where a subset of patients treated with mini-dystrophin gene therapy harbored dystrophin-specific T cells [77]. Flanigan and colleagues subsequently confirmed these observations in their study of a larger population of DMD patients, where they found that 50% of untreated patients harnessed dystrophin-specific T cells, in contrast to 20% in patients treated with glucocorticoids [76]. It remains to be determined whether any of the observed T cell responses in mdx mice are dystrophin-specific. However, studies have shown that

dystrophin-specific antibodies are elicited in animal models of DMD and patients following myoblast transplantation or dystrophin gene therapy [75]. Collectively, these studies support a lack of immunological tolerance to dystrophin in DMD, leading to the generation of dystrophin-specific B and T cells. Dystrophin-specific immune responses will likely compromise the long-term efficacy of dystrophin gene therapy due to immune-mediated rejection of myofibers expressing the dystrophin protein. Future investigations aimed at defining the immunoregulatory mechanisms operating in muscular dystrophy will provide the field with an increased understanding to better design future therapies that specifically augment dystrophin tolerance.

7.4 The Role of the Immune System in Myopathies

Similar to DMD, activation of the immune system is also a feature of other muscle diseases. However, the immune cell populations that promote pathogenesis and their functional attributes differ among these disorders. This section explores immune responses in chronic myopathies, primarily focusing on sporadic inclusion body myositis (sIBM) as an example of a muscle disease with suspected autoimmune etiology. We describe the role of the immune system in these muscle disorders and highlight the Treg response in these muscle diseases.

7.4.1 Immune Responses in Myopathies

Distinctions in the localization and function of immune cells can be made among muscle disorders. In DMD, inflammatory cells surround and invade necrotic muscle fibers but spare non-necrotic muscle fibers [78]. In contrast, in facioscapulohumeral muscular dystrophy (FSHD), dermatomyositis (DM), polymyositis (PM), and sIBM, immune cells infiltrate the endomysium to form inflammatory halos that surround non-necrotic muscle fibers [78, 79]. We speculate that the inflammatory halos, primarily composed of $CD8^+$ cytotoxic T lymphocytes (CTLs) and macrophages, are responsible for the injury of healthy muscle fibers found in these diseases [78, 79]. In support of this interpretation, perforin-expressing $CD8^+$ CTLs invade non-necrotic fibers that express MHC class I in PM and sIBM patients [79]. Studies showing that perforin deficiency in mdx mice reduces myofiber injury further support perforin-dependent cytotoxicity as an important mechanism for T cell-mediated injury of muscle [80].

Several studies suggest that antigen-dependent activation of $CD8^+$ CTLs underlies the pathogenesis of several chronic myopathies. In sIBM, circulating and muscle $CD8^+CD28^{null}$ and $CD4^+CD28^{null}$ T cells are expanded [81, 82]. A percentage of these cells expressed CD107a (a marker of degranulation and cytotoxicity) and IFNγ (the prototypical Th1 effector cytokine). Given that CD28 is decreased when T cells are exposed to antigen [83], the expression of CD107a and IFNγ on $CD8^+$

T cells likely reflects an undefined antigen-dependent activation of $CD8^+$ CTL responses during sIBM. Evidence of clonal expansion of T cells in sIBM was provided by Greenberg et al., which showed that $CD8^+CD57^+$ T cells were increased [84]. In human disease, $CD8^+CD57^+$ T cells represent a population of oligoclonally expanded T cells generated in response to chronic antigenic stimulation [83]. Corroborating an antigen-dependent activation of T cells in sIBM, Pandya and colleagues showed a restricted usage of T cell receptor Vβ chains in sIBM patients, indicating clonal expansion of T cells following antigen exposure [82]. Although these data do not indicate that $CD8^+$ T cells dominate the immune response, they do suggest that inflammation in chronic muscle disorders encompasses a CTL response that promotes muscle injury. However, further research is necessary to determine whether CTL activation is a result of muscle degeneration or an intrinsic defect in the immune system causing disease.

In several autoimmune diseases, a feature of autoimmunity is the generation of autoantibodies [85]. Similarly, in sIBM 70% of patients were seropositive for NT5c1A autoantibodies, which was associated with a more severe clinical outcome as determined by a significantly longer timed get-up test [86, 87]. Seropositive patients were also more likely to require a walker or wheelchair, present symptoms of dysphagia, and demonstrate facial weakness [87]. Retrospective studies based on data from European sIBM registries identified NT5c1A seropositive patients as having a higher mortality risk [88]. Thus, the presence of autoantibodies directed against NT5c1A supports a means of stratifying sIBM patients into clinical groups to predict prognosis.

In addition to distinct immune effector cells and antibody responses associated with myopathies, the upregulation of pro-inflammatory cytokines and mediators may also enhance pathogenesis. Peripheral blood mononuclear cells in FSHD patients showed higher expression of pro-inflammatory cytokines including IL-12, IFNγ, and TNFα [89]. The role of IFNγ was also highlighted in sIBM and DM, where IFNγ induced greater expression of CXCL9, promoting systemic Th1 immune activation [81, 90, 91]. Furthermore, it was demonstrated in sIBM that high mRNA expression of IFNγ, CXCL9, and CCL3 positively correlated with β-amyloid-associated protein expression in myofibers, suggesting that the accumulation of this protein induces the expression of pro-inflammatory factors [92]. The prevalence of pro-inflammatory cytokines, in concert with activated immune cells and autoantibodies, is collectively important in potentially enhancing pathogenesis in some muscle diseases.

7.4.2 Regulatory T Cells as Immunosuppressors of Chronic Myopathies

Similar to acute injury and muscular dystrophy, Treg responses also appear to operate in additional muscle disorders. Previous studies revealed a decrease in peripheral Tregs in PM, DM, and sIBM that may underlie the elicitation of an autoimmune response specific for undefined muscle antigens [62, 81, 93]. Although a subset of

patients with active juvenile DM displayed a higher frequency of Tregs in inflamed muscle, Treg suppressive activity was defective in these patients [61]. Also observed in DM was a reduction in the serum levels of IL-10 and TGF-β, effector cytokines produced by peripheral Tregs that suppress inflammation [94, 95]. Conversely, studies in the mdx mouse model of DMD have collectively shown that Tregs are elevated in dystrophic muscle [60] and IL-10 inhibited M1-like macrophages, reducing muscle injury and improving strength [65]. The consequence of reduced Treg frequencies and IL-10 expression may be an underlying cause contributing to autoimmunity in DM, PM, and sIBM. In the context of inflammation, understanding the roles of effector T cells, Tregs, and their cytokine expression profiles may elucidate new targets for the treatment of chronic myopathies.

7.5 Potential Immunological Targets for the Treatment of Muscle Disorders

A great precedence should be placed on the advancement of treatment options aimed at improving the quality of life of DMD patients while a cure is discovered. Understanding how the immune system contributes to dystrophinopathy may lead to the development of novel immunological therapies that ameliorate disease severity by inhibiting degenerative inflammation. Moreover, immune-based interventions may be used to tolerize DMD patients to the dystrophin gene therapy protein product. Although numerous strategies exist, NF-kB inhibitors, direct cytokine targeting, and Tregs show great promise to address these unmet clinical needs and will be discussed in this section.

7.5.1 Blockade of NF-κB

The NF-κB pathway is an attractive target because of its well-described role in transcriptionally regulating the inflammatory cascade. The NF-κB family consists of p50, p52, p65, RelB, and c-Rel proteins, which form hetero- and homodimers that regulate the transcription of numerous inflammatory genes [96]. During homeostasis, the majority of NF-κB dimers are sequestered by IκBα and kept in an inactive state. Upon inflammatory stimuli, IκBα is phosphorylated by IKK (IκB kinase) and degraded, allowing NF-κB to translocate to the nucleus and activate the transcriptional program that drives inflammation [96].

The therapeutic potential of targeting this pathway was shown by genetic studies where NF-κB was deleted in macrophages or myotubes, resulting in reduced inflammation and improved regeneration, respectively [97]. Moreover, treating mdx mice with NEMO-binding domain (NBD) peptides, an NF-κB-specific inhibitor, increased muscle fiber regeneration and decreased macrophage accumulation in muscle [97]. As an alternative approach, Sun and colleagues used sulforaphane (SFN) to inhibit

NF-kB [98]. SFN increased muscle mass while decreasing nuclear accumulation of NF-κB and the expression of pro-inflammatory cytokines such as IL-1β, TNFα, and IL-6 [98]. Of particular interest, a new experimental steroid drug, VBP15, has gained clinical interest due to its ability to inhibit the NF-kB pathway without activating glucocorticoid receptor (GR) target transcripts [99]. The potential clinical benefits for DMD patients are of high impact given that the side effects of anti-inflammatory glucocorticoids are suspected to act through classical steroidal transactivation [100]. Targeting the NF-κB pathway has proved successful in preclinical models of muscular dystrophy and currently stands as a highly plausible form of immunological treatment for DMD.

7.5.2 Cytokine Targeting

A prominent feature of the inflammatory infiltrate in muscular dystrophy is the secretion of pro-inflammatory cytokines that potently induce degenerative inflammation. Although multiple cytokines should be considered as potential targets for muscular dystrophy, here we highlight TNFα and IL-6 for their well-known role in muscle inflammation [101, 102]. TNFα is secreted mainly by macrophages and is a potent activator of immune cells and the NF-κB pathway. Studies using cVq1, an anti-TNFα antibody, showed a decrease in necrosis and contractile dysfunction after exercise-induced muscle damage in mdx mice [103]. TNFα, in addition to its ability to activate NF-κB, is potent amplifier of IFNγ-induced activation of M1 macrophages. Thus, the ameliorative effect of cV1q treatment in mdx mice likely involves the inhibition of M1 macrophages that are known to induce muscle injury in this setting [54]. Given the preclinical success of TNFα blockade, the FDA-approved human-specific TNFα antibody, infliximab, may serve as a potential immunological therapy for DMD [104].

IL-6 is an inflammatory cytokine with pleiotropic activity, showing anti- or pro-inflammatory effects in skeletal muscle ailments [105]. It was recently postulated that the functional dichotomy of IL-6 is attributed to classic versus trans-signaling [106]. In trans-signaling, IL-6/soluble IL-6 receptor complexes bind gp130 in trans to activate signal transduction, promoting the transition from acute to chronic inflammation [106, 107]. Alternatively, in classic signaling IL-6 binds membrane-bound IL-6 receptor and gp130 to activate anti-inflammatory cascades. IL-6 is overexpressed in mdx mice and DMD patients, and blockade of the IL-6 pathway had beneficial effects in preclinical settings [108, 109]. Treatment of mdx mice with monoclonal IL-6 receptor-blocking antibodies reduced muscle inflammation and necrosis after exercise-induced injury [109]. It is important to note, however, that some studies have shown that neutralizing IL-6 ligand reduced inflammation in mdx mice without improving muscle function [110]. It remains to be addressed whether targeting the IL-6 ligand or blocking the receptor has differential effects on trans and classical IL-6 signaling. We propose that tocilizumab, an IL-6 receptor antagonist approved for rheumatoid arthritis, may be used as an anti-inflammatory treatment for DMD. However, further research is required to determine the effect of tocilizumab on trans- and classical signaling.

7.5.3 Treg-Promoting Therapies

Their immunosuppressive nature makes Tregs an attractive therapeutic target in DMD, because of their strong potential to suppress muscle inflammation and promote dystrophin tolerance. The therapeutic potential of Tregs in DMD is supported by studies showing an accumulation of Tregs in mdx skeletal muscle, and their depletion exacerbated muscle injury, inflammation, and fibrosis [14, 60]. Importantly, mdx mice treated with IL-2 and anti-IL-2 antibody complexes (IL-2c) that potently induce Tregs in vivo reduced inflammation and myofiber injury. The amelioration of muscular dystrophy in mdx mice treated with IL-2c was associated with an increased frequency of Tregs and expression of IL-10 in mdx skeletal muscle [60]. Further supporting the potential of Tregs in muscular dystrophy, Gazzerro and colleagues found that inhibitors of the extracellular ATP/P2X purinergic signaling pathway significantly ameliorated disease severity in mdx mice. Inhibition of the ATP/P2X axis increased muscle Treg numbers and muscle strength while reducing muscle inflammation and injury [111]. It remains to be addressed whether the ameliorative role of P2X inhibitors was attributed specifically to an inhibition of the ATP/P2X axis in Tregs or other cellular targets. Eghtesad et al. showed previously that targeting the mTOR pathway with the immunosuppressant rapamycin (RAPA) ameliorated dystrophic pathology in mdx mice by increasing the frequency of Tregs in dystrophic muscle. However, it appeared that the mechanism of action for RAPA involved the targeting of effector T cells (Teff), effectively increasing the Treg/Teff ratio [112]. However, it should be noted that inhibition of the mTOR pathway causes atrophy [113]. Thus, further research is required to determine the consequences of long-term RAPA treatment on dystrophic muscle. Nonetheless, RAPA remains an attractive therapy because of its ability to inhibit dystrophin immunity [114], which may outweigh the negative consequence of mTOR inhibition on muscle growth.

7.6 Conclusion

Understanding the functional outcome of immune cell and muscle interactions that promote injury may lead to the development of novel treatments that prevent or delay muscle disease. Corticosteroids delay the deterioration of muscle that is exacerbated by cytotoxic inflammatory cells. However, chronic corticosteroid use is associated with side effects, and the complete inhibition of inflammatory cells may lead to the silencing of protective mechanisms that promote muscle repair. The growing body of evidence that specialized subpopulations of immune cells which contribute to muscle injury or regeneration sets forth a precedence on delineating the mechanisms that promote cytotoxic inflammatory responses from those that promote repair. Research directed at addressing this goal will lead to the development of safer treatments that target cytotoxic mechanisms while leaving intact those that are protective. In addition, the findings of these future lines of investigation may provide the DMD research community with the insight required to address the clinical challenge of promoting tolerance to dystrophin.

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Chapter 8
Design of Muscle Gene Therapy Expression Cassette

Yi Lai and Dongsheng Duan

Abstract The first gene therapy drug approved by the European Regulatory Commission involves the transfer of a therapeutic gene to the muscle by adeno-associated viral vector (AAV). Now, muscle gene transfer is quickly becoming a therapy of choice for muscle and non-muscle diseases. Successful muscle gene therapy requires efficient expression of therapeutic proteins in the muscle without causing any toxicity and side effects. To achieve this, the expression cassette of therapeutic proteins needs to be designed rationally. A typical expression cassette usually contains a promoter to initiate transcription, the coding sequence of a transgene, and a termination signal to terminate transcription. Other *cis*-regulatory elements can be added into the 5′- and 3′-untranslated regions. In this chapter, we review the development of the components of the expression cassette in the context of muscle gene therapy.

Keywords Muscle · Gene therapy · Promoter · Transgene · Termination signal AAV

Y. Lai (✉)
Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri, Columbia, MO, USA
e-mail: laiy@health.missouri.edu

D. Duan
Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri, Columbia, MO, USA

Department of Neurology, School of Medicine, University of Missouri, Columbia, MO, USA

Department of Biomedical Sciences, College of Veterinary Medicine, University of Missouri, Columbia, MO, USA

Department of Bioengineering, University of Missouri, Columbia, MO, USA
e-mail: duand@missouri.edu

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8.1 Introduction

Muscle gene therapy is becoming a therapy of choice for muscle and many non-muscle diseases, as exemplified by the first gene therapy drug (Glybera) approved by the European Regulatory Commission, which involves adeno-associated virus (AAV)-mediated expression of a therapeutic gene, lipoprotein lipase, in the muscle [1]. Gene transfer to muscle has many advantages. First, muscle is the most abundant tissue in the body, comprising 30–40% of the body mass. The abundance of the muscle tissue could possibly represent a barrier for gene transfer in muscle genetic disorders. However, on the other hand, a large quantity of muscle cells are advantageous for gene therapy of non-muscle diseases, since abundant muscle cells are able to produce sufficient amount of secretive proteins for systemic distribution or vaccination. Second, muscle gene transfer is easy to manipulate as muscle is readily accessible to multiple gene delivery routes. Third, due to slow turnover of the muscle cells, the expression of a transgene in the muscle usually persists for a long period.

In general, gene transfer includes viral (such as adenovirus, AAV, herpes simplex virus (HSV), lentivirus, and retrovirus) and non-viral gene transfer (such as naked plasmid DNA). Irrespective of the gene transfer methods, there is usually a basic gene expression unit (or the expression cassette) to express a therapeutic protein. Sometimes, a therapeutically relevant RNA or oligonucleotides can also be expressed from the basic gene expression unit. In this chapter, we focus our discussion on the design of the protein expression unit.

The expression cassette comprises a default structure, composed of a promoter to initiate transcription, a transgene coding sequence, and a termination signal to terminate transcription. Also, other *cis*-regulatory elements can be added to facilitate transgene expression (Fig. 8.1). To induce safe and efficient transgene expression in the muscle, the expression cassette needs to be designed rationally by carefully evaluating and assembling each component in the expression cassette. Generally, several factors should be considered. First, the size of the expression cassette should fit in the gene transfer vector. For example, the packaging capacity of AAV is <5 kb.

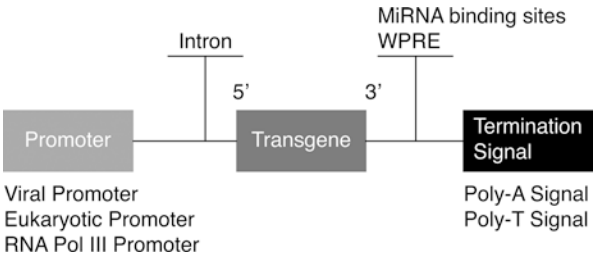

Fig. 8.1 The structure of a typical expression cassette in the gene transfer vector**.** The expression cassette is composed of the promoter to initiate transcription, the coding sequence for a transgene, and the termination signal to terminate transcription. Other *cis*-regulatory elements can be added into the 5′- and 3′-untranslated region (UTR) of the transgene coding sequence

So the size of the expression cassette for AAV cannot be beyond 5 kb. Second, the efficiency of the expression cassette should be high enough to produce sufficient therapeutic proteins in muscle. Third, duration of the transgene expression should be long enough to generate therapeutic effects but without causing any toxicity and side effects. Below we discuss each components of the expression cassette.

8.2 Promoter

A good promoter can drive robust transcription of a transgene in the muscle. The commonly used promoters include the RNA polymerase II-dependent promoter (Pol II promoter) and RNA polymerase (Pol) III-dependent promoter. The Pol II promoter is the most commonly used one, and it is usually used to express a protein-coding sequence. The commonly used Pol II promoters include viral promoters, eukaryotic promoters, and synthetic promoters.

8.2.1 Viral Promoter

The most commonly used viral promoters are the ubiquitous cytomegalovirus (CMV) immediate-early promoter, Rous sarcoma virus (RSV) promoter, simian virus 40 (SV40) promoter, and retroviral LTR promoter. Because of their high potency and easy availability, the majority of gene transfer vectors utilized viral promoters during the early period of gene therapy development. Both CMV and RSV promoters can induce a higher level of transcription than SV40 and retroviral LTR promoter in the muscle [2, 3]. Therefore, CMV and RSV promoters are more widely used in muscle gene therapy and have been shown to induce the expression of many transgenes in mouse muscle [4–9]. In addition to the murine studies, CMV and RSV promoters are also widely used in large animal studies. For example, alkaline phosphatase (AP) has been expressed in whole body muscles by an AAV.RSV.AP vector in neonatal dogs [10], and AAV.CMV.μ-dystrophin vectors have been used to drive efficient expression of therapeutic micro-dystrophin proteins in the dystrophic muscle of canine muscular dystrophy models [11–13]. Moreover, CMV promoter has been reported in three clinical trials of muscle gene transfer. Two therapeutic proteins, α-sarcoglycan and follistatin, have been successfully expressed using AAV.CMV vectors in the muscle of the patients with muscle genetic diseases, including limb-girdle muscular dystrophy (LGMD) type 2D [14], Becker muscular dystrophy (BMD) [15], and sporadic inclusion body myositis [16].

In addition to efficient transgene expression in the muscle, as ubiquitous promoters, viral promoters also can induce high-level expression in other organs, such as the liver, lung, pancreas, kidney, testes, retina, and brain [17–21]. Although universal activity makes viral promoters attractive for transgene expression in multiple tissues, untoward expression in nontargeted tissues may cause cytotoxicity and

immune responses. Eukaryotic cells are equipped with mechanisms to inactivate and silence viral gene expression. Hence, transgene expression from viral promoters may encounter promoter shutoff, in which viral promoters are inactivated and silenced in eukaryotic cells. The immune mechanisms, including cytokine production and promoter methylation, are responsible for CMV promoter shutoff [22]. Interestingly, the RSV promoter is not prone to silencing by methylation [23]. As a result of untoward expression and promoter shutoff, transgene expression from viral promoters is often transient [24–26].

8.2.2 Eukaryotic Promoter

The common eukaryotic promoters include elongation factor 1α (EF-1α) promoter; CAG promoter, which is composed of CMV immediate-early enhancer, chicken β-actin promoter, and the splicing acceptor of rabbit β-globin; phosphoglycerate kinase (PGK) promoter; ubiquitin c (UBC) promoter; and tissue-specific promoters. In an in vitro study, the strength of ubiquitous eukaryotic promoters was compared with viral promoters in the myoblast C_2C_{12} cells. Using lentivirus-mediated GFP expression as a readout, the CAG promoter is shown to have the highest transcription activity, followed by EF-1α, CMV, SV40, PGK, and UBC promoter [27]. In an in vivo study using the dog as the model, intracardiac injection of AAV.CAG.GFP led to efficient GFP expression in the smooth muscle of the heart microvessels [28].

Similar to viral promoters, ubiquitous eukaryotic promoters can also lead to widespread transgene expression, which may cause immune responses and cytotoxicity. To restrict transgene expression in the muscle, muscle-specific promoters have been developed. Most of these promoters are developed based on our understanding of gene regulation in muscle-specific proteins such as creatine kinase (CK), desmin, troponin, α-myosin heavy chain (α-MHC), and myosin light chain 2 (MLC-2). Another approach is to develop synthetic muscle promoter. By randomly joining multiple copies of four myogenic elements, E-box, MEF-2, TEF-1, and SRE, synthetic promoter libraries have been established. The transcriptional activities of synthetic promoter libraries have been screened in vitro and in vivo. One synthetic promoter C5–12 has been shown to have better transcriptional activity than endogenous muscle-specific promoters and CMV promoter in both skeletal and cardiac muscle [29]. In the following studies, the C5–12 promoter was used to drive the robust expression of therapeutic genes, dystrophin and neuronal nitric oxide synthase (nNOS), in both skeletal and cardiac muscles [30, 31]. In another line of studies, by taking advantage of the enhancer and promoter of the genes expressing muscle-specific proteins, including creatine kinase (CK), desmin, troponin, α-myosin heavy chain (α-MHC), and myosin light chain 2 (MLC-2), muscle-specific promoters have been identified to confine transgene expression to the muscle.

The CK promoter successfully drives efficient expression of Lac Z and dystrophin in the muscle [3, 32]. Compared to the ubiquitous CMV promoter, CK-driven transgene expression is significantly higher in the muscle [33, 34]. Further modification

by joining the enhancer of the α-myosin heavy chain gene to the CK promoter dramatically increases transcription efficiency in the heart [35] and enables the CK promoter for treating muscle genetic disorders with severe cardiac symptoms, such as Duchenne muscular dystrophy (DMD). The desmin promoter can mediate transgene expression specifically in skeletal and cardiac muscle [36, 37]. However, one study found that the desmin promoter is less efficient than the CK promoter in skeletal muscle [14]. The troponin promoter is mainly used for cardiac gene transfer [38–42], and it successfully induces robust expression of transgenes (such as SOD, GFP, and Cre recombinase) in the heart. One study compared the transcription activity of CMV, desmin, α-MHC, MLC-2, and troponin promoters in the heart and found that the CMV promoter resulted in the highest expression [37]. Since both C5-12 and CK promoters are more efficient than the CMV promoter in muscle [29, 33, 34], these two promoters may be more appropriate for muscle gene transfer.

Contrary to viral promoters, muscle-specific promoters result in longer periods of transgene expression and less severe immune reaction. The use of the CK promoter prevented the transgene expression in dendritic cells and prolonged the persistence of transgene expression in the muscle [43]. One study compared CK- and CMV-driven γ-sarcoglycan expression in the muscle and found that CMV-driven γ-sarcoglycan expression was much lower than that from the CK promoter. Further, the authors found the CMV promoter induced the immune response to γ-sarcoglycan [44]. Given the success of muscle-specific promoters in limiting the transgene expression in the muscle and reducing non-specific expression in murine studies, now muscle-specific promoters have been expanded to canine studies, and they were shown to successfully induce robust expression of a series of therapeutic genes, such as myotubularin and micro-dystrophin, in dog muscles [45–48].

To date, the synthetic C5-12 promoter, the CK promoter, the desmin promoter, and the troponin promoter are the most commonly used muscle-specific promoters in gene therapy studies. At present about 4806 eukaryotic RNA Pol II promoters have been discovered and confirmed by experiments. The information of these promoters can be found in the Eukaryotic Promoter Database (EPD) (http://epd.vital-it.ch/). Facilitated by promoter-specific high-throughput data analysis, more and more eukaryotic promoters have been added and expanded the EPD to EPDnew, which accommodates 27,233 promoters in *Homo sapiens* and 21,239 promoters in *Mus musculus*. These two databases are valuable resources for eukaryotic promoters. The wealth of information about eukaryotic promoters in these databases should be very useful for the development of the next-generation muscle-specific promoters.

8.2.3 RNA Pol III-Dependent Promoter

Both viral and eukaryotic promoters require RNA Pol II to synthesize mRNAs, which encode proteins. Although RNA Pol II promoters also synthesize noncoding RNAs, such as microRNAs, RNA Pol III promoters, including U6 and H1, are mainly used to express small RNAs, such as guide RNAs (gRNAs) for clustered

regularly interspaced short palindromic repeat (CRISPR)-Cas9 gene editing, small interfering RNAs (siRNAs), and short hairpin RNAs (shRNAs) to inhibit target gene expression. Transcription from the U6 promoter usually starts at the +1 position, which is 23 nucleotides (nt) downstream of the TATA box and G as the favorite initiation nucleotide. However, one study has identified the region from −3 to +4 in the mouse U6 promoter as the putative initiation site. Transcription by the U6 promoter can start with A or G, from the −1 to +2 positions. The sequences around the putative initiation site of the U6 promoter are critical for the accuracy of the 5′-end sequence of small RNAs. For another RNA Pol III promoter, H1 promoter, initiation consistently starts from multiple sites from −3 to +1 position. Hence, small RNAs transcribed by H1 promoter may have variable 5′ ends, which may influence the processing of small RNAs and knockdown efficiency [49]. It was reported that human U6 promoter-mediated shRNA knockdown is more potent than that of the mouse U6 promoter in both human and murine cells [50]. Knowledge on the typical features of the RNA Pol III promoter will be helpful in engineering the expression cassettes of small RNAs in muscle gene therapy.

The application of small RNAs in muscle gene therapy mainly includes four categories. The first category is the small nuclear RNA (snRNA) driven by the U7 promoter. Delivery of AAV.U7.snRNA to the muscle successfully induced exon skipping and restored the expression of dystrophin in dystrophic mice. AAV.U7.snRNA resulted in widespread expression of functional dystrophins in the dystrophic muscle and improved muscle function [51, 52]. However, with the loss of viral genomes, dystrophin expression decreased significantly [53]. Future studies are needed to optimize AAV doses to induce higher expression of dystrophin in order to stabilize myofibers and prevent the loss of viral genomes. The second category is the shRNAs driven by the U6 promoter. AAV.U6.shRNA SOD1 delivery led to significant reduction of the SOD1 protein in both skeletal and cardiac muscles, and no apparent side effects were observed when AAV was injected to neonatal mice [54]. Knockdown of NF-kappaB/p65 by injection of AAV.U6.shRNA.NF-kappaB/p65 reduces the muscle pathology of *mdx* mice, the mouse model of DMD [55]. VEGF expression in the muscle was knocked down by 91% by AAV.U6.shRNA.VEGF delivery [56]. AAV.U6.shRNA-mediated knockdown was also used in a dog study. Reduction of phospholamban (PLB) expression in the heart of the dog was achieved with AAV.U6.shRNA.PLB. However, AAV.U6.shRNA.PLB delivery is associated with cardiac toxicity possibly due to saturation of endogenous miRNA pathways caused by overexpression of shRNAs [57]. To overcome this issue, the third category of the small RNA expression cassettes was developed by embedding synthetic shRNA stems into the context of endogenous miRNAs (shRNAmir). The resulting shRNAmir can be expressed from RNA Pol II promoters [58]. In the myotubes isolated from DMD patients, knockdown of phosphatidylinositol transfer protein-α (PITPNA) has been successfully achieved by the shRNAmir cassette driven by a doxycycline-inducible promoter [59]. AAV-mediated shRNA expression, which targets dystrophin and was driven by a modified CMV promoter, resulted in efficient and specific knockdown of the dystrophin expression in muscle [60].

The fourth category involves the expression of gRNAs driven by either the U6 or the H1 promoter in CRISPR-Cas9 gene editing. CRISPR-Cas9 is a rapidly developing gene editing tool because of its easy manipulation and versatility. With the help of gRNAs, the Cas9 endonuclease recognizes and cleaves the DNA targets in a sequence-specific manner. CRISPR editing has been used to treat two muscle genetic diseases (DMD and congenital muscular dystrophy type 1A) in mouse models. In *mdx* mice, a point mutation causes a premature stop codon in exon 23 of dystrophin and results in dystrophin deficiency. With the CRISPR-Cas9 technology, the mutated exon 23 was removed by cleavage and the subsequent nonhomologous end-joining of the broken DNA ends. Removal of exon 23 restored dystrophin expression in the muscle and improved muscle function [61–63]. These studies involved the CMV-driven Cas9 expression. In another study, to reduce the off-target risk and minimize the immune response, the expression of Cas9 was induced by the muscle-specific CK8 promoter. Muscle-targeted Cas9 also successfully corrected dystrophin mutation in *mdx4cv* mice, another DMD mouse model [64]. In these studies, due to the large size of the Cas9 coding sequence, CRISPR-Cas9 system was delivered to the muscle by two AAV vectors: one carrying the Cas9 expression cassette and the other containing the gRNA expression cassette. In a recent study, a smaller Cas9 was identified. Combined with the small-size muscle-specific promoter C5-12, both expression cassettes of Cas9 and gRNAs can be engineered into a single AAV vector. Delivery of the single AAV vector carrying both Cas9 and gRNA expression cassettes successfully created indels at the target site in the muscle cells [65]. Besides treating skeletal muscle disease, a recent study revealed that systemic delivery of AAV.CK7.Cas9 and AAV.U6.gRNAs reconstituted the expression cassette of dystrophin and improved the heart function of mdx/utro+/− mice, a severe DMD mouse model [66]. Another muscle genetic disease, congenital muscular dystrophy type 1A, is caused by the mutation in the splice site of the Lama2 gene, leading to the exclusion of exon 2 and the expression of a truncated the Lama2 protein. Systemic delivery of AAV.CMV.Cas9 and AAV.U6.gRNAs led to cleavage of the region containing the mutation, restored the expression of the full-length Lama2 protein, and improved muscle histology and function [67].

In muscle gene therapy with CRISPR-Cas9 system, Cas9 endonuclease is expressed either by the CMV promoter or by a muscle-specific promoter. These promoters can drive long-term expression of Cas9 in the muscle. Although until now no phenotype of off-target effects was reported by CRISPR-Cas9 muscle gene therapy, it still raises the concern on long-term and unregulated expression of Cas9 endonuclease. One way to overcome this issue would be to replace constitutive CMV or muscle-specific promoters with regulable promoters, such as ligand-inducible systems (tetracycline on/off system, hormone response system, and rapamycin system) or physiologically responsive autoregulatory promoters [68], in which transcriptional activity of the autoregulatory promoter is responsive to the specific physiological change. These regulable promoters will confer the extra control of the Cas9 expression and increase the safety of CRISPR-Cas9 in muscle gene therapy.

8.2.4 Promoterless Cassette

Conventional expression cassettes in muscle gene therapy usually contain promoters to drive transgene expression. Recently, in vivo gene targeting involved with the insertion of a promoterless therapeutic gene into an endogenous locus has been successfully used in liver gene transfer. Without the use of any nucleases, through homologous recombination, the promoterless therapeutic cDNA was integrated into the albumin locus. As a consequence, transgene expression is controlled by the robust liver-specific albumin promoter, an endogenous promoter [69, 70]. As a postmitotic tissue, muscle has lower efficiency of homologous recombination than the liver. To date, there is no study with the use of promoterless gene targeting in the muscle. Further improvements in our understanding of homologous recombination will help us design the promoterless gene targeting vector for muscle gene therapy.

8.3 Termination Signal

The termination signals for RNA transcription are usually downstream of the transgene sequence (Fig. 8.1), which include polyadenylation (poly-A) signal for RNA Pol II-mediated transcription and poly-T signal for RNA Pol III-mediated RNA transcription. The major function of the poly-A signal is to terminate transcription, increase the stability of mRNA, and facilitate the export of mRNA from the nucleus to the ribosomes in the cytosol. The poly-A signal is an essential component of the gene transfer vector containing RNA II promoters. For the RNA III promoter, the termination signal is the poly-T signal containing four to six thymidine residues [49, 71]. The most common poly-A signals used in gene transfer are SV40 late poly-A (SV40 pA) and bovine growth hormone poly-A signals (bGHpA). When SV40 pA is present, transgene expression is increased 3–6.5-fold under the control of either CMV or EF-1α promoter [72]. The common poly-A signals have the size ranging from 600 to more than 1000 bp. Since AAV has a limited packaging capacity, a minimal synthetic poly-A (SPA) signal (49 bp) is becoming more popular in AAV expression cassettes. Although SPA has been used to induce the transgene expression in neurons [73] and airway epithelia [74], the potency of SPA is controversial since one study suggests that SPA is equal to bGHpA in the transgene expression [74], while some studies suggest that SPA is less efficient than SV40 pA in inducing the transgene expression [73, 75, 76].

Typically, the RNA II poly-A signal is composed of an upstream sequence element (USE), the central poly-A sequence motif AAUAAA, and a downstream sequence element (DSE) with GU-rich sequence [77]. Several studies suggest that inclusion of two copies of USE resulted in better transgene expression in the mouse brain [73, 78]. It remains to be determined whether optimizing poly-A signal by including more USE will improve transgene expression in the muscle.

8.4 Transgene Sequence

Optimization of the transgene sequence plays an important role in improving transgene expression in muscle gene therapy. Depending on the packaging limit of the gene transfer vector, usually a full-length or a truncated version of the coding sequence (cDNA without intron sequence) is engineered in the gene transfer vector. However, addition of an intron sequence in the expression cassette has been shown to result in increased transgene expression. In hemophilia gene therapy, inclusion of human factor IX (F9) intron 1 at the 5′-UTR of the human factor IX coding sequence significantly improved the expression of factor IX when AAV was delivered to the skeletal muscle or the liver [79–81]. The role of intron sequence in improving transgene expression was further revealed by a study that an intron of a non-coding exon, which contains bacterial replication origin and selection marker, improves transgene expression in the liver when the intron is placed at 5′-UTR of the transgene [82]. It is worthwhile to explore whether inclusion of the intron sequence in the expression cassette will improve transgene expression in other muscle gene therapies.

The sequence surrounding the start codon of an mRNA is critical for ribosome recognition in eukaryotic genes. To this end, investigators often add the Kozak sequence for the optimal translation of mammalian genes. The translation efficiency can also be influenced by codon usage since different species may preferentially use different transfer RNAs for the same codon. If gene therapy is for a clinical trial, then codon usage and transfer RNA frequencies of the transgene coding region should be optimized for use in human. Codon optimization of microdystrophins and human factor IX has shown increased expression in animal models [12, 13, 81, 83–85].

To increase mRNA stability, the transgene sequence can be optimized to increase the GC content, since GC-rich genes are more efficiently expressed than their GC-poor counterparts [86]. However, increasing the GC content should be balanced with avoiding adding more CpG motifs in the transgene sequence. TLR9 can recognize unmethylated CpG motifs in the expression cassette and activate innate and adaptive immunity. In a muscle gene transfer experiment, depletion of CpG motifs in the transgene allowed escape from the adaptive immune response and resulted in long-term transgene expression in muscle [87].

8.5 Other *Cis*-Regulatory Elements

MicroRNAs (miRNAs) regulate protein expression posttranscriptionally. Endogenous miRNAs bind to their complementary target sequence at the 3′-UTR of mRNA and repress protein expression. More importantly, the expression of some miRNAs is cell-specific. Taking advantage of these features, engineering of the targeting sites of a cell-specific miRNA into the 3′-UTR of the expression cassette will

allow for de-targeting or transgene expression inhibition in a cell-specific manner. For example, when four copies of hematopoietic-specific miR-142-3p and hepatocyte-specific miR-122 target sequences were engineered into a lentiviral vector, the transgene expression in Kupffer cells and hepatocytes was significantly reduced [88, 89]. Oncolytic viruses are promising for cancer therapy. However, oncolytic viruses can cause severe organ toxicity, such as myositis and liver toxicity. Inclusion of the target sequence of liver-specific miR-122 into an oncolytic virus led to up to 80-fold reduction of viral protein expression in the liver and abrogated liver toxicity [90]. Insertion of the binding sites of muscle-specific miR-133 and miR-206 into the 3′-UTR of oncolytic viruses also prevented severe myositis and attenuated toxicity of oncolytic viruses [91]. AAV serotype 9 (AAV9) leads robust transgene expression in the heart. However, AAV9 also efficiently transduces the skeletal muscle and liver. To achieve heart-specific transgene expression by AAV9, binding sites of skeletal muscle-specific miRNA-206 and liver-specific miR-122 were included at the 3′-UTR of an AAV vector, and the transgene expression in both the skeletal muscle and liver was strongly repressed, but robust transgene expression in the heart was maintained [92]. Adding the binding sites of miR-122 at the 3′-UTR of AAV.Luciferase and AAV.LacZ vectors led to 50- to 70-fold lower transgene expression in the liver [93]. Incorporation of miR-142-3p target sequences into an AAV vector expressing the highly immunogenic protein ovalbumin (OVA) prolonged the expression of OVA in the muscle after AAV.OVA was intramuscularly injected into the muscle [94]. The size of miRNA binding sites is small and can be conveniently engineered into viral vectors. This technique can modify the viral tropism, de-target transgene expression from nontarget tissues, and decrease tissue toxicity and immune response.

Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) is important for viral gene expression and responsible for nuclear export of intronless viral RNA and for mRNA stability. Compared to the parental AAV vector without WPRE, inclusion of WPRE at the 3′-UTR of the transgene in the AAV.GFP vector increased GFP expression in the muscle [95]. Also AAV.hFIX.WPRE led to efficient expression of human factor IX in the muscle [96]. However, another study suggests that there is no significant difference of the transgene expression between the viral vectors with and without WPRE [97]. Since WPRE is derived from woodchuck hepatitis B virus, and it contains a viral enhancer and some residues of a viral protein, there is a concern for potential oncogenesis [98]. Further studies are needed to clarify this issue.

8.6 Summary

The expression cassette for muscle gene therapy usually contains a default structure including the promoter, the coding sequence, and termination signal. Depending on the requirement of gene transfer, other *cis*-regulatory elements can be added at 5′- or 3′-UTR. To achieve safe and efficient gene transfer, design of the expression cassette must be tailored to the specific need of muscle gene therapy.

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

Serge Braun

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

Keywords Naked · Complexes · Muscle · Vaccines · Hydrodynamic delivery

9.1 Introduction

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

S. Braun (✉)
AFM-Telethon, Evry Cedex, France
e-mail: sbraun@afm-telethon.fr

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

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

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

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

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

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

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

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

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

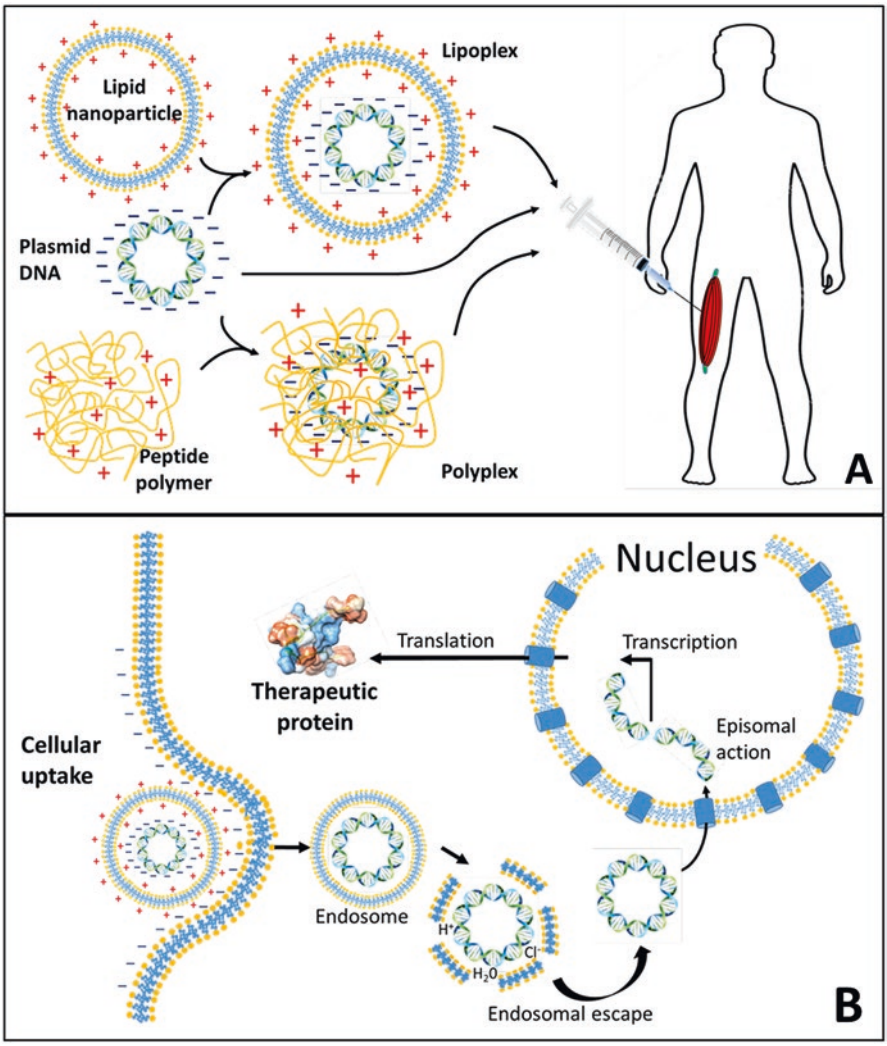

Fig. 9.1 Delivery options of non-viral vectors into skeletal muscles. (**a**) Examples of non-viral vectors, including negatively charged naked plasmid DNA (or polynucleotides) delivered either directly or combined with physical methods (ultrasound, electroporation) or complexed with various chemical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

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

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

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

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

9.3 "Naked" DNA

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

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

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

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

(continued)

Table 9.1 (continued)

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

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

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

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

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

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

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

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

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

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

9.4 Pressure-Mediated Gene Transfer

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

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

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

9.5 Conclusion

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

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

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Chapter 10
Viral Vectors for Muscle Gene Therapy

Dan Wang, Alexander Brown, and Guangping Gao

Abstract Muscle is a major target tissue for gene therapy, in part because various gene delivery vector platforms enable in vivo gene delivery to muscle tissues. Among them, recombinant adeno-associated virus (rAAV) stands out as one of the most safe and effective vectors for human applications. Many AAV strains isolated from nature collectively constituted the vector toolbox for muscle gene delivery during the field's early development through animal studies. Caveats emerged as these vectors were carefully evaluated in clinical applications. As the research community has accumulated knowledge about basic AAV biology and the nature of human-specific hurdles to translational therapy, AAV capsid engineering has emerged as a powerful approach for modifying naturally occurring AAV to better address the challenges in human muscle gene therapy. In this chapter, we first introduce basic AAV biology that pertains to the vectorology of AAV for gene therapy. Next, we summarize how AAV vectors based on natural isolates contributed to the continuing success of human muscle gene therapy. Finally, we discuss the protein engineering approaches that have been applied to AAV capsid to develop better clinical vectors, namely, rational design and directed evolution.

Keywords AAV · Capsid engineering · Gene therapy · Muscle

Authors Dan Wang and Alexander Brown have contributed equally for this chapter.

D. Wang · A. Brown
Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA, USA
e-mail: dan.wang@umassmed.edu; alex.brown@umassmed.edu

G. Gao (✉)
Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA, USA

Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, MA, USA
e-mail: guangping.gao@umassmed.edu

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10.1 Introduction

Gene delivery technology empowers the development of human gene therapy. In general, gene delivery vectors are broadly categorized into two classes, non-viral and viral vectors [1]—both of which have been explored for muscle gene therapy. The most commonly used non-viral vectors for gene therapy include circularized DNA (plasmid or minicircle) and oligonucleotide [2]. These have several advantages as compared to viral vectors, such as ease of design, manufacturing, storage, distribution, and less immunogenicity. However, these vectors are frequently less efficient in gene delivery to human cells than viral vectors and are not suitable for sustained gene expression. To improve gene transfer to human cells/tissues, vectors have been developed from several viruses which inherently possess a natural capacity to infect mammalian cells, such as lentivirus (LV), adenovirus (AdV), adeno-associated virus (AAV), and herpes simplex virus (HSV) [1, 3, 4].

Vectorology of these viruses calls for the creation of "gutted" recombinant viral particles that carry therapeutic genes in place of native viral sequences, which also improves safety and immunogenic profile. LV vector has an RNA genome and delivers its genetic payload, ultimately an integrating proviral complementary DNA, into the host genome. Therefore, gene delivery by LV vector is stable but also carries risks of genotoxicity and oncogenicity due to non-targeted genomic integration. Both AdV and AAV vectors contain a DNA genome that resides in the target cell nucleus as an episomal DNA. Although the episomal vector genome can be gradually diluted by losses during cell division, gene expression can be quite long-term in terminally differentiated cell types such as myocytes. Adverse immune reaction within the human body is a concerning feature of AdV vector, whereas AAV vector has much lower immunogenicity. HSV vector retains the parental virus's natural ability to infect neural cells and has been used for neurological disease gene therapy. The design, production, clinical application, and recent development of the various types of gene delivery vectors have been reviewed in depth in the literature [1, 3–5]. In this chapter, we focus on AAV vector, for it is particularly well suited for muscle gene therapy, as discussed below [6].

AAV is a small virus with a simple architecture. It is composed of a single-stranded DNA genome of ~4.7 kb encapsidated within a protein capsid ~26 nm in diameter [7]. The posttranslational modifications of the capsid are just starting to be explored [8]. The wild-type (WT) AAV genome carries two genes and is flanked by two inverted terminal repeats (ITRs) on the ends. The *rep* gene encodes four replication-associated (rep) proteins that are involved in various steps of WT AAV life cycle, such as genome replication, viral assembly, and site-specific integration into host genome [9]. Notably, because recombinant AAVs (rAAVs) are devoid of the *rep* gene, their genomes remain predominantly episomal when delivered to the host cell nucleus. The *cap* gene encodes three viral protein (VP) subunits, VP1, VP2 and VP3, that form the icosahedral capsid consisting of 60 copies of VP at 1:1:10 ratio [10]. The assembly-activating protein (AAP) is transcribed and translated from the *cap* gene and has been shown to promote capsid assembly [11]. The ITRs serve as structural signals for genome packaging and therefore are the only viral DNA

kept within the rAAV genome, while the *rep* and *cap* genes are replaced with a recombinant gene of interest. During rAAV production, sequences encoding the Rep proteins and VPs, along with other helper elements, are provided in *trans* by genetic means [12].

Muscle gene therapy usually requires in vivo gene delivery, although ex vivo gene therapy is also a potential means to treat certain conditions [13]. Depending on the route of delivery, rAAV must overcome several barriers within the human host before successfully delivering its DNA cargo to the target cell. This process mainly relies upon features of the capsid. For example, following systemic delivery such as intravenous injection, the host immune system may recognize the rAAV capsid by pre-existing neutralizing antibodies (NAbs). These NAbs are present in the general human population at various degrees, depending on the serotype, and they are believed to result from natural infection by WT AAV [14]. The rAAV particles escaping neutralization must then traverse endothelial barriers to achieve close proximity to target cells. Once localized to targeted cells, crossing the cell membrane is initiated by interaction of capsid and cell surface receptors [15]. This interaction is crucial for determining the tropism of rAAV packaged within a particular capsid. Receptor recognition leads to a series of events, including endocytosis, intracellular trafficking, endosomal escape, nuclear entry, and capsid unfolding-which eventually allows for the release of the DNA cargo into the nucleus [16].

So far, a handful of naturally occurring AAV serotypes and hundreds of variants have been isolated, each being unique in the capsid sequence [17, 18]. Although their capsids share common features, some subtle differences in amino acid residues are sufficient to confer distinctive properties when used as gene delivery vectors [10]. Therefore, solving the three-dimensional structures of various AAV capsids and understanding the molecular interactions between capsid and host are among the major efforts in vector development for gene therapy. For example, characterizing the mechanism of capsid-NAb and capsid-receptor interactions has provided crucial information for engineering novel capsids to better suit specific clinical needs.

In the following sections, we first summarize the rAAVs used in muscle gene therapy that are based on naturally occurring WT AAV serotypes. Although numerous animal studies and several clinical applications have demonstrated their therapeutic efficacy, improvement is needed for several aspects, such as tissue specificity and immunogenicity. We next discuss capsid engineering for developing tailored rAAV to better address these clinical needs. It should be noted that the rAAV genome harboring a therapeutic gene is also an important vectorology aspect for muscle gene therapy. This topic is discussed in the other chapters in this book.

10.2 Muscle Gene Therapy Vectors Based on Naturally Occurring AAV Capsids

Since the initial cloning of AAV, numerous naturally occurring AAV genome sequences have been isolated and adapted for use as recombinant viral vectors. While WT AAV genomes encode two genes, *rep* and *cap*, and require helper virus

genomic sequences for replication and vector production, these elements can be readily supplied in *trans* within an appropriate production cell line to achieve packaging of transgenes up to approximately 4.7 kb in length, when flanked by two ITRs. In this fashion, rAAV viral vector is produced that largely maintains serotype-specific capsid structure and functionality [19, 20]. These pseudotyped rAAVs collectively comprise a major portion of the modern vector-driven gene therapies in use and under development today.

The exceptionally low immunogenicity of naturally occurring AAV capsid has made rAAV an attractive choice in designing gene therapies for a host of pathologies, including those originating within muscle and those in which muscle may serve as a factory to produce therapeutic proteins. Since in vivo transgene delivery to muscle tissue by rAAV2 was well established over 20 years ago [21], a variety of serotypes have been employed, displaying a wide array of tropism and gene expression profiles within muscle fibers of all types [22, 23]. As AAV2 was among the first serotypes discovered, possesses broad tissue tropism, and was noted to transduce muscle fibers, it formed the basis for initial investigative treatment strategies.

AAV2 vectors have been investigated within several notable muscle disease animal models, including the cardiomyopathic hamster and *mdx* mouse models of Duchene muscular dystrophy [24, 25]. Additionally, rAAV2 has been used to target muscle tissues for the systemic expression of therapeutic transgenes, such as the alpha-1-antitrypsin gene and the factor IX gene [26, 27].

Despite achieving only limited measures of therapeutic efficacy during various phases of clinical investigation, the vector's observed preference for slow-twitch muscle fiber, as well as high titer requirement for both systemic and direct injection of the virus, has helped establish the relative safety of rAAV therapeutic strategies and drive the search for other naturally occurring capsids with improved muscle transduction. Since these initial studies, rAAV2 transduction and expression in muscle has consistently been determined to be among the lowest performing vectors. Furthermore, pre-existing immunity to AAV2 appears relatively common in humans, which may in turn lead to adverse immune reactions in a portion of potential patients [28]. Nevertheless, rAAV2 remains among the most well-characterized viral vectors and may continue to form the basis for muscle-targeted gene therapies in development, especially where localized intramuscular (IM) injection provides for stable transgene expression and therapeutic efficacy.

Among the remaining and most frequently adapted pseudotyped rAAVs, rAAV1, 6, 7, 8, and 9 have demonstrated consistent and high-level transduction vastly superior to rAAV2 when delivered via direct or systemic means [23, 29–31]. In 2012, IM injection of a rAAV1 treatment for lipoprotein lipase deficiency, Glybera, became the first AAV gene therapy approved for use in Europe [32]. Additional studies for AAV1-based recombinant therapeutic targeting of muscle are currently in clinical trials for treatment of a variety of diseases including a number of muscular dystrophies [33]. AAV1 treatments appear safe and effective thus far; however, the prevalence of AAV1 neutralizing antibody across the human population is only marginally improved over AAV2. While patient immunological response can be managed to some degree, these features of AAV1 and 2 will likely limit the effectiveness and availability of therapies to some patients.

While direct intramuscular injection offers a potential benefit in that it may reduce the amount of vector needed to achieve efficient transduction, it is important to note that muscle comprises approximately 40–50% of total mass in humans, with widespread distribution throughout the body. Therefore, targeting muscle via systemic delivery of viral gene therapy vectors is frequently desired when maximizing therapeutic transgene expression is necessary to achieve therapeutic effect. As compared to the other muscle-transducing vectors, rAAV8 and 9 appear to exhibit superior patterns of systemic muscle transduction [29, 30, 34]. Additionally, rAAV8 and 9 display broad tissue tropism, with particularly high efficiency of transduction in non-muscle tissues such as the liver [29, 31]. Therefore, deliveries via systemic routes of these serotypes are expected to target multiple tissues simultaneously. It is important to note that direct IM administration of rAAV8 and 9 also displays greatly enhanced transduction, a feature that is currently under clinical consideration for treatment of muscular dystrophies.

While rAAV8 and 9 appear most well suited for broadly targeting muscle, pathology-specific considerations must be made during AAV serotype selection, and the ideal vector may not be immediately clear. Furthermore, new naturally occurring serotypes, including minor variants of established structural clades, are continuously being discovered and characterized for their tissue-specific tropisms [35, 36]. Large-scale profiling of these variants for tissue-specific patterns of transduction holds great promise for informing new strategies targeting muscle-specific diseases.

10.3 AAV Capsid Engineering to Meet Clinical Needs

Although naturally occurring AAV capsids collectively provide a valuable toolbox for in vivo gene transfer and prevail in clinical applications [37], each has potential drawbacks for human use. For muscle gene therapy, the most concerning imperfections include off-targeting to non-muscle tissues and an undesirable immunological interaction profile with the host. Therefore, engineering of AAV capsid to meet specific clinical needs is a major research endeavor. Two main approaches have proven to be fruitful in developing clinically relevant capsids: rational design and directed evolution. Rational design is "capsid-driven," in that it aims to exploit the existing knowledge base concerning AAV capsid structure and function, from which a specific hypothesis guides the design of a novel capsid. In contrast, directed evolution is "phenotype-driven," in that it is experimentally designed to generate selective pressure for isolation of a desired capsid phenotype, without prior knowledge about capsid structure.

10.3.1 Rational Design of Novel AAV Capsid

Grafting a cell-targeting molecule on the external surface of capsid is a straightforward concept to alter AAV vector tropism. AAV capsid can tolerate insertion of peptides and polypeptides at many sites, such as the N-terminus of VP2 subunit and

exposed flexible loop regions of the outer capsid [38–40]. These sites were initially identified by biochemical screening approaches and later verified through analyzing the three-dimensional structure of AAV capsid. For example, a fusion protein consisting of DARPin (designed ankyrin repeat protein; an antibody-like protein that exhibits high-affinity target binding) linked to the N-terminus of VP2 can be incorporated into a fully assembled capsid, redirecting vector tropism according to the specific binding properties of DARPin [41, 42]. The position between N587 and R588 (AAV2 VP1 numbering) is also commonly used to insert short peptide sequences without compromising vector packaging. Several studies have successfully exploited this site's structural flexibility to insert muscle-targeting peptides for engineering of modified vectors with enhanced gene delivery to muscle [43, 44]. When a peptide ligand is displayed on AAV capsid in this fashion, it is important to note that its function may be compromised by spatially neighboring capsid residues. Interestingly, Boucas et al. demonstrated that it is possible to recover the targeting functionality of the inserted peptide ligand by further mutating the interfering residues [45]. In general, the N-terminus of VP2 can tolerate larger polypeptides [46, 47], whereas the nonterminal sites allow for displaying shorter peptides, although Judd et al. successfully inserted sequence encoding mCherry (240 residues) in place of deleted sequence 453-GTTTQSR (AAV2 VP1 numbering) [48].

In addition to genetic manipulation, biochemical methods are also useful to physically modify AAV capsid for cell targeting. Recently, a novel method to covalently couple large proteins to pre-assembled AAV capsid has emerged, utilizing split intein-mediated protein *trans*-splicing [49]. This method provides flexibility in generating rAAVs with desired binding specificity and affinity, in part because larger targeting molecules can be used, such as single-chain variable antibody fragments (scFvs). Another versatile approach to rationally modify AAV capsid involves click chemistry [50, 51]. In this technique, an unnaturally occurring, chemically reactive amino acid containing azide is co-translationally incorporated into assembled capsid to serve as a biochemical hook, enabling covalent coupling with alkyne-linked effector molecules. The modification can be geared toward not only altered tropism but also improved immunological properties and genome editing [50, 51].

As the structure-function relationship for several naturally occurring AAV capsids have been elucidated [52–56], this information has proven crucial in guiding the development of novel capsids by rationally altering capsid residues. Notably, the capsid residues located at or near the threefold symmetry protrusions appear to play key roles in determining some clinically important features of AAV capsid, such as tissue tropism and immunogenicity [9]. Therefore, altering these residues can simultaneously yield several benefits, such as improved on-targeting to muscle, reduced off-targeting to the liver, and decreased vector recognition by pre-existing NAbs.

AAV2i8 is a chimeric capsid variant generated by replacing a receptor-binding hexapeptide motif in AAV2 capsid with corresponding residues from AAV8 capsid [57]. When delivered into mice following isolated limb perfusion [57] or into non-human primates [58] by a systemic route, AAV2i8 vector shows selective tropism to cardiac and skeletal muscles throughout the whole body and reduced sequestration

in the liver. AAV2i8 is also less likely to be neutralized by human sera than the parental capsids [57]. Therefore, AAV2i8-based vector can be used to treat muscular dystrophies that usually require body-wide muscle gene delivery. Similarly, AAV2.5 was generated from replacing five residues in the AAV2 capsid with the corresponding orthogonal residues of AAV1 [59]. The resulting chimeric capsid shows an improved muscle transduction profile and reduced antigenic cross-reactivity, compared with both parental serotypes. Intramuscular injection of an AAV2.5 vector expressing a minidystrophin transgene was safe and well tolerated when administered to DMD boys, supporting its potential use as a muscle gene therapy vector [59].

A series of engineered AAV capsids carry mutated surface-exposed residues, notably tyrosine and serine. The rationale behind this modification is that these residues are readily phosphorylated, which induces capsid degradation through the ubiquitin-proteasome pathway [60]. Mutating these residues can not only stabilize the capsid to improve transduction but also confers immunological benefits by reducing capsid-derived antigen presentation. Tyrosine-mutant versions of several naturally occurring muscle-tropic capsids have been generated and tested for local or systemic muscle gene delivery in mice or dogs such as AAV1, AAV6, and AAV9 [61]. In general, these rationally designed rAAVs showed robust transgene delivery and expression in various muscle types. However, as of yet, their immunological advantages remain to be fully characterized when used for muscle gene therapy.

In addition to the structural information, the primary capsid sequences of extant AAVs are also helpful for rational design of new rAAVs by ancestral sequence reconstruction [62]. This novel approach relies on sequence analysis and bioinformatics tools and aims to infer the ancestral AAVs (ancAAVs) that gave rise to the modern AAV variants. These ancAAVs are hypothesized to possess distinctive properties that are absent in their modern derivatives, such as evading neutralization by the modern AAV NAbs [62]. In addition, such ancAAVs may have higher tropism to certain cell types than the extant AAVs [63].

10.3.2 Capsid Selection by Directed Evolution

Directed evolution starts with the creation of a diverse capsid gene library of high complexity, populated through genetic means. The library is used in AAV production to generate a mixture of assembled AAV particles, each possessing a unique capsid, and packaged with the corresponding capsid protein-encoding DNA sequence. This AAV mixture is subsequently subjected to certain selective pressure, such as preferential transduction of muscle tissue, so that only the AAV particles passing through the selection can be recovered. To retrieve and identify the capsid sequences of those AAV particles capable of withstanding the selective pressure, the corresponding capsid genes embedded in the packaged AAV genomes must be amplified by viral replication or PCR and sequenced at depth. Furthermore, the capsid

diversification and/or library selection procedure can be performed for multiple rounds to progressively enrich for the AAV capsids with a desirable feature.

The naturally occurring AAV capsid sequence collectively provides the foundation to create a capsid gene library by various molecular methods, such as mutagenesis and DNA shuffling [64]. Regardless of the molecular cloning methods used in mutating the capsid gene, the resulting AAV particle library usually carries three types of capsid modifications, namely, random amino acid mutations, recombined capsids generated from several natural AAV capsid sequences, and insertion of degenerately encoded peptides [65]. In one particularly interesting adaptation of directed evolution, an ancestral AAV capsid gene library was computationally designed, based upon extant sequences, and constructed by chemical synthesis [66]. This library comprises combinatorial variations at 32 amino acid residues. Several variants were identified to have higher muscle transduction than AAV1, a muscle gene delivery vector commonly used in clinical applications. In general, the more diversity contained within a library, the more likely it is to yield variants that meet the selection criteria.

The AAV packaging process imposes the first selection, because many mutations in the capsid proteins are not tolerated for viral assembly. To select for muscle-tropic AAV capsid variants, Yang et al. generated a capsid library by shuffling the capsids of natural AAV1 to AAV9, delivered the AAV mixture to adult mice by intravenous injection, and analyzed the vectors enriched in the heart and muscle [67]. They isolated a chimeric capsid designated as AAV-M41 that shows enhanced muscle targeting and reduced liver transduction. Choudhury et al. also conducted a similar library selection experiment focusing on the central nervous system (CNS) after intravenous delivery to mice. Interestingly, the lead vector, AAV-B1, showed higher tropism in both CNS and muscle than AAV9 [68]. A potential caveat of selecting tissue-tropic capsid variants in a model organism, prior to human application, is that the utility of the resulting variants may be limited to the experimental species examined. To address this concern, selection can be performed in xenograft models implanted with human cells in vivo [69] or surgically resected human cells ex vivo [70]. A human skeletal muscle xenograft model has been developed [71], but its utility for AAV directed evolution remains unproven. Surgically resected primary human skeletal muscle cells have been used to evolve AAV capsids with increased transduction in human surgical explants ex vivo [70]. Meanwhile, the evolved vectors have the advantage of reduced seroreactivity against pooled human sera, compared to existing serotypes.

Being able to escape pre-existing NAbs is another commonly employed selective pressure. To this end, initial studies were aimed to select AAV variants resistant to neutralizing sera or pooled intravenous immunoglobulin (IVIG) [72, 73]. Both approaches yielded novel capsids that can escape neutralization and retain transduction capability. Grimm et al. combined tissue tropism and NAb escape in directed evolution of a shuffled capsid library, culminating in the isolation of a variant that shows enhanced liver transduction and resistance to IVIG neutralization [74]. Recently, resistance to individual patient-derived neutralizing serum was used as the selective pressure [75]. In this study, AAV mutants isolated in vivo were better at

evading NAbs derived from the cognate serum than those from the others, suggesting that NAb-escaping AAV variants can be developed in a patient-specific manner if necessary. Furthermore, when muscle tropism was applied as another selective pressure, the isolated AAV mutants showed the combined benefits of NAb escaping and enhanced muscle transduction [75].

10.3.3 Combinatorial Approaches

Usually, a particular aspect of structure-function relationship is only partially understood, and there is insufficient knowledge to solely rely on rational design for capsid engineering. Similarly, it may accelerate directed evolution or library selection if the diversity is rationally designed at a certain capsid region. Therefore, a combinatorial approach has emerged as a powerful means for AAV capsid engineering. Pulicherla et al. hypothesized that altering the amino acid residues within the GH loop would confer a novel binding profile, because the surface-exposed structure is highly variable among AAV strains and is thought to influence tissue tropism of different AAV serotypes [76]. These authors focused random mutagenesis only to the GH loop of AAV9 and characterized a handful of candidates based on sequence analysis, structural modeling, and in vivo screening. Through such a comprehensive workflow, they isolated novel AAV9 variants that retain AAV9's capability of efficiently transducing the heart and skeletal muscle following systemic delivery in mice but display diminished transduction in the liver [76].

In an effort to engineer novel AAV capsids for immune evasion, Tse et al. first performed structural analysis of antigenic epitopes on AAV1 capsid to identify the residues in contact with multiple monoclonal antibodies (mAbs) [77]. These antigenic footprints were then mutated in synthetic capsid variants, which were subjected to iterative evolution to yield highly divergent antigenic motifs. One lead AAV variant, CAM130, consisting of multiple evolved antigenic sites evades neutralizing polyclonal anti-AAV1 antibodies derived from multiple species, including 1:5-diluted human sera. Notably, seronegativity to a therapeutic AAV vector capsid at this dilution is a common criterion for patient inclusion in clinical trials, indicating that CAM130 is potentially compatible with a large population of patients.

10.4 Conclusion

The gene delivery technologies based on rAAV has played a pivotal role in the success of muscle gene therapy. Vectors derived from several natural AAV strains have been extensively tested in animal studies for muscle gene delivery and proven efficacious in human applications. Although these vectors have high muscle tropism following various routes of administration, they lack some desired features for safe and efficacious human muscle gene therapy—such as tissue specificity and

escaping host immune surveillance. In the future, comprehensive screenings of new AAV isolates, with novel capsid properties, appear poised to address many of these basic delivery issues—or at least serve to better inform the rational design and directed evolution of capsids with robust muscle-specific tropism and limited immunological intolerance. Because these features are largely determined by the capsid, protein engineering has already been employed to modify the AAV capsid to produce better clinical vectors. These "designer" capsid-based rAAVs have just begun to enter the clinical arena and are expected to further drive the development of muscle gene therapy toward the clinic.

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Chapter 11
Development of Next-Generation Muscle Gene Therapy AAV Vectors

Michael E. Nance and Dongsheng Duan

Abstract Recombinant adeno-associated virus (AAV)-based gene delivery is a promising approach to treat muscle diseases. However, body-wide muscle delivery and pre-existing immune responses pose significant challenges to AAV muscle gene therapy. While the determinants of tissue tropism and immunogenicity of AAV are amenable to traditional molecular engineering, the development of a muscle-specific, immunosilent AAV vector has remained elusive. Recent advances in understanding the relationship between capsid structural motifs and functional domains have created exciting developments in the search for a muscle-specific AAV. Novel approaches to generate unique AAV properties through forced evolution have resulted in capsids with improved immune properties and/or muscle-targeting efficiency. Optimization of the gene cassette to restrict expression to mature muscle fibers provides another level of control. These reengineered AAV vectors have the potential to greatly increase efficacy and reduce off-target effects for body-wide muscle gene therapy. In this chapter, we discuss recent advances in the development of a next-generation, muscle-specific AAV vector.

Keywords AAV · Capsid · Muscle · Directed evolution · Rational design

M. E. Nance (✉)
Department of Molecular Microbiology and Immunology,
School of Medicine, University of Missouri, Columbia, MO, USA
e-mail: mengxb@health.missouri.edu

D. Duan
Department of Molecular Microbiology and Immunology,
School of Medicine, University of Missouri, Columbia, MO, USA

Department of Neurology, School of Medicine, University of Missouri, Columbia, MO, USA

Department of Biomedical Sciences, College of Veterinary Medicine, University of Missouri, Columbia, MO, USA

Department of Bioengineering, University of Missouri, Columbia, MO, USA
e-mail: duand@missouri.edu

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11.1 Introduction

Muscular dystrophies are a diverse group of inherited muscle diseases characterized by muscle weakness and dystrophic muscle pathology. Histologically, dystrophic muscle shows degeneration/regeneration, inflammation, and fibrotic/fatty infiltration. These microscopic changes result in variable clinical manifestations involving skeletal, smooth, and cardiac muscle and sometimes non-muscle organs such as the brain or skin. While our understanding of disease pathogenesis is still evolving, many genetic determinants underlying muscular dystrophies have been identified [1]. Thus far, defects associated with muscular dystrophies are primarily related to genes that encode extracellular matrix proteins, sarcolemma-associated proteins, nuclear membrane proteins, sarcomeric proteins, and proteins with enzymatic functions [1]. Before the 1990s, treatment options for muscular dystrophies were limited to mainly supportive therapies without hope for correcting the underlying gene mutation. This therapeutic gap has its origins in our incomplete understanding of the genetics of each disease and, also, our limited ability to modify the genetic landscape. In more recent years, exciting progress in the areas of gene replacement, gene supplementation, and gene editing have increased potential for a viable genetic treatment in the near future.

The ability to efficiently and stably deliver the therapeutic gene via a vehicle to the diseased muscle all over the body is a premise for muscular dystrophy gene therapy. This vehicle can be either a viral or non-viral vector. From a pharmacokinetic standpoint, the vector must traffic through the muscle vasculature, traverse the endothelium and extracellular space, and then, finally, enter the target cell. For an effective therapy for many muscular dystrophies, the target cells would include not only mature muscle fibers but also muscle progenitor cells. Gene delivery to muscle is further complicated by off-target uptake in the reticuloendothelial organs such as the spleen and liver and activation of local and systemic immune responses [2].

After decades of research, adeno-associated virus (AAV) is now considered the premier gene delivery vector for muscle. AAV is a dependent virus and requires a helper virus such as adenovirus or herpesvirus for productive infection. In the absence of a helper virus, AAV establishes a latent infection capable of stable long-term gene expression in dividing and nondividing cells. AAV was discovered more than 50 years ago and has been studied as a gene transfer vector in mammalian cells for over 30 years [3–6]. To date, there are 12 known AAV serotypes with hundreds of AAV variants having been engineered in the laboratory or isolated from nature [7, 8]. Despite this diversity, none of the existing AAV can fulfill all the needs of muscle gene therapy such as selective and body-wide muscle targeting and the capability to efficiently transduce muscle progenitor cells. In this chapter, we introduce AAV biology in the context of muscle gene therapy and then discuss current progress toward developing next-generation, muscle-targeting AAV vectors. Additionally, we highlight key issues and potential solutions in the field of AAV capsid engineering.

11.2 AAV Biology

11.2.1 The Basic Biology of AAV

AAV is a 25 nm non-enveloped, icosahedral particle with a 4.7 kb single-stranded DNA genome. The AAV genome is flanked by 145 base pair inverted terminal repeats which are necessary for AAV replication and packaging [9]. Within the genome, there are two major open reading frames (ORFs) that encode nonstructural Rep proteins and structural Cap proteins. Rep proteins are involved in viral replication and packaging. Cap proteins are responsible for forming the viral capsid. Recently, a small ORF was discovered in the AAV genome. This ORF encodes a protein called AAV assembly-activating protein [10]. An intact AAV particle consists of 60 monomers formed by Cap proteins. Each monomer contains a conserved core structure with an alpha helix and eight antiparallel beta sheets (βB–βI) with intervening hypervariable loops [11, 12]. The hypervariable loops represent the primary source of diversity and convey the tropic/immunological properties. For this reason, these loops have been the focus of AAV capsid modification.

11.2.2 AAV Infection Biology in the Context of Muscle and Muscle Stem Cells

While the mechanisms of AAV gene transfer have been extensively studied over the past couple of decades, detailed knowledge of AAV infection in muscle remains incomplete [13, 14]. At the cellular level, AAV infection is initiated with the attachment of the viral particle to the cell surface followed by entry and trafficking to the nucleus for gene expression. Various types of cell surface glycans have been identified as the primary binding receptor for different AAV serotypes. Of particular interest is the discovery of galactose as the AAV-9 receptor because this may contribute to the superior cardiotropic property of AAV-9 [15–19]. AAV entry is mediated by a secondary binding event to a co-receptor (often a transmembrane protein such as integrin) that recycles between the endosomal compartment and plasma membrane. More recently, a generic AAV receptor named AAVR was discovered [20]. AAVR interacts with multiple AAV serotypes through its extracellular immunoglobulin domains. Characterization of the expression profile of AAVR in muscle will help to better understand AAV transduction in muscle and, if needed, to increase AAVR expression for enhanced muscle transduction. AAV internalization is primarily through clathrin-independent carriers/GPI-enriched endocytic compartment (CLIC/GEEC) endocytic pathway [21]. Following endocytosis, AAV appears to utilize the microtubule network to transport to the perinuclear region prior to nuclear entry. This may be important in muscular dystrophies where the microtubule network may be disrupted [22, 23]. In the absence of a helper virus, AAV establishes a latent infection in muscle as a double-stranded circular episomal molecule [24, 25].

Following intramuscular injection, AAV gradually spreads through the endomysial compartment between muscle fibers [26]. By 4 h, AAV particles are readily detected three myofibers away from the injection site. AAV capsids become detectable as early as 2 h after injection at the injection site. Nucleus-associated AAV particles increase by tenfold at 4 h after injection. However, by 6 days after injection, AAV capsids become largely undetectable in myonuclei although they are still readily visible in the endomysial compartment [26]. A study in human patients suggests that AAV capsid can persist in myonuclei for at least 12 months after local injection [27]. In contrast to AAV capsid proteins, the AAV vector genome can persist for many years after gene transfer in muscle. This leads to continuous transgene expression [28].

Skeletal muscle has the unique property of high regeneration upon chemical/physical injuries or in diseased status (such as muscular dystrophy). Satellite cells are responsible for muscle regeneration [29]. Effective targeting of satellite cells has clear advantage for gene editing therapy because genetic defects in all regenerated muscle cells should theoretically be corrected. AAV gene transfer to satellite cells has been the topic of great interest in past several years, yet the ability of various AAV serotypes to efficiently infect satellite cells remains uncertain. Satellite cells are a challenging target due to their (1) sub-laminar location, (2) cellular quiescence, (3) differential gene expression including cellular receptors and downstream trafficking molecules, and (4) low frequency in adult muscle. To study AAV-mediated satellite cell transduction, several labs used reporter AAV vectors [30, 31]. A study in adult mice from the Chamberlain Lab suggests that AAV-6 and AAV-9 do not transduce satellite cells and AAV-8 transduces ~5% satellite cells following local injection in mice [30]. However, AAV-8 did not appear to transduce satellite cells to a detectable level following systemic delivery [30]. Stitelman et al. tested in utero transduction of satellite cells with AAV-9 in mice and observed an efficiency reaching ~28% when AAV was delivered on embryonic day 16 [31]. More recently, Tabebordbar and colleagues used CRISPR editing to track AAV-9 transduction in satellite cells. Using fluorescence-activated cell sorting, they found slightly over 3% of satellite cells were transduced irrespective of AAV delivery route (intramuscular injection or intraperitoneal injection) [32]. Collectively, except for in utero delivery, postnatal AAV delivery using existing AAV serotypes appears very inefficient in transducing satellite cells.

11.3 Rational Design of Muscle-Targeting AAV Capsids

11.3.1 Naturally Existing Muscle-Tropic AAV Serotypes

Since the initial isolation of AAV as a contaminant of adenoviral stocks nearly 50 years ago [6], the explosion of knowledge and isolation of novel AAV serotypes have propelled muscle gene therapy forward from bench to clinic in a relatively short period of time. While many initial studies focused on AAV-2 [5], the discovery

of AAV serotypes with inherent tropism for muscle tissue has been instrumental in the development of vectors for treating muscular dystrophies [33].

While a number of AAV serotypes (such as AAV-1, AAV-5, AAV-6, AAV-7, AAV-8, and AAV-9) have been shown to efficiently transduce muscle following local injection [33], recent studies suggest that tyrosine-mutated AAV-6 may likely represent the most potent serotype for local muscle delivery [34]. Efficient muscle transduction has been detected following systemic administration of AAV-1, AAV-6, AAV-7, AAV-8, AAV-9, rh10, and rh74 in rodents and large mammals [33, 35]. Among these, AAV-8, AAV-9, and rh74 have received particular attention for human use to treat neuromuscular diseases [36]. Several systemic AAV gene therapy clinical trials have been initiated using these serotypes to treat type 1 spinal muscular atrophy, X-linked myotubular myopathy, and Duchenne muscular dystrophy [36]. The recent report on the spectacular clinical efficacy and high tolerability of systemic AAV-9 treatment in type 1 spinal muscular atrophy patients is a historical milestone for the entire field of gene therapy [37].

11.3.2 Improving Muscle Targeting with Rational Design

Despite robust muscle transduction with naturally existing AAV serotypes, these vectors usually display broad tropism to a number of tissues. This makes it a challenge to achieve muscle-specific transduction. Rational design of muscle-tropic AAV uses knowledge of high-resolution AAV capsid structure, receptor and co-receptor binding footprints, and known muscle-homing peptides. Most commonly, a muscle-targeting peptide is inserted to a specific location on the surface of a naturally existing AAV serotype. In this regard, the hypervariable loops located on the capsid threefold protrusions have been found extremely effective. Yu et al. inserted muscle-targeting peptide ASSLNIA following either 587 or 588 amino acid residues in AAV-2 [38]. Interestingly, the authors found that insertion after 587 ablated the intrinsic heparin binding of unmodified AAV-2, while insertion after 588 did not. Systemic delivery of a modified capsid inserted after 587 resulted in significantly improved heart and skeletal muscle transduction as well as reduction in non-muscle tissues. Work et al. inserted the peptide EYHHYNK following 587 and achieved great targeting of AAV-2 to human venous and arterial smooth muscle cells [39]. Ying et al. tested several cardiac-targeting peptides they discovered through in vitro screening of a random peptide library [40]. Insertion of these peptides after residue 588 in AAV-2 indeed significantly increased myocardial transduction in mice [40].

Besides muscle-homing peptides, swapping the tissue tropic footprint from one serotype to another has also resulted in dramatic change in tissue tropism. AAV-2 cannot efficiently transduce muscle. The AAV-2 heparan sulfate receptor footprint is a hexapeptide located between residues 585 and 590. The Asokan lab replaced the AAV-2 footprint with the corresponding residues from AAV-8, a serotype that can efficiently transduce muscle following systemic delivery. The resulting chimeric capsid AAV2i8 displayed significantly enhanced muscle and heart transduction [41].

Adachi et al. took a more radical approach in designing a muscle-tropic AAV [42]. They performed a double alanine scan in AAV-9 and identified residues critical for the tissue tropic phenotype. Based on this knowledge, they successfully reprogrammed AAV-2 for muscle targeting using a minimum number of noncontiguous mutations.

11.4 Evolving Novel Muscle-Tropic AAV Capsids Through Directed Evolution

Despite the success of rational design for improving muscle targeting of AAV capsids, it is suggested that the determinants of the AAV tropism are likely spread widely over the entire capsid surface. Modification in one location, or even one residue, may lead to unexpected changes in AAV properties. This often makes structure-function correlation difficult. For this reason, investigators have turned to directed evolution [43, 44]. The fundamental concept in directed evolution is the isolation of desired individuals from a diverse population to meet desired selection criteria. Similar to natural selection, selective pressures drive the emergence of beneficial traits, which at the protein level amounts to desirable amino acid polymorphisms. In-depth discussions on the applications of directed evolution to AAV and DMD therapy are reviewed in [45]. Here, we limit our discussion to a brief introduction of basic concepts, and we then focus on the application of directed evolution for AAV muscle gene therapy.

11.4.1 Basic Concepts

The methods of AAV plasmid library construction and selection platform are of utmost importance in the initial planning phase for a directed evolution experiment. Traditional library construction has typically focused on one approach for diversification. Moving forward however, combining multiple approaches is becoming more popular to further increase diversity (e.g., point mutations, small insertions, and DNA shuffling [45]). Point mutations are introduced with conventional molecular biology techniques such as error-prone PCR. Small insertions can be achieved by inserting a small stretch of random nucleotides at a particular location. DNA shuffling leverages sequence homology between parental AAV serotypes to derive novel, diverse capsid genes through in vitro recombination [46]. Theoretically, higher diversity correlates with a greater probability in isolating a beneficial mutation(s). However, it also increases the risk of defective variants, for example, variants that contain nonsense mutations and variants that affect assembly and packaging. According to an estimation by PacBio, sequencing a plasmid library with ~10^7 diversity may lose around 99% of its diversity resulting in ~10^5 in the packaged virus library [47].

Following diversification and virus production, the virus library is applied in recursive cycles to a selection platform for the desired phenotype. For muscle targeting, in vivo selection will likely provide the most realistic selection parameters. Screening methods should take into account the requirements of (1) intravenous delivery, (2) endothelial barrier translocation, (3) body-wide muscle targeting, (4) reduce non-muscle, especially liver, uptake, and (5) immune evasion. Another criteria may include species, which is difficult to address since viral libraries cannot be directly screened in human patients.

11.4.2 Retaining Muscle Tropism While Avoiding the Liver

From a reductionist perspective, primary muscle cells are the most straightforward platform for selecting muscle-targeting AAV capsids. However, in vitro screening methods often fail to address key desired traits for in vivo application. A vector that performs well in cell culture may be dependent upon a surface expression profile that does not occur naturally in the intact muscle and vice versa. For this reason, in vivo selection is preferred.

Following systemic delivery, the vast majority of AAV accumulates in the liver. Hence, de-targeting from the liver will allow more AAV to transduce muscle. Although, theoretically, minimizing liver retention should enhance muscle transduction, it may not always be the case. For example, Yang and colleagues identified a variant called AAVM41 from a DNA-shuffled virus library [48]. AAVM41 displayed reduced liver tropism but also had reduced muscle transduction when compared to AAV-9. Focusing on mutations to the threefold protrusion, Pulicherla and colleagues isolated an AAV-9 variant, AAV-9.45. This variant displayed reduced expression in the liver [49]. The muscle tropism of AAV-9 was retained but not enhanced. These studies suggest that there may be a trade-off in terms of cost-benefit when the entire capsid gene is mutated. It should be pointed out that although these variants did not show increased muscle transduction, they are still highly very useful for systemic muscle gene therapy due to reduced toxicity from liver de-targeting.

In a recent study from the Sena-Esteves laboratory, Choudhury et al. described a novel variant called AAV-B1 [50]. This variant was isolated from a shuffled library consisting of 11 parental serotypes. Similar to AAVM41 and AAV-9.45, AAV-B1 showed reduced liver transduction. However, it also showed robust transduction in the central nervous system and motor neurons. Of relevance to muscle gene therapy, AAV-B1 displayed skeletal muscle and heart transduction at least tenfold higher than that of AAV-9 in mice. These results were extended and consistent following systemic delivery in cats. If these results can be extended to human patients, AAV-B1 may become a preferred vector for systemic muscle gene therapy in clinical trials in the future.

11.4.3 Evading Immunity to Enhance Muscle Delivery

Muscle-specific and systemic humoral and cellular immune responses are impediments to muscle gene delivery with AAV vectors. It is estimated that 30–80% of the human population has pre-existing neutralizing antibodies from childhood exposure with a high cross-reactivity between serotypes [51]. IgG appears to be the most important neutralizing antibody. In one of the earliest studies investigating the evolution of vectors capable of escaping antibody neutralization, Maheshri et al. developed a screening method by preincubating shuffled virus with increasing concentrations of rabbit anti-AAV-2 sera with each round of selection in HEK-293 cells. Interestingly, this approach identified mutant capsids with improved gene expression in the mouse hind-limb muscle compared to wild-type following preincubation with rabbit anti-AAV-2 sera [52]. While these results were promising, an important question is how relevant are antigenic variants isolated when screening with pooled animal sera versus human sera and, more stringently, to patient-specific sera? Further is preincubation with sera contextually relevant to a viral-antibody encounter within the vasculature? In a recently published study by Tse et al., the authors used a combined rational and directed evolution approach focused on mouse monoclonal antibody epitopes to derive antibody-resistant AAV vectors [53]. The resulting vector (AAV-CAM130) displayed improved antibody resistance to not only mouse anti-sera but pooled nonhuman primate and human anti-sera while maintaining the tissue tropism of parental AAV-1. Therefore, there is likely antigenic conservation between species to an extent.

The capsid composition, in terms of the individual structural motifs, derived from each parental serotype may have a large impact on the ability of chimeric capsids to avoid antibody neutralization. Conspicuous epitopes, such as the threefold protrusions, are formed by the apposition of GH loops from adjacent monomers. In theory, GH loop segments could derive from different serotypes in an evolved chimeric AAV capsid. Such a capsid may still react to the neutralizing antibody against the parental serotype. For example, a capsid containing segments of GH loop from AAV-1 may not be able to evade neutralizing serum against AAV-1. Li et al. observed that shuffled vectors screened with human sera performed variably depending on a patient-to-patient basis [54]. This phenomenon appeared to be related to the parental origin of certain segments of the VP3 viral protein monomer. Therefore, the origin of the micro-architectural domains within the capsid could have a significant influence on important vector properties. With this notion, another consideration would be the ability of shuffled vectors to retain the tissue transduction efficiency of native vectors. Several approaches may help overcome this limitation. One solution is to co-inject the shuffled capsid library and neutralizing serum into skeletal muscle. While this approach incorporates two selective pressures, it fails to mimic the natural route of infection and neutralization, i.e., intravascular delivery. Furthermore, it would be important to consider the effect of injecting high doses of serum into the muscle and how this alters the environment and muscle transduction. Indeed, muscle-specific vectors isolated from this approach failed to exceed the heart and

muscle transduction of parental serotype AAV-9 [54]. Another solution is to pre-infect the host with a parental AAV capsid, then deliver the virus library and anti-sera intravenously. This approach incorporates more realistic selection. Tse et al. identified capsid variants with improved antibody evasion and robust muscle expression using this approach [53].

While one of the driving principles behind directed evolution is the ability to achieve improved vectors without prior knowledge of the structure of the evolved vector, analysis of the primary amino acid sequence from advantageous capsids can help to rationally identify capsid segments responsible for functional improvements. Maheshri identified a T716A mutation in the C-terminus of VP-1 to confer improved antibody resistance [52]. Likewise, muscle tropism in chimeric vectors may be enhanced by inserting the AAV-6 VP-1 sequence from amino acid 347–446 [54].

To summarize, directed evolution is a powerful platform to develop AAV variants with enhanced muscle targeting and immune evasion. However, despite the immense effort to evolve novel vectors, the preclinical results have, thus far, not translated to the clinic. Likely combined approaches or, at the least, focused evolution of specific capsid regions will be an important area of research development for muscle gene therapy.

11.5 Future Directions and Conclusions

AAV capsid engineering is an alembic process whereby years of research are beginning to produce promising novel vectors for muscle gene therapy. In a sense, the immense power of directed evolution and multitude of potential rational modifications provides gene therapists with an open canvas for endless creative possibilities. However, there are several additional questions (among many others) that remain for capsid engineers.

11.5.1 Can We Use Capsid Engineering to Expand the AAV Capsid Packaging Capacity?

While capsid engineering approaches have been applied to retargeting vectors and reducing immune responses, it is relatively unknown if these techniques may be utilized to enhance AAV packaging capacity. Increasing the packaging capacity opens the door to more complex gene expression cassettes which may accommodate larger genes and more sophisticated elements for selective muscle expression. Several studies since the early 2000s [55–57] determined that the packaging capacity of AAV is limited to ~5.2 kb, although larger packaging size was reported. Interestingly, AAV genomes may also be cross packaged in other human parvovirus capsids including parvovirus B19 (up to 5.6 kb) [58] and human bocavirus-1 (up to 5.5 kb) [59]. The molecular interactions, specifically protein and nucleic

moieties, involved in cross packaging AAV genomes to the capsids of other parvoviruses have yet to be determined. Identification of these signal/interaction domains may allow rational cross packaging to other, larger icosahedral capsids. A high degree of homology exists between AAV serotypes, underlying the fundamental recombinogenic mechanism in directed evolution. The ability of AAV genomes to cross package to other human parvovirus capsids suggests the path to an effective muscle-targeting vector with increased packaging may lie in the choice of human parvovirus B19 or human bocavirus-1 as starting platforms for directed evolution or rational design. In this scenario, endogenous tropism may be ablated and vectors retargeted through in vivo bio-panning or retargeting with muscle-specific ligands.

11.5.2 Is Directed Evolution in Large Animal Models Possible?

When considering library selection in animal models, the amount of library injected should be large enough that each individual clone is represented multiple times [60]. Similarly, injecting too much virus results in saturation and isolation of nonspecific variants. The use of approximately 1000-fold more particles than the estimated library diversity has been reported as ideal for a mouse [60]. This factor provides a limitation on the use of larger animal models such as dogs, pigs, or nonhuman primates. Inevitably, directed evolution in larger animals may be justified, despite the costs. In an attempt to bypass this limitation, translational models such as human xenografts in immune-deficient mice may allow selection in a more relevant in vivo model while limiting the amount of virus needed for screening [61].

In conclusion, next-generation muscle-targeting vectors may dramatically improve the translation of gene therapy efforts for treating muscle diseases. Not only will more selective vectors improve uptake in the muscle tissue, but they may reduce unwanted toxicity in the other tissues and curtail untoward immune responses that have significantly hindered moving preclinical success into the clinic. With the increase in our understanding of AAV biology and the need to treat different muscle diseases using a tailored vector, capsid engineering certainly warrants dedicated research in the coming years.

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Chapter 12
Histological and Biochemical Evaluation of Muscle Gene Therapy

Michael W. Lawlor, Joel S. Schneider, Martin K. Childers, and Kristy J. Brown

Abstract The histological and biochemical evaluation of muscle tissue can be of critical importance for the establishment of safety and efficacy in muscle gene therapy studies. While specific pathological and biochemical endpoints vary greatly with respect to disease, most gene therapy studies encounter common challenges associated with study planning, tissue triage, and tissue preparation. This chapter discusses a number of issues related to study planning for the performance of histological and biochemical studies, highlighting our own experience and lessons learned from other studies. Additionally, we illustrate some current approaches using our own experiences in gene therapy studies of X-linked myotubular myopathy (XLMTM) and Duchenne muscular dystrophy (DMD), both of which are now in the human clinical trial stage. The evaluation of endpoints related to important histological features and the expression of key proteins by immunohistochemistry, western blot, and mass spectrometry are discussed in the context of these studies.

Keywords Pathology · Western blot · Mass spectrometry · X-linked myotubular myopathy · Duchenne muscular dystrophy · XLMTM · DMD · Immunohistochemistry

M. W. Lawlor (✉)
Division of Pediatric Pathology, Department of Pathology and Laboratory Medicine and Neuroscience Research Center, Medical College of Wisconsin, Milwaukee, WI, USA
e-mail: mlawlor@mcw.edu

J. S. Schneider · K. J. Brown
Solid Biosciences, Inc., Cambridge, MA, USA
e-mail: joel@solidbio.com; kristy@solidbio.com

M. K. Childers
Department of Rehabilitation Medicine, Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle, WA, USA
e-mail: mchilders@askbio.com

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The evaluation of tissue morphology and biochemistry is often essential in determining the therapeutic efficacy of gene therapy agents. The selection of key tissues for evaluation depends on the disease and experimental system, but many muscle gene therapy preclinical studies incorporate some combination of skeletal muscle and organ histology. Multiple approaches including histological examination, western blot, and mass spectrometry can assess the effects of a new therapeutic agent, dose, or manufacturing technique. This chapter will outline some lessons learned with respect to the performance of histological, western blot, and mass spectrometry assays in our experience of translating gene therapy studies for X-linked myotubular myopathy (XLMTM) and Duchenne muscular dystrophy (DMD) to the human clinical trial stage, while also touching on issues related to other disease states where gene therapy is being pursued.

12.1 Tissue Collection Planning

The appropriate collection of tissue is essential in studies of skeletal muscle disease, and careful planning of tissue collection can accelerate the pace of a study. It is extremely important to understand the possible endpoints of a study prior to planning animal numbers and tissue collection strategies. Factors that may impair (or entirely prevent) the use of specific assays may include (1) the amount of tissue, (2) freezing or fixation technique used, and (3) delay between tissue collection and freezing/fixation.

Common endpoints in muscle gene therapy studies include organ histology (usually performed on formalin-fixed tissue); skeletal muscle histology (often performed on isopentane-frozen tissue); DNA, RNA, and protein expression studies (usually requiring frozen tissue); and possibly electron microscopy (usually performed on glutaraldehyde-fixed tissue). Some of these techniques require specialized buffers to be used at the time of tissue collection, further decreasing the ability to use a given tissue fragment for multiple experimental indications. Thus, thorough planning is essential to allow parallel evaluation of structural and biochemical endpoints that are relevant to gene therapy. In the following sections, we discuss considerations for tissue collection.

12.1.1 Natural History of the Disease State

Primary muscle disorders display a range of degenerative and non-degenerative phenotypes associated with weakness. In some of the non-degenerative disorders like XLMTM, a given fragment of muscle tissue will contain a large number of myofibers, but the overall tissue size available for a study may be very small. In contrast, more degenerative disorders like DMD may produce

myofiber loss and replacement with fibrous and fatty tissue, to the extent that only a subset of the collected tissue is suitable for further study. These considerations related to disease natural history may affect the range of studies proposed on a given tissue type or the study timepoint at which tissue collection is performed.

12.1.2 Method of Tissue Collection

The appropriate method of tissue collection can depend on the size of the experimental model in use. Murine studies are frequently associated with postmortem tissue collections and some flexibility with respect to tissue use. Some mouse muscles, however, have an asymmetric distribution of oxidative and glycolytic fibers. In such cases, the most appropriate tissue triage strategy involves the subdivision of muscle fragments transversely, as a longitudinally divided muscle may be excessively enriched in one fiber type and skew experimental results.

Larger animal (such as canine) studies offer the opportunity for longitudinal studies of treatment effects with serial muscle biopsies. Important areas of decision-making include the site(s) of biopsy and the tissue collection method used. Selection of biopsy site(s) can be complicated by variation in pathology and fiber-type distribution between muscles or even between different areas of the same muscle. There is also some likelihood of sampling old/healed biopsy sites if muscles are repeatedly biopsied, and the histological features of muscle regeneration (endomysial fibrosis, fiber size variation, internally nucleated myofibers) may complicate the evaluation of treatment efficacy or primary disease pathology.

12.1.3 Freezing Versus Fixation

At the time of tissue acquisition, it is often necessary to either freeze or fix tissue in what may correspond to an irreversible commitment to certain assays. Many assays require frozen tissue, but the freezing technique may impact assay results. For instance, frozen tissue collected for the purpose of RNA expression studies should be collected quickly and in RNase-free conditions, whereas frozen muscle histology studies require freezing in isopentane that can take considerably longer time [1]. In both of these instances, using tissue prepared for the other indication is likely to significantly impact the quality of the results. Additionally, it is easier to subdivide muscle while it is in the fresh (rather than frozen) state, so effective collection plans should also integrate likely tissue distribution plans into the initial collection and freezing strategy.

12.1.4 Fixation for Histological Studies

A variety of fixatives are available for the preservation of tissues, and the correct choice depends on the study endpoints and the tools intended for use. For general surveys of organ histology, it is generally sufficient to fix organs in 10% neutral-buffered formalin with subsequent paraffin embedding, sectioning, and staining. This approach may not be ideal for the evaluation of skeletal muscle in a given study, however, as fixation and processing affect fiber size and shape while also rendering the muscle tissue enzymatically inactive. Thus, isopentane-frozen skeletal muscle is most often used for histological studies due to the ability to preserve morphology and enzymatic activity in an appropriately frozen specimen. In some cases, however, fixed muscle tissue may be preferable because some antibodies and stains are designed for fixed tissue and do not work in frozen tissue. Additionally, if electron microscopy (EM) studies are planned, then optimal tissue quality is obtained by glutaraldehyde fixation fairly quickly after tissue excision [1]. While it is possible to perform electron microscopy on glutaraldehyde-fixed tissue that has previously been frozen or formalin-fixed, there are often significant preparation artifacts, and this approach should be avoided when possible.

12.2 Pathological Assessments in Skeletal Muscle Disease

Skeletal muscle histology is typically evaluated using hematoxylin and eosin (H&E) or other histochemical stains on sections of frozen muscle tissue. The H&E stain is a mainstay of most studies, as it allows the identification of myofiber size, areas of inflammation, myofiber degeneration, and myofiber regeneration [2]. The selection of additional stains will depend on the disease under study but may include Gomori trichrome staining to identify a variety of aggregates and inclusions, oxidative stains (NADH, COX, SDH) to identify abnormalities of organelle distribution, ATPase stains or myosin subtype immunostains to assess oxidative vs. glycolytic fibers, or substrate-specific stains (PAS, Oil Red O) to identify abnormal material in storage diseases [2]. An enormous number of antibodies are also available for immunostaining, and these techniques can be used to document expression of specific proteins, identify structural abnormalities that are not clearly distinguishable using histochemical stains, or generate datasets that are more easily interpretable using automated quantification algorithms.

12.2.1 Histochemical Stains

The selection of pathological endpoints to assess disease progression or recovery is usually simplified by natural history studies in the experimental models proposed for study. Some studies also use constructs that provide enzymatic activity (such as

alkaline phosphatase) to serve as a marker of tissue distribution of the therapeutic agent [3–6]. Even when using well-established experimental models and techniques, however, it is important to validate the measurements and tools proposed for use in the animal colony proposed for study. Differences between published phenotypes and the phenotype of the animal colony intended for use should be identified during the design period of treatment studies. It is also helpful to perform comparative studies of different models before pursuing treatment studies in a new model or species, as this can affect endpoint selection. For instance, in our work on murine, canine, and human XLMTM, we have observed that species differences cause different pathological patterns of abnormality despite the fact that similar biological processes are likely responsible [7]. When translating studies from the murine to the dog and human stage, such differences must be taken into account when planning study endpoints, or there will be a danger of missing key disease- or recovery-related phenotypes.

In our work on XLMTM and DMD, the construction of pathological grading systems has been extremely helpful for recognizing treatment or dose-related differences in muscle pathology. These grading systems are most reliably constructed following a blinded evaluation of a full range of pathologies, so the study design should include non-diseased samples and samples displaying severe disease phenotypes. Assessment of abnormalities with respect to the estimated proportion of fibers involved is often very useful in correlating disease pathology to treatment doses or groups. For instance, in our studies of XLMTM, the percentage of fibers involved would be an estimation of fibers displaying central nucleation, myofiber smallness, and/or organelle mislocalization [7, 8] (Fig. 12.1a). In DMD, the percentage of fibers involved would be a reflection of the percentage of fibers or area where myofiber degeneration, active regeneration, and inflammation are present [9] (Fig. 12.1b). In our studies of both of these diseases, divisions between different grades of pathology (ranging between 0 and 4) were made based on the distribution of data from our blinded assessments, and then each sample was given a score. This somewhat simple approach allows significant flexibility for different experimental designs, as it allows (1) the comparison or pooling of pathological grading data across multiple muscles from a given animal and (2) some flexibility in the definition of "active disease" to account for differences in the phenotype of different experimental models.

12.2.2 Immunohistochemistry

Immunohistochemical studies offer an opportunity to establish the expression of key proteins or highlight features that are not apparent on routine histochemistry. Antibodies (if they are available) are useful in the assessment of disease-related proteins (such as dystrophin in DMD) to monitor the degree of protein loss in the disease state or the degree of restoration following gene therapy (Fig. 12.1b). Immunohistochemistry has been useful in demonstrating low levels of

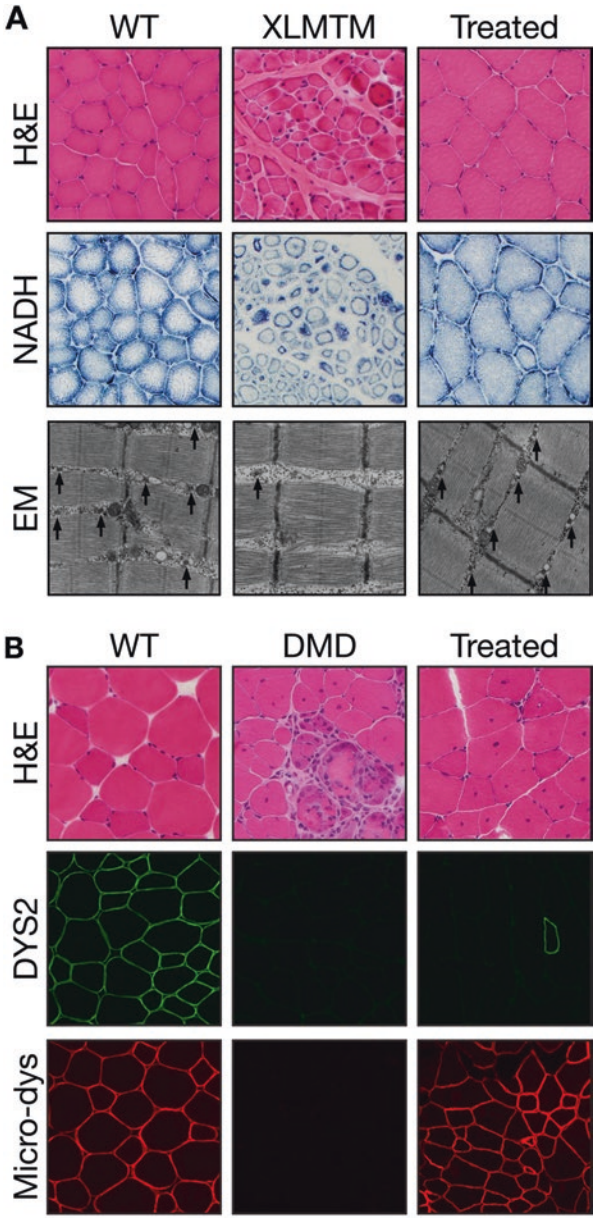

Fig. 12.1 Skeletal muscle histology and immunohistochemistry in evaluating therapeutic efficacy. (**a**) Comparison of pathological features between wild-type (WT) and XLMTM canines in the presence and absence of AAV gene therapy. WT muscle shows limited variation in fiber size and peripherally located nuclei on H&E staining. NADH staining illustrated an even distribution of organelles (primarily mitochondria and t-tubules) across the myofiber cytoplasm. Electron microscopy in longitudinal section can identify well-constructed triads (black arrows). Untreated XLMTM canines at similar ages show marked myofiber smallness and internally located nuclei on

microdystrophin expression in early human gene therapy studies of DMD [10], as well as more promising positive expression patterns of dystrophin in other DMD studies [3, 4, 6, 11–21], alpha sarcoglycan in limb-girdle muscular dystrophy (LGMD) type 2D [22–24], dysferlin in LGMD type 2B [25–28], and FKRP in LGMD 2I [29]. While this approach can be straightforward, the selection of the appropriate antibodies for study can at times be challenging. Using the example of dystrophinopathy, the usefulness of immunohistochemistry is highly dependent on the impact of a given mutation on dystrophin levels and structure. Dystrophin is a very large protein (approximately 427 kDa and 79 exons) [25], and mutations in this protein can cause a loss of nearly all dystrophin expression or changes in only a subset of dystrophin epitopes. Additionally, as microdystrophin and minidystrophin gene therapy strategies restore only a portion of the full-length dystrophin molecule [4, 25, 30], the successful expression of these constructs will only restore immunoreactivity for antibodies that bind to the microdystrophin or minidystrophin protein (Fig. 12.1b). This creates a situation where multiple anti-dystrophin antibodies can be used to evaluate minidystrophin/microdystrophin expression and distinguish it from fibers that express dystrophin even in the context of significant mutations (revertant fibers).

With respect to the quantification of immunohistochemical findings, this is an area of recent debate and technological development, and once again the DMD field provides some useful examples. There is evidence that dystrophin levels in the range of 20–30% of normal can lead to significantly milder clinical severity [31–34], resulting in a goal of at least 20% dystrophin restoration as a therapeutic target for gene therapy [35]. Immunofluorescence intensity can be measured in a variety of ways, but standardization of approaches can be problematic because most microscopes and slide scanners have not been designed with the quality control of immunofluorescence intensity in mind. When immunofluorescence intensity is a desired study endpoint, extensive quality control evaluation and response strategies are recommended to ensure the acquisition of high-quality data that can be compared across multiple measurement dates. A more straightforward semiquantitative measurement used in numerous studies is the estimation of dystrophin-positive fibers within a sample [3], but this also suffers from subjectivity due to potential variations in the threshold for positive fibers. This was a concern in a recent trial of exon skipping using eteplirsen in a clinical trial for DMD [36], where additional data collection on dystrophin positivity was required in response to Food and Drug

Fig. 12.1 (continued) H&E staining. NADH staining shows marked mislocalization of organelles, and triads are rare or may appear abnormal on EM. Successful gene therapy with AAV reverses these pathological abnormalities. (**b**) Comparison of muscle histology between WT mice and DMD mice (in the presence or absence of AAV gene therapy). H&E staining shows dystrophic pathology including myofiber degeneration and basophilic fibers consistent with active regeneration in the mdx mouse model of DMD. The DYS2 antigen which is absent in microdystrophin is used to illustrate the number of naturally occurring fibers expressing dystrophin (revertant fibers) in these DMD mice. AAV gene therapy of these mice results in a decrease in dystrophic pathology and a marked upregulation of micro-dystrophin expression by immunofluorescence staining, without affecting the expression of DYS2

Administration (FDA) review comments [37]. In our experience, quantitative or semiquantitative expressions of immunohistochemical results can be extremely informative but are also best counterbalanced by other quantitative assays such as western blot or mass spectrometry. Additionally, assessments of the percentage of dystrophin-positive fibers may be strengthened by counts performed by multiple blinded investigators in parallel in the generation of datasets.

Immunohistochemistry can also be used to assist in the quantification of general structural features in muscle, such as myofiber size, fiber type, or the percentage of fibers with internally located nuclei. Measurement of myofiber size can be of particular importance when evaluating disorders where myofiber smallness is a primary pathological finding (such as XLMTM) [7, 38–40] (Fig. 12.1a) or where myofiber growth is a principal mechanism of action (such as follistatin gene therapy) [41–43]. Recognition of myofiber edges for these measurements can be improved using antibodies against proteins located at or adjacent to the sarcolemma, such as laminin or dystrophin. Once the fibers are clearly defined, myofiber size is often measured through determinations of MinFeret diameter (the diameter along the minor axis of the fiber cross section) or cross-sectional area. While both of these measurements provide useful information in well-controlled situations where myofibers are cut in well-oriented cross sections, the cross-sectional area will vary dramatically in areas where longitudinally or obliquely oriented fibers are present. As suboptimal orientation has a much less dramatic impact on MinFeret diameter, our work has usually focused on this measurement when determining myofiber size. Integration of additional antibodies or stains can also be useful in determining whether specific fiber size populations correlate with fiber types (using anti-myosin antibodies) or for nuclear position (using 4′6-diamidino-2-phenylindole, or DAPI). As far as quantification of myofiber size and internal nucleation is concerned, it is feasible to perform these measurements either manually or using automated software. To allow optimal efficiency of measurement and quality control, we use automated measurement with rigorous manual comparisons between the measured values and the quantified images.

12.2.3 Electron Microscopy (EM)

EM can be useful when determining the structural impact of gene therapy in some disease states. However, in comparison to light microscopy, EM can be considerably more difficult to perform in a systematic way. Issues related to sampling are more of a concern in EM studies because tissue fragments are much smaller. Also, while the entire fragment of tissue for EM can be evaluated at the light microscopic level on 1-micron-thick “scout sections,” it can often be difficult to ensure that the entire specimen has been viewed at higher magnifications using the EM scope. For some diseases (such as Pompe disease and some peripheral nerve disorders) [44–48], the processing and Epon embedding used for EM is more useful than other processing methods, and so these scout sections can be stained to evaluate for

specific structures. In these instances, the processing may be more useful than the magnification, and there may be little real need for EM studies. In other situations (such as XLMTM), EM is performed to identify the organization of structures that cannot be adequately visualized at the light microscopic level, and so systematic approaches must be used. Our EM work in XLMTM has focused on counts of triad structures in longitudinal sections of muscle [7, 49, 50]. To provide some means of reproducible quantitation, our approach involved photographing a single well-oriented area in each fiber in a longitudinally oriented muscle specimen at several magnifications. Manual counts of triads, T-tubules, and L-tubules at a standard magnification were capable of distinguishing XLMTM animals from wild-type animals when the specimens were processed appropriately. This approach was also capable of identifying restoration of triad structures with gene therapy [8, 51] and targeted enzyme replacement therapy [38] in several studies. However, even this relatively simple measurement strategy has been difficult to maintain as studies have expanded to include laboratories that were not experts in EM tissue collection. Variations in tissue handling practices, age of fixative, and duration of fixation can complicate the evaluation of many organelles at the EM level. Our experience has identified a need for significant training of tissue collection sites and caution in endpoint selection when multi-site studies involving EM are proposed.

12.2.4 Histological Assessments Related to Inflammation

Tissue pathology also plays an important role in the evaluation of possible immune or inflammatory reactions due to muscle gene therapy (the mechanisms of which are well-reviewed in [30, 52]). While the potential for immune responses in muscle gene therapy is dependent on the vector, promoter, transgene, and species involved, surveillance for immunological reactions is a necessary component of study planning. Fortunately, the evaluation of inflammatory reactions in muscle tissue is fairly straightforward, owing greatly to the numerous sensitive, non-histological methods for evaluating immune activation. In general, histology-focused studies have investigated (1) whether inflammation or myofiber degeneration is associated with a given experimental condition and (2) whether this inflammation shows any relationship to transgene expression. Additional immunohistochemical studies are possible to better understand the nature of a given inflammatory infiltrate, but these studies are usually performed in support of techniques that more clearly define the status of immune signaling and humoral versus cellular immune responses.

In the context of prior muscle gene therapy studies, several general categories of immune response exist, and an appropriate pathological evaluation strategy would vary in each instance. In a disorder where muscle inflammation is not part of the characteristic disease pathology (such as XLMTM [51, 53], hemophilia B [54, 55], or lipoprotein lipase deficiency [56]), evaluation of H&E stained sections or screening with a pan-lymphocyte marker (CD3) may be sufficient to evaluate immune cellular infiltrates. In a disorder such as DMD, however, it can be challenging to

distinguish inflammation and myofiber degeneration associated with the disease itself from similar changes that may be the result of treatment. A number of studies have demonstrated the potential for marked cellular infiltration after gene therapy for DMD, although these inflammatory reactions could be mitigated by alternate vector, construct, or immunosuppression strategies [4, 30]. These studies differentiated treatment-induced inflammation from DMD-associated inflammation because the treatment responses tended to be much more diffusely spread than one typically sees in DMD. Additionally, some studies of microdystrophin therapy using well-tolerated AAV constructs show a marked decrease in myofiber degeneration and inflammation as a result of successful correction of the underlying disease process (Fig. 12.1b).

When inflammation is present, it may be necessary to determine whether an inflammatory response is affecting transgene expression. Several studies in DMD canines have identified severe inflammatory reactions on histology occurring at 2–4 weeks posttreatment [57–61]. While this reaction is accompanied by a loss of treatment efficacy in some cases [58–61], there are other instances where there was no significant impact on transgene expression [20, 59, 60]. The distinction between these two subtypes of cellular inflammatory reaction should be straightforward in cases with good histological endpoints for transgene expression. It may also be useful to further characterize the inflammatory infiltrate with further immunohistochemical testing, which can be done with inflammatory cell markers including CD3, CD4, CD8, CD11b, CD20, CD68, granzyme B, and Fas ligand. Additionally, other limited assessments of immune function can be performed through evaluations of major histocompatibility complexes (I and II) and the C5b-9 membrane attack complex. Practically speaking, most evaluations of inflammatory tissue reactions in DMD gene therapy have focused on H&E and immunostaining for CD4 and CD8 cells (used in support of more complex serological and biochemical testing), but histological evaluations of inflammatory infiltrates are likely to be expanded as exploratory endpoints in muscle gene therapies continue to progress to the human clinical trial stage.

12.3 Planning Biochemical Assays and Endpoints

As the use of histological methods for quantitative endpoints can be problematic, it is very useful to have additional, more quantitative techniques such as western blot and mass spectrometry to provide additional correlative data. For some disorders related to enzymatic function (such as Pompe disease), it may also be possible to use biopsy tissue to demonstrate restoration of biochemical processes with successful therapy [62]. This section will focus specifically on the use of western blot and mass spectrometry assays to provide complementary information to muscle histology using our recent work in DMD as an illustration.

12.3.1 Western Blot

While advances have been made recently to allow the use of immunofluorescence staining intensity as a quantitative or semiquantitative measure of protein expression, western blot has also been used as a supplementary and more easily quantifiable measurement of protein expression in some gene therapy studies [63, 64], as well as in the recent human clinical trial of eteplirsen for DMD [36]. As with any rigorous quantitative study, obtaining optimal quantitative data requires the use of appropriate control conditions. This section will focus on the lessons learned in our experience of using western blot to quantify dystrophin intensity following microdystrophin gene therapy in DMD.

12.3.1.1 Critical Decisions in Western Blot Design

Optimal western blot performance requires a thorough knowledge of the target proteins, antibodies, tissues under evaluation, and the disease state under study. Careful planning of assay strategy can significantly improve the quality of the data and the overall pace of data acquisition. It is also essential to identify the optimal antibodies and quality control conditions, as this will allow consistent measurement throughout the study.

The isolation of high-quality protein for western blot studies can be performed after either crushing or cryosectioning the tissue. Cryosectioning is more labor-intensive, but it does offer the potential to fine-tune the amount of tissue collected, while also offering the opportunity for histological/western blot correlations. The method of protein extraction can also affect the amount of dystrophin extracted relative to other proteins or may impact the background observed in high molecular weight regions, so it is useful to evaluate different extraction protocols (including protocols comparing manual vs. blender-assisted homogenization and using standard extraction buffers vs. those containing urea) to optimize signals in a given study.

With respect to the western blot technique, it may be necessary to fine-tune the conditions under which gels are run (amount of protein loaded, type of gel used, voltage settings, etc.) based on the protein of interest. Large proteins like dystrophin often run better on lower percentage tris-acetate gels, but this may not offer an ideal resolution of lower molecular weight proteins in some situations. Similar considerations can affect the transfer of proteins, and we have found that some strategies providing adequate transfer of low molecular weight proteins do not work well for large proteins. In addition, blocking conditions may be of particular importance in situations requiring the detection of very low levels of dystrophin. Blocking agents containing sodium azide as a preservative can impact chemiluminescence signals, and so exposure of these agents to the secondary antibody should be avoided. Blocking solutions made using 5% milk in the laboratory do not have this issue, but may have increased background in comparison to some commercially available

agents. Once again, the correct choice for a given study requires an assessment of tissues and conditions relevant to the needs of that study.

Testing of western blot conditions prior to beginning a gene therapy study is highly recommended, as troubleshooting western blot protocol in the middle of the study can exhaust tissue quickly. Additionally, if a scaling-up of sample numbers is planned, we would recommend testing western blot procedures at the highest sample number planned to ensure that the workflow and equipment can handle the sample volume. In some cases, western blot protocol that works well with low numbers of samples may run or transfer poorly when both the size of the gels and the number of gels running in parallel are increased. The assessment of protein transfer using Coomassie blue staining of gels and Ponceau S staining of membranes can be useful to determine whether proteins are being fully transferred.

12.3.1.2 Factors Impacting Western Blot Quantification

When designing western blot experiments for quantitative comparisons across multiple blots, it is important to have a rigorous quality control system in place. Most western blot experiments incorporate some sort of "loading control" assessment to account for errors in lane loading (Fig. 12.2). In many cases, the post-transfer myosin heavy chain band (by Ponceau S membrane staining or Coomassie gel staining) can be used as a reliable loading control that does not appear to be affected by disease process, although it should be noted that it is not suitable to use this band when comparing different tissue types. Other commonly used loading controls include skeletal muscle actin and GAPDH, and a comparison of these bands between lanes allows the identification of lanes with too much or too little protein. The expression of proteins used as loading controls may be affected by disease processes or may be tissue-specific, however, so the most appropriate loading control may depend highly on the experimental situation. Additionally, it can be difficult to use high-abundance proteins (like actin) as loading controls for low-abundance proteins because of dramatic differences in signals between the proteins of interest. For western blot studies where quantitation is key, it is additionally advisable to incorporate standard curve conditions on the blot (Fig. 12.2b). The inclusion of standard curve conditions allows optimal comparison between gels in a study and helps account for variability associated with antibody reactivity and chemiluminescence from one blot to another. In our DMD work, we include a standard curve of wild-type dystrophin, using mixtures of wild-type and dystrophin-deficient protein. The proportion of wild-type to dystrophin-deficient protein on a given gel depends on the likely range of

Fig. 12.2 (continued) in comparison to a calibration curve of full-length dystrophin using canine tissue. Full-length dystrophin is detected by the same antibody at 427 kDa in the control curve using canine tissue. Microdystrophin is shown at 150 kDa, and the bottom of the membrane was probed for loading using GAPDH at 37 kDa. (**c**) Mass spectrometry of samples from the same experiment, each spiked with a constant internal standard (blue), reveals a similar dose-dependent increase in microdystrophin (red) as doses increase from dose level 1 to dose level 6. The intensity seen in wild-type (WT) mice is also shown

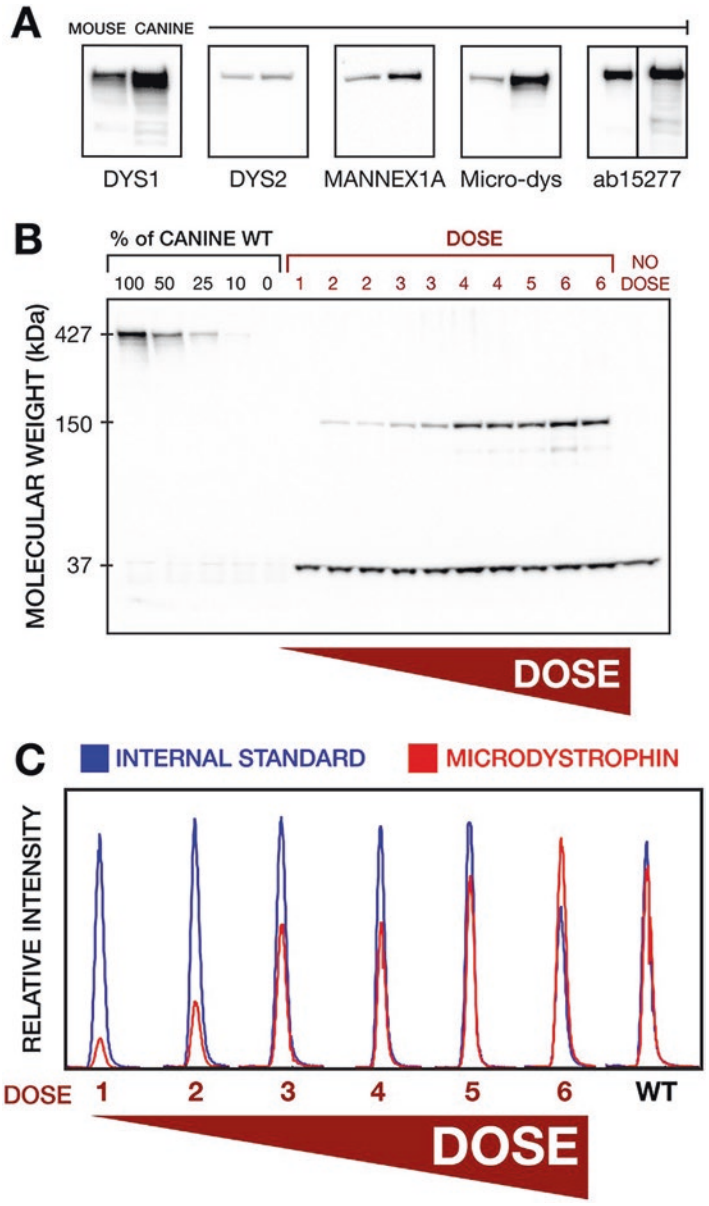

Fig. 12.2 Use of western blot and mass spectrometry in the quantitative evaluation of dystrophin expression. (**a**) Comparison of various antibodies for the detection of mouse and canine full-length dystrophin, loaded at an equivalent protein concentration. Mass spectrometric analysis confirms that the amount of dystrophin is comparable in each species, so differences reflect species-dependent differences in antibody affinity. A black bar for ab15277 denotes lanes that were not adjacent on the original blot. (**b**) Western blot studies of a dose-response experiment show increasing micro-dystrophin levels with increasing doses of AAV (across increasing doses labeled 1–6),

dystrophin expression within the set of samples. A 5-point curve ranging from 0 to 100% is useful if high levels of dystrophin expression are expected, whereas a 5-point curve ranging from 0 to 25% may be more useful when sensitivity for low levels of expression is desired. The use of this standard curve and the application of a clear quality control standard (such as the visibility of the lowest control curve condition, adequate signals in four of the five control curve conditions, and/or R-squared values of the standard curve greater than 0.9) have allowed us to maintain a high level of reproducibility and comparability to our other quantitative studies.

As in immunohistochemistry, primary antibodies for western blot must be carefully selected to obtain high-quality data. The identical antibody can sometimes be used for the detection of proteins by both immunohistochemistry and western blot, but an independent assessment of other antibodies can often be highly informative. One area for caution is the potential for species-related differences in immunoreactivity with certain antibodies, as this can be much more of a factor on western blot in comparison to immunohistochemistry. While this is not a particular area of concern when using a dystrophin construct from the same species as the experimental animal, it is a particular concern in the gene therapy field because human or canine dystrophin constructs may first be tested in mice. For instance, certain anti-dystrophin antibodies have higher affinity for canine and human dystrophin in comparison to mouse dystrophin (Fig. 12.2a). As a result, the signal obtained on western blot for human or canine microdystrophin constructs is very high when compared to mouse wild-type dystrophin, whereas other antibodies with more equivalent affinity will display more equivalent levels of immunoreactivity. When such a situation occurs, consideration should be made to switching the antibody of choice or switching the source of tissue for control curve lanes to be of equivalent affinity to the dystrophin construct of interest. Sequence homology at the reactivity region of the antibody should be mapped and accounted for with respect to cross species western blotting.

12.3.2 Mass Spectrometry

Mass spectrometry, typically coupled online to liquid chromatography, is widely used in drug development. The liquid chromatography-mass spectrometry (LC-MS) platform is capable of separating analytes by retention time and mass with high analytical accuracy, precision, and reproducibility that far exceed the immunofluorescence staining or western blot approaches. The vast majority of the assays in use are small molecule quantitation, but the field has been rapidly expanding to include biomolecules. Quantitative mass spectrometry requires the inclusion of an internal standard, which is typically a stable isotope-labeled version of the analyte of interest. A quantitative mass spectrometry assay for a protein typically requires the digestion of the protein into smaller peptides, and the peptides are measured to report the protein level. The application of LC-MS to muscle tissue has been slow to develop due to the inherit limitations of muscle, i.e., large dynamic range, limited solubility, and limited sample sizes. These limitations are being overcome, however, with recent improvement to modern mass spectrometers for sensitivity, speed, and

resolution [65, 66]. To date, the majority of muscle mass spectrometry assays have been academic. For example, dystrophin was readily quantified using a full-length stable isotope standard in both normal and dystrophic muscle tissues [67, 68]. But, despite steps to reduce dynamic range limitations and using state-of-the-art instrumentation, the limit of detection was approximately 5% of normal and therefore not applicable for Duchenne reverent fiber dystrophin levels or low-level restoration therapies. The immunofluorescence and western blot techniques remain the optimal approach for dystrophin evaluation at these low levels. Gene therapy approaches now hold promise for restoring dystrophin to easily quantifiable levels, however, making quantification using mass spectrometry highly relevant.

For our studies, the mass spectrometry platform applied to the quantification of micro-dystrophin has been invaluable as a stand-alone assay or as confirmation of immunofluorescence and western blot efforts (Fig. 12.2c). The wide-dynamic range enables clear dose-response measurements from 5% to manyfold above normal without saturation effect. Mass spectrometry is a direct detection approach that does not involve the use of antibodies, so all species and mutations can be evaluated equivalently. Currently, immunofluorescence, western blot, and mass spectrometry all report data as a percentage of normal, but mass spectrometry serves as a unique tool to fully characterize "normal." Since every sample analyzed is spiked with the same internal standard, this generates an endogenous-to-standard ratio for every analysis. There is therefore no limitation on the number of possible comparisons, whereas comparisons using western blot may be limited by tissue availability for multiple blots. For instance, mass spectrometry enables comparison across multiple donors and species to better understand the level of normal dystrophin in different muscle types, ages, and genders. Such studies will be critical in benchmarking normal ranges and potential variability seen in restorative clinical trials.

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Chapter 13
Optical Polarization Tractography Imaging of Structural Changes in the Skeletal and Cardiac Muscles of the mdx4cv Mice

Gang Yao

Abstract Optical polarization tractography (OPT) is a recently developed imaging technology that can quantitatively evaluate the three-dimensional fiber organization in tissue with microscopic resolution. In this chapter, we first introduce the basic principle and system design of this technology. We then show its applications for imaging skeletal muscle damage and heart structural remodeling in the mdx4cv mice, a mouse model for Duchenne muscular dystrophy. Because of its relatively low system cost, high imaging speed, and cellular-level resolution, OPT may become an effective tool for phenotype assessment in the research of neuromuscular diseases.

Keywords Imaging · Fiber · Tractography · Muscle · Heart · Mouse · Remodel Polarization

13.1 Introduction

Duchenne muscular dystrophy (DMD) is the most common and severe muscle disease caused by mutations in the dystrophin gene [1]. It affects approximately every one of ~5000 male infants. The absence of the dystrophin gene affects the integrity of the muscle cell membrane, which leads to body-wide muscle degeneration and necrosis. Both skeletal and cardiac muscles are affected in DMD. Most patients eventually die from respiratory and/or cardiac failure. Although a cure is still unavailable, several treatment options are under active investigation and have shown great promise [2].

The ultimate function of the muscular tissue is to produce mechanical force. This is realized by a sophisticated biophysiological mechanism that coordinates and integrates the actions from all tissue constituents. In particular, the structural organization and integrity of the myofibers play a key role in effective force generation. Structural changes in muscle such as fiber branching have important pathological implications in dystrophinopathies [3]. The myocardial fiber structure is especially

G. Yao (✉)
Department of Bioengineering, University of Missouri, Columbia, MO, USA
e-mail: yaog@missouri.edu

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critical for the heart to function normally [4, 5]. The cardiac myofibers are organized in a delicate helix architecture which changes in response to the progression of heart disease as a part of the cardiac remodeling process [6]. Clinical evidence indicates that progressive remodeling is always pathogenic and reverse remodeling is associated with a better prognosis. It has now been recognized that an ideal therapy should slow or/and reverse the remodeling rather than simply relieving local symptoms [6].

Due to the importance of disease-related myofiber structural changes, visualizing the myofiber organization may provide valuable information for elucidating pathogenic mechanisms and evaluating treatment outcomes. Histological staining-based examination is conventionally used to visualize tissue structures. However, it requires serial sections and is only practical for imaging a very small area. In addition, myofiber structural features are inherently three-dimensional (3D), which makes them difficult to assess using current 2D-based histological methods. Diffusion magnetic resonance imaging (MRI) methods such as diffusion-tensor MRI (DTI) and diffusion spectrum MRI have achieved great success in imaging myofiber structure [7, 8]. Unfortunately, the spatial resolution of MRI-based methods is limited to 100~200 μm or worse [9, 10], which makes it impossible to reveal important cellular-level details.

A high spatial resolution becomes especially important for imaging small animal tissues. Animal models are indispensable for the understanding of and for developing treatment for DMD [11]. In particular, mouse models are widely used due to their good accessibility and low cost. Preclinical mouse studies provide crucial information on potential risks and effectiveness of an experimental therapy, which is a critical step prior to human clinical studies. Due to the small size of mouse tissues, a high-resolution imaging modality is needed to reveal detailed structural changes in muscular tissues in mice.

Optical polarization tractography (OPT) is an emerging optical imaging technology that shows potential for routine imaging of fibrous tissue structure [12, 13]. OPT can image large tissue samples with cellular-level spatial resolution at high imaging speed. Its applications have been recently demonstrated in a variety of tissues such as the muscle [13], heart [14–16], artery [17], and cartilage [18]. In this chapter, we will introduce this technology and show its potential application for visualization of myofiber organization in the skeletal muscle and heart of the mdx4cv mouse, a widely used DMD mouse model.

13.2 An Overview of the OPT Technology

13.2.1 Optical Birefringence and OPT

Fibrous tissues such as muscle often show a strong optical birefringence, where the light experiences different optical refractive indices depending on the relative direction between the polarization of the light and the orientation of the fiber axis. The

refractive index is referred to as the "ordinary" index (n_o) when the light polarization is perpendicular to the fiber axis, whereas it is referred to as the "extraordinary" index (n_e) when the light polarization is parallel to the fiber axis. The optical birefringence is defined as the difference $\Delta n = n_e - n_o$ which has a positive value in myofibers. A higher optical refractive index results in a slower light speed. Therefore, the birefringence Δn can be calculated from the relative "phase delay" (δ) measured between the parallel and perpendicular polarization components of the incident light:

$$\Delta n = \frac{\delta}{\Delta z}\frac{\lambda}{2\pi}, \tag{13.1}$$

where λ is the optical wavelength and Δz is the light path length. The fiber orientation (or "optic axis") can be determined based on the polarization direction that leads to the longest delay, i.e., when it is parallel to the fiber.

The major advantage of the OPT technology lies in its capability to image the 3D fiber orientation and birefringence inside the tissue with high resolution and speed. OPT is based on polarization-sensitive optical coherence tomography (PSOCT) [19]. Optical coherence tomography (OCT) is an emerging tissue imaging modality using low-coherence optical interferometry [20, 21]. OCT is analog to conventional "pulse-echo"-based ultrasonic imaging but uses light instead of sound to achieve significantly better resolution. OCT can achieve a micrometer-scale spatial resolution with an extremely high imaging speed that is sufficient for in vivo applications. Conventional OCT uses the optical reflectance as the image contrast to differentiate various tissue structural features at different depths inside the tissue. Polarization-sensitive OCT (PSOCT) extends conventional OCT by providing additional image contrast from the polarization properties of the tissue [22, 23].

A challenging issue in PSOCT imaging is that the light backscattered from a particular depth is affected by the sample properties from the tissue surface to the signal depth. Therefore, the measured optical birefringence and fiber orientation do not accurately represent the true fiber organization at that particular depth. This is especially troublesome when the tissue fiber orientation changes with depth, which is the case in most biological samples. In this case, the directly measured phase delay in PSOCT cannot simply be used to infer the "local" birefringence or optic axis.

OPT provides an effective way to solve this issue and can accurately derive the true local, depth-resolved fiber orientation in tissue. It images the tissue Jones matrix [24] which uses a rigorous mathematic framework to describe the optical polarization properties in materials. The measured Jones matrix provides a comprehensive characterization of tissue optical polarization properties. In OPT, the tissue is modeled using a series of general Jones matrices from the surface to a specific imaging depth [19]. Jones calculus-based algorithms are then used to derive the depth-resolved birefringence [14, 25] and fiber orientation [14, 26].

The smallest tissue volume (i.e., a single image pixel) that can be imaged in OPT is determined by the system resolution. It is likely that the fiber content and direction may be inhomogeneous within this small volume. In this case, the OPT measures the

assemble-averaged fiber orientation which represents the predominant fiber orientation. Similarly, the birefringence obtained represents the mean value of the overall fiber distribution. Therefore, a small birefringence value is an indicator of an "isotropic" region with either no directional fibers or randomly distributed fibers.

13.2.2 OPT System Implementation

The OPT methodology can be implemented in any Jones matrix-based PSOCT systems. Figure 13.1 illustrates an example single-camera Fourier domain Jones matrix PSOCT system that was used to obtain the images shown in Fig. 13.2 in this chapter [19, 27]. This imaging system is a spectral/Fourier domain bulk-optical OCT system using an 847.8 nm wavelength ($\Delta\lambda$ = 58.3 nm) superluminescent diode as the light source. At the sample arm, a 5× telecentric scan lens (LSM03-BB, Thorlabs, Newton, NJ) is used as the objective lens. The light intensity at the sample surface is 5.0 mW. This imaging system has a measured lateral resolution of 12.4 μm and a measured axial resolution of <5.9 μm in tissue within a 1.5 mm imaging depth.

The incident light can be scanned over the sample using a 2D galvanometer scanner (Fig. 13.1a). To image the Jones matrix of the sample, the polarization state of the incident light is modulated using an electric optical modulator to achieve alternating right- and left-circular polarizations [19]. At each incident polarization, the backscattered light from different depths inside the sample is combined with the

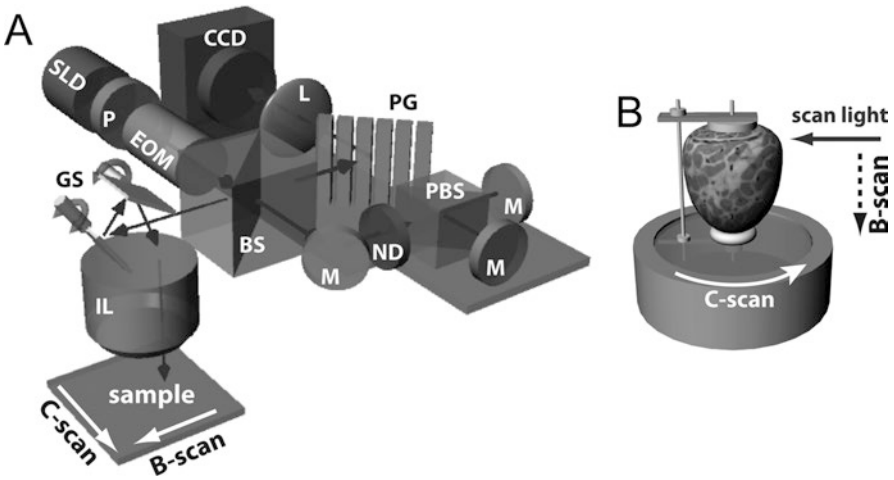

Fig. 13.1 Single-camera Fourier domain Jones matrix PSOCT system. (**a**) A schematic illustration of the 3D OPT system. *BS* beam splitter, *CCD* charge-coupled device (line-scan camera), *EOM* electro-optic modulator, *GS* galvanometer scanner, *IL* image lens, *L* lens, *M* mirror, *P* polarizer, *PBS* polarized beam splitter, *PG* phase grating, *SLD* superluminescent diode. (**b**) An alternative scanning scheme for imaging a whole mouse heart

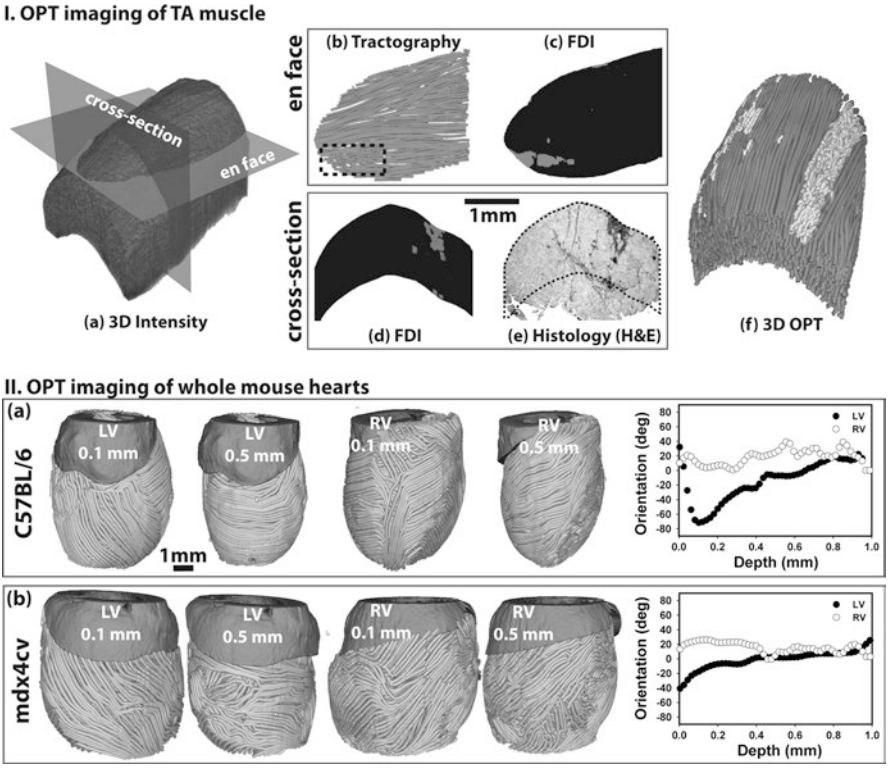

Fig. 13.2 OPT imaging of mouse skeletal muscle and heart. (**Panel-I**) OPT imaging of the mdx4cv tibialis anterior (TA) muscle. (a) 3D intensity images. (b) Tractography and (c) fiber disarray index (FDI) image within the en face projection plane shown in (a). (d) FDI image and (e) H&E histology (red-filtered) of the cross-sectional plane shown in (a). (f) The 3D OPT image with muscle damaged areas highlighted. (**Panel-II**) OPT imaging of freshly excised whole hearts from (a) a C57BL/6 mouse and (b) an mdx4cv mouse. The curves show the change of myocardial fiber orientation with depth from a representative 100 × 100 μm^2 region of interest in the left ventricle (LV) and right ventricle (RV) of the heart, respectively

two co-aligned reference lights with orthogonal linear polarization states (horizontal and vertical polarization). The interference signals are then acquired at the detection arm using a custom spectrometer equipped with a 1024-pixel line-scan CCD camera (Fig. 13.1a). Fourier transform is then applied to extract the two orthogonal polarization components of the interference signals for each incident polarization [27]. From these four signals, the Jones matrix at each pixel can be constructed [19], and the OPT algorithms described above are applied to derive the birefringence and fiber orientation images.

As a convention, the depth-resolved signal acquired at each scanning position on the sample surface is referred to as an A-scan. By scanning the light over a 1D line on the sample surface, multiple A-scans are acquired to form a 2D B-scan image. The second mirror of the 2D galvanometer scanner (Fig. 13.1a) can change the posi-

tion of the B-scan lines to form a C-scan so that multiple B-scans can be acquired to form a 3D image. This standard imaging scheme can be also modified to accommodate different samples. Figure 13.1b shows a setup for imaging the whole heart [15]. The excised heart can be mounted on a rotational stage via a 20-gauge needle passing through the long axis of the heart (between the apex and center of the base). The needle is fixed on the base of the stage and aligned with the rotational axis. The stage can rotate continuously to form a C-scan while repeating the synchronized B-scans along the long axis of the heart (Fig. 13.1b).

13.3 Applications of OPT in Studying Myofiber Structural Changes in a Mouse DMD Model

We show in the following two subsections some example applications of OPT for imaging the tibialis anterior muscle and heart of a mouse. All animal experiments were approved by the institutional animal care and use committee. All the imaging processing was implemented in MATLAB. The tractographic image was constructed using the fiber orientation data and visualized using the 3DSlicer software available from www.slicer.org [28].

13.3.1 Imaging the Whole Tibialis Anterior (TA) Muscle of the mdx4cv Mouse

Figure 13.2-I shows an example OPT result obtained in a freshly excised whole TA muscle from a 7-m-old mdx4cv mouse. The experimental details have been described in a previous publication [13]. The entire 3D image dataset had a total 280 × 2000 × 1000 pixels along the A-, B-, and C-scan directions and covered an imaging area of 1.1 × 8.0 × 8.0 mm^3 (A × B × C). Images were acquired at a speed of 50 k A-lines/s which was limited by the speed of the line-scan CCD used (Fig. 13.1). The final 3D datasets of local optical properties were resized using cubic spline interpolation to produce the same pixel size of 3.9 μm in A-, B-, and C-scan. The 3D image data was filtered using a 3 × 3 × 3 (pixel) median filter to improve the signal-to-noise ratio. When visualizing and analyzing the en face images, a 5 × 5 median filter was applied to further reduce noise. Figure 13.2-I(a) shows 3D intensity image which can also be obtained from current OCT technology. Although the intensity image shows the overall morphology of the TA muscle, it does not reveal any obvious abnormalities.

The 3D image volume can be examined in detail by computationally sectioning through the entire image dataset. Figure 13.2-I(b) shows a tractographic illustration inside an en face plane (Fig. 13.2-I(a)). In most areas, the myofibers appeared well-organized. However, the fiber organization at the lower-left part of the image

(dotted box) showed signs of disruption with randomized fiber orientation. Such "randomness" feature can be quantified using image processing [17]. For example, the "fiber disarray index" (FDI) can be calculated using the standard deviation of the local optical axis within a small 3D evaluation window [13]:

$$\mathrm{FDI} = \sqrt{\sum_{i,j,k=1}^{N} \left(\theta_{i,j,k} - \overline{\theta_{i,j,k}}\right)^2 / N^3}, \tag{13.2}$$

where $\theta_{i,j,k}$ is the fiber orientation at the pixel location of (i,j,k) and $\overline{\theta_{i,j,k}}$ is the average fiber orientation within the evaluation window. A "fiber disarray" image can then be constructed using the FDI values calculated for each image pixel while "sliding" the evaluation windows over the entire 3D image volume.

Figure 13.2-I(c) shows the quantification result by assigning a single gray color to all image pixels with a FDI higher than 16° calculated using small evaluation windows of 35 × 35 × 35 μm^3. Areas with organized fibers (i.e., small FDI values) are shown in black. The small region with disrupted (with a high FDI) myofibers can now be clearly visualized. Figure 13.2-I(d) shows the FDI image obtained in a cross-sectional cutting plane (Fig. 13.2-I(a)). Using the same segmentation threshold as in Fig. 13.2-I(c), a small disrupted region was revealed close to the upper-right side of the tissue.

The OPT images obtained in the cross-sectional planes (Fig. 13.2-I(d)) can be directly compared with the conventional histology results acquired at the same sectioning position. Figure 13.2-I(e) shows the corresponding histology image (H&E stain). The histology image was red-filtered for display in the gray scale. The histology result revealed striking abnormalities at the same location with higher FDI. A close examination of the histology results indicated significant muscle damages including necrosis and inflammation within the segmented regions with high FDI values.

The results shown in Fig. 13.2-I demonstrated that the 3D OPT images can be conveniently analyzed in any evaluation plane in the software. As a comparison, conventional histology evaluation is destructive and can only be obtained in a specific cutting plane. Figure 13.2-I(f) further illustrated the power of 3D OPT for visualization 3D muscle damages in the entire TA muscle, where the damaged regions are highlighted using a lighter gray shade. Once all damaged areas within the entire 3D TA muscle were segmented using FDI, additional quantitative assessment can be performed. Table 13.1 shows a comparison of the fiber disarray index and optical birefringence obtained in normal C57BL/6 mice and the damaged and non-damaged regions of mdx4cv mice. The results were group-averaged from the TA samples excised from four mdx4cv mice and four C57BL/6 mice (Table 13.1).

Damaged mdx4cv muscles appeared to have slightly lower imaging intensities (Table 13.1). However, the difference did not reach statistical significance due to the variations in the intensity. In contrast, damaged mdx4cv muscles showed a significantly higher FDI than those of non-damaged mdx4cv muscles and muscles from C57BL/6 ($p < 0.0001$, one-way ANOVA with Bonferroni's post hoc test). Damaged

Table 13.1 Quantitative comparison of OPT results (intensity, birefringence, and FDI)

	Intensity (dB)	Δn ($\times 10^{-4}$)	FDI (°)
C57BL/6	55.1 ± 8.4	8.1 ± 0.2	8.1 ± 0.7
mdx4cv (damaged)	48.1 ± 8.1	3.1 ± 0.1[a]	32.8 ± 2.0[a]
mdx4cv (non-damaged)	55.4 ± 9.1	7.3 ± 0.6	7.8 ± 1.3

Values are represented as mean ± standard deviation
[a]Significantly different from the other groups ($p < 0.001$)

mdx4cv muscles also had a significantly smaller birefringence value than non-damaged mdx4cv muscles and C57BL/6 muscles ($p < 0.0001$). Both FDI and birefringence values had higher variations in non-damaged mdx4cv muscles than in muscles from normal C57BL/6 mice. No significant difference ($p > 0.05$) was found in either the fiber disarray index or local birefringence between non-damaged mdx4cv muscles and muscles from normal C57BL/6 mice.

13.3.2 Imaging the Whole Heart of the mdx4cv Mouse

Figure 13.2-II shows the example OPT images obtained in freshly excised mdx4cv and C57BL/6 hearts. Both animals are 7-month-old males. The experimental details have been described previously [15, 16]. Briefly, these results were obtained using the scanning scheme illustrated in Fig. 13.1(b). During imaging, the stage was rotated continuously over 360° at a speed of 1.25°/s while the incident light repeatedly scanned along the long axis of the heart (as the B-scan). Each B-scan covered 8 mm with 2000 A-scans. A total of 3600 B-scans were acquired at a recording speed of 12.5 B-scans/s. It took 288 s to image a whole heart.

The constructed 3D dataset of fiber orientation had 280 × 2000 × 3600 pixels (in A × B × C scans) and covered a corresponding imaging area of 1.1 mm × 8.0 mm × 360°. The 2D "planar" tractography was first built at each en face plane using MATLAB streamline functions [15]. A 5 × 5 (pixels) median filter was applied to reduce the speckle noise in the calculation. The stack of the "planar" 2D tractography was then polar-transformed into the 3D coordinates from the measured heart diameter and heart surface as described previously [15]. The accuracy of OPT technology in imaging fiber orientation in heart tissues has been rigorously validated by comparing directly with histology images in a previous study [12].

Figure 13.2-II(b) shows that the mdx4cv heart was clearly enlarged. In addition, OPT can reveal global fiber architecture at various transmural depths inside the ventricular wall. Figure 13.2-II(a), II(b) shows example images of fiber orientation after "peeling" off 100 μm and 500 μm cardiac tissue from the surface. These image results revealed that the cardiac fiber orientation changes with the depth. In all tractographic images, the zero degree was aligned in the horizontal direction toward the right. In the left ventricle (LV), the myocardial fibers had negative orientation close to epicardium; the orientation transited to positive angles toward the endocardium.

This trend was in good agreement with the unique cross-helical fiber structure expected in the LV of the heart. Detailed examination suggested that the overall fiber orientation in LV appeared to be similar in both the mdx4cv and C57BL/6 hearts although the fiber structure had more variations inside the mdx4cv heart (Fig. 13.2-II(b)) [16].

The RV of the mdx4cv heart showed more differences in fiber organization from the C57BL/6 RV. Some vertically oriented fibers were observed in the C57BL/6 RV at ~100 μm depth from the heart surface. This RV location coincided with the right ventricle vein in the mouse heart. Such a feature was not as clear in the mdx4cv RV. In addition, the fiber organization in the mdx4cv RV had much greater variations than the C57BL/6 RV at both 100 μm and 500 μm depths from the heart surface.

To further quantitatively assess the fiber organization, Fig. 13.2-II also shows the representative curves of fiber orientation changes with transmural depth in both the LV and RV of the heart. The "zero" depth was at the epicardium of the heart. These curves indicated that the fiber orientation over the depth was greater in the LV than in the RV. There was a reversed trend within the first 100 μm beneath the epicardium in the C57BL/6 LV which was reported in a previous OPT study and was confirmed in histology. This pattern was absent in this mdx4cv heart. The quantitative curves also suggested that the slope of the orientation change with depth was smaller in the mdx4cv heart than the C57BL/6 heart (Table 13.2).

The above observation was confirmed in a total of six mdx4cv hearts and six C57BL/6 hearts (Table 13.2). As a whole, the mdx4cv mice had a larger heart than the C57BL/6 mice ($p = 0.013$, Student's t-test). Table 13.2 also shows the group comparison of the slope of fiber orientation with depth obtained inside a large evaluation window (2.8 mm × 70°, or 700 × 700 pixels in B × C) located at the center of the LV and RV [16]. The data indicated that the fiber orientation increased with depth in the LV of all hearts in both groups, resulting in "positive" slopes (i.e., the classic double-helical profile). The slope values had significantly bigger variations in the mdx4cv LV than in the C57BL/6 LV ($p = 0.018$, Levene's test). The slopes were smaller in both the LV and RV of the mdx4cv heart than those of the C57BL/6 heart. Such a difference in slope reached statistical significance in the RV ($p = 0.004$, Student's t-test). The overall observation of greater myofiber organization changes in the mdx4cv RV is consistent with the emerging evidence that DMD cardiomyopathy in mouse starts from the right ventricle (RV), which is likely a result of the severe diaphragm dystrophy in the mouse DMD model [29, 30].

Table 13.2 Group comparison from six mdx4cv hearts and six C57BL/6 hearts

		Slope of orientation (°/mm)	
	Diameter (mm)	RV	LV
mdx4cv	6.9 ± 0.5[a]	20.0 ± 28.4[b]	86.4 ± 20.4
C57BL/6	6.2 ± 0.3[a]	67.9 ± 12.6[b]	104.4 ± 7.0

Values are represented as mean ± standard deviation

[a,b]The difference between mdx4cv and C57BL/6 was significant ($p < 0.05$)

13.4 Conclusion

We show in this chapter that the newly developed OPT technology can effectively visualize fiber structural changes in the skeletal and cardiac muscles of a mouse DMD model. OPT can rapidly and nondestructively examine the entire sample in 3D and with histology-like resolution. We demonstrated that "virtual" histology can be realized using the 3D OPT data to evaluate and quantify the muscular structures in great details. This technology may find potential applications in muscle disease and muscle gene therapy research as a practical tool for phenotype assessment. It can be used for routine assessment of the biopsied muscle specimen, which helps to improve the current labor-intensive histology practice. Because OPT is nondestructive, the same tissue sample can still be used for other standard histochemical examinations. The capability of visualizing muscle damages nondestructively is valuable for researchers/clinicians to better allocate the proper tissue segments for further detailed and targeted analysis.

In addition to the skeletal muscle and heart, fibrous tissues exist in many other parts of the body, for example, in neural fibers, dental tissues, skin, and cartilage. Disruption of the normal fibrous structure in these tissues is an important indication of tissue dysfunction, whereas the recovery of a normal fiber organization can be indicators of positive treatment outcome. Therefore, OPT may find many important biomedical applications in many tissues and diseases beyond imaging mouse skeletal and cardiac muscle as demonstrated in this chapter.

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Chapter 14
Biomarkers for Muscle Disease Gene Therapy

Yetrib Hathout, Kristy J. Brown, Kanneboyina Nagaraju, and Eric P. Hoffman

Abstract Molecular biomarkers are becoming increasingly attractive in drug development programs for muscle diseases especially for Duchenne muscular dystrophy (DMD). Robust and validated blood and muscle biomarkers that are sensitive to drug treatment (e.g., pharmacodynamic biomarkers) and that can predict later clinical outcomes (e.g., surrogate biomarkers) will likely aid in developing effective therapies for DMD at several levels. Peripheral blood biomarkers can serve as more objective and acute readouts of drug effects relative to clinical outcome measures. Furthermore, they may minimize the burden on patients and families regarding participating in trials and can help with go-no-go decisionmaking at early stages of drug development possibly reducing the length and cost of the clinical development program. Lastly, they may provide insights into pathobiochemical pathways and help define novel therapeutic targets. In this chapter, we will focus mainly on molecular biomarker advances in DMD with the emphasis on their utility to assess efficacy of gene therapy in this disease.

Keywords Duchenne muscular dystrophy · Gene therapy · Dystrophin Pharmacodynamic biomarkers · Surrogate biomarkers

14.1 Introduction

The term biomarker in the clinical setting remains broadly defined and often refers to a measurable indicator of the health status of an individual. Depending on the disease and/or the condition, these indicators could be functional, physiological, biological, molecular, or imaging. The intent of use is often for diagnosis or

Y. Hathout (✉) · K. Nagaraju · E. P. Hoffman
Binghamton University School of Pharmacy & Pharmaceutical Sciences, Binghamton, NY, USA
e-mail: yhathout@binghamton.edu; nagaraju@binghamton.edu; ehoffman@binghamton.edu

K. J. Brown
Solid Biosciences, Cambridge, MA, USA
e-mail: kristy@solidbio.com

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prognosis, but recently drug developers have found biomarkers, especially molecular biomarkers, useful to assess safety and efficacy of new drugs. In the case of DMD, a number of biomarkers and outcome measures have been developed and implemented by clinicians since the discovery of the disease [1]. Throughout the years, these biomarkers have evolved from an observational nature to more precise diagnostic biomarker such as DNA genotyping for *DMD* gene mutations [2], dystrophin protein testing for biochemical diagnosis [3], and imaging using NMR [4]. However, over the past 5 years, clinicians and regulatory agencies such as US Food and Drug Administration (FDA) and European Medicines Agency (EMA) have recognized the need of more robust and reliable outcome measures to aid drug development programs in DMD and other disorders. As highlighted by the US Congress in the recent twenty-first century Cures Act—Provisions to Promote Drug Development (https://www.ropesgray.com/newsroom/alerts/2016/December/21st-Century-Cures-Act-Provisions-Relating-to-Promoting-Drug-Development.aspx), the FDA is willing to consider novel clinical trial designs and surrogate endpoints, such as molecular biomarkers, in the drug development process. This is especially true for DMD where numerous examples of very expensive DMD clinical trials have failed despite the fact that the investigational drugs showed efficacy in animal models. This paradox leads families, their physicians, and scientists scratching their heads: "What is going on?" Unfortunately, it is often not known if the failed trial was due to the drug simply not working or, alternatively, that the clinical trial was unable to clearly detect a beneficial effect. For example, the key clinical test used in most DMD trials to date is the "6-min walk test" [5], where a weak and easily tired young child is asked to walk up and down a hospital hallway for 6 min. Parents of most any 5-year-old boy will suggest this seems a better test for the mood of the child than their strength and improvement by a drug! Furthermore, the 6-min walk test can only be administered to cooperative boys that are still walking—this excludes very young children 1–4 years of age (generally uncooperative) and older boys 10–18 years (who may not be able to walk).

For these reasons, there is a need for additional unbiased outcome measures that are more sensitive and reliable to detect whether or not a drug is working days or weeks after treatment (e.g., detecting "acute" responses compared to clinical outcomes that may take a year or more of treatment to show significant change). In this context, serum molecular biomarkers might help overcome some of these issues: (1) early readout to detect response to a drug and go-no-go decisionmaking at early stages of a clinical trial; (2) cost reduction in clinical trials, which is an important consideration given that development of new drugs has become increasingly challenging and resource intensive as defined in section 2021(e) of the twenty-first Century Cures Act—enacted December 2016; (3) decrease invasiveness and minimize burden on patients; (4) determination of optimal dosing; (5) easy implementation across different stages of the disease and across different preclinical and clinical studies; and (6) the need for further insights about the mechanism of disease progression and novel therapeutic targets. It should be noted these biomarkers are not intended to replace the 6-min walk test or other meaningful clinical endpoints but rather used as additional outcome measures to help gauge decisionmaking and especially assess if a treatment has engaged its target.

In this chapter, we provide readers with a comprehensive review of biomarker development in DMD with a special emphasis on the development of blood and/or urine accessible biomarkers in DMD and how they can help assess gene therapies. Other biomarkers such as functional, physiological, and imaging are described in more details elsewhere and are not covered in this chapter.

14.2 Status of Molecular Biomarker Development and Implementation in DMD

Thanks to the advances in "omics" technologies, a comprehensive list of candidate serum biomarkers has been identified for dystrophinopathies. These included miRNAs, proteins, and metabolites [6–10].

A key distinction when discussing biomarkers is whether or not the biomarker is directly related to disease pathogenesis or a downstream secondary change associated with the disease. It is well accepted that lack of expression or expression of an aberrant dystrophin protein in skeletal muscle is the primary cause of two well-known dystrophinopathies, Becker muscular dystrophy (BMD) and DMD [3]. Dystrophin protein is present in very low amount or completely absent in skeletal muscle of DMD patients and expressed in varied truncated forms and amounts in BMD, hence the difference in disease severity between DMD and BMD patients [3]. While diagnosis of these diseases is done via genetic analysis these days, assessing levels of restored dystrophin protein in skeletal muscle has become a primary outcome measure in clinical trials of dystrophin restoration therapies. Below, we begin with the status of using dystrophin protein in muscle as a biomarker. We then discuss serum proteins that reflect more downstream disease pathogenesis and/or drug efficacy.

14.2.1 Dystrophin Levels in Muscle as a Pharmacodynamic Biomarker and Surrogate Outcome Measure for DMD

Restoration of dystrophin expression in DMD patient muscle is targeted to the primary defect of this disease (namely, dystrophin deficiency). Strategies for dystrophin restoration have included gene therapy, exon skipping, and stop codon read-through and have been shown to be effective in animal models for DMD. These strategies are covered in more detail by other chapters in this book, and we only briefly mention them in this chapter in the context of biomarker studies. When a biomarker, such as dystrophin, is the cause of the disease and it is the intended target in dystrophin replacement therapies, it makes complete sense to check its levels before and after treatment.

Ataluren, a stop codon read-through drug [11], and eteplirsen (Exondys 51), an exon-skipping antisense oligonucleotide [12], have received conditional approval

from EMA and FDA, respectively [13, 14]. Both drugs faced significant challenges in gaining approval due to the very low amount of restored dystrophin in DMD clinical trials (<1% of the levels seen in healthy muscle). This modest increase raised questions whether it is clinically meaningful or not. How much of dystrophin is enough remains an ongoing debate among clinicians, the DMD community, and regulatory agencies. However, one may argue some dystrophin is better than none.

Another setback during approval of these two drugs was associated with the methods used to measure levels of restored dystrophin in muscle biopsies. Dystrophin was measured mainly by western blot and immunofluorescence staining in these clinical trials. While straightforward, these methods suffer from large variability from laboratory to laboratory and even within the same laboratory [15] making it challenging to meet FDA draft guidance for industry "Bioanalytical Method Validation" [16] that requires a bioassay to have less than 15% CV to be valid.

In March 20, 2015, a scientific workshop cosponsored by the FDA and NIH discussed dystrophin protein quantification methodologies and gaps. The workshop was open to the public, and about 180 people including scientists in the field of muscular dystrophy research as well as patients and families attended the workshop. Twenty investigators were invited to present the different methods to quantify dystrophin followed by questions/answers moderated by FDA and NIH representatives. The three methods that were highlighted and discussed during the workshop included western blot, immunofluorescence staining, and liquid chromatography tandem-mass spectrometry (LC-MS). A panel of experts in these fields facilitated the discussion and concluded that the three methods are complementary, and there are strengths and weaknesses associated with each one (Table 14.1). A fourth dystrophin assay included in Table 14.1, which was not extensively discussed during

Table 14.1 Existing dystrophin quantification methods

Dystrophin assay	Advantages	Disadvantages	Literature
Western blot	• Simple • Sensitive	• Semiquantitative • Challenge balancing between detection and saturation • Large CVs	[15, 17]
Immunofluorescence staining	• Sensitive • Spatial localization • Differentiate between positive and negative muscle fibers	• Requires several steps • Background issues • Poor linearity • Large CVs	[15, 18]
LC-MS	• Specific • Accurate • Excellent linearity • Good CVs	• Technically complex • Requires state-of-the-art instrument and expertise	[19]
Reverse transcriptase PCR	• Simple • Very sensitive	• Risks of sample degradation • May not reflect the amount of functional dystrophin	[20–22]

LC-MS liquid chromatography tandem-mass spectrometry, *PCR* polymerase chain reaction, *CV* coefficient of variation

the workshop, relies on measuring mRNA transcripts of the restored dystrophin. While this assay is simple, it has not been widely implemented in clinical studies perhaps due the fact that mRNA levels may not directly reflect functionality while dystrophin protein does.

Key steps in implementing a specific assay for any specific biomarker include method development, a validation plan, and method validation (completing the plan). This provides information on selectivity, sensitivity, accuracy, reproducibility, and stability of the assay. Each dystrophin bioanalytical assay, whether it is an affinity-based assay such as western blot and immunofluorescence staining or a chromatographic-based assay such as LC-MS, should be validated prior to use in a clinical trial setting. As described in more details in the FDA draft guidance for industry "Bioanalytical Method Validation" [16], selectivity refers to the ability of an analytical method to specifically detect and quantify the intended target in a complex mixture. Sensitivity refers to the lowest amount of the target that can be accurately measured by the assay, while accuracy refers to how close the measured value is to the actual value in the samples. Reproducibility of the assay refers to repeatability of the assay. Furthermore, a good assay should give the same results on replicate analysis, a parameter that is also dependent on the stability of the target analyte. For example, the stability of the analyte or target in a given matrix under different conditions for a given time is often influenced by sample handling, shipment, and storage and should ideally be consistent over time.

14.2.2 Circulating Molecular Biomarkers to Monitor Dystrophin Replacement Therapies in DMD

Defining the success of dystrophin replacement in DMD patient muscle typically requires a muscle biopsy. Muscle biopsies are a significant burden on DMD boys and their families and are considered quite invasive. Furthermore, there is significant sampling error in all muscle biopsies, where the very small muscle biopsy specimen may not represent what is happening to muscle body-wide. Circulating biomarkers may not directly measure dystrophin in muscle but may provide an indirect readout of overall skeletal muscle health. However, such circulating biomarkers must be shown to be associated with the disease and respond to dystrophin restoration.

Circulating serum biomarkers identified in DMD have been studied for many years and include cytoplasmic enzymes of the myofibers that become released into the circulation due to the dystrophic process (membrane leakage and degeneration of myofibers). These include creatine kinase (CK) [23], myoglobin (MB) [24], and carbonic anhydrase III (CA3) [25]. These biomarkers are found at their highest levels in DMD patients at younger ages and then gradually decrease with age toward the levels seen in healthy subjects [9]. The age-related normalization of these myofiber enzymes reflects the progressive loss of skeletal muscle and replacement with

fibrotic and adipose tissue. As the patient ages, there is less and less skeletal muscle to release these biomarkers into the peripheral circulation.

CK is a useful diagnostic biomarker for DMD at younger ages, inclusive of newborn screening [26, 27]. The utility of CK as a prognostic biomarker in drug trials is less well-supported, as levels are quite variable and are highly age-related. Nevertheless, in preclinical drug trials in the *mdx* mouse model of DMD, CK has been shown to respond very well to some experimental therapies. It significantly decreased by more than 50% in mdx mice treated with AAV-U1#23 (an adeno-associated virus-mediated antisense exon-skipping therapy) relative to untreated mice [28] and also decreased in mdx mice treated with phosphorodiamidate morpholino oligomers (PMO) for exon skipping [29]. In this latter study, the CK decreased in a dose-dependent manner with PMO. It was moderately decreased without reaching significance in mice treated with a clinical dose of PMO (30 mg/kg) but significantly decreased by more than 70% when mice were treated with a higher dose of PMO (1.5 g/kg). This suggests that more systemic dystrophin restoration is needed to see meaningful change in the level of circulating CK.

Preclinical studies testing AAV micro-dystrophin gene therapy often use dystrophin expression as a surrogate endpoint because it directly reflects the drug mechanism of action. Micro-dystrophin shows expression in skeletal muscle body-wide, and levels of expression correlated well with the improvement in muscle strength in treated animals [30, 31]. Nevertheless, CK could still prove to be a useful pharmacodynamic biomarker to assess efficacy of dystrophin replacement therapies and for dose selection as shown in the mdx PMO study above. This will require a careful correlation between the levels of restored dystrophin and decrease in CK levels to validate CK as a pharmacodynamic biomarker. Future integration of dystrophin, CK, and other biomarkers into the clinical development of drugs may provide sufficient evidentiary data as required by the FDA to expedite the translation of preclinical drugs.

14.2.3 Other Serum Biomarkers to Assess Gene Therapies in DMD

Over the last few years, “omics” technologies, such as next-generation sequencing, metabolomics, and proteomics (mass spectrometry and SOMAscan technologies), have been applied to both DMD patients and the mdx mouse model, leading to comprehensive discovery of circulating molecular biomarkers. These biomarkers are now being implemented as candidates for aspects of safety, efficacy, and drug mechanism of action in clinical drug development programs for DMD.

These candidate biomarkers provide instruction on different pathobiochemical pathways involved in the dystrophic process in patient muscle. These biomarkers are expected to respond to different treatment modalities targeting different aspects of the disease (e.g., sarcolemma stabilization, inflammation, and fibrosis). Logically,

one would expect that biomarkers associated with sarcolemma leakage should respond acutely to dystrophin replacement therapies. Dystrophin replacement directly addresses the primary genetic and biochemical defect, as well as the cellular phenotype of membrane leakage and CK release. On the other hand, anti-inflammatory drugs such as glucocorticoids and vamorolone may show acute responses of inflammatory biomarkers [32].

14.2.3.1 Response of Serum Circulating MicroRNA (miRNA) to Gene Therapy in DMD

miRNAs are small noncoding RNAs composed of approximately 22 oligonucleotides. They regulate gene expression at the posttranscriptional level. miRNAs are increasingly viewed as an important aspect of DMD muscle pathogenesis and thus potential therapeutic targets [33]. Muscle miRNAs are also released by dystrophic muscle into the blood and are seen as candidate serum biomarkers for DMD and drug treatment [34–36]. Most of these circulating miRNAs increased with age in young DMD patients, from 4 to 6 years of age, and then gradually decreased with age in DMD patients older than 6 years [37, 38]. Thus, it is important to take age into consideration when assessing miRNAs as pharmacodynamic biomarkers.

To provide specific examples in the context of gene therapy, there was a marked decrease in the serum levels of miR-1 and miR-206 in mdx mice treated with AAV-U1#23 exon skipping [34]. However, these same miRNAs showed only a moderate trend toward normalization without reaching statistical significance in DMD patients treated with the antisense oligomer eteplirsen for 12 weeks [38]. This difference in outcomes could be due to an actual difference in dystrophin restoration in an individual muscle, the ability of each treatment to treat all muscles in the body, and other stochastic effects of the respective therapies. Indeed, a low amount of dystrophin was restored by the dose of eteplirsen used in the clinical trial (<1% of the normal) [13], compared to the high levels of dystrophin resulting from mdx mice treated with AAV-U1#23. Specifically, AAV-U1#23 showed not only higher levels of dystrophin but dystrophin restoration was systemic [34].

14.2.3.2 Response of Serum Circulating Protein Biomarkers to Dystrophin Replacement Therapies

Comprehensive studies of serum protein biomarkers in DMD patients and the mdx mouse model have been published [6, 9, 10, 39]. The pharmacodynamic responses of these serum protein biomarkers have been reported in a limited number of studies (mostly preclinical). Serum protein biomarkers that responded to dystrophin replacement therapies in mdx mice treated with Pip6a-PMO (12.5 mg/kg) have been reported [39, 40], and these are listed in Table 14.2 with their relative response and their change with age in DMD patients.

Table 14.2 List of serum protein biomarkers that responded to dystrophin replacement therapies in the mdx mouse model

Protein biomarker	Fold change (mdx vs wt)	Response to Pip6a-PMO treatment	Decline with age in DMD
Myomesin 3	>100	↓↓	Yes
PGAM1	136	↓↓	ns[a]
TNNI3	53	↓↓↓	Yes
MB	15	↓↓	Yes
LDHB	8.4	↓↓	Yes
FABP3	7.8	↓↓	Yes
CAMK2B	5.3	↓↓	Yes
ANP32B	5.7	↓↓	Yes
CYCS	3.6	↓↓↓	Yes
CAMK2D	3.6	↓↓	Yes
PCNA	3.3	↓↓	ns
HTRA2	3.1	↓↓↓	Yes
ADAMTS5	2.8	↓↓↓	ns[a]
PTPN11	2.8	↓↓	Yes
LYN	2.7	↓↓	ns[a]
Calpain I	2.4	↓↓↓	Yes
THBS4	2.2	↓↓	Yes
EDA2R	2.1	↓↓	ns[a]
SFN	−3.2	↑↑	ns[a]

[a]ns: not significant when comparing DMD to age matched healthy controls. Number of arrows refers to degree of response of the biomarker to Pip6a-PMO treatment [39, 40]

Cross-referencing these mdx mouse pharmacodynamic biomarkers to human DMD natural history data shows that the same biomarkers that responded to dystrophin replacement therapy in the mdx mouse model are also decreased as a function of patient age (Fig. 14.1). Thus, if these biomarkers were to be used in a DMD drug development program, the decreases due to drug effect would need to take into account the decreases due to advancing age. Although some biomarkers declined less sharply than others (see left and right panel, Fig. 14.1), it may be challenging to differentiate between the decline due to the natural course of the disease and the decline due to the actual benefit of a treatment. So far, only cytochrome C (CYCS) seems to show longitudinal stability over time in young DMD boys.

In this case an ideal pharmacodynamic biomarker candidate for gene therapy is a biomarker that is altered in its levels in DMD relative to healthy controls but has a trajectory that remains stable or positively correlated with disease progression over a period of time suitable to conduct a clinical trial. Such biomarkers remain to be selected and tested longitudinally in both preclinical and clinical studies.

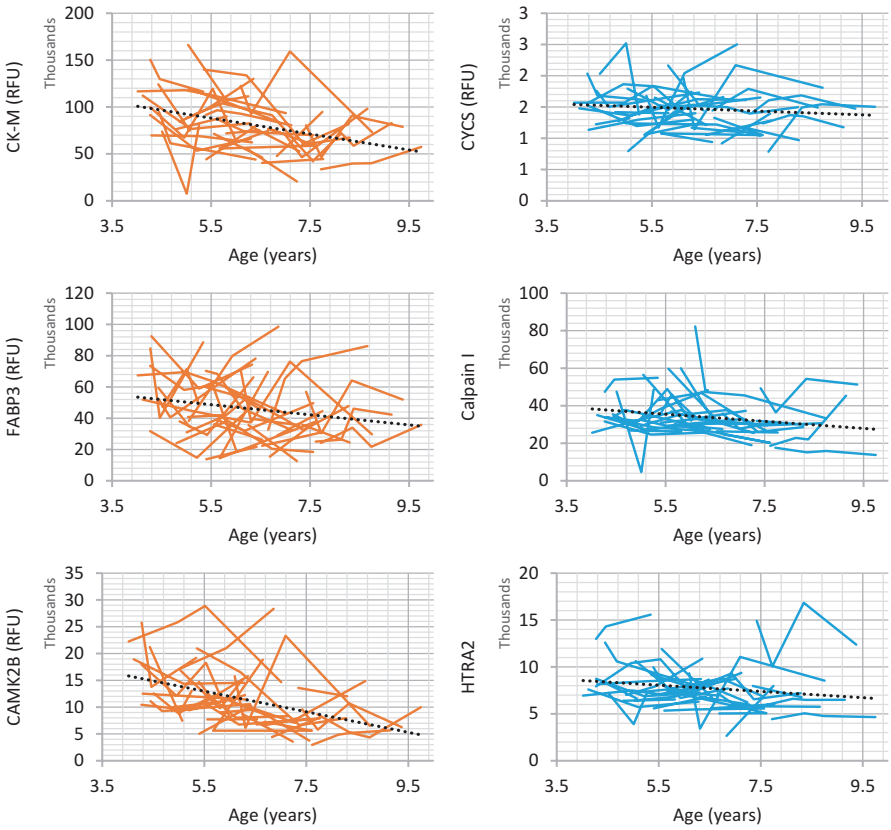

Fig. 14.1 Longitudinal trajectory of potential dystrophin replacement pharmacodynamic biomarker candidates in the natural history study of DMD patients. The dashed trend line shows the overall trajectory of the biomarker. Left panel list biomarkers that declined sharply with age and right panel lists those that declined slowly with age. Unpublished data

14.3 Validation and Qualification of Biomarkers for DMD Gene Therapy

The regulatory terms "validation" and "qualification" can be disorienting to investigators in the field of biomarker development. From a regulatory point of view, the terms validation and qualification of a biomarker do not mean replicating the biomarker in another study or a cohort but instead defining a specific context or use of the biomarker in a clinical drug development program and then carrying out studies that ensure the reliability of the assays used to measure the biomarker. For more detail about the definition of these terms, see a review article about development of biomarkers for osteoarthritis [41] and the draft "Guidance for Industry and FDA Staff Qualification Process for Drug Development Tools" assembled and continuously updated by the FDA's Center for Drug Evaluation and Research (FDA-CDER) (https://www.fda.gov/downloads/drugs/guidances/ucm230597.pdf).

14.3.1 Biomarker Validation

Validation of a biomarker is a process to ensure that a method used to measure the biomarker is reliable (will provide accurate and reproducible data). To validate a biomarker, it is important to first define the type of the biomarker, the context of use, and the method or assay planned to measure it. The method or assay to measure the biomarker needs to be validated in terms of dynamic range, selectivity, specificity, sensitivity, limit of detection and quantification, accuracy, linearity, repeatability, reproducibility, and stability. If a biomarker is to be utilized as a clinical trial endpoint in a quantitative manner, then demonstrating the precision of the assay is critical. As outlined in Fig. 14.2, validation of a biomarker is one part of the qualification process.

14.3.2 Biomarker Qualification

According to the FDA definition, qualification of a biomarker refers to a regulatory process to transition an exploratory biomarker from the laboratory bench to a well-defined use of that biomarker in a clinical drug development program (context of use and fit for purpose [42]). A qualified biomarker should be reliable in providing a specific interpretation within the stated context of use. The context of use could be prediction of disease progression, prediction of safety and efficacy of new treatment, dose selection, or use as a surrogate outcome (endpoint). Thus, the qualification process of a biomarker is tightly tied to its context of use. The strategic paths to qualify a biomarker are clearly described in the draft guidance under

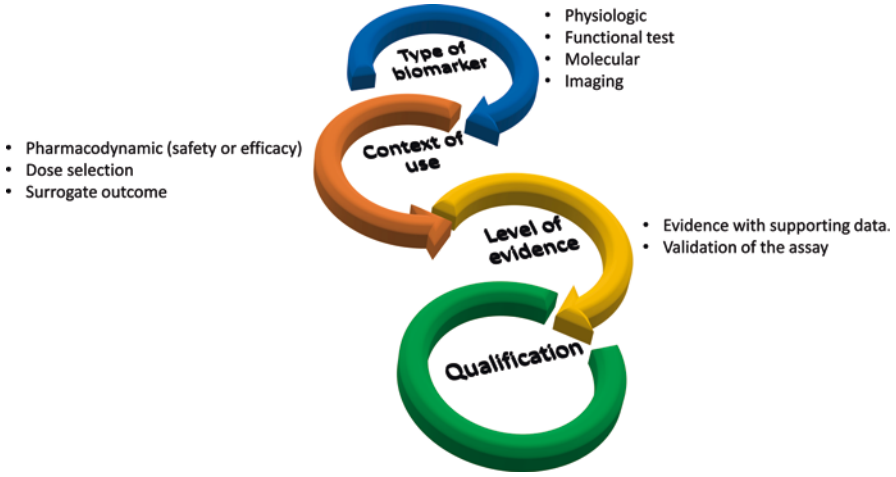

Fig. 14.2 Biomarker qualification process

Biomarker Qualification Program that is maintained and periodically revised by CDER of the FDA guidance web page. From the time of the submission of letter of intent to the final qualification and clearance of a biomarker, it takes an average of 2–3 years [42].

There is often the perception that a biomarker must be "qualified" before integration of that biomarker into a drug development program. This is not true—for example, measurement of the dystrophin protein in patient muscle biopsies after exon skipping has been accepted by the FDA as a surrogate endpoint in DMD without qualification. However, the assays utilized to measure dystrophin (or other non-qualified biomarkers) should still be validated for reliability of the assays used for detection. That said, a qualified biomarker might be expected to show consistent and robust clinical utility having gone through the 2-year qualification process. Successful completion of the qualification process, inclusive of rigorous validation and tests that adhere to predefined regulatory guidelines, is more likely to be accepted as a robust endpoint by regulatory authorities in drug development. Furthermore, a validated and qualified biomarker can be used in multiple drug development programs as long as the same original context of use is used according the FDA guidance for industry.

14.4 Conclusion

Currently, there is no qualified molecular biomarker for dystrophin replacement therapies in DMD. But with the variety of biomarkers identified in DMD and the number of preclinical studies testing new gene therapies, we are in a position to select the best candidate biomarkers and evaluate their utility to accurately access safety and efficacy of various gene therapies. A validated and qualified biomarker can be implemented not only to aid the therapy development but can also be implemented in a long-term standard of care once the drug or a therapy is approved. Overall, validated and qualified biomarkers will speed up drug development programs, reduce the cost of clinical trials, and most importantly improve the public health.

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Chapter 15
Large-Scale Clinical Manufacturing of AAV Vectors for Systemic Muscle Gene Therapy

Nathalie Clément

Abstract Gene therapy targeting the muscle using adeno-associated vectors (AAV) has a long track record starting from the first vector design in the 1980s until today where systemic delivery to the muscle mass has become perhaps one of the most sought-after therapy designs. The unparalleled efficacy of AAV vectors to deliver robust and long-term expression of the desired therapeutic gene into the different muscle cell types remains one of the top assets of this viral drug. However, it also created one of its biggest challenges: manufacturing recombinant AAV stocks to scale sufficient to fulfill the needs of preclinical studies and phase I to III clinical trials. Ultimately, commercial manufacturing remains a major hurdle, if not a roadblock, toward its full implementation for clinical uses in humans. Nevertheless, robust processes have recently emerged to produce phase I- to III-enabling, clinical-grade AAV drugs and present with promising paths toward commercial use.

Keywords AAV · Gene therapy · Manufacturing · Clinical

15.1 Introduction

Muscle is perhaps the most suitable organ for gene therapy applications using adeno-associated vectors (AAV) or recombinant AAV (rAAV). Quickly upon discovery of AAV serotype 2 (AAV-2) and its engineering as a recombinant vector, investigators explored using AAV-2 as a vector for muscle gene delivery. Compared to other viral and non-viral vectors, AAV-2 has resulted in high and persistent transduction in muscle [1]. The isolation and cloning of numerous capsid sequences, which is responsible for the virus cellular tropism, has further improved the portfolio of biological delivery tools to achieve high and sometimes highly specific, muscle transduction efficacy. Among them, serotypes 1, 6, 8, and 9 have become the

N. Clément (✉)
Department of Pediatrics, Powell Gene Therapy Center, University of Florida, Gainesville, FL, USA
e-mail: nclement@peds.ufl.edu

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leading vectors to promote gene transfer to muscle fibers from rodents to non-human primates and are currently being evaluated in humans for a variety of genetic diseases [1, 2].

On a sidenote, but perhaps meaningful, the AAV life cycle is also intimately intertwined with muscle cell biology, although indirectly. AAV-2 is the only human virus capable of site-specific integration in the human genome during its latent phase. Molecular characterization of the AAV-2 integration locus (AAVS1) revealed that it lies within the coding region of the human myosin-binding subunit 85 (MBS85) gene. MSBS85 is a protein expressed in muscle cells and involved in actin-myosin fiber assembly [3]. Interestingly, integration of AAV-2 within AAVS1 does not disturb the cell phenotype or survival [4]. Recombinant AAV, on the other side, does not integrate site specifically due to the lack of the Rep protein expression. AAV is widely accepted as one of the safest vectors available to date for clinical gene therapy.

While several serotypes support efficient muscle transduction, each presents with specific features that make the choice of AAV vector even more resourceful for gene therapists. AAV serotype transduction capability may differ in terms of the muscle type (skeletal or cardiac), myofiber subtype, kinetics of expression, and ability to spread to other organs [1, 5]. In some cases, serotype features also vary across species and gender [6, 7]. Further, AAV muscle transduction profile can be orchestrated by the use of various regulatory sequences, including but not limited to muscle-specific promoters such as the muscle creatinine-kinase promoter [8] and the desmin promoter [9, 10], as well as the vector genome design (single-stranded or double-stranded forms) [11].

Altogether, this panoply of AAV vectors creates an unrivaled source of delivery tools for successful gene transfer in human patients. As a result, AAV is one of the most sought-after delivery vehicles to support muscle gene transfer. Consequently, the demand for high-titer, high-quality AAV vector preparations, both for preclinical and clinical applications, is booming. The biggest, and perhaps the least expected, challenge facing AAV gene therapy applications to the muscle lies in our ability to manufacture at scales to meet the demand [12].

The purpose of this chapter is to review the current processes that have been utilized to manufacture clinical-grade AAV and to provide an updated overview of the methods currently developed and implemented both by academic and industry groups to support the needs for muscle gene therapy.

15.2 Manufacturing for Clinical Applications: Needs and Challenges

Whether the muscle tissue is specifically targeted to treat a muscle disorder, or alternatively, to provide a consistent and long-term expression of a systematically secreted therapeutic protein for treating metabolic disorders, the goal is often identical: deliver AAV to most of the body muscle mass. Muscular and cardio-muscular dystrophies, such Duchenne muscular dystrophy (DMD), Becker muscular

dystrophy, limb-girdle muscular dystrophy (LGMD), Pompe disease, myotubular myopathy (MTM), Crigler-Najjar disease, spinal muscular atrophy (SMA), or Barth syndrome, will require high-dose body-wide correction targeting most of the affected muscles, with systemic administration via intravenous delivery. Metabolic diseases, such as hemophilia A and B, alpha-1 antitrypsin deficiency (AAT), diabetes, atherosclerosis, or even vaccines for cancer or infectious diseases, also require high-dose delivery via local intramuscular administration. The needs for high doses and systemic delivery approaches, combined with the first clinically relevant data established during the first trials of the past decade, further exacerbated the needs for high-titer AAV production at large scales.

The first clinical trials using AAV for muscle delivery date back to the mid-1990s and early 2000, notably for AAT, LGMD, Pompe disease, and DMD. In these trials, clinical doses ranged between 1×10^{11} and 1×10^{13} vector genome (vg) units per patient with doses as low as 2×10^{10} vg/kg [8–10, 13–16]. The phase I/II trial for Pompe disease initiated in 2009 at the University of Florida required about 2×10^{14} vg of rAAV1-CMV-hGAA to support the trial, toxicology, and biodistribution studies and subsequent multi-year stability study. The vector was prepared by transient transfection in human embryonic kidney 293 (HEK293) cells at the University of Florida Powell Gene Therapy Center (UF PGTC, Gainesville, FL) and requested approximately 50 10-layer CellSTACKs (CS10®) for a period of about 12 months for the preclinical and clinical manufacturing [9, 17]. The production of clinical-grade rAAV1-CB-hAAT generated close to 4×10^{14} vg from 120 CS10® in about 12 months [16]. Similar production scales were performed at other academic and industry facilities at the time (Clinical Manufacturing Facility at Nationwide Children's Hospital, Columbus, OH; Targeted Genetics, Seattle, WA; Asklepios BioTherapeutics, Chapel Hill, NC, among others). The St. Jude Children's Hospital (Memphis, TE) processed as many as 432 CS10® to generate approximately 2×10^{15} vg GMP-grade scAAV8-factor IX clinical vector [18]. Despite encouraging clinical outcomes, increasing the clinical doses became one critical requirement to ensure a path toward clinical efficacy and larger cohorts. In that line of effort, our group had implemented a full-GMP scale process for clinical manufacturing of AAV9 [19], which led to the production of about 2×10^{15} vg of clinical drug product from 120 CS10® over a 12-month period (Cleaver B.D., Clément N., et al.; unpublished data). The associated preclinical studies utilized close to 1×10^{16} vg, generated by two entities in several test article lots over a 2-year period (UF PGTC, University of Pennsylvania Vector Core).

Clinical doses proposed for muscle dystrophies, such DMD, MTM, or Pompe disease, are approaching, and may soon exceed, the 1×10^{16} vg/patient range, and demands for clinical batches exceeding 1×10^{17} vg are not so uncommon anymore. In other words, in the lapse of two decades, the manufacturing needs for clinical AAV have increased by almost three logs, while the development of technologies capable of supporting these scales has not followed this steady pace. The field is now faced with an unprecedented technical challenge to develop the production platforms and purification processes that will support the clinical needs for research and commercial requirements for the next decade.

15.3 Current Manufacturing Methods for Clinical-Grade AAV Drug Products

In the next few paragraphs, the main protocols utilized for producing AAV at the clinical grade will be presented. For the purpose of simplification, this review will mainly focus on processes utilized or currently developed for clinical manufacturing of AAV drug products. Many variations and alternative protocols have been utilized to make research-grade AAV and have been previously reviewed extensively [20–24].

AAV production methods all rely on a simple biological mechanism: AAV genome replication and packaging into a virion require both the AAV *trans* functions, that consist in the AAV Rep and Cap proteins (aka VPs), as well as the AAV helper virus functions, mostly from adenovirus or herpesvirus, to be introduced to the same host cell. AAV production methods can be classified into three major groups based on the mechanism used to introduce the genetic material to the producer cells: (1) chemical, by transfection of plasmid materials; (2) biological, by viral infection (HSV, baculovirus); and (3) stable cell lines which may combine some steps of the other two approaches [2, 22–26].

15.3.1 Transient Transfection

15.3.1.1 Adherent Platform

In transient transfection protocols, AAV Rep/Cap and helper functions are provided from plasmids, either combined onto one large plasmid molecule such pDG or pXYZ [27–29] or separated onto a Rep/Cap plasmid (pAAV Rep/Cap type) and a helper plasmid containing the adenovirus serotype 5 functions (pXX6-80) [23]. The transfection process relies on chemical precipitation, mostly by calcium phosphate (C_aPO_4) [17, 18, 25, 30] or polyethylenimine (PEI) [30]. The most utilized cell lines for AAV production by transfection are the HEK293, HEK293-derived HEK293-T that expresses SV40 T antigen, and HeLa cells. The advantage of HEK293 is that they express the Ad5 E1A/B gene which is required for AAV replication.

Transient transfection protocols are by far the most utilized to support AAV clinical manufacturing with more than 75% clinical trials using drugs generated by transfection [2]. Among the clear advantages of this method are (1) safety, with the longest track records to date; (2) simplicity, with materials and reagents commercially available, including GMP-grade plasmid stocks; (3) versatility, with plasmid engineering and production relying on rapid and low-cost procedures; (4) relatively low cost associated with the material and reagent production; and (5) protocols that are free of intellectual property restrictions, a consideration that could favor its choice for the industry setting.

The majority of the trials for muscle-targeted gene therapy have been achieved by transfection, which demonstrated this process capability as well as safety, both for intramuscular delivery approaches, as for DMD [14], LGMD [8], and Pompe disease [31], and, more recently, by systemic delivery for Sanfilippo syndrome (ABO-101 and 102, Abeona Therapeutics, Cleveland, OH) or SMA [32]. Other major trials utilizing the transfection method for muscle delivery include the AAT trial and the hemophilia B trial [2, 13, 18, 33]. The biggest challenge for this method is its inherent lack of scalability. Until recently, transfection of HEK293, or HeLa cells, has always been performed on adherent cell lines grown in culture flasks, generally Cell factories (Nunc) or CellSTACKs (Corning). In this setting, the amount of AAV produced is a linear relationship with the number of flasks or cell growth surface area. The GMP-grade scAAV9 vector from St. Jude Children's Hospital reported using 432 CS10® to generate 2×10^{15} vg or ~ 4.6×10^{12} vg per flask [18]. We produced the same amount from 120 CS10® for an AAV9 product or ~ 1.7×10^{13} vg per flask (Cleaver, Clément et al., UF PGTC, unpublished). These manufacturing scales typically require months if not years for completion, adding significant cost for facility maintenance and personnel dedication to one project. When considering the current needs for midsize preclinical and phase I/II clinical studies and for patient dose ranging from 5×10^{13} to or above 2×10^{14} vg/kg, each patient could receive between 3.5×10^{15} and 1.4×10^{16} vg per dose. If we assume that an average of $1–2 \times 10^{13}$ vg of purified clinically ready AAV drug can be generated from each CS10® (PGTC, AAV9 [34], and unpublished), each patient would necessitate a production scale of 500 to as many as 1000 CS10®. Production at such a scale could take several years to complete at an academic facility or even at a contract research/manufacturing organization (CRO/CMO). Transfection using adherent platforms has proven simply not a feasible approach for AAV clinical manufacturing for scales exceeding $1–5 \times 10^{15}$ vg per patient.

15.3.1.2 Suspension Platform

More recently, the transfection model has been adapted for suspension cell cultures that provide a far more convenient, less cumbersome, and scalable platform. Suspension-adapted HEK293 may be derived either from an in-house cell line [30] or commercially available (EXPI293F®, Thermo Fisher, or similar derivatives). Triple transfection in suspension platforms is mediated by the use of PEI and cells that are grown in disposable WAVE GE Healthcare bioreactors [30]. This method was utilized for multiple clinical drugs [30]. Reported yields from multiple serotypes range from as low as ~ 3×10^{12} to as high as 3×10^{13} vg/L of purified product [30] or ~ 1×10^{14} vg/L in crude harvests for the better producers. The major benefit of this method is its scalability. However, the overall yield may prove challenging for manufacturing scales above 1×10^{17} vg since it would be in excess of 3000 L. Another potential drawback for this method is the significant amount of GMP-Source plasmid required. It is to date, however, the best option for transfection-based AAV manufacturing and has had significant appeal for both academic and

industry facilities (Vector Core, University of North Carolina, Chapel Hill, NC; Bamboo-Pfizer, Chapel Hill, NC; Audentes, San Francisco, CA; and Généthon, Every, FR, among others).

15.3.2 Packaging and Producer Cell Lines for AAV Clinical Manufacturing

Several clinical AAV products have been successfully manufactured using stable cell lines (for review [2]). Detailed manufacturing protocols or production data are not readily available for review, rendering a side-by-side comparison with other methods difficult. Typically established in HeLa cells, stable cell lines are stably transformed by integrating either the AAV vector genome (producer cell lines) or the AAV Rep/Cap sequences (packaging cell lines). AAV production is triggered by delivering the complementing sequences, AAV Rep/Cap and helper adenovirus (typically with a recombinant adenovirus/AAV hybrid virus), or by introducing the AAV genome sequences (typically by transfection or infection by adenovirus/AAV hybrid virus) (reviewed in [24, 35–38]). The main advantage to the stable cell lines is the presence of the AAV genetic material in every cell, statistically increasing the number of cells producing AAV. For this reason, it is widely thought that stable cell lines are capable of generating higher AAV yield per cell when compared to other systems. However, a side-by-side comparison of the same construct in different platforms is still lacking. HeLaS3, a variant of HeLa cells that can be grown both in adherent and suspension formats, has been adopted for AAV production. The use of HeLaS3 cells greatly facilitates production scale-up. Several trials have been completed using stable cell lines by groups including the Clinical Manufacturing Facility at Nationwide Children's Hospital (Columbus, OH).

A major drawback (Table 15.1) of this approach is that, it requires a significant upfront effort to establish and screen the cell lines and generate and fully qualify the master and working cell banks for GMP production, which includes demonstrating the cell line stability during cell passaging [39]. Complementing helper viral stocks also need to be produced and fully characterized. Lastly, the use of either wild-type or recombinant adenovirus variants warrants powerful purification protocols to remove this highly immunogenic virus and assays to demonstrate the absence of adenovirus-derived impurities.

15.3.3 Infection Using Recombinant Viral Vectors

The genetic information required to produce AAV, namely, the AAV vector genome containing the gene of interest (GOI) and the AAV Rep/Cap, can also be biologically introduced into the host cell by viral infection. The two currently utilized viruses are the human herpesvirus type 1 (HSV-1) and the insect baculovirus (BV).

Table 15.1 Overview of methods used for producing clinical-grade AAV

Cell type	Mammalian cells							Insect cells
Cell growth platform	Adherent			Suspension				
Cell line	HEK-293 or HEK293T	HEK-293	HeLa	HEK293	HeLaS3	sBHK21	EXPI293F	Sf9
DNA delivery	Transfection	Infection	Infection	Transfection	Infection	Infection	Infection	Infection
	C_aP0_4, PEI	rHSV	rAd or wtAd	PEI	rAd	rHSV	rHSV	rBV
Production vessel	CS10® HS12 or HS36 Roller bottles	CS10®	CS10® Flasks	Shaker flasks Wave	CS10® Shaker flasks	Wave	Shaker flasks	Shaker flasks Wave Bioreactors
Raw material	Plasmids	rHSV	Ad	Plasmids	Ad	rHSV	rHSV	rBV
Cell banks	HEK293	HEK293 V27	HeLa HeLa/AAV	HEK293	HeLaS3 HeLaS3/AAV	sBHK21	Expi293F	Sf9 Sf9/AAV
Scale performed	20–432 CS10®	10–100 CS10®	N/A	10–20 L	N/A	100 L	15 L	200–500
US INDs	Yes	Pending	Yes	Yes	Yes	Yes	Pending	Pending
Yield harvest (vg/L[a])	$1–6 \times 10^{13}$	$1–6 \times 10^{14}$	1×10^{14b}	1×10^{14}	1×10^{14b}	1×10^{14}	$3–5 \times 10^{14}$	1×10^{14}
Yield product (vg/L[a])	$4 \times 10^{12}–2 \times 10^{13}$	$5 \times 10^{13}–2 \times 10^{14}$	N/A	$8 \times 10^{12}–3 \times 10^{13}$	N/A	2×10^{13}	$1–3 \times 10^{14}$	$2–3 \times 10^{13}$
Advantages	Versatility Cost Track record	Yields Potency	Yields Potency	Scalability Versatility Potency	Yields Scalability Potency	Yields Scalability Potency	Yields Scalability Potency	Yields Scalability BV amplification

(continued)

Table 15.1 (continued)

Cell type	Mammalian cells							Insect cells
Cell growth platform	Adherent			Suspension				
Cell line	HEK-293 or HEK293T	HEK-293	HeLa	HEK293	HeLaS3	sBHK21	EXPI293F	Sf9
Challenges	Scalability	Scalability HSV creation HSV production HSV testing	Scalability CL creation Ad creation Ad production Ad testing	Yields Plasmid production	CL creation CL testing CL stability	HSV creation HSV production HSV testing		BV creation BV testing BV stability AAV Potency
Current producing facilities[c]	UPENN PGTC Nationwide St Jude GMP Harvard Initiative Cornell	PGTC	Targeted genetics Nationwide Dimension	UNC AskBio Bamboo-Pfizer	Nationwide Dimension	AGTC	PGTC	UniQure Voyager Adverum Abeona Agilis Virovek Lacerta

CL stable cell line with AAV GOI and/or Rep/Cap

Note: Yields are provided for reference, but may vary depending on the serotype or AAV constructs produced, the analytical assay used, and may not be a true reflection of a direct method comparison

[a]For adherent format 1 CS10 = 1 L

[b]Accepted value—not from publication

[c]For clinical and non-clinical production

There are at least three major advantages to the infection-based approach: (1) a highly efficient delivery mechanism that relies on natural infection of a host cell by the virus, (2) the shuttle virus (HSV or BV) also serves as the helper virus, and (3) infection is a highly scalable platform that works very efficiently in cell suspension formats.

Though the HSV and BV procedures are very similar in principle, they differ by at least three major biological aspects: (1) HSV uses eukaryotic mammalian cell lines, the natural host species for wild-type AAV, while the BV system uses insect cell lines; (2) HSV is a genuine helper virus for wild-type AAV, closely mimicking the AAV natural cycle, while BV uses an insect virus; and (3) HSV system is a replication-deficient system in that the recombinant HSV used to produce AAV does not replicate during the production phase, while the BV may use a self-amplifying approach where the recombinant BV inputs can be amplified during AAV production. Both systems have been used to produce clinical AAV.

15.3.3.1 HSV System

Three clinical trials have been initiated in the USA using AAV produced by the HSV system for retinal diseases (AGTC), AAT [40] and very recently Duchenne Muscular Dystrophy (Solid Bio.), and several additional INDs are in preparation for systemic diseases targeting the muscle and/or the CNS, including but not limited to, Pompe disease (Byrne et al., UF), Friedreich's ataxia (Byrne et al., UF), and other retinal diseases (AGTC). The first trial using this method was initiated in 2010 for AAT [40–42], in which the muscle was the primary organ to support a continuous production and secretion of alpha-1 antitrypsin for body-wide therapy. Currently, there are two ongoing trials for achromatopsia and X-linked retinoschisis, two retinal diseases with intraocular administration [43–46]. To date, the trials confirmed safety of the clinical drugs often with little or no side effects, further validating the use of this method when combined with appropriate purification processes.

HSV-mediated production relies on coinfection with two engineered recombinant HSV viruses that carry either the rAAV vector genome with gene of interest (rHSV-GOI) or the AAV Rep and Cap sequences (rHSV-AAVR/C). AAV production is initiated by coinfection of the host cells, either baby hamster kidney cells or HEK293 cells grown in adherent layer or in suspension, with an appropriate multiplicity of infection [34, 47–49]. AAV virions are recovered from the cells [34] or the cell and supernatants [41, 48] at 48–72 h post-infection and purified by multistep filtration and chromatography processes [34, 41, 48].

The first established benefit of this method was the noticeable yield increase, independent of the cell platform used. In suspension-cultured baby hamster kidney cells, yields as high as 1×10^{14} vg/L in crude harvests were reported [48], and a 100 L clinical batch in the WAVE bioreactor was performed for the AAT trial [41], generating about 1×10^{16} vg in crude harvest (or 1×10^{14} vg/L) and 2×10^{15} vg of clinical material (2×10^{13} vg/L). We were able to increase the

overall yields between 3.5 and 4.7 × 10^{14} vg/L in crude harvests when using optimized production conditions in suspension-adapted EXPI293F and in excess of 1 × 10^{14} vg/L of purified AAV9 [47] (Clément et al., unpublished) and are in the process of implementing this method for clinical manufacturing (PGTC, Gainesville, FL). Based on such yields, clinical batches of 1 × 10^{16} vg could easily be achieved in a laboratory-scale format (50–100 L), and multiple 250–500 L batches would suffice for generating >1 × 10^{17} vg in the industry/CROs settings. To date, scalability has been demonstrated in various formats, from spinner flasks to shaker flasks [47, 48] and to single-use bioreactors such as the WAVE [48].

The second major benefit is that the biological potency of the purified AAV appears improved for all the serotypes tested to date when directly compared to transfection-made material, the gold standard for AAV production [34, 41, 48]. AAV-1 showed a fivefold increase infectivity when compared to transfection-made product [41], and AAV-9 was shown to have ~ three- to fivefold in vitro potency increase [34]. This is in sharp contract with other methods, notably the BV system, that often results in the loss of AAV particle infectivity. The HSV system appears, therefore, more versatile and lenient to support production of various AAV serotypes without the need to modify the expression cassettes, an often lengthy and cumbersome effort aimed at tailoring the expression ratio of the various AAV Rep and Cap proteins in the BV system.

Another benefit of the HSV method is the apparent increase in the percentage of full capsids as compared to empty capsids [34, 41] (Clément et al., to be published). It is yet to be demonstrated whether the increase in the number of full particles and potency is mechanistically related. The basis for improved potency in HSV-produced AAV batches is yet unknown. However, the clinical relevance of these findings could be major since it may result in lowering clinical doses to achieve a therapeutic benefit in patients.

The major drawback of the HSV method is the need to produce master and working viral banks for both rHSVs, sometimes in significant amounts. The current production platform for rHSV relies on adherent VERO-derived complementing V27 cells, and its scalability is limited due to the challenges of implementing suspension-based production, such as the use of microcarriers [50]. However, the current achievable scales using adherent V27 in multilayer flasks largely support the current AAV needs 1 × 10^{16}– 1 × 10^{17} [47] (Clément et al., unpublished). Due to its relative simplicity, rHSV production protocols could easily be subcontracted from CROs upon technology transfer. Importantly, rHSV stocks have been shown stable over a period of several years (Clément et al., unpublished) when stored frozen and genetic stability has been demonstrated over at least ten serial passages [47, 50, 51] (Clément et al., unpublished). This amplification series would support the production of thousands of liters of rHSV batches well beyond the current needs for rAAV manufacturing. Lastly, rAAV stocks must be thoroughly tested for the presence of process-derived impurities as further described in the section below. To date, preclinical and clinical studies have confirmed the safety profile of this method [34, 42, 45, 52–54].

15.3.3.2 BV Expression System for AAV Clinical Manufacturing

The first BV system for AAV production was developed at the NIH by Kotin et al. [55] and relied on a somewhat unexpected finding that the insect baculovirus could serve as helper for AAV replication in producing cells. This original system used three rBVs to deliver the AAV functions to *Spodoptera frugiperda* (Sf9) insect cells: one carrying the AAV vector genome with GOI, one with AAV Rep (typically from AAV2), and the third with AAV Cap of a chosen serotype. The production phase starts by infecting the Sf9 cells with these three rBVs at the appropriate multiplicity of infection. The first market-approved AAV gene therapy drug Glybera was produced using the BV system. Glybera was developed by uniQure. It is an AAV-1 vector for treating familial lipoprotein lipase deficiency by intramuscular injection [56]. To support their commercial needs, uniQure moved from the HEK293 transfection method they used in phase I/II to the BV system to produce Glybera. The clinical product Glybera met all the release criteria per the European Medicines Agency (EMA) policy and was used once in one patient in May 2016. Production protocols and data such as the yield of physical particles and the biological potency are not publicly available at this time. The design of Glybera phase I/II reveals that patients treated at a dose 1×10^{12} vg/kg would respond positively to the gene therapy [57–59]. For an average size adult of 70 kg, this would require 7×10^{13} vg per patient, which is within the capability of this method at mid-scale (<100 L) based on other studies (see below). However, this drug recently qualified as the world's most expensive medicine with one single patient treated for a cost of $1 million [60]. This first commercial production provided significant knowledge and paved the way for future products, notably in the USA (uniQure, Lexington, MA).

Despite this pioneering success, the BV production platform has suffered many setbacks since its first description in 2002 [55]. Among the more significant hurdles were (1) the instability of the rBV during amplification to generate viral stocks, resulting in loss of functions [61], and (2) partial or complete reduction of AAV particle infectivity for almost every serotype tested [61–63], mostly due to the low expression of VP1. There is another challenge for the BV system: unlike in mammalian cells, endogenous AAV promoters are not fully active in insect cells and had to be replaced with BV promoters or regulatory sequences [55, 62]. These challenges have been partially overcome in recent designs that include genetic engineering of various expression cassettes with modified ATG start codons, various baculoviral promoters and introns, or other regulatory sequences, to restore the level of VP1 expression similar to that observed in mammalian cells [22, 24, 61, 62, 64, 65]. In addition, the number of rBV was reduced to two or one when stable insect cell lines carrying the AAV Rep/Cap functions were created [62, 63, 66]. Another unique aspect of this method is the amplification of the rBV during the AAV production run when using BV-infected Sf9 cells (BIIC system) which could reduce the need for large stocks of rBV [24, 26, 67, 68]. Current yields of the BV system approach 1×10^{14} vg/L in crude harvests [68] with infectivity in most cases comparable to that of transfection-generated AAV [63] and with reduced amount of empty capsids. While none of these recent developments have been implemented to GMP

for clinical manufacturing, their potential benefits, as well as the lack of major roadblocks related to intellectual properties, have rendered this approach attractive to several companies that are in the process of implementing this platform to support their commercial-scale manufacturing in the years to come (uniQure, Voyager Therapeutics, Abeona Therapeutics, Adverum, etc.).

15.4 Purification of Clinical AAV

A successful manufacturing platform must guarantee that the final biological drug is sterile and of high purity for use in humans. Ideally, AAV clinical product should also meet two additional biological features: high titer and high potency, to deliver the most effective therapeutic drug to the patient in a low volume. Requirements for final product release testing are well documented in the FDA guidelines (Table 15.2) [21]. The purification process should guarantee the removal of process-derived impurities and contaminants, such as serum, antibiotic or Benzonase® residuals,

Table 15.2 Final product release tests

Transfection HEK293	Cell lines	Infection rHSV System	Infection rBV System
Sterility, bacteriostasis and fungistasis Benzonase® residual In vitro adventitious agents[a] Host cell DNA (293, HeLa, BHK, Sf9) Host cell proteins (293, HeLa, BHK, Sf9) Endotoxins Mycoplasma[a] Vector genome titer Infectious titer Identity Purity Osmolality or conductivity pH Vector genome sequencing rcAAV Transgene activity/identity Total capsids (when available) % Empty capsid BSA residual Gentamycin residual Host cell proteins			
N/A	N/A	rHSV residual rcHSV residual HSV DNA HSV proteins V27 DNA V27 proteins	rBV residual rcBV residual BV DNA BV proteins

[a]Performed in unprocessed harvest or intermediates

host-cell proteins and DNA, helper virus proteins, DNA and replication-competent viral particles (HSV, adenovirus, BV), as well as product-related impurities, such as empty capsids or replication-competent AAV. The sterility of the final product and process intermediates is guaranteed by adhering to strict aseptic conditions through the purification steps under the GMP setting.

One unique challenge is the diversity of AAV serotypes, whether naturally occurring or laboratory-engineered, with different physical properties that necessitate highly capsid-specific protocols. The FDA identifies each new AAV drug, including those with changes in their capsid, as a new product, warranting exhaustive preclinical evaluation and release testing for each new variant. Capsid-tailored protocols typically include one capture step, based on their natural binding receptors if known, and/or chemo-physical properties of the capsids (pH, pI, stability, size, etc.), followed by subsequent polishing steps to increase product purity. Processes resulting in high overall recovery from crude material to final purified product will have the most chances of success for large-scale manufacturing and commercial production. Current clinical manufacturing protocols are characterized by overall low recovery, ranging between 5% and 25% [18, 30, 34, 40]. The challenge in balancing recovery versus purity is evident and must be evaluated carefully.

15.4.1 Harvest

There are two main strategies to harvest AAV-containing production material. AAV particles can either be extracted from the producer cells, upon harvest by traditional centrifugation, or alternatively from the entire production pool, combining cells and media [2]. Historically, AAV was purified from cell harvests after simple media removal from adherent cells, limiting the overall volume to be processed. Removal of the media may have also reduced the need for highly stringent purification processes with this upfront removal of the majority of process-derived impurities (serum, antibiotics, plasmids, helper virus, etc.). However, with the recent implementation of suspension culture platforms, extraction of virus particles from the cell and media pool harvest has become more popular, eliminating the need for long and cumbersome centrifugation steps, and may increase the overall AAV yield. Suspension harvests require stringent clarification by serial filtration steps to remove cell debris, as well as concentration steps to generate workable volumes for downstream purification. The risk of vector loss, by direct volume loss or particle loss, increases with each step added. Proper storage conditions must be evaluated for each step. During harvest, the cells, whether isolated or in culture media, must be disrupted to release AAV virions. This is achieved either chemically, with the use of detergent (triton) or high osmolality with the use of salt, or physically, by successive freeze-thaws, microfluidization, and sonication or in combination [17, 23, 25, 41]. Typically, crudes are subjected to stringent enzymatic treatment such Benzonase® to remove the majority of cellular DNA/RNA and plasmid DNA, which additionally helps release the membrane-bound virus prior to clarification. Harvests can be stored frozen.

15.4.2 Bulk Purification

The particle extraction from the crude lysate, or harvest, relies on a series of purification steps that promote binding of the AAV capsids to chromatography resins or, inversely, binding of the non-AAV proteins and recovery of AAV particles in the flow through. To date, only a handful of AAV serotypes have been produced for the clinic: AAV-1, AAV-2, AAV-5, AAV-8, AAV-9, and AAV-rh10 and capsid variants 2i8, 2.5, and AAV-2 tyrosine mutant (reviewed in [2]). Capsid variant typically can rely on protocols already in place for the serotype they are derived from with no or minor changes. The AAV-2 triple trypsin mutant was purified using the protocol established at UF PGTC for AAV-2 (Cleaver, Clément et al., unpublished).

For serotype of known cellular receptors, affinity chromatography is a standard and powerful first capture step. Heparin affinity chromatography was used to purify clinical AAV-2 and hydroxyapatite for AAV-1. AVB sepharose high performance using Camelidae-derived single-domain anti-AAV capsid antibody fragments was used to purify AAV-1 (GE Healthcare Life Sciences) [17, 18, 30, 41]. More recently affinity resins derived from AAV-8 or AAV-9 immunized llamas (POROS CaptureSelect AAV8, POROS CaptureSelect AAV9, Thermo Fisher) were specifically developed for serotypes 8 and 9. Published data on established protocols is still very limited at this time [69], but these resins will likely be used for future clinical AAV manufacturing.

Until recently, AAV-9 was considered one of the most challenging serotypes for its inability to efficiently bind to any commercially available resin, before the POROS CaptureSelect method was developed. We developed a one-column clinical process for AAV-9 based on protein flocculation as pre-purification step followed by anion exchange under acidic conditions [19, 34] which was used for the production of two clinical AAVs (Byrne, Cleaver, Clément et al., unpublished).

Historically, a chromatography-only approach prevented complete removal of AAV empty capsids. Density gradient centrifugation based on cesium chloride or iodixanol has been used to remove empty capsids for some clinical products. This approach has multiple disadvantages with the first and foremost being product loss (often as high as 50–90% of infectious particles), inherent lack of scalability, challenge to perform under strict aseptic conditions, and poor consistency since it relies on highly trained operators. For these reasons many clinical products used to date do contain a significant amount of empty capsids [2]. It is noteworthy that the need for AAV empty capsid removal is still actively debated, as it has not been formerly demonstrated that the presence of empties is detrimental related to safety and/or efficacy of the drug. It seems accepted however that the FDA may soon request empties to be removed in the majority of products and much effort is invested toward the development of optimal chromatography removal. Noteworthy methods such the BV or HSV system have been shown to increase the percentage of full capsids in final AAV stocks, which may render the need for empty capsid removal less critical.

15.4.3 Bulk Concentration and Final Product

The last step of any purification process is concentration of the particles, often combined to a formulation or buffer-exchange step. The most utilized method is tangential flow filtration (TFF), which may be combined with a dialysis step [9, 18, 30, 34]. The choice of excipient is critical to the success of the product potency and stability over time. Current clinical drugs have been formulated in PBS, PBS with various supplements such as magnesium, calcium, pluronic acid, salt, detergent, balanced salt solution, lactated Ringer's solution, or citrate buffer, that ensure multi-year stability and reduce capsid aggregation for highly concentrated stocks. If the storage excipient is not ideally suited for in vivo administration, additional process and development work must be in place to demonstrate product stability and buffering of the drug upon thaw and dilution in a more suitable excipient.

15.5 Quality Control and Stability Testing

Clinical product safety and biological parameters must be fully tested prior to FDA-approved release. Testing guidance is provided by the FDA (for the USA), or equivalent agencies in different countries, and must be conducted in compliance with cGMP (Table 15.2) (also reviewed in [2, 70, 71]. The final release testing results are summarized on the certificate of analysis and include an extensive list of parameter assessment that covers safety, product concentration, purity, potency, and stability. In-house or subcontracted assays are conducted using qualified procedures or standard operating procedures and include internal parameters to assess the validity of each independent assay based on pre-established criteria, such as positive and negative controls. Full qualification should include an extensive assay characterization to include linearity, reproducibility, specificity, sensitivity, and robustness.

Safety assessments are determined from a series of product-independent assays, including sterility, endotoxins, mycoplasma, adventitious agents, in process reagent-residual when applicable (antibiotic, Benzonase, and serum residual when applicable), pH, conductivity or osmolality, and appearance. Purity is a critical assessment that is highly dependent on the processes utilized to both produce and purify AAV drug products. Process-related impurities are by definition process-specific and include host-cell DNA and protein residuals (293, HeLa, BHK, etc.), helper virus DNA and protein residuals, when applicable (HSV, BV, adenovirus), as well as the presence of helper virus and/or replication-competent helper viruses (Table 15.2).

AAV-specific, or product-specific, testing includes identity, GOI expression and activity, vector genome titer, infectious titer, and ratio of full-versus-empty capsids, when possible. Product-related impurities will consist of replication-competent AAV (rcAAV) that would have been generated via homologous recombination between the AAV-GOI cassette and the AAV Rep/Cap helpers. In some instances, packaging of nonspecific sequences in AAV capsids can also be quantified and

determined, although this is not yet a prerequisite for phase I/II drugs. Product identity is finally confirmed by full genome sequencing [71, 72].

As mentioned above, viral-based production systems, like the HSV, BV, or adenovirus system, must undergo further assessments with additional assays to evaluate the presence of virus-derived impurities. As the clinical program progresses toward phase III and commercial production, these methods also warrant extensive viral clearance studies to demonstrate the inactivation and/or elimination of the helper viral particles. A thorough viral clearance study was published for the HSV platform demonstrating the complete removal of the HSV virus during downstream purification steps, further consolidating the safety of this method [73].

It is obvious however that in addition to a strong in vitro assessment and characterization of any drug product, the product safety is largely determined during extensive preclinical toxicology and bio-distribution studies. To date, most if not all preclinical test articles or clinical AAV drug products have met the safety requirements, with each of the production methods described in this chapter.

In addition to final product release testing, each IND must be subject to formal short-term and long-term stability studies, as required by the FDA. These quality control assessments must demonstrate the product stability upon pre-defined storage conditions (excipient, temperature, pH). Long-term stability studies are often carried multiple years to cover the duration of the clinical trial. Standard time points are 3, 6, 9, and 12 months post-manufacturing and then 18 and 24 months and every year thereafter until the last patient is dosed.

One of the current challenges for the field is the lack of standardized analytical procedures. Past and current clinical AAV drugs have been evaluated using in-house or outsourced methods that are highly specific to each manufacturing facility. The lack of standardized assays clearly prevents an objective and thorough comparison of the production and purification processes used to date in the clinic, as well as the final product characterization. Of the most variable assays across laboratories are the vector genome titers (quantitative PCR, droplet digital PCR, dot blot or slot blot) as well as the infectious titers (TCID50 or infectious center assay). Stability protocols typically include evaluation at 3, 6, 9, 12, 18, and 24 months and then every year thereafter, until the last patient is dosed. In our laboratory, we have conducted stability of multiple products for up to 7 years post-manufacturing (Clément, Cleaver, Byrne et al., unpublished). Additional stability evaluation must include clinical handling and dosing preparation, as well as, when applicable, during temperature-controlled drug shipment.

15.6 Conclusion

Clinical manufacturing for AAV gene therapy started over two decades ago and has supported several hundreds of clinical trials to date. Until recently, the most utilized method was based on transfection of adherent cell lines. In the light of several recent clinical successes, the demand for high amounts of clinical AAV quickly surpassed

the manufacturing capability, both due to technologies that could not meet the needs and the limited number of manufacturing facilities specialized in AAV production and testing. Today, multiple companies, academic cores, and CROs are specializing in AAV production to meet the demand and are investing, often jointly, in the development of novel methods to support large-scale manufacturing, with commercial production in mind. Methods based on suspension platforms have proven robust enough for the next-generation products and are by far the most promising, but it is unclear whether one of these methods or their variants will be used, as each presents with advantages and disadvantages.

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Chapter 16
Genome Editing for Muscle Gene Therapy

Alan O'Brien and Ronald D. Cohn

Abstract Gene editing is defined as creating targeted changes in the genome using sequence-specific nucleases. The CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated protein 9) system provides a gene editing tool that has led to significant advances in this field and the development of potentially curative strategies for a variety of disorders. Most of the research on gene therapy and gene editing for muscle disorders has focused on Duchenne muscular dystrophy (DMD), a fatal, progressive X-linked neuromuscular disorder resulting from the absence of dystrophin. The molecular aspects of DMD present challenging obstacles to gene therapy; however, the versatility of the CRISPR system is providing ingenious ways of circumventing those obstacles. In this chapter, we review gene editing tools, notably zinc-finger nucleases, transcription activator-like effector nucleases, and CRISPR. We then discuss the uses of CRISPR in muscle disorders, focusing on DMD, as well as challenges inherent to gene editing of muscle cells.

Keywords Duchenne muscular dystrophy · DMD · CRISPR · Gene editing

16.1 Introduction

Gene therapy is a broad term that encompasses multiple strategies, notably gene replacement, gene addition, and alteration of gene expression [1]. Gene editing is another such strategy and refers to the making of targeted changes to the genome

A. O'Brien (✉)
Division of Clinical Genetics, Department of Paediatrics,
University of Toronto, The Hospital for Sick Children, Toronto, ON, Canada
e-mail: alan.obrien@sickkids.ca

R. D. Cohn
Department of Paediatrics, University of Toronto, The Hospital for Sick Children,
Toronto, ON, Canada
e-mail: ronald.cohn@sickkids.ca

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using sequence-specific nucleases that induce double-strand breaks at specific loci, which are then repaired by homologous recombination (HR) or nonhomologous end joining (NHEJ).

Gene editing has distinct advantages compared to the other abovementioned gene therapy strategies. The changes made to the genome are potentially permanent (compared to the transient expression obtained with some other methods), and there is no loss of transgene expression in highly mitotic cells. Also, because the changes are made directly to the target gene, the surrounding regulatory sequences and environment are kept, allowing more optimal gene expression. There is also no risk of insertional mutagenesis, but there are risks of off-target cuts, as will be discussed below.

16.2 Gene Editing Nucleases

Nucleases used for gene editing include meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)-associated nuclease 9 (CRISPR/Cas9) [2]. Meganuclease use has been restricted by their long and challenging engineering process. In this chapter, we will briefly review ZFNs and TALENs and then focus more extensively on CRISPR/Cas9 and its uses for muscle gene editing, mainly for Duchenne muscular dystrophy.

16.2.1 Zinc-Finger Nucleases

Developed in the early 2000s, ZFNs are artificial constructs comprised of (1) a zinc-finger protein domain, composed of a tandem arrangement of zinc-finger motifs (which are naturally occurring DNA-binding motifs in eukaryotic cells), and (2) a nuclease domain derived from the *Fok1* restriction enzyme (present in *Flavobacterium okeanokoites*), the two separated by a short inter-domain linker [2, 3]. The Fok1 nuclease domain must dimerize to cleave DNA; thus two ZFN monomers, each binding adjacent half-sites, are required to form an active nuclease. Zinc-finger motifs each recognize a 3 bp DNA sequence; thus each ZFN dimer recognizes an 18–36 bp sequence depending on the number of motifs present (generally 3–6) [4].

The heterodimer arrangement of ZFNs increases their binding specificity. It is possible to customize the binding specificity of ZFNs by altering the type of zinc-finger motifs present in the DNA-binding domain. However, designing ZFNs is difficult and time-consuming. Furthermore, the incomplete repertoire of recognized triplet sequences and the requirement of guanine-rich 5′ GNN 3′ motifs in the target sequence limit the flexibility of this method [2, 5].

In terms of preclinical studies on muscle disorders, Ousterout et al. [6] showed that ZFN-mediated removal of dystrophin's exon 51 restored proper dystrophin

expression in patient cells harboring a deletion of exons 48–50. Clinical trials using ZFNs are being developed, however not for muscle disorders at the time of writing of this chapter. One particular trial is currently underway for HIV patients using the strategy of ZFN-mediated disruption of the CCR5 co-receptor in CD4+ T-cells [7].

16.2.2 TALENs

Transcription activator-like effector nucleases are composed of (1) a Fok1 nuclease, as described above, and (2) a DNA-binding domain called a transcription activator-like effector (TALE), derived from *Xanthomonas* spp. bacteria, which is itself composed of a tandem array of 33–35 amino acid modules, each of which recognizes a specific nucleotide in the major groove [8]. This recognition is made possible by the presence of two amino acids at positions 12 and 13 of each module, named the repeat variable diresidues (RVDs). Four different RVD modules are used to recognize the four nucleotides in DNA, making it possible for TALENs to recognize any desired sequence. TALENs, like ZFNs, need to dimerize, and each pair recognizes a target sequence of approximately 30–40 base pairs. The targeting flexibility of TALENs is an advantage over ZFNs; however their design does remain challenging and time-consuming [2].

TALENs have notably been used for generation of CAR T-cells used in recent clinical trials [9]. They have not been used in clinical trials for muscle disorders; however, preclinical studies have shown their capacity to restore proper dystrophin expression. The reading frame is restored by the creation of indels of specific lengths (stochastically) [10, 11] or by indel-mediated disruption of a splicing-acceptor site, inducing skipping of exon 45 [11].

16.2.3 CRISPR/Cas9

The CRISPR system is a naturally occurring bacterial immune mechanism whose purpose is defense against bacteriophages and plasmids. Its discovery, characterization, and development as a biotechnological tool span close to three decades. For a detailed historical overview, we refer the reader to published review articles, such as the one from Hsu et al. [12].

CRISPR systems are based on RNA-directed endonuclease cleavage of foreign nucleic acid sequences. The invading foreign sequence is initially captured and inserted into the genome of the host organism at a CRISPR locus, which is composed of CRISPR-associated (Cas) genes (coding for endonucleases) followed by the CRISPR array, itself consisting of a series of direct repeat sequences interspaced by "spacer" sequences [2, 12]. These spacers correspond to the sequences of incorporated foreign nucleic acids, which are themselves called protospacers before their incorporation. The CRISPR locus is then transcribed and processed and serves as a

guide for endonucleases to target foreign nucleic acids based on complementarity with the RNA. Three different types of CRISPR systems have been described [13], although the basic principles outlined above apply to all of them. They differ, among other things, by the amount and types of associated proteins and the processing of the CRISPR locus. The type II CRISPR system is the simplest version and has been the one harnessed as a biotechnological tool to date.

In the type II system, the CRISPR array is transcribed and then processed into smaller CRISPR RNA's (crRNA), consisting of one direct repeat and one spacer sequence. The direct repeat portion then hybridizes with a trans-activating CRISPR RNA (tracrRNA). The crRNA-tracrRNA complex is further processed by RNase III and complexes with endonuclease Cas9; this potentially releases Cas9's self-inhibitory conformation [14] and guides it toward the target DNA sequence to be cleaved. Cas9 contains two nuclease domains, RuvC and HNH, each responsible for nicking one of the two strands of DNA at the target site. Cas9 searches target sites by looking for and binding to protospacer-adjacent motifs (PAMs) which are short sequences flanking the 3′ end of the DNA target site. These PAM sequences are absolutely necessary for Cas9 binding and cleavage of DNA and are absent from the endogenous CRISPR loci in bacteria, allowing self- versus nonself-discrimination [12].

The most commonly used version of CRISPR/Cas9 for gene editing requires Cas9 from *Streptococcus pyogenes* (SpCas9) and a chimeric single-guide RNA (sgRNA) made up of a crRNA fused with a tracrRNA. Generally, guide sequences are 17–20 bp long. Target sequences must have at their 3′ end the PAM sequence for SpCas9: 5′ NGG 3′. Once expressed in cells, the Cas9 nuclease and the sgRNA form a complex, bind to the target sequence, and create a double-strand break. The break can then be repaired via NHEJ, which is an error-prone process that introduces insertions and deletions (indels) into the target sequence. Another repair pathway, named homology-directed repair (HDR), can be used to introduce targeted mutations, by co-transfecting single- or double-stranded DNA templates [2, 12]. One strategy is to use an engineered Cas9 with one of its two nuclease domains inactivated, thus resulting in a "nickase" that can only create a single-strand break. A single DNA nick, in the presence of a template strand, can be used to promote HDR instead of NHEJ [15].

There are several common variations to the above-outlined method. For example, the Cas9 from *Staphylococcus aureus* (SaCas9) is frequently used because of its smaller size compared to SpCas9, which makes packaging into viral vectors or other delivery vehicles easier. However, its PAM sequence (5′ NNGRRT 3′) is longer, which can make targeting of a specific region more difficult [16]. A novel CRISPR-associated endonuclease named Cpf1 (for CRISPR from *Prevotella* and *Francisella* 1) was described in 2015 [17] and has several specificities, notably its use of a single shorter gRNA (instead of a chimeric sgRNA or crRNA-tracrRNA hybrid), its use of a T-rich PAM at the 5′ end of the target sequence (making it more useful to target A-T-rich regions), and its creation of double-strand breaks with sticky ends rather than the blunt ends created by Cas9.

The CRISPR/Cas9 tool also allows the regulation of gene expression. This approach requires the use of a catalytically inactive or "dead" (dCas9), with both the

RuvC and HNH domains inactivated, which when bound to DNA elements represses transcription by sterically hindering the RNA polymerase machinery [18]. Another strategy is the conversion of Cas9 into a synthetic transcriptional activator by fusing it to multiple copies of VP16 activator [19].

The targeting specificity of the CRISPR/Cas9 system is not perfect, and several studies show that Cas9 will tolerate several base pair mismatches [12]. This raises concerns because this tool potentially leads to permanent modification of the genome. If Cas9 binds to sites with sequence similarity but which are not the intended target site, this could lead to what is called "off-target" cuts in other regions of the genome. One method used to detect those off-target cuts is to assess the sequence of genomic regions which have been predetermined based on sequence similarity to the desired target locus. This type of assessment is biased, however, because off-target cuts will only be found if they occur in one of the specific regions of the genome that the research team analyzes. Unbiased, nontargeted search methods for off-target cuts, based on whole-genome sequencing or other strategies, will be needed. Tsai et al. [20] and Kim et al. [21] have already demonstrated the feasibility of this. There are strategies to minimize the risk of off-target cuts, for example, the use of paired guide RNAs coupled with a nickase mutant of Cas9 (described above and reviewed in Hsu et al. [12]), the titration of Cas9 dosage (as high concentrations of Cas9 lead to increased mismatch tolerance), and the use of tissue-specific expression cassettes, which prevents off-target cuts in cell types that are not necessary targets of gene editing.

In recent years, CRISPR-Cas9 has been used as an efficient gene editing tool in a variety of species and cell types. Contrary to ZFNs and TALENs, which depend on protein-DNA interaction, CRISPR/Cas9 sequence recognition depends on DNA-RNA Watson-Crick base pairing. Targeting of a specific DNA sequence thus only requires modification of the sgRNA, a relatively easy and straightforward process, while the Cas9 nuclease needs no modification. The ease of use and versatility of the CRISPR system are major advantages over other methods of gene editing.

16.3 Use of CRISPR/Cas9 for Muscle Gene Editing

Muscle cells present specific challenges for gene therapy and gene editing. Importantly, HDR is very inefficient in postmitotic cells such as myofibers, leaving NHEJ as the preferred mechanism to repair double-strand breaks [22]. Satellite cells, which are muscle-specific stem cells, are however not affected by this problem. At the current time, muscle disorders are not amenable to ex vivo gene therapy, unlike other disorders affecting cells that are hematopoietically derived. Hence, delivery vehicles must be used. Naked DNA delivery, such as electroporation and hydrodynamic injection, is not ideal for in vivo human gene therapy. Viral vectors are generally used to deliver the CRISPR/Cas9 components to target cells. Several types of viral vectors have been used, such as lentivirus or adenovirus; however recombinant adeno-associated virus (AAV) vectors are often preferred because of

their safety profile, lack of genome integration, and wide variety of available subtypes with different tissue tropisms [3, 23, 24]. We refer the reader to other chapters in this book that describe viral vectors and delivery methods in more detail.

16.3.1 Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is a fatal X-linked disorder affecting between 1/3500 and 1/5000 males, characterized by progressive deterioration of skeletal and cardiac muscle due to the absence of dystrophin, coded by the *DMD* gene [25]. Dystrophin is present in muscle cells as part of the dystrophin-associated glycoprotein complex (DGC), which anchors the intracellular cytoskeleton to the extracellular matrix [26]. The absence of dystrophin and the DGC causes sarcolemmal instability, eventually resulting in muscle degeneration and fibrosis. The clinical consequences include loss of ambulation, respiratory weakness, and dilated cardiomyopathy. Loss of ambulation typically occurs during adolescence, and death, usually from respiratory or cardiac complications, occurs in the third decade. DMD results in dystrophin mutations causing an out-of-frame transcript leading to the absence of the protein. Approximately 60–70% of patients have a large deletion encompassing one or more exons; most of them occur in a mutation "hotspot" spanning exons 45–55. Becker muscular dystrophy (BMD) is a milder form of DMD resulting from mutations leading to an in-frame transcript, yielding a truncated but functional protein [27].

DMD is the largest human gene, spanning 2.4 Mb and 79 exons, which encode 14 kb of cDNA. This large size creates challenges for gene delivery. Importantly, AAVs can only carry up to 4.7 kb of DNA [28]. The fact that a truncated dystrophin can still be functional, as seen in BMD patients, has prompted the strategy of using truncated versions of DMD (mini-/microdystrophin) for delivery by AAVs, and the removal and/or inducing of skipping of exons harboring mutations, using antisense oligonucleotides or other means as outlined below [29]. Another approach has been to use NHEJ to create indels, creating frameshifts which restore the original reading frame; however, as described earlier in this chapter regarding Ousterout's study with TALENs, the stochastic nature of this process indicates that not all cells will be edited with indels of the appropriate length needed to restore the reading frame.

Out of all the animal models used for studies of DMD, the *mdx* mouse is the most widely used and known. It harbors a nonsense point mutation in exon 23 of the *DMD* gene, which disrupts transcription and causes the absence of dystrophin. Various authors have shown how CRISPR can be used to remove the *DMD* mutation from *mdx* mice, using AAV dual vector approaches, with one vector carrying Cas9 and another vector carrying the sgRNAs. Nelson et al. [30] used an AAV8 system to deliver SaCas9 and two sgRNAs, targeting introns 22 and 23, and were able to remove exon 23 in the *mdx* mouse, leading to expression of truncated but functional dystrophin in cardiac and skeletal muscle, as well as increased skeletal muscle func-

tion. Tabebordbar et al. [31] used AAV9 to deliver SaCas9 and sgRNAs flanking the 5′ and 3′ terminal regions of exon 23, showing similar results, as well as the successful targeting of satellite cells, which also showed recovered dystrophin expression without loss of their regenerative capacity. Long et al. [32] used AAV9 to deliver a humanized SpCas9 and two sgRNAs (targeting the 3′ end of exon 23 and the mutant sequence in exon 23); the resultant NHEJ-mediated disruption of the 3′ splice site resulted in skipping of exon 23.

Bengtsson et al. [33] used a different mouse model (*mdx*^{4cv}, harboring a nonsense mutation in exon 53) and used sgRNAs targeting intronic regions to excise exons 52 and 53, resulting in a shortened but in-frame transcript and recovered dystrophin expression leading to increased muscle function. They also showed that HDR can occur in muscle cells, although at a low frequency, and did not succeed in increasing muscle force. It was also not clear if these HDR-edited cells were postmitotic myofibers or proliferating precursors.

Zhang et al. [34] showed similar results using the Cpf1 endonuclease and patient fibroblast-derived-induced pluripotent stem cells that harbor a deletion of exons 48–50 (creating a premature stop codon in exon 51) and were differentiated into cardiomyocytes. They used a single-gRNA "reframing" strategy to target the splice acceptor site of exon 51, causing indels through NHEJ, thus restoring the appropriate open reading frame in part of the targeted cells. They also used a two-gRNA strategy to disrupt the acceptor splice site of exon 51, thus restoring the reading frame in a truncated dystrophin with joining of exons 47 and 52. They also show rescue of dystrophin expression in a variety of tissues after HDR-mediated correction in *mdx* mouse zygotes.

Other studies have shown the development of a research pipeline for genome editing of various genetic conditions, by demonstrating CRISPR-mediated correction of cells acquired from patients with genetic conditions. A paper from 2016 details some of those strategies, including two for DMD [35]. One of those two strategies is to upregulate utrophin in cells of a DMD patient. Utrophin is a paralog of dystrophin present in humans and mice which can compensate for the loss of dystrophin at the DGC. Studies have already shown that the increase of utrophin expression in mice decreases the severity of the pathology, and clinical trials are underway using small molecules to pharmacologically upregulate utrophin production. In the abovementioned article, myoblasts of a DMD patient harboring a deletion of exons 45–52 are transfected with a catalytically inactive SpCas9 fused to ten tandem repeats of transcriptional transactivator VP16, guided to the utrophin promoter A or B. The results show increased utrophin expression, especially with targeting of promoter B. Interestingly, a combination of several sgRNAs targeting promoter B was able to increase utrophin expression up to 15 times the basal amount (Fig. 16.1). These data suggest the feasibility of using CRISPR for modulating the expression of disease-modifying genes as a treatment for various disorders, including DMD. A second approach for DMD described in this study refers to the removal of a tandem duplication of exons 18–30 in *DMD* in myoblasts differentiated from patient fibroblasts, by using a single sgRNA targeting a sequence well inside the

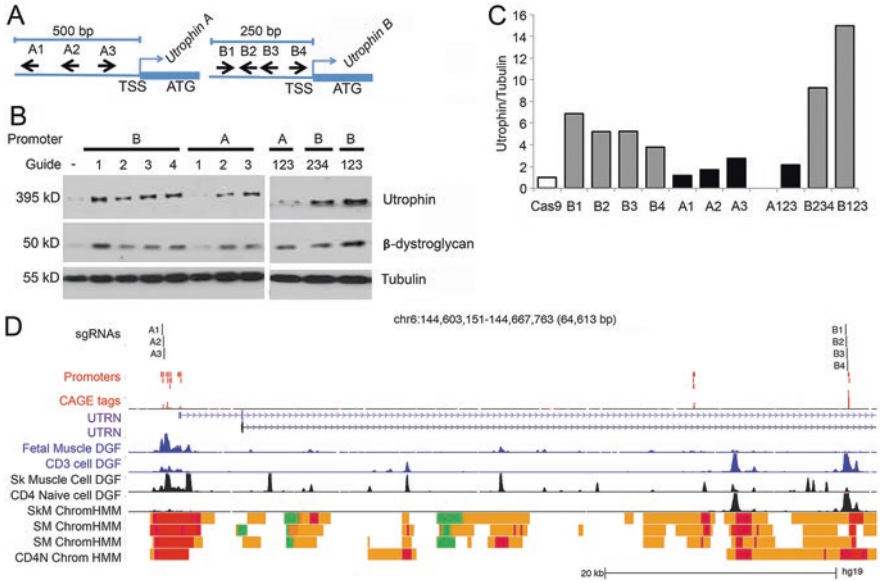

Fig. 16.1 Utilizing CRISPR/Cas9 to modulate expression of *UTRN*, a disease-modifying gene in DMD myoblasts. (**a**) Schematic diagram (not to scale) of sgRNAs targeting regions upstream of *UTRN* A (A1–A3) and B (B1–B4) TSSs. (**b**) CRISPR-/Cas9-mediated transcriptional activation of *UTRN* in DMD myoblasts. Amounts of utrophin, β-dystroglycan, and tubulin were analyzed by Western blot 4 days after transfection with dCas9-VP160 plasmid containing each sgRNA. (**c**) The amount of utrophin was normalized to that of tubulin by densitometric analysis of four different experiments. (**d**) Location of sgRNAs in relation to *UTRN* TSS, DNase I hypersensitivity footprints, and chromatin-state maps. sgRNAs are plotted above experimentally determined TSSs obtained from a FANTOM5 assay of over 300 primary tissues. The maximum signal at each promoter region is shown below the TSSs (CAGE tags). Digital DNase Footprinting (DGF) assays for fetal muscle and primary CD3 cells are shown in blue (ENCODE). DGF assays for skeletal muscle cells, skeletal muscle, and naive CD4 cells are shown in black. Chromatin-state maps from the Roadmap Epigenomic Consortium are shown for skeletal muscle cells (SkM), skeletal muscle (SM), and naive CD4 cells (CD4N). Red indicates TSSs, and yellow indicates enhancer states. The A guides all fall within muscle promoter regions. The B guides fall into an enhancer region immediately upstream of an annotated promoter region. In CD4 cells, this region is considered an active promoter. At promoter B, the DGF footprint in muscle cells is weak in comparison to that in CD4 cells. Data were plotted according to positions from the UCSC Genome Browser. FANTOM5, DGF, and chromatin-state data were obtained from UCSC "Track Hubs" (Reproduced from Wojtal et al. [35])

duplication. This resulted in restoration of full-length dystrophin in the treated cells (Fig. 16.2), suggesting that this could be a feasible treatment approach for the approximately 10–15% of DMD patients that have a duplication of one or more exons [36].

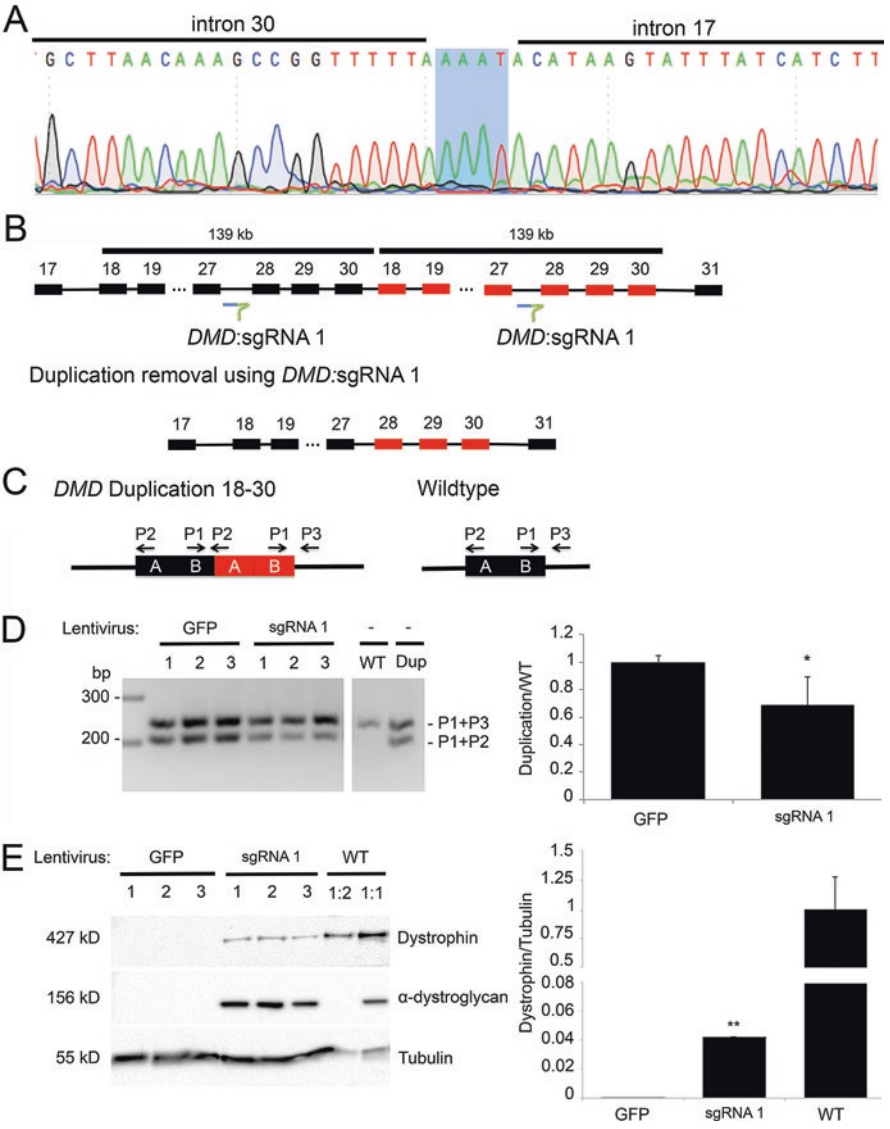

Fig. 16.2 Genome-editing strategies for individuals with duplication of *DMD* exons 18–30. (**a**) Electropherogram of the junction of the duplication of *DMD* exons 18–30; highlighted in blue is the insertion of AAAT at the junction. (**b**) Schematic of the position of *DMD* sgRNA 1 and the duplication-removal strategy. (**c**) Schematic of the three-primer duplication-removal strategy. (**d**) Targeted deletion of a 139 kb duplication in *DMD*. PCR was performed on DNA from three replicate experiments in which affected myoblasts were transduced with LentiGFP or lentiCRISPR Cas9 nuclease with *DMD* sgRNA 1. The top band was amplified with universal primers (P1 + P3) to both an allele with the duplication and a control. The bottom band is specific to alleles harboring the duplication (P1 + P2). A decrease in the bottom band, indicating removal of the duplicated region, was only observed when Cas9 and sgRNA 1 were present. (**e**) Western blot with antibodies Fig. 16.2 (continued) against dystrophin, α-dystroglycan, and tubulin as a loading control. The amount of dystrophin was normalized to that of tubulin by densitometric analysis. $^{*}p < 0.05$, $^{**}p < 0.01$ (Student's t-test from three independent experiments) (Reproduced from Wojtal et al. [35])

16.3.2 Congenital Muscular Dystrophy Type 1a

Congenital muscular dystrophy type 1a (MDC1a) is a severe, neonatal-onset disorder, characterized by significant hypotonia, weakness, dysmyelinating neuropathy, and CNS white matter lesions. Respiratory failure is common, as well as feeding difficulties, aspirations, and recurrent respiratory infections. MDC1a is caused by biallelic mutations in *LAMA2*, which encodes the α2 chain (LAMA2) of the laminin-211 (formerly merosin) complex, an essential component of the basement membrane of muscle and Schwann cells. Its deficiency leads to loss of integrity of those cells, resulting in muscle degeneration and fibrosis. There is good genotype-phenotype correlation, with the degree of LAMA2 deficiency being related to the severity of clinical presentation [37]. Approximately 40% of patients with MDC1a have splice-site mutations, leading to exon skipping and a truncated protein [37, 38].

A mouse model for MDC1a, dy^{2J}/dy^{2J}, has a splice-site mutation (c.417 + 1 g → a) at the beginning of intron 2 of *Lama2*, leading to skipping of exon 2 and a truncated N-terminal domain [39, 40]; this causes muscle atrophy and hind limb atrophy. The g → a mutation in the first nucleotide of intron 2 leads to disruption of the consensus splice-site sequence A/C-A-G-g-t-a/g-a-g-u, where the first intronic g-t nucleotides are invariant.

An AAV-based gene replacement approach might not be feasible in the case of MDC1a, given the large size of the LAMA2 coding region, which exceeds the carrying capacity of AAV vectors. A recent paper described a CRISPR-/NHEJ-based approach to fix the splice-site mutation in the abovementioned mouse model of MDC1a [41]. The study team hypothesized that removal of the proximal part of that intron by cutting just immediately before that g → a mutation would allow reconstitution of the consensus splice site by joining with a g-t nucleotide sequence downstream in the intron. This required the design of two sgRNA. Three-week-old dy^{2J}/dy^{2J} mice were treated with an intramuscular or intraperitoneal injection of AAV9 encoding hemagglutinin-tagged SaCas9 and either sgRNA1 or sgRNA2. This leads to recovery of full-length LAMA2 in muscles as well as improvement of muscle morphology; however, no improvement of locomotion or paralysis was shown, and no full-length LAMA2 recovery occurred in the sciatic nerve. Systemic delivery through the temporal vein resulted in LAMA2 recovery in muscle and sciatic nerve, improved muscle morphology as well as decreased paralysis and improved locomotion. Overall, this study reveals the feasibility of CRISPR-mediated correction of splice-site mutations without the use of homology-directed repair, paving the way toward therapeutic applications in patients with such mutations, either with MDC1a or other genetic disorders.

16.4 Conclusion

In summary, the ease of use and flexibility of the CRISPR method has led to exciting developments in the field of gene editing. Challenges remain, such as diminishing the risk of off-target cuts or working around the inefficiency of HDR in muscle

cells, but significant progress has already been made in those areas. Regarding DMD, the proof of concept has already been established for treatment of a variety of different mutations, paving the way toward clinical trials in the future. However, multiple issues need to be resolved before this technology moves to the clinic, among other things the risk of off-target effects. Unbiased, comprehensive genome-wide search methodologies will need to be used to assess for off-target cuts. Furthermore, research into delivery vehicles that are nonviral in nature may be necessary to develop in the near future. However, new developments in CRISPR methodology are opening up a number of potential treatment avenues, and the next few years will likely see the number of genetic disorders amenable to CRISPR-mediated correction expand significantly.

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Part II
Preclinical Muscle Gene Therapy

Chapter 17
Considerations on Preclinical Neuromuscular Disease Gene Therapy Studies

Dongsheng Duan

Abstract Numerous neuromuscular and non-neuromuscular diseases are amenable to gene therapy. Rigorously designed and carefully conducted preclinical studies are essential to translate these muscle gene therapies to human patients. Many general guidelines have been published in recent years on how to enhance reproducibility and improve predictive value of preclinical studies. These are excellent guidelines to follow in preclinical gene therapy studies. However, they are not tailed specifically for muscle gene therapy. In this chapter, I discuss considerations in the design of a preclinical neuromuscular disease gene therapy study based on our experience in the preclinical development of adeno-associated virus (AAV) micro-dystrophin gene therapy. I also discuss adapting the design of phase III clinical trials to animal studies to improve their reproducibility. This chapter is not intended to be all-inclusive and to cover all possible scenarios. Due to the complexity of the candidate diseases that can be treated by muscle gene therapy, it is critical to consider disease-specific issues in the design of each preclinical muscle gene therapy study.

Keywords Preclinical study · Gene therapy · Muscle disease · Experimental design · Adeno-associated virus · Animal model · Dystrophin · Muscular dystrophy

D. Duan (✉)
Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri, Columbia, MO, USA

Department of Neurology, School of Medicine, University of Missouri, Columbia, MO, USA

Department of Biomedical Sciences, College of Veterinary Medicine, University of Missouri, Columbia, MO, USA

Department of Bioengineering, University of Missouri, Columbia, MO, USA
e-mail: duand@missouri.edu

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17.1 Introduction

Muscle gene therapy refers to therapies that are based on delivering genetic materials (nucleic acids) to muscle to treat diseases. While the majority of muscle gene therapy aims at treating neuromuscular diseases (such as muscular dystrophy), investigators have also explored treating non-muscle diseases with muscle gene therapy. In the latter case, the muscle is used as a platform to produce and secrete a therapeutic protein such as factor IX, antitrypsin, or lipoprotein lipase [1–4]. In this chapter, I limit the discussion to preclinical gene therapy studies for treating neuromuscular diseases. Currently, clinical muscle gene therapy has only been conducted in a few neuromuscular diseases. Among these, Duchenne muscular dystrophy (DMD) gene therapy, specifically systemic adeno-associated virus (AAV)-mediated micro-dystrophin gene therapy, has attracted significant attention in recent years [5]. For this reason, I focus on experiences gained and lessons learned in the preclinical development of AAV micro-dystrophin gene therapy to illustrate bench-to-bedside translation of muscle gene therapy. Specifically, I discuss issues related to animal models, gene delivery vectors, and outcome measurements. I also highlight some common misconceptions and important precautions in statistical analysis and data interpretation. Finally, I discuss the preclinical randomized controlled trial (pRCT), a new strategy to conduct late-stage animal studies using best practices learned from human trials.

17.2 Overview of Preclinical Muscle Gene Therapy

Broadly speaking, preclinical studies can be divided into early explorative studies and late investigational new drug (IND)-enabling studies. Early explorative studies establish the proof of principle. Late IND-enabling studies define the risk-benefit profile of the therapy to support regulatory approval of a human trial. In the context of neuromuscular disease gene therapy, early explorative studies have four major goals. The first goal is to identify the therapeutic gene. This can be the disease-causing gene (a gene causing disease when mutated, e.g., the DMD gene, the gene that encodes dystrophin), a homologue of the disease gene (e.g., the utrophin gene), or a disease-modifying gene (e.g., the follistatin gene) [6–8]. This gene can also be an endogenous gene (e.g., the alpha-sarcoglycan gene) or an engineered synthetic gene (e.g., the micro-dystrophin gene) [9, 10]. In some neuromuscular diseases, the disease-causing mutation occurs in the untranslated region (UTR). For example, type I myotonic dystrophy is due to CTG repeat expansion in the 3′-UTR of the DMPK gene. For these diseases, the goal should include the identification of disease-causing mutations.

The second goal of early explorative studies is to determine the optimal gene therapy strategy. The most commonly used gene therapy strategies are gene replacement, gene knockdown, gene repair, and disease-modifying gene therapy. Gene replacement therapy is used to treat a recessive disease by providing a functional

copy of the mutated gene (e.g., treating autosomal recessive limb-girdle muscular dystrophy 2D by expressing the alpha-sarcoglycan gene) [9]. Gene knockdown therapy is used to treat dominant muscle diseases (e.g., treating autosomal dominant limb-girdle muscular dystrophy 1D with RNA interference-mediated silencing of the mutated myotilin gene) [11]. Gene repair therapy aims to remove or correct the mutation. The recently developed clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) editing has yielded promising results in removing the dystrophin gene mutation in animal models of DMD [12, 13]. It should be pointed out that gene repair therapy is not limited to DNA level modification. RNA-level modifications such as RNA trans-splicing and exon skipping are powerful gene therapy tools to bypass the DNA mutation and produce a functional protein [14, 15]. Disease-modifying genes are genes that do not cause the disease themselves but rather they modify disease severity [16]. Disease-modifying gene therapy hence does not target the mutated disease gene directly. Instead, it aims at genes that regulate downstream pathogenic events such as degeneration/regeneration, muscle atrophy, necrosis, and fibrosis. Cytosolic calcium overloading is a common feature in muscular dystrophies. Supraphysiological levels of calcium kill muscle cells by activating proteases and phospholipases. Overexpression of the calcium pump, the sarco/endoplasmic reticulum calcium ATPase (SERCA) gene, or silencing of the SERCA inhibitor sarcolipin has been shown to mitigate muscular dystrophy in mice [17–19].

The third goal of early explorative studies is to select the gene transfer vector and the delivery route. A number of non-viral and viral vectors have been explored for muscle gene therapy. After several decades of research, AAV now stands out as the leading vector for muscle gene therapy (see Chaps. 10 and 11 for additional discussion on AAV) [20]. The delivery route of muscle gene therapy has evolved from early direct intramuscular injection to regional perfusion and now to whole-body systemic delivery. The muscle is one of the most widely distributed tissues in the body. Many muscle diseases not only affect the skeletal muscle but also compromise the heart. Hence, body-wide systemic delivery via intravenous injection is the preferred delivery route for many neuromuscular disorders [21, 22]. A small number of muscle diseases only affect a selective set of muscles (e.g., oculopharyngeal muscular dystrophy). In these cases, direct muscle injection will meet the therapeutic need [23].

The fourth and perhaps the most important goal of early explorative studies is to demonstrate therapeutic efficacy in a relevant animal model. Ideally, this should include demonstration of the intended molecular and biochemical changes, amelioration of histopathology, improvement of physiological functions, and/or the survival rate or lifespan following administration of the candidate gene therapy vector. Early explorative studies are often conduced in academic laboratories. These studies establish the proof of principle for treating a particular neuromuscular disease with a tailored gene therapy approach.

IND-enabling studies are prompted by the positive findings from early explorative studies. The aim is to generate adequate data to support the safety and the scientific basis for an early-phase clinical trial (see Chap. 39 for additional discussion on IND-enabling study). These studies are usually designed with significant

input from the regulatory agency such as the Pre-IND Consultation Program at the Center for Biologics Evaluation and Research (CBER) and the Food and Drug Administration (FDA) (https://www.fda.gov/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/ApprovalApplications/InvestigationalNewDrugINDApplication/Overview/default.htm) [24]. Some IND-enabling studies are conducted in accordance with good laboratory practice (GLP). IND-enabling studies are expected to generate efficacy, pharmacology, and toxicology data. The pharmacology includes pharmacodynamics (PD) and pharmacokinetics (PK). PD refers to the effects a drug has on the body, or simply, drug effects. PK describes how a drug moves through the body, including absorption, distribution, metabolism, and excretion. In the context of gene therapy, these often include but are not limited to dose finding, vector biodistribution in target and nontarget tissues, vector replication, vector shedding, transgene expression in target and nontarget tissues, immune response (T-cell and B-cell responses to the viral capsid and/or transgene product and the innate immune response), blood chemistry, histopathology, insertional mutagenesis, germline transmission, and, in certain cases, long-term evaluation [25]. For gene therapy studies, IND-enabling studies also include characterization and validation of the method used for vector production and purification [25].

17.3 Preclinical Development of AAV Micro-Dystrophin Gene Therapy for DMD

17.3.1 Dystrophin and DMD

DMD is caused by frameshift or frame-aborting mutations in the dystrophin gene. These mutations eliminate production of the dystrophin protein, a subsarcolemmal cytoskeletal protein that stabilizes the muscle cell membrane and protects muscle from contraction-induced injury. In the absence of dystrophin, muscle undergoes degeneration and necrosis and eventually is replaced by fibrofatty tissue. DMD is an X-linked recessive disease. It occurs at a frequency of 1 in 5000 newborn boys [26]. Without medical intervention, patients die prematurely in their 20s to 30s from respiratory and/or cardiac muscle failure. Currently, there is no cure for DMD.

17.3.2 Animal Model

The dystrophin gene was discovered in 1987 [7]. Right around the time of dystrophin gene cloning, two naturally existing dystrophin-deficient animal models were identified, including the mdx mouse and the golden retriever muscular dystrophy (GRMD) dog [27, 28]. Subsequently, a large collection of additional DMD models were discovered in nature or developed using chemical induction or genetic engineering. Currently, ~100 DMD models exist in a broad range of

species including invertebrates (worms and flies), zebrafish, mice, rats, cats, rabbits, dogs, pigs, and nonhuman primates [29, 30]. For mouse and dog models, there also exists a great variety of strains/breeds that differ either in the genetic background or in the mutation type (see Chap. 3 for additional discussion on the DMD model). In addition to the existing models, new DMD models are continuously being generated [31–33]. The existence of so many options allows for a maximal coverage of the entire spectrum of the human disease and makes it possible to meet different experimental needs. However, this also makes it challenging to decide on the best model to use.

In the development of AAV micro-dystrophin gene therapy, several parameters have been considered in model selection. The most important one is the study purpose or the study question. The chosen animal model should allow investigators to answer the question being asked in the study. Selection is further narrowed by the cost, availability, genetic background, age, sex, and existing knowledge on the model (e.g., do we know the natural history of the model? Are there robust outcome measurement assays to study the model? Has the model being utilized previously in testing other experimental therapies?). It should be pointed out that DMD is not the only neuromuscular disease that has multiple animal models. This is also the case for many other neuromuscular diseases such as myotonic dystrophy, facioscapulohumeral muscular dystrophy, and spinal muscular atrophy. The considerations discussed here may also help model selection in designing preclinical gene therapy studies for other neuromuscular diseases.

At the early exploratory stage of AAV micro-dystrophin gene therapy, the primary question is whether the highly abbreviated micro-dystrophin gene can treat muscle disease [10]. In this context, young adult mdx mice become the most appropriate model because (a) the model is readily available, easy to handle and breed, and low-cost (hence, no problem for designing experiments with a meaningful sample size); (b) an array of outcome measurements have been developed to study biochemical, histological, and physiological changes following experimental intervention; (c) mdx mice have been extensively used in transgenic studies to demonstrate therapeutic benefits of the full-length dystrophin gene and various truncated dystrophin genes; (d) mdx mice have been widely used in cell therapy studies and adenoviral vector-based gene therapy studies; (e) mdx mice have also been used to test various drug therapies; (f) the natural history data is available for mdx mice; and (g) there exists a background-matched normal control, the C57Bl/10 mouse. Indeed, all early AAV micro-dystrophin gene therapy studies were performed in mdx mice [34–41].

Despite positive results from young adult mdx mice, there are caveats with using this model. For example, these mice do not manifest the dystrophic clinical phenotype and do not model classic dilated cardiomyopathy of DMD. To determine whether AAV micro-dystrophin therapy can ameliorate disease in a symptomatic model, investigators turned to more severe mouse models such as aged mdx mice, dystrophin/utrophin double knockout mice, dystrophin/MyoD double knockout mice, and DBA/2J-mdx mice [39, 42–46]. Cardiac function of young mdx mice is well preserved. These mice also lack heart pathology. Interestingly, the heart of the aged female mdx mouse displays dilated cardiomyopathy similar

to that seen in DMD patients [47]. Aged female mdx mice are thus used to determine whether AAV micro-dystrophin therapy can mitigate Duchenne cardiomyopathy [48, 49].

Following the proof-of-principle studies in mice, the next challenge is whether AAV micro-dystrophin therapy can be scaled up to treat affected boys. This question is addressed in the canine DMD model [50]. The body weight of a boy is ~700- to 800-fold higher than that of a mouse but only ~2- to 4-fold higher than that of a dog. Importantly, the clinical course and manifestations of dystrophin-deficient dogs are similar to those of DMD patients [29]. AAV micro-dystrophin gene therapy in the dog model bridges the gap between mice and human patients [50–55]. Together, efficacy and pharmacokinetic and dose-finding studies in different animal models pave the way to the regulatory approval of three early-phase systemic AAV micro-dystrophin gene therapy trials in DMD patients [10].

It is worth pointing out that preclinical studies are not only limited to the disease model. They are also performed in normal animals. For example, FDA requires toxicology data from at least two species (one rodent and one non-rodent). In the case of systemic AAV micro-dystrophin gene therapy, normal nonhuman primates were used as the non-rodent species for toxicology and biodistribution study [56].

The discussion above mainly focused on animal models used in the development of systemic AAV micro-dystrophin gene therapy. However, these models may not be ideal for testing other DMD gene therapy approaches. The most common mutations in DMD patients are deletion mutations between exons 43 and 55 [57]. However, the majority of early DMD models carry point mutations outside this hotspot region. For example, mdx mice carry a nonsense point mutation in exon 23, and GRMD dogs carry a point mutation in intron 6 that disrupts splicing [27, 28]. For exon skipping and CRISPR/Cas9 gene-editing therapy, models that share mutations similar to those of DMD patients would be more useful for translational purposes. To meet these needs, several new models carrying a deletion mutation in this region were generated recently [30–33]. To study the human dystrophin gene in the mouse genome, a transgenic hDMD mouse, which carries the full-length human dystrophin gene, was generated on the background of the mdx mouse [58, 59]. Subsequently, a deletion mutation was introduced in the mutation hotspot region (exons 43–55) in the human dystrophin gene [31, 33]. There is a high likelihood that gene repair reagents developed in these models can be directly used in human studies because of the sequence identity.

Some DMD gene therapy-related questions can also be addressed without applying gene therapy to diseased animals. For example, one of the fundamental questions in dystrophin gene replacement therapy is the level of expression. Will low-level expression offer any benefit? Will high-level expression cause toxicity? To study whether low-level dystrophin expression can ameliorate DMD, we used the chemically induced mdx3cv mice which express 4–5% dystrophin in all muscle cells [60–62]. The Aartsma-Rus lab crossed mdx mice with $Xist^{\Delta h}$ mice. Due to random X-chromosome inactivation, the resulting mdx-$Xist^{\Delta hs}$ mice showed a wide range of low-level dystrophin expression [63–65]. These studies echo well with clinical findings and suggest that marginal level dystrophin expression is still ben-

eficial [66, 67]. The toxicity of supraphysiological dystrophin overexpression was studied in transgenic mdx mice [68, 69]. These studies suggest that 50-fold overexpression is not toxic. However, ≥100-fold overexpression is associated with cardiac toxicity.

The age, gender, and genetic background are known factors that greatly affect study outcome [70–75]. Mdx display an acute necrotic phase between the age of 2 and 6 weeks. This phase does not exist in human patients [29]. It is thus advisable to avoid performing gene therapy studies in this age range. It has been demonstrated that mouse sex may greatly impact skeletal muscle function, cardiomyopathy manifestation, and inflammation [76–79]. We also noticed that sex profoundly influences disease severity in mdx mice. Male mdx mice show more severe skeletal muscle disease, while female mice display more advanced cardiomyopathy [47, 80]. It is worthwhile to point out that animal sex may not only modify disease manifestations but may also change transduction profile of the AAV vector [81, 82]. Hence, depending on the study question, animal sex should be carefully considered in experimental design. One often made mistake is mixing male and female animals in the same experimental group. This is especially problematic when the ratios of male and female animals are not equal between the gene therapy group and control groups (untreated controls and normal controls). Such a design can greatly confound data interpretation.

There are ample publications documenting the influence of the genetic background on animal behaviors, activities, organ/tissue functions, and disease phenotype [70, 71, 83–88]. Of particular interests to muscle gene therapy are the publications that demonstrate the impact of the genetic background on muscle and heart function [75, 89–93]. In the context of mdx mice, it has been shown that the genetic background profoundly influences the frequency and expansion of revertant fibers, the rarely occurring dystrophin positive fibers seen in dystrophin-deficient muscle [94]. Tachycardia is a characteristic feature in DMD patients. Interestingly, we found that this phenotype is preserved in C57Bl/10 background mdx mice but not in FVB background mdx mice [95, 96].

Collectively, these findings underscore the importance of model selection as a critical aspect in the design of preclinical muscle gene therapy studies. As a rule of thumb, one should keep in mind that no model is perfect because humans are not animals. The other point that can never be overemphasized is the nature history study. Such studies should begin as soon as the model becomes available and continue through the course of preclinical development and after the completion of a preclinical gene therapy study.

17.3.3 Gene Delivery Vector

Gene therapy vectors can be broadly classified as viral and non-viral vectors. Viral vectors can be further divided into DNA viral vectors and RNA viral rectors [97]. All these vectors have been tested in human patients for different gene therapy needs [98]. A number of factors are considered when determining which vector is

the best for a particular application. These include, but are not limited to, the nature of the disease (recessive or dominant, affected organ(s) and tissues(s), disease gene and gene product, etc.), biological features of the vector (carrying capacity, tropism, replication competence, persistence, immunogenicity, genotoxicity, etc.), and vector manufacture (production, purification, storage, etc.).

DMD gene therapy faces several unique challenges. First, the dystrophin gene is one of the largest genes in the genome, and the dystrophin coding sequence exceeds 11 kb. Second, the disease affects both skeletal and cardiac muscle. Skeletal muscle accounts for ~40% body weight and spreads throughout the body. The heart is located in the chest cavity, and it beats continuously. Third, the dystrophic muscle is highly inflamed. Fourth, DMD is a chronic disease, and an effective therapy requires lifelong expression of a therapeutic dystrophin gene. To meet these needs, an ideal vector for DMD gene therapy should have a large packaging capacity, have the ability to efficiently transduce all body striated muscle, result in persistent transduction, and be non- or minimally immunogenic.

Since the discovery of the dystrophin gene in 1987, many different vector systems have been tested for DMD gene therapy. The non-viral plasmid vector did not meet the needs because it only led to poor and transient transduction in the muscle [99, 100]. The retroviral vector did not work because it could not transduce postmitotic muscle tissue [101]. The adenoviral vector failed because of its immune toxicity [102]. AAV seems to meet most of the needs of DMD gene therapy. AAV can lead to high-level, persistent, whole-body muscle transduction without inducing a strong cellular immune response. Further, AAV is now considered a medicine [103]. Several AAV-based gene therapy drugs have received regulatory approval for commercial use, such as Luxturna for treating an inherited childhood blindness disease called Leber congenital amaurosis and Glybera (also called Alipogene tiparvovec) for treating lipoprotein lipase deficiency. The only shortcoming of the AAV vector is its less than 5 kb packaging capacity. Fortunately, this problem is solved with the development of the micro-dystrophin gene ($\leq$ 4 kb).

The wild-type AAV virus is composed of an approximately 4.7 kb single-stranded DNA genome and a ~25 nm icosahedral capsid. An intact AAV virus genome has two inverted terminal repeats (ITRs) at the 5′ and 3′ end. In the middle are the expression cassettes for making AAV replication proteins, capsid proteins, and the assembly-activating protein. In a recombinant AAV vector, viral gene expression cassettes are replaced by a transgene (reporter gene or therapeutic gene) expression cassette. The ITR serves as the AAV replication origin and packaging signal. The ITR is the only viral element present in an AAV vector.

Two decisions are made in AAV vector selection, one for the vector genome and the other for the viral capsid. For the vector genome, we need to decide on the genome configuration and expression cassette. Two genome configurations exist including single-stranded AAV (ssAAV) and self-complementary AAV (scAAV) [104]. The ssAAV has the exactly same single-stranded genome configuration as that of wild-type AAV. In the scAAV vector, the terminal resolution site from one ITR is deleted [105]. This allows packaging of a double-stranded vector genome with two wild-type ITRs at the ends and the mutated ITR in the middle. This con-

figuration bypasses single-strand to double-strand conversion, a rate-limiting step in AAV transduction, and yields rapid-onset and higher-level transduction in the muscle [104, 106, 107]. The trade-off of the scAAV vector is the reduction of the packaging capacity from 5 kb to 2.5 kb. A number of therapeutic genes have been packaged in scAAV such as the factor IX gene for treating hemophilia B and the survival motor neuron gene for treating spinal muscular atrophy [108, 109]. The size of a functional micro-dystrophin gene is in the range of 3.8–4 kb [10]. This exceeds the 2.5 kb packaging capacity of scAAV. Hence, only ssAAV is used for micro-dystrophin gene therapy.

A basic expression cassette is composed of a promoter, a transgene, and a polyadenylation (pA) signal. Sometimes, additional regulatory elements (such as the microRNA target site) are included to fine-tune transgene expression (see Chap. 8 for additional discussion on expression cassette design). At the early exploratory stage, investigators often use a ubiquitous promoter such as the cytomegalovirus (CMV) promoter. However, tissue-specific promoters (if available) should be used for large animal studies and IND-enabling studies. In the case of AAV micro-dystrophin gene therapy, a variety of muscle-specific promoters have been used including abbreviated promoters derived from the muscle creatine kinase gene (e.g., CK6, CK8, MHCK7, and minimized MCK) and synthetic promoters (e.g., Spc5-12) [10, 110, 111]. The transgene refers to the cDNA rather than the intron-containing full-length gene. Several strategies have been used to improve expression. These include codon optimization of the cDNA sequence [40], inclusion of a small intron between the promoter and the transgene [112], and utilization of a gene variant that shows an increased activity (e.g., the Padua variant of the factor IX gene for hemophilia B gene therapy) [113]. To minimize the immunogenicity of the transgene, one may also consider removing CpG motifs by introducing silent mutations [114, 115]. A unique issue for AAV micro-dystrophin gene therapy is the configuration of the microgene. In the microgene, ~70% of the dystrophin coding sequence is removed. More than 30 different microgene configurations have been published [10]. The difference in the constitution of the microgene may greatly impact its biological activity. For example, inclusion of dystrophin spectrin-like repeats 16 and 17 in the microgene allows restoration of neuronal nitric oxide synthase to the sarcolemma [39, 116]. Polyadenylation is critical for nuclear export of the RNA transcript and the stability/translation of mRNA. A variety of pA signals are available [117]. SV40 virus late pA (SV40 pA) and bovine growth hormone pA (bGH pA) are commonly used strong pA signals [118]. In the case of the AAV micro-dystrophin vector, a 49 bp synthetic pA is used due to the packaging limit of the AAV vector [119].

Another important decision to make in AAV vector selection is the viral capsid. Capsid not only determines tissue tropism but also influences intracellular trafficking and processing of the viral particle. The triangulation number T reflects the size and complexity of the capsid. Although the T-number for AAV is merely 1, the AAV capsid still comes with many different “flavors.” Twelve AAV serotypes and hundreds of AAV variants have been published [120]. Newer AAV capsids are continuously being generated.

Among existing AAV serotypes, AAV1 and AAV6 (especially tyrosine-modified AAV6) are the best for direct intramuscular injection [121]. AAV6, AAV8, and AAV9 are the top serotypes for intravascular delivery [21]. For the purpose of systemic muscle gene therapy, a capsid with enhanced muscle transduction and reduced non-muscle transduction would be very appealing. Hence, there have been continuous efforts from many academic laboratories as well as industry to further improve the existing serotypes. Using AAV8 and/or AAV9 as the benchmark, several new muscle tropic AAV capsids were developed in the last few years. These new capsids were engineered either by rational design or by forced evolution [122]. They include AAV2i8, AAVB1, AAV587MTP, AAVM41, AAV9.45, AAV9.61, AAVMyo, and AAVNP22 and AAVNP66 [123–129]. Except for AAVNP22 and AAVNP66 [128], most of these newly developed muscle tropic AAVs are developed and tested in mice. It should be mentioned that AAV tropism may exhibit significant interspecies difference. For example, AAV8 is the best for the mouse liver, but AAV3B is the best for human liver cells [130, 131]. AAV9 is considered as a cardiotropic capsid in murine studies, but AAV8 outperforms AAV9 in the neonatal dog heart [132–134]). Because the same AAV serotype may exhibit distinctive behavior in different species, additional studies are needed to determine whether these newly developed muscle tropic AAV variants can outperform existing serotypes (AAV8 and AAV9) in large animals and human patients.

At the time when the first micro-dystrophin gene was engineered [135], the best studied AAV was AAV serotype 2 (AAV2). Methods have also been developed for the production and purification of AAV2 vectors. Hence, AAV2 was used in early micro-dystrophin studies [34–36]. The discovery of the systemic transduction property of several newer AAV serotypes (AAV6, AAV8, and AAV9) opens the door to whole-body muscle therapy [136–138]. These serotypes (especially AAV8 and AAV9) are now not only the most popular AAV capsids in preclinical muscle gene therapy studies but are also the chosen serotypes in several ongoing human trials. For example, AAV9 was used in type 1 spinal muscular atrophy patients and DMD patients [10, 22], AAV8 was used in X-linked myotubular myopathy patients and hemophilia B patients [139, 140], and AAVrh74 (a serotype similar to AAV8) was used in DMD patients [10, 141].

Additional issues to consider in AAV capsid selection are the pre-existing neutralizing antibody (NAb) titer, AAV purity, AAV production methods, and AAV titer determination. The preinjection NAb titer is usually not measured in murine studies (unless the study goal is to test readministration). However, it is essential to measure the preinjection NAb titer in large animal studies because of the high prevalence of NAb in these animals and the high cost of the large animal study [142–145]. It has been shown that even a relatively low NAb titer may compromise AAV transduction [146–148]. AAV purity has been shown to significantly influence its transduction efficiency in a serotype- and tissue-independent manner [149]. For this reason, the stock AAV vector should be checked for purity, at least, by silver stain for capsid purity and by quantifying the endotoxin level for contamination before dosing large animals [150]. Numerous methods have been developed to produce and purify AAV [151–153]. Research grade AAV is usually made using the transient transfection

method in adherent 293 cells. For systemic delivery in human patients, scalable methods (such as baculovirus production system, herpes simplex virus production system, and transient transfection in suspension 293 cells) are often used to generate clinical grade AAV [154–156]. Research grade AAV can be used in most preclinical studies. However, clinical grade AAV should be used for IND-enabling toxicity study. The AAV titer is usually determined by quantitative PCR (qPCR) and expressed as viral genome particles per milliliter (vg particles/ml or vg/ml). Despite a seemingly simple technique, significant inter- and intra-laboratory variations have been reported [157–160]. Some technical issues in the design of the qPCR protocol may have contributed to this inconsistency. For example, using ITR-specific qPCR may lead to the overestimation of the titer [158]. The conventional method that is used for titrating ssAAV may underestimate the scAAV titer [161]. Hence, optimization and validation of the AAV titration protocol should be an integral component of a preclinical muscle gene therapy study [162].

17.3.4 Outcome Measurements

In addition to the correct animal model, vector and delivery route, rigorous outcome measurements, and data analysis are also essential to get meaningful results and correct conclusions (either positive or negative). For some outcome measurements, there are established methods/assays widely used in the field. In this case, it is essential to strictly follow published protocols or use established standard operation procedures (SOPs). For protocols described only in the method section of research articles, it is always helpful to contact the authors and get the working protocol. The working protocol often contains more details that are critical for conducting the assay. Sometimes, a detailed protocol may have already been published in a different format in method journals such as *Methods in Molecular Biology* (Springer protocols; published by the Humana Press) [163–165]; *Journal of Visualized Experiments* (JoVE; published by MyJove Corporation) [166–168]; *Current Protocols* series such as *Current Protocols in Molecular Biology*, *Current Protocols in Mouse Biology*, and *Current Protocols in Immunology* (published by John Wiley & Sons Inc); and *Nature Protocols* (published by Nature Publishing Group) [169–172]. A number of SOPs have been established for studying neuromuscular diseases. These SOPs are freely accessible at the Treat-NMD Neuromuscular Network website (http://www.treat-nmd.eu/research/preclinical/overview/) and Parent Project Muscular Dystrophy website (http://join.parentprojectmd.org/site/PageServer?pagename=Advance_researchers_sops) [173–176].

While many functional assays exist for studying neuromuscular diseases in mice, few are available for studying large animals. To fill the gap, we and others have begun to develop protocols for evaluating muscle function in canines over the last few years. These include dog gait analysis [177–180], noninvasive dog activity quantification [177, 181], electrical impedance myography [182], in situ single muscle force measurement [183], and, most recently, a physiological assay to study

functional ischemia in dog muscle in vivo [184]. Several points should be considered in the development of a new assay. These include selectivity (the new method is specific for the intended purpose), sensitivity (the lowest value the new method can accurately measure), accuracy (the value obtained from the new method should reflect the actual value in the sample), and reproducibility (the new method can yield the same results on replicates). It should be pointed out that the development, optimization, and validation of a new protocol should not be considered as a trivial undertaking. It often requires multidisciplinary collaboration among investigators who have expertise in different fields including, but not limited to, neuromuscular disease, muscle physiology, veterinary medicine, computer science, bioengineering, and statistics.

Failure to validate/authenticate experimental reagents contributes to irreproducibility in biomedical research. Authentication of key biological and chemical reagents has now attracted a lot of attention from major funding agencies (such as NIH) and publishers [185–189]. Online guidelines and checklists are readily available on NIH (https://grants.nih.gov/grants/guide/notice-files/NOT-OD-17-068.html), journal, academic society, and institution websites and will not be discussed in depth in this chapter. However, I'd like to emphasize the importance of antibody validation. Antibodies are among the most commonly used reagents in research. Unfortunately, many commercially available and self-generated antibodies are poorly validated and/or characterized. This has become a major concern in the research community because data generated with these antibodies vary greatly and conclusions drawn are often misleading due to frequent false-positive and false-negative results.

In the case of DMD, we recently evaluated 65 epitope-specific dystrophin monoclonal antibodies in murine and canine muscles by immunostaining and Western blot [190]. These antibodies were originally developed for diagnostic use in human patients [191, 192]. Their research use in the mouse and dog DMD models has never been validated. Interestingly, we found some antibodies work well for immunostaining/Western blot in murine/canine muscle (e.g., Mandys 1), some antibodies work well only for immunostaining in murine/canine muscle (e.g., Mandys 105), some antibodies work well only for Western blot in murine/canine muscle (e.g., Mandra 13), some antibodies work well for immunostaining/Western blot in murine muscle only (e.g., Mandnex 45B), and some antibodies work well for immunostaining/Western blot in canine muscle only (e.g., Mannex 4850D) (Fig. 17.1). There are also antibodies that do not work for immunostaining/Western blot in murine/canine muscle (e.g., Mandhinge 2C) (Fig. 17.1).

Various strategies have been suggested on how to validate an antibody [193–197]. Several working groups and consortium have developed online resources that allow users to search for validation information of an antibody. These online antibody databases include, but are not limited to, Antibodypedia (https://www.antibodypedia.com/) [198, 199], Human Protein Atlas (https://www.proteinatlas.org/) [200], CiteAb (https://www.citeab.com/) [201], AbMiner (https://discover.nci.nih.gov/abminer/) [202], EuroMAbNet (https://www.euromabnet.com/guidelines/example1.php) [203], Biocompare (https://www.biocompare.com/Antibodies/)

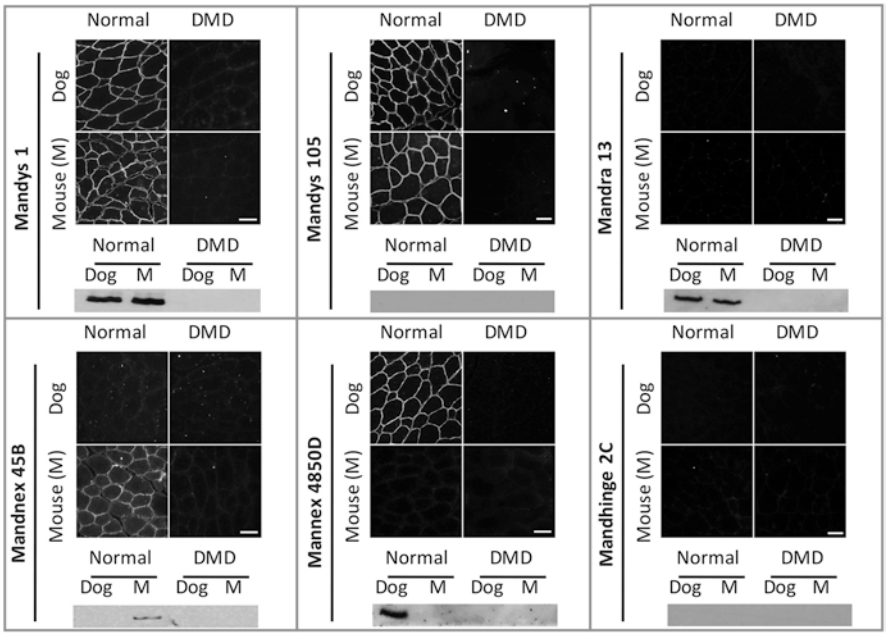

Fig. 17.1 Validation of dystrophin monoclonal antibodies in the muscle of normal and affected mice and dogs by immunostaining and Western blot. Representative photomicrographs illustrate different behaviors of different antibodies. Some (e.g., Mandys 1) can recognize dystrophin in both murine and canine muscle irrespective of detection methods. Some (e.g., Mandys 105, Mandra 13, Mandnex 45B, and Mannex 4850D) recognize dystrophin in a species- and method-dependent manner. In extreme cases (e.g., Mandhinge 2C), an antibody may completely fail to recognize dystrophin in mouse and dog muscle

[204], SelectScience (https://www.selectscience.net/antibodies/product-directory/) [204], and BenchSci (https://www.benchsci.com/).

17.3.5 Statistics

Statistical considerations for preclinical muscle gene therapy studies are not different from those used for other studies. Several recent review articles have thoroughly elaborated these considerations [205, 206]. Here I only outline some important points.

Statistical methods should be introduced at the study planning stage rather than just for data analysis. Prior to animal experiments, the sample size should be determined using the power analysis. Methods for sample size calculation can be found in publications cited here [207–211]. A number of free online programs are also available such as G*Power (https://download.cnet.com/G-Power/3000-2054_4-10647044.html) and Sample Size Calculator (http://powerandsamplesize.com/) [212].

Randomization is another important strategy to improve the stringency and reproducibility of preclinical studies [213, 214]. Depending on the study design, randomization can be achieved with a variety of methods such as simple randomization, block randomization, stratified randomization, and covariate adaptive randomization [215]. Similar to the power analysis used for sample size calculation, several online programs such as Randomization (http://www.randomization.com/) and Research Randomizer (https://www.randomizer.org/) are available for randomization.

The *t*-test and analysis of variance (ANOVA) are commonly used statistic methods to compare the mean value between two groups and among multiple groups, respectively [216, 217]. It should be noted that these methods are only applicable to parametric data. When the sample size is small, data may not always follow normal distribution. Most animal studies have a sample size of $\leq$10 in each experiment group. Therefore it is important to check if the data is parametric before applying the *t*-test or ANOVA. Sometimes skewed data can be converted to parametric by logarithm transformation. If data are not parametric or if there are outliers, nonparametric tests should be used. The Wilcoxon rank-sum test (also called the Mann–Whitney test) and the Wilcoxon signed-rank test are nonparametric tests for two non-paired and paired group comparison, respectively. The Kruskal–Wallis test is the nonparametric test for multiple group comparison [218]. Very often, multiple outcomes are measured in the same study, or a single outcome is measured repeatedly in a longitudinal study. In the former, the Bonferroni adjustment should be used. In the latter, mixed models should be considered. In these cases, it is always advisable to consult with a statistician.

The *p* value is perhaps the most commonly used but also the most controversial and misused statistical concept [219–223]. In statistical analysis, one usually concerns two types of errors, false positive (type I error) and false negative (type II error). The *p* value quantifies the probability of making a type I error, i.e., the chance of rejecting a null hypothesis that is actually true. The smaller the *p* value, the smaller the chance of the results being false positive. The *p* value does not reflect clinical significance. Interpretation on clinical implications of a statistically significant finding requires input from clinicians and experts in the related field. For example, in hemophilia B gene therapy, $\geq$5% factor IX activity is required for a clinically meaningful improvement. A candidate gene therapy may result in a statistically significant increase of 0.5% activity (e.g., from 1% activity to 1.5% activity). Although this is statistically significant, we cannot consider this gene therapy successful because the improvement is not therapeutically relevant. The *p* value should always be considered with the type II error or power (1-type II error). A *p* value of larger than 0.05 does not necessarily suggest a therapy is not effective. The 0.05 threshold of the *p* value is only based on a somewhat arbitrary convention. A *p* value of >0.05 can also be caused by a small sample size or a lack of statistical power. The power reflects the probability of making (or avoiding) a type II error. The higher the power, the lesser the likelihood of the result being a false negative.

17.3.6 Data Interpretation

Misinterpretation and over-interpretation of animal study results contribute to the poor translation of preclinical research findings to clinical benefits [224–226]. Below I illustrate some commonly encountered issues in preclinical muscle gene therapy studies.

The creatine kinase (CK) level is a commonly used blood biomarker for muscle injury. The enzyme is released from muscle to the circulation when muscle is injured. The reduction in the CK level is often considered as an indication of muscle protection by the candidate therapy. However, the CK level can be skewed by a number of factors such as the method used in blood collection, body condition and age of the animal, and the genetic background of the animal [227–230]. For example, the CK level can be artificially raised because of dehydration or inappropriate handling of the animal at the time of blood collection [231]. It should also be noted that the CK level will go down with age in dystrophic subjects even in the absence of any therapy (likely due to the replacement of muscle by fibrofatty tissue) [227].

The context of the experimental setting (such as the strain, age, and sex of the animal or detection method) should be carefully considered in data interpretation. For example, optical imaging of cathepsin activity in live mice is an excellent tool to sensitively monitor muscle inflammation in a noninvasive way [232]. The value in mdx limb muscle is two- to threefold higher than that of aged-matched BL10 mice at 7 weeks. However, the difference between two strains becomes negligible at 52 weeks although mdx mice still have muscle inflammation [233]. Conclusions drawn without the consideration of the mouse age will be misleading in this example. In dystrophin restoration gene therapy, an increase in the percentage of the dystrophin level may have different meanings depending on the detection method (Western blot or immunostaining). Western blot reflects total dystrophin in the whole muscle lysate, while immunostaining reflects dystrophin expression in individual myofibers. A moderate increase detected by Western blot could suggest a uniform low-level increase in all myofibers or a high-level increase in a subset of myofibers (mosaic expression).

Muscle histology is essential for the evaluation of the therapeutic effect. However, there could be significant individual variations among strain-, age-, and sex-matched animals (Fig. 17.2a). Further, since muscle damage is not uniform in mdx mice, there are often huge differences in different regions of the same section from the same muscle (Fig. 17.2a). Correct interpretation can only be obtained from a thorough evaluation of the entire muscle section from all experimental animals.

Degeneration and regeneration are characteristic features in muscular dystrophy. This is frequently evaluated by quantifying the percentage of centrally localized nuclei (% CN). A reduction in the % CN after gene therapy is often considered as an indication of improved muscle protection. In other words, the candidate gene therapy is effective in treating muscle disease. However, this may not always hold true because the % CN will also decrease if the candidate therapy negatively impacts muscle regeneration (a detrimental effect).

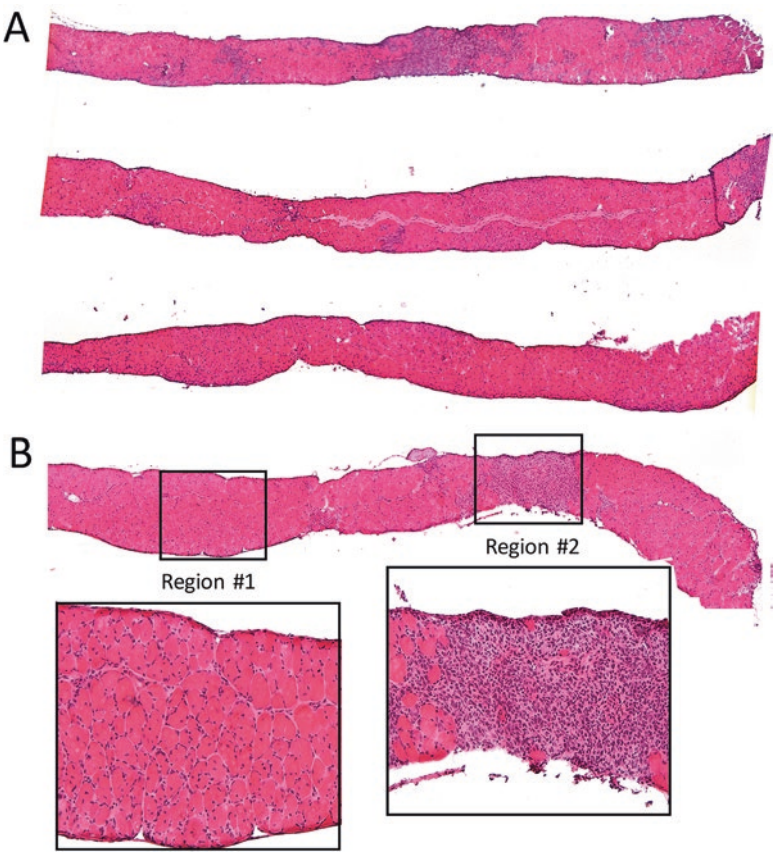

Fig. 17.2 Heterogeneity of the pathology in dystrophic muscle. (**a**) Diaphragm HE staining from three age- and sex-matched untreated young adult mdx mice. (**b**) HE staining revealing dramatic differences of muscle pathology in different regions of the same muscle section in the diaphragm of a young adult mdx mouse

A commonly held misconception by inexperienced researchers is that "muscle histology should always correlate with muscle function (and vice versa)." This might be true in some occasions, but there are many exceptions. For example, removing collagen VI from γ-sarcoglycan-deficient mice (a model for limb-girdle muscular dystrophy 2C) attenuates muscle pathology, but muscle force is not improved [234]. Marginal level dystrophin expression improves skeletal muscle function, heart function, and survival in mouse DMD models, but it does not improve muscle histology [60–62].

17.4 Phase III: A New Concept in Preclinical Study

Clinical studies are divided into different phases. Phase I evaluates the safety. Phase II establishes the efficacy. Phase III confirms the safety and efficacy in a large group of patients through a multicenter randomized controlled trial (RCT). Phase IV is post-marketing surveillance. The FDA makes the decision on whether a candidate drug should be approved or not based on the results from the first three phases of the trial (since a go no-go decision has to be made after each phase, the final FDA decision is usually based on the results of the phase III trial). To improve translation from animal studies to human trials, a similar paradigm was proposed recently to divide preclinical studies into four phases [235, 236]. Preclinical phase I is the discovery phase. At this phase, the focus is to elucidate disease mechanism(s) and to identify therapeutic targets (drug targets). Preclinical phase II refers to initial efficacy studies performed by independent (often individual) academic laboratories. Traditionally, a preclinical exploratory study will stop here and move to the stage of the IND-enabling study. It is believed that this approach has contributed to the reproducibility crisis in translational research. To address this issue, a new concept called "preclinical phase III study" is introduced. When positive results are obtained in a preclinical phase II study, the study moves to a large-scale preclinical phase III study instead of an IND-enabling study. In preclinical phase III, multicenter preclinical randomized controlled trials (pRCT) are conducted to confirm and validate the efficacy data obtained from preclinical phase II [235–238]. Preclinical phase IV is initiated based on positive findings from preclinical phase III studies. In preclinical phase IV, IND-enabling PK, PD, and toxicology studies are carried out by academia, industry, or academia/industry collaboration to pave the way for IND application.

Numerous clinical trials that were based on the efficacy data of animal studies have failed to yield a therapeutic drug [224–226]. Among many factors that have caused this translational crisis is poor reproducibility. In fact, it was suggested that more than 70% of published studies cannot be reproduced [239–242]. It is believed that the high failure rate of replication studies is due to the limited sample size (lack of power), lack of blinding and/or randomization, inadequate data report, and flaws in statistical analysis. Two approaches have been used to address this issue, systemic review/meta-analysis and pRCT [237, 243, 244]. In systemic review/meta-analysis, individual published studies are reviewed, and data from each study are pooled together and reanalyzed as a whole [244–248]. Systemic review/meta-analysis provides a less biased opinion on a candidate therapy (or drug). However, its conclusion is limited by the lack of a predefined study protocol, lack of inclusion/exclusion criteria, heterogeneity in materials and methods, inconsistence in data collection and reporting, and difference in study end points [249, 250]. This is further aggravated by publication bias in favor of positive data.

The recently introduced pRCT approach is modeled after the RCT, the gold standard in clinical trials. The pRCT utilizes the same rigorous design and standardized protocols as the RCT. The pRCT utilizes a centralized management structure. It has a study steering committee, a coordinating center, a data monitoring committee, an adjudication committee, a statistical center, and a training center. The study is designed by the statistical center in collaboration with participating laboratories. All participating laboratories use identical animal models, (strain/genetic background, age, sex, animal supplier, and husbandry), reagents (chemical and biological), standardized methodologies, key equipment, and animal protocols. All participating researchers receive centralized training on procedures (data generation, blood/tissue collection and transportation). All participating researchers agree to every aspect of the study design such as blinding, randomization, placebo control, concealment of allocation, and sharing of the original data. Data generated in all participating laboratories are sent to the statistical center for data analysis. Inclusion and exclusion criteria for animals and data points are determined prior to the study. All participating researchers agree on publication (even if results are negative) [236, 237]. The pRCT addresses a number of issues that have contributed to the reproducibility crisis such as the small sample size, inconsistent data handling, publication pressure and bias, and deficiency in methodology reporting. However, the pRCT also faces significant challenges including coordination, standardization, and financial cost. The first pRCT was published recently [251]. This study establishes feasibility of the pRCT. Despite the pRCT being considered a major advance in the field of translational research, it is yet unknown whether and how much the pRCT will improve validity of animal studies and prevent unnecessary (or even harmful) clinical trials in the future. A recent publication argues that excessive standardization in animal studies may undermine translation from bench to bedside due to ignorance of inherent biological variations in animals and humans [252].

Preclinical muscle gene therapy is at the dawn of a rapid growing phase. Incorporating the concept and practice of the pRCT may give us a better chance of success when translating from animal studies to human trials.

17.5 Conclusion

Irreproducibility in preclinical research has placed a significant socioeconomic burden on therapeutic drug development. It is estimated that ~$28 billion is spent every year in the United State of America on preclinical studies that cannot be reproduced [253]. Concerns on the reliability of preclinical studies have sparked intense discussions in the biomedical research field. To address this critical issue, many excellent general guidelines have been published in recent years on how to rigorously design a preclinical study to improve reproducibility and enhance predictive value for clinical trials [188, 254–258]. These papers have thoroughly discussed fundamental principles, commonly encountered problems, and solutions regarding sample size determination, randomization, blinding, data handling, statistical analysis,

reporting, biological variables, standard operating protocols in outcome measurement, and validation/authentication of experimental reagents [205, 259–266]. These guidelines should always be strictly followed in a preclinical muscle gene therapy study.

According to the gene table of neuromuscular disorders (www.musclegenetable.fr/), nearly 500 disease genes have been identified for ~900 neuromuscular diseases [267]. It is conceivable that many of these diseases are eligible for muscle gene therapy. The considerations discussed in this chapter may serve as a starting point to shape the design of a preclinical gene therapy study for treating these neuromuscular diseases. However, it should be pointed out that every preclinical muscle gene therapy study has its unique features. There is no one-size-fits-all. Disease-specific issues should always be considered in the design and implementation of a preclinical muscle gene therapy study. There are numerous examples where costly clinical trials have failed despite promising efficacy data in animal models. In light of this, perhaps it is more important to carefully design and rigorously conduct preclinical studies to exclude ineffective candidate gene therapy drugs from going to more expensive and potentially detrimental human trials.

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Chapter 18
Gene Replacement Therapy for Duchenne Muscular Dystrophy

Katrin Hollinger, Julie M. Crudele, and Jeffrey S. Chamberlain

Abstract Duchenne muscular dystrophy (DMD) is a fatal, X-linked disease caused by mutations in the massive dystrophin gene that lead to extremely low or non-detectable levels of dystrophin. Conversely, Becker muscular dystrophy (BMD) is a highly variable and significantly less severe disease that results from truncated or poorly expressed dystrophin variants. Based on the insights from BMD patient mutations and knowledge of the working domains of dystrophin, various miniaturized mini- and micro-dystrophin constructs have been developed for gene therapy and tested in preclinical animal models. Much of the central rod domain can be deleted with minimal loss of function, provided that spectrin-like repeats 16 and 17, which contain the neuronal nitric oxide synthase localization domain, are maintained. The N-terminal actin-binding domain and the C-terminal dystroglycan-binding domain (covering parts of "hinge 4" and the cysteine-rich domain) provide important functions and stability, while the function of the C-terminal domain appears redundant. While a range of viral vectors expressing these miniaturized genes have been utilized for DMD gene therapy, the recent focus has been on recombinant adeno-associated viral vectors (rAAV), which have now been tested extensively in *mdx* mouse and DMD dog models, and have own entered clinical trials. These vectors have shown significant improvement in the DMD pathology of mice and dogs, although complete correction has yet to be attained. Gene editing through exon-skipping oligonucleotides and CRISPR/Cas9 is also being developed, with varying success and a sense that both technologies are still in their infancy. While promising rAAV clinical trials have begun, there is still work to be done to advance the field of gene replacement for DMD.

Keywords Micro-dystrophin · Mini-dystrophin · Dystrophin · AAV · Duchenne muscular dystrophy · DMD · BMD

Authors Katrin Hollinger and Julie M. Crudele have contributed equally for this chapter.

K. Hollinger · J. M. Crudele · J. S. Chamberlain (✉)
Department of Neurology, Senator Paul D. Wellstone Muscular Dystrophy Research Center, University of Washington, Seattle, WA, USA
e-mail: katrin.hollinger@gmail.com; julie.crudele@gmail.com; jsc5@uw.edu

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18.1 Introduction

Duchenne muscular dystrophy (DMD) is the most common fatal, genetic, childhood disease, affecting 1:5000 newborn boys. It is caused by mutations in the X-linked dystrophin gene that lead to a complete or near complete absence of the dystrophin protein. DMD leads to progressive muscle damage resulting in wheelchair dependency around the ages of 8–13 and ultimately death due to respiratory or cardiac failure, usually in the third decade of life [1, 2]. Conversely, Becker muscular dystrophy (BMD) is a milder disease caused by mutations in the same gene that generally result in 5–20% levels of full-length dystrophin or by expression of an altered dystrophin protein, usually the result of an internal deletion. BMD disease severity varies widely, ranging from patients with almost no motor impairment to those with impairments similar to that of DMD.

The dystrophin gene is 2.2 megabases in length, has a cDNA of 14 kb, and contains 79 exons (not including 6 additional non-muscle first exons), making it one of the largest genes in nature [3]. Full-length dystrophin is expressed primarily in the muscle, explaining the severe muscle pathology involved in DMD. The dystrophin gene has seven different promoters, which, together with alternative splicing, lead to several isoforms of different lengths in different tissues [4]. The full-length, muscle-specific dystrophin is a 427 kDa protein consisting of four distinct regions: the N-terminal domain, central rod domain, cysteine-rich (CR) domain, and C-terminal (CT) domain [5]. Each domain has its own functional role. The N-terminal domain is the primary binding site for cytoskeletal F-actin [6]. The rod domain is postulated to give some flexibility to the protein through 24 spectrin-like repeats (R) and 4 hinge regions [7, 8]. There is also an additional F-actin-binding site in the rod domain that is necessary for full dystrophin function [9, 10], and more recently it has been established that spectrin-like repeats 16 and 17 in the rod domain facilitate neuronal nitric oxide synthase (nNOS) localization to the sarcolemma [11, 12]. The cysteine-rich domain together with a "WW" motif in hinge 4 participates in the interaction of dystrophin with β-dystroglycan [13]. Finally, the C-terminal domain facilitates protein-protein interactions with multiple isoforms of α-dystrobrevin and syntrophin [14, 15].

Through these linkages with F-actin, β-dystroglycan, α-dystrobrevin, and syntrophin, dystrophin nucleates the assembly of and anchors the dystrophin-glycoprotein complex (DGC), which serves to connect the intracellular and extracellular environments [16]. This link allows for lateral force transmission from the inside of a muscle cell to the extracellular matrix during muscle contractions as well as outside-in signal transduction [17]. Without functional dystrophin protein, as in DMD, the DGC will not assemble [18] leading to loss of its signaling function and inadequate force transduction.

Interestingly, some BMD patients with mild disease progression have substantial deletions in the dystrophin gene [19, 20], resulting in proteins as small as half the size of full-length dystrophin. These findings suggested that "mini-" and "micro-"

versions of the dystrophin gene—developed based on BMD patients' truncated dystrophins and the known functions of the dystrophin domains—can be utilized in gene therapy applications to ameliorate a DMD phenotype, converting severe DMD patients into mild BMD patients. This is especially relevant for recombinant adeno-associated viral vector (rAAV)-based gene therapy, the first gene therapy for DMD to reach the clinic.

18.2 Mini-dystrophin Constructs

The first mini-dystrophin used in preclinical studies was discovered in a family of BMD patients with very mild disease, including an ambulatory 61-year-old [20]. The patients' dystrophin gene had a central rod domain deletion of exons 17–48 (5106 bp of coding sequence corresponding to spectrin-like repeats 3–19 and the second hinge), which maintained the open reading frame and reduced the dystrophin mRNA to approximately 8.8 kb. Removal of most of the 2.7 kb 3′ untranslated region of the mRNA enabled generation of 6.3 kb synthetic cDNAs that were based on this "BMD" gene [21]. Extensive preclinical studies utilizing this mini-dystrophin were conducted in *mdx* mice. Direct intramuscular injection of plasmids encoding the mini-dystrophin gene led to expression in approximately 1% of myofibers and reduced the number of centrally located nuclei [22]. Intramuscular injection of retroviruses with the same transgene was associated with expression in approximately 6% of myofibers and reconstitution of components of the DGC [23]. Adenoviral vectors were also used, leading to protection against degeneration from natural progression [24] and stretch-induced damage during tetanic contractions [25] while also increasing muscle force generation compared to untreated mice [26]. Transgenic *mdx* mice expressing the patient mini-dystrophin at near-normal levels had almost complete phenotypic rescue, similar to that of the full-length protein [21, 27], while those expressing only 20–30% of normal levels still had significantly corrected phenotypes [28], an especially encouraging result for gene therapy prospects.

While the patient-derived mini-gene had demonstrated that much of the central rod domain was expendable, additional transgenic *mdx* lines were developed to interrogate the necessity of the N- and C-terminal regions. Guided by the fact that the main dystrophin isoform in the brain lacks exons 71–74 (encoding the major syntrophin-binding domain), a transgenic mouse expressing the murine mini-gene in muscle was developed [29]. These mice had normal muscle physiology, morphology, and expression and localization of the DGC with no signs of muscular dystrophy. Further studies showed that the dystrobrevin-binding domains (exons 75–78) could also be deleted with no loss of function [30], and the entire C-terminal domain was shown to be redundant with other portions of the DGC [31]. Thus, the CT domain can be removed from therapeutic constructs, reducing the overall size by 819 bps [31, 32]. While deletion of the C-terminal domain was not detrimental, deletion of the cysteine-rich region resulted in severely dystrophic muscles [30];

this latter region has therefore been included in all preclinical iterations of mini- and micro-dystrophins. Transgenic *mdx* mice expressing a dystrophin without amino acids 45–273 (encoding 2 of the 3 actin-binding sites and part of hinge 1, similar to a BMD exon 3–7 deletion) had greatly reduced pathology provided the expression levels were near normal [33]. However, N-terminal domain deletions are associated with reduced protein levels in patients [34], likely due to protein instability, and at low levels of expression, the transgenic mice exhibited severe disease. Therefore, the N-terminal actin-binding domain (ABD1) has likewise been a standard inclusion in mini- and micro-dystrophins.

18.3 AAV Micro-dystrophin Constructs

While some vectors derived from viruses, such as retroviruses, lentiviruses, and adenoviruses, can accommodate the large size of mini-dystrophins, each has its drawbacks. Meanwhile, one of the most promising viral vectors, rAAV, has its own challenge: a packaging capacity of approximately 5 kb, which must include the transgene as well as promoter, regulatory, and polyadenylation sequences. Thus, labs began developing micro-dystrophins, demonstrating their feasibility through improved pathology and force transduction in animal models.

An early micro-dystrophin construct, ΔR3−R21 + H3/ΔCT, also called 3990, contains the ABD1; 5 spectrin-like repeat; hinges 1, 3, and 4; and CR domains [35]. rAAV delivery of 3990 led to dystrophin expression that protected myofiber membranes from Evans blue dye infiltration in *mdx* mice. Unfortunately, in an rAAV clinical trial delivering 3990 micro-dystrophin to DMD patients by an intramuscular injection, dystrophin expression was detected in only 2 out of 6 patients, with only 1–3 positive fibers detected in those patients' biopsies [36]. An immune response to dystrophin epitopes may have eliminated transgene expression. As was covered in a previous chapter, cytotoxic T-cell responses to both the transgene and the viral vector can result in loss of transgene expression following AAV gene therapy [37, 38].

One of the first constructs developed by our lab was ΔR4−R23/ΔCT, also called hinge 2-micro-dystrophin (H2-μDys), which contains the ABD1; 4 spectrin-like repeat; hinges 1, 2, and 4; and CR domains [32]. This construct led to functional and morphological improvements in *mdx* mice and canine models [32, 39, 40], including in a large-scale canine study by Genethon meant to pave the way to the clinic [41]. However, it was later discovered that the inclusion of hinge 2 leads to ringbinden and smaller muscle fibers in a subset of muscles [42]. Exchanging hinge 2 for hinge 3, which is less proline rich, resolved these issues. The resulting construct, called hinge 3-micro-dystrophin (ΔH2-R23 + H3/ΔCT; H3-μDys), significantly enhanced muscle fiber size, maintenance of neuromuscular junctions, and protection against muscle degeneration compared to that of hinge 2 micro-dystrophin in mice [42].

After the discovery that spectrin-like repeats 16 and 17 anchor nNOS to the DGC [12], many newly developed constructs included R16 and R17. The 5 spectrin-like repeat construct ΔR3–15/ΔR18–23/ΔCT, which included the nNOS-binding domain in addition to the ABD1, hinges 1 and 4, and CR domain, was able to successfully localize nNOS to the sarcolemma in dystrophic mice [11]. A smaller 4 spectrin-like repeat construct ΔR2–15/R18–19/R20–23/ΔCT, also containing the nNOS-binding domain, was systemically transduced in DMD dogs [43], resulting in body-wide dystrophin expression and reduced histopathology. Localization of nNOS was unfortunately not evaluated. Finally, the newest construct from our lab also contains nNOS-localizing repeats 16–17: ΔR2–15/Δ18–22/ΔCT, also called μDys5, contains the ABD1, 5 spectrin-like repeats, hinges 1 and 4, and CR domains [44]. μDys5 was able to localize nNOS and improve muscle function in *mdx* mice [44, 45]. It has also been used extensively in preclinical studies in numerous DMD dogs [46, 47] in conjunction with Solid Biosciences, who is currently evaluating it in the clinic. Examples of some of these micro-dystrophin constructs are illustrated in Fig. 18.1.

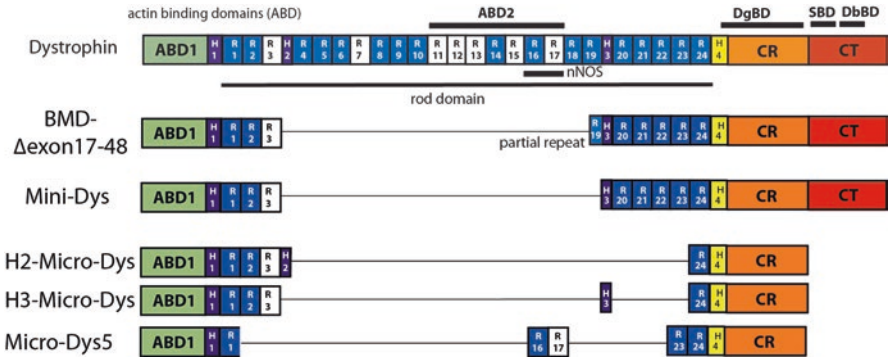

Fig. 18.1 Structure of full-length dystrophin and various mini- and micro-versions of dystrophin. "Dystrophin" is the structure of the full-length protein. BMD-Δexons17–48 illustrate the structure of the protein made from the exon 17–48 deletion in a patient with a mild case of BMD [20]. Note that R19 is encoded by both exons 48 and 49, such that this dystrophin carries a partial R19. Dystrophins with partial spectrin-like repeats can have reduced function [32]. Mini-Dys is a synthetic construct derived from the exon 17–48 deletion but lacking all of R19, improving overall stability and function [32]. Also shown are three different micro-dystrophins. Abbreviations are *H* hinge, *R* spectrin-like repeat, *ABD* actin-binding domain, *CR* cysteine-rich domain, *CT* C-terminal domain, *nNOS* neuronal nitric oxide synthase localization domain, *DgBD* dystroglycan-binding domain, *SBD* syntrophin-binding domain, *DbBD* dystrobrevin-binding domain

18.4 Delivery of Full-Length Dystrophin

Ideally larger, even full-length dystrophins would be delivered to dystrophic muscle. To address that, multiple AAVs have been administered simultaneously to create a larger dystrophin gene via homologous recombination or trans-splicing (e.g., Fig. 18.2) [48–51]. In all studies, longer transcripts, including full-length dystrophin [50, 51], were observed. However, efficacy was limited due to the requirement that multiple vectors must co-transduce each cell and then successfully recombine. While the original 6.3 kb "BMD" mini-dystrophin was made at about 90% of the levels of μDys, the full-length protein was produced at levels around 5% of normal.

An alternative way to deliver full-length dystrophin is through the utilization of larger vectors. In proof of principal studies, both lentiviral vectors and foamy viral vectors have been tested. Both systems were able to deliver full-length dystrophin to patient-derived cells in vitro. However, the large size of the cDNA, which is approaching the upper packaging limit of both vectors, leads to markedly reduced vector production titers [52, 53]. In contrast, "gutted" adenoviral vectors (gAd) achieved higher titers and were able to deliver full-length dystrophin to *mdx* mice via intramuscular injection; unfortunately, these gAd vectors have not been adapted for systemic delivery due to high uptake by the liver and residual immunogenicity [54].

Editing of the mutant dystrophin DNA and RNA can also lead to the production of near-full-length dystrophin. One such example is exon skipping, where antisense oligonucleotides are continuously administered to induce alternative splicing and restoration of the open reading frame in patients with frameshift mutations. To avoid frequent readministration, AAV could be used to deliver the antisense oligonucleotides; this approach was successfully tested in DMD dogs

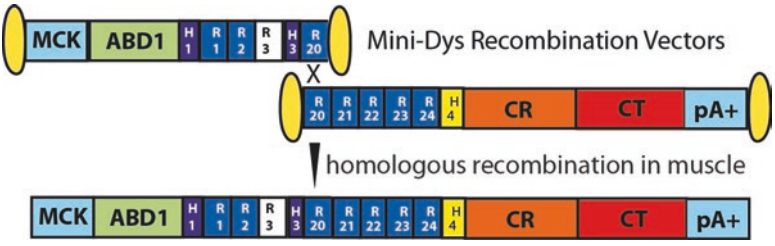

Fig. 18.2 Generating mini-dystrophins by a dual rAAV delivery strategy. In this example, two rAAV (top) that each carries one half of a mini-dystrophin gene are delivered to the muscles via systemic infusion. After the single-stranded vector DNA is released from the rAAV particle inside of a muscle cell, the two half-dystrophin genes can undergo homologous recombination via a small region of shared sequence identity (here the sequence around spectrin repeat 20 is used) to generate a larger piece of DNA carrying the entire mini-dystrophin gene (bottom). The various dystrophin domains are as described in the legend to Fig. 18.1. Yellow ovals represent the AAV inverted terminal repeats (ITRs); MCK, mouse muscle creatine kinase enhancer plus promoter element; pA+, polyadenylation signal. Adapted from [49]

[55]. In an alternative approach, recent gene editing with CRISPR/Cas9 was successful in restoring near-full-length dystrophin in *mdx* and *mdx*^{4cv} mice [56–59]. The nuclease is targeted to the site of action on the DNA by guide RNAs and causes a double-stranded brake. The double-stranded brake gets fixed by nonhomologous end joining, restoring the reading frame.

Both gene-editing techniques only work for known mutations and for a limited number of patients, since the antisense oligonucleotides and the guide RNA recognize a specific site and thus restore frameshifts caused by mutations in specific exons. Therefore, new antisense oligos or guide RNAs need to be designed and tested for different mutations, but even then they will be limited in which mutations can be treated, especially in patients lacking portions of the essential dystroglycan-binding domain.

18.5 Methods of Delivery

In large animal models, vector delivery to multiple muscles had been challenging. In some earlier studies, vector delivery was localized to a limb by excluding blood flow to the rest of the animal and perfusing the vector in the limb, resulting in localized muscle transduction. A dialysis system seemed to improve local muscle transduction in nonhuman primates [60]. However, in order to achieve lifesaving and quality-of-life-improving treatment levels, systemic transduction needs to occur. In 2-month-old dogs, intravenous injection of AAV9 expressing the alkaline phosphatase reporter construct Y731F leads to body-wide gene expression [43]. The possibility of direct intravenous administration in large animals opens the door to clinical trials treating all the muscles in patients at once.

18.6 Summary and Future Direction

Since the first miniaturized dystrophins were tested in mice, the design has been continuously improved upon. Current micro-dystrophins contain an nNOS-binding site that had yet to be discovered when the first mini-dystrophin was made and show significant improvements in muscle pathology, morphology, and force transduction. At the time of this writing, Solid Biosciences, Sarepta and Pfizer are all conducting clinical trials with rAAV micro-dystrophins (μDys5 for the former and variations of H2-μDys for the two latter). However, even with the advances made over the previous decades, the micro-dystrophins currently in use do not completely restore wild-type dystrophin function, especially in the heart. At best, one would be converting DMD disease pathology to a mild BMD.

Larger dystrophins delivered with multiple rAAV vectors are less successful, mainly due to low recombination efficiency, and alternative vectors are limited by severe immune responses (adenoviral vectors), the risk of insertional mutagenesis

(retroviruses), and production limitations (lentiviruses). Gene editing with CRISPR/Cas9, while intriguing, is still in its infancy and has safety concerns that must be addressed before potential human trails. Off-target effects need to be carefully characterized, and the immunologic risk of expressing a bacterial enzyme in humans needs to be thoroughly assessed. Meanwhile, exon skipping has shown some limited success in patients; Sarepta's Exondys 51™ (eteplirsen) became the first drug ever approved to directly treat DMD. Unfortunately, skipping exon 51 with Exondys 51 can be used to treat just 13% of DMD patients.

While multiple promising clinical trials are underway, advances in gene replacement therapy for DMD must continue to be made.

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Chapter 19
Recent Advances in AON-Mediated Exon-Skipping Therapy for Duchenne Muscular Dystrophy

Xianjun Gao, Gang Han, and HaiFang Yin

Abstract The accelerated approval of *eteplirsen* (renamed *Exondys51*™) by US Food and Drug Administration (FDA) garnered renewed enthusiasm for antisense oligonucleotide (AON)-mediated exon-skipping therapies within the Duchenne muscular dystrophy (DMD) community. However, this approval is not without dissent, particularly from within the FDA committee, originating from the discordance between functional improvements seen in patients and lack of efficacy at the cellular level. Undoubtedly, improvements in exon-skipping efficiency and delivery of AONs would go a long way to quell doubts on the applicability of this approach in DMD. Several novel strategies have been developed to enhance exon-skipping efficiency. These include modification of the backbone chemistry of AONs (e.g. tricyclo-DNA and peptide nucleic acid) or conjugation of a peptide (e.g. cell-penetrating peptide or muscle-targeting peptide) with AON and the use of adjuvants including hexose and dantrolene. Here, we examine recent developments in these areas and discuss the likelihood of future clinical application and limitations of these approaches.

Keywords Exon skipping · Antisense oligonucleotide · Peptide · Duchenne muscular dystrophy · Adjuvant

19.1 Introduction

Duchenne muscular dystrophy (DMD) is one of the most prevalent and devastating muscular dystrophies, and it is caused by frame-disrupting deletions (65%), duplications (15%), nonsense and other mutations (20%) in the dystrophin gene [1]. To the credit and hard work of clinicians, research groups and the DMD community, particularly TREAT-NMD network, a DMD mutation database has been established and made available to the public. Detailed genetic dissection of the dystrophin gene and deepening understanding of DMD pathologies provide a cornerstone for the development of therapeutic interventions. Different modalities of

X. Gao · G. Han · H. Yin (✉)
Department of Cell Biology, Tianjin Medical University, Tianjin, China
e-mail: gaoxj_2010@tmu.edu.cn; hangang@tmu.edu.cn; haifangyin@tmu.edu.cn

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therapeutic approaches for DMD have been attempted, including gene replacement [2, 3], terminal codon read-through [4, 5], cell therapy [6–8], genomic correction with the CRISPR-Cas9 genome editing system [9–11], utrophin up-regulation [12, 13] and antisense oligonucleotide (AON)-mediated exon skipping [14–21]. After more than a decade of research, AON-mediated exon-skipping therapy has moved into the clinic with the first AON drug receiving accelerated approval from the US Food and Drug Administration (FDA) in 2016 [22, 23].

Although the approval is encouraging and brings the first ever DMD nucleic acid drug to reality, insufficient systemic delivery efficiency of PMO and lack of activity in the heart mean that there is plenty of room for improvement. This necessitates the development of more potent AON chemistries and tools to enhance AON activities. In this chapter, we focus on progress achieved for AON-mediated exon-skipping therapy in the past decade. Emphasis is placed on alternative AON chemistries such as tricyclo-(tc)-DNA [24], peptide nucleic acid (PNA) [18] and peptide-modified PMO [25–29] (Table 19.1). We also discuss the use of adjuvants, particularly hexose and dantrolene, to enhance AON efficacy and clinical safety [30, 31]. Finally, we touch on early development stage nanoscale carriers for AON delivery [32–39].

19.2 AON Chemistries Used in DMD Exon-Skipping Therapy

Since the first proof-of-concept demonstration for AON-mediated exon skipping in the mouse model of DMD, a number of different chemistries have been developed and tested. Two lead compounds including 2′-O-methyl phosphorothioated RNA (2′OMe) and phosphorodiamidate morpholino oligomer (PMO) have undergone clinical trials. The most representative 2′OMe and PMO are *PRO015* (also called *drisapersen* or *kyndrisa*) and *eteplirsen* (originally known as AVI-4658), respectively [40, 41]. PNA and tc-DNA AONs are the most promising next-generation nucleic acid backbones that are likely to improve exon-skipping activity in future clinical trials. We focus our discussion on these two chemistries in this section.

PNA uses a peptide backbone to replace the ribose-phosphate backbones in RNA and is characterized by high binding affinity to PNA, DNA and RNA, sequence specificity and protease and nuclease resistance [42–44]. PNAs are also preferentially localized to the nucleus, and they can be easily modified with peptides [45–48].

Table 19.1 Oligonucleotide and peptide nomenclature and sequence

	Sequence	Ref.
PMO	GGCCAAACCTCGGCTTACCTGAAAT	[14]
B-PMO	$(RXRRBR)_2$XB-PMO	[25]
P007-PMO	$(RXR)_4$XB-PMO	[25]
B-MSP-PMO	$(RXRRBR)_2$-ASSLNIAX-PMO	[26, 27]
Pip5e-PMO	RXRRBRRXR-ILFQY-RXRBRXRB-PMO	[29]
Pip6a-PMO	RXRRBRRXR-YQFLI-RXRBRXRB-PMO	[57, 58]

Initial assessment of PNA AONs in *DMD* cells and *mdx* mice by intramuscular injection demonstrated an activity comparable to that of PMO but much higher than that of 2′OMe AONs at the same dose [18]. The nuclease-/protease-resistant PNA AONs result in persistent exon skipping, peaking 4 weeks after single injection into the tibialis anterior (TA) muscle in *mdx* mice. This suggests that a single dosing with PNA AONs may result in relatively persistent dystrophin restoration. If this is confirmed in human patients, it will greatly reduce the frequency of dosing and hence become clinically much more appealing to patients [19]. Similar to PMO, PNA AONs were shown to be length-dependent with longer PNA AONs outperforming shorter ones; however the length of PNA AONs is limited by inefficient synthesis resulting in an exponential increase in price for each extra base added; thus there will be always a compromise between efficacy and cost [19]. Recent studies demonstrated that up to 40% of normal level of dystrophin could be induced in peripheral muscles in *mdx* mice with repeated intravenous administration of PNA AONs (20 mer) at the dose of 100 mg/kg/week for 5 weeks, without any detectable toxicity [20]. This level of dystrophin restoration is similar to what was achieved with PMO at the dose of 100 mg/kg/week for 7 weeks [16]. When PNA AONs were compared with PMO side by side, comparable systemic activities were observed under identical conditions, indicating the potential of PNA AON as an alternative chemistry candidate for treating DMD. Intriguingly, although both PMO and PNA are neutral AONs, peptides that enhanced delivery of PMO after covalent conjugation lost function when conjugated to PNA AONs [49]. The reason for this remains a mystery, but it is consistent across a number of peptides tested, so perhaps the nature of the amide bond results in some form of interaction between the PNA and peptides. A potential concern of PNA use in the clinic is that the synthesis and purification of PNA requires the presence of trifluoroacetic acid (TFA). TFA makes PNA AON solution acidic upon resuspension of the lyophilized powder.

A conformationally constrained oligonucleotide analogue, tc-DNA, was able to cross the blood-brain barrier and elicit dystrophin restoration in the brain with improved cognitive ability in *mdx* mice after repeated intravenous injections at the dose of 200 mg/kg/week for 12 weeks [24]. The cost of manufacturing tc-DNA is unclear as it is not commercially available; thus it is difficult to predict its potential cost to patients for clinical application. As the monomers are not easily available, the cost per treatment for patients would likely be much higher than the established 2′OMe and PMO backbones. However, this may be offset by lower doses required, but it is unknown if tc-DNA is effective at lower doses. The inaccessibility of this chemistry also prevents other labs from easily modifying the AON with targeting peptides and other moieties that could improve delivery and efficacy. Without a commercial manufacturer that can supply the chemistry to research labs, the progress of tc-DNA's development may be limited.

In summary, both PNA and tc-DNA appear to be promising second-generation AONs for clinical translation and are in the initial assessment stages. A thorough investigation on their efficacy and safety profiles needs to be established prior to their clinical use. Beyond these chemistries, the development of other novel backbone chemistries for DMD may result in surprising improvements in efficacy and delivery; thus this is a good direction for future studies.

19.3 AON Nanoparticles

Low systemic delivery efficiency is the biggest challenge and hurdle for clinical translation of AONs. It was assumed that the low molecular weight of AONs results in rapid renal clearance and this partially accounts for its low systemic efficacy. Complexing nucleic acids into nanoparticles can lead to a longer circulation half-life and the enhanced permeability and retention (EPR) effect. For these reasons, AON nanoparticles have been extensively applied to cancer therapeutics [50]. More recently, investigators have begun to apply this strategy to DMD AONs. Negatively charged 2′OMe AONs were complexed with copolymers of cationic poly(ethylene imine) (PEI) and non-ionic poly(ethylene glycol) (PEG) and delivered into TA muscles of *mdx* mice, resulting in sixfold more dystrophin-positive fibres compared to AON alone [33]. Buoyed by these results, PEI2K-PEG550 and PEI2K-PEG5K copolymers were conjugated to nanogold (NG) or adsorbed onto colloidal gold (CG) to improve cellular uptake. Disappointingly, no significant improvement was found [32]. In order to improve the bio-distribution of PEG-PEI-AON polyplex, Sirsi and coworkers encapsulated this polyplex into degradable polylactide-co-glycolic acid (PLGA) nanospheres and tested efficacy in TA muscles of *mdx* mice. Intramuscular administration of nanospheres loaded with PEG-PEI-AON polyplexes showed a 3.4-fold higher number of dystrophin-positive fibres than AON alone without any overt toxicity [34]. However, the efficacy of PLGA and PEG-PEI-AON polyplex was much lower than PEG-PEI2K copolymer. Approaches to improve uptake were attempted by incorporating oxidation- or hydrolysis-sensitive controlled release with block copolymers PEG polycaprolactone (PCL), PEG-polylactic acid (PLA) and inert PEG polybutadiene (BR) to form polymersomes. The rationale of this was to reduce degradation in circulation. These polymersomes were able to deliver negatively charged 2′OMe AONs but only showed marginal improvement over AON alone in vitro and in *mdx* mice by intramuscular injection [35]. These results suggest that getting AONs into muscle cells, rather than degradation of AONs in body fluids, was the bottleneck. Other nanomaterials such as cationic polymethyl methacrylate (PMMA) were used for systemic intraperitoneal delivery of 2′OMe AONs in *mdx* mice but only improved delivery slightly over AONs alone [36]. The PMMA cores of PMMA-based nanoparticles were then modified with a random copolymer shell, derived from N-isopropil-acrylamide+ (NIPAM), and reactive methacrylate-bearing cationic groups to form PMMA/N--isopropil-acrylamide+ (NIPAM) nanoparticles (ZM2). Strikingly, ZM2 could efficiently absorb 2′OMe AONs to form ZM2-AON complexes, and this significantly increased the level of dystrophin expression in peripheral muscles of *mdx* mice with some effects in the heart [37]. The effect persisted for 90 days after systemic administration of ZM2-AON complexes in *mdx* mice at the dose of 7.5 mg/kg/week for 7 weeks [38]. Based on the encouraging results, the authors attempted oral delivery for ZM2-AON complexes in *mdx* mice but failed to achieve any effect [39].

Unlike siRNAs that are prone to degradation, AONs typically are stable in the blood and do not require protection for degradation in the form of nanoparticles. Furthermore, neutral nucleic acid backbones will not be complex with cationic

polymers; thus this restricts the choice of AON chemistry. Therefore, the researchers involved in developing AONs have carefully balanced the pros and cons of the complexed AONs. While complexation improves AON uptake nonspecifically, the bigger challenge is to ensure that as much of the available AON ends up in the organs that require it, namely, the muscle and brain. Thus, the likely direction for the development of AON nanoparticles for DMD will be towards adding targeting moieties on these nanoparticles. If that can be achieved, the combination of AON and a targeted nanoparticle shell may prove to be a winning combination for DMD patients.

19.4 Peptide-Conjugated AONs

Cell-penetrating peptides (CPPs) are positively charged peptides that can penetrate the negatively charged cell membrane and thus facilitate the uptake of AONs conjugated to them [51, 52]. CPP conjugation is synthetically easy for neutral PMO and PNA, but negatively charged AONs are hard to modify, and the resultant conjugates can flocculate due to intermolecular interactions. The first CPP used to modify PMO (namely, PPMO) and tested in *mdx* mice was $(RXR)_4$ peptide (also named as P007 or R peptide, Table 19.1) [25]. Intraperitoneal injection of R-PMO in neonatal *mdx* mice at the dose of 25 mg/kg restored near-normal level of dystrophin expression in the diaphragm and peripheral muscles. Further, dystrophin restoration persisted for approximately 26 weeks. Despite these encouraging results, no dystrophin expression was detected in the heart. The inefficiency of R-PMO in restoring dystrophin expression in the heart suggests that there are endogenous barriers in the heart for this AON delivery approach. Follow-up studies from three different groups suggest that this problem may be due to the injection route. Switching from intraperitoneal injection to intravenous injection greatly enhanced exon skipping in the heart. Following a single intravenous injection of R-PMO at the dose of 25 mg/kg, investigators not only observed near-normal levels of dystrophin restoration in body-wide peripheral muscles but also found approximately 20% of normal level of dystrophin expression in the heart of *mdx* mice [25]. The same effect was also seen with B-PMO, a variant of R peptide (Table 19.1), with sustainable expression of dystrophin protein in skeletal and cardiac muscles after repeated administration at 12 mg/kg/day for 4 days [53]. Functional improvement in *mdx* heart was also demonstrated by Wu and coworkers after repeated administration of B-PMO at the dose of 30 mg/kg biweekly for 12 weeks, which is the first evidence showing exon skipping can improve cardiac function [54]. These findings suggest that CPP may have solved a nagging issue (i.e. inefficiency in the heart) plaguing AON-mediated exon-skipping therapy for DMD.

Although PPMO shows great promise in restoring dystrophin in skeletal and cardiac muscles, investigations in healthy non-human primates (rhesus monkey) showed detectable nephritic tube degeneration with 6 mg/kg B-PMO [55], which hints at the potential toxicity of PPMOs. Although CPPs are effective in penetrating cell membrane, the lack of targeting is a drawback. To confer CPPs' muscle-

targeting property and thus reduce toxicity, a new form of chimeric peptide, consisting of CPPs and muscle-targeting peptides (herein B-MSP), was conceived and assessed in *mdx* mice systemically [26]. The chimeric peptide-modified PMO (B-MSP-PMO) demonstrated greater activity and induced significantly higher level of dystrophin restoration in body-wide muscles than its counterpart (B-PMO) in *mdx* mice at the dose of 6 mg/kg/week for 3 weeks intravenously [27]. Notably, the incorporation of MSP into B peptide increased cellular uptake in muscles and reduced amounts of PMO in non-muscle tissues [56]. An interesting observation is that chimeric peptides function in a context-dependent manner with one orientation outperforming the other [56]. However, there was no dystrophin found in the heart with B-MSP-PMO at the dose of 6 mg/kg, which can possibly be attributed to the low doses used and the low affinity of MSP to the heart [26]. Subsequently, more efforts have been directed to search for more potent peptides with heart-targeting properties such as the PNA/PMO internalization peptide (Pip) [29, 57, 58]. The Pip series of peptides are characterized by a central ILFQY hydrophobic motif flanked on each side by Arg-rich domains containing only arginine, aminohexyl (X) and beta-alanine (B) residues [29]. After systematic screening and evaluation, Pip5e showed the strongest heart homing property in *mdx* mice. Much higher level of dystrophin expression was induced in the heart with Pip5e-PMO compared to B-PMO and other Pip-modified PMOs under identical conditions [59]. Interestingly, one of Pip5e variants, Pip6a containing reverse order of the hydrophobic core, presented a stronger heart-targeting property than Pip5e in *mdx* mice [57, 58]. Despite the unique heart-targeting feature conferred by the hydrophobic core, enrichment of positively charged amino acids remains a concern for the potential toxicity.

Guanidinium head groups of arginine-rich peptides are principally responsible for the uptake of PMO into cells; thus a tri-functional triazine was used as a core scaffold to assemble non-peptide dendrimers by presenting guanidinium head groups in a nonlinear pattern with a total of eight guanidine head groups at the end of each side chain. The dendrimeric octaguanidine moiety (named as Vivo porter) was coupled to PMO (Vivo-morpholino) and tested in *mdx* mice systemically [60, 61]. Effective dystrophin expression was achieved in body-wide peripheral muscles at the dose of 6 mg/kg, though negligible level of dystrophin expression was detected in the heart [60, 61].

An increasing appreciation of muscle-targeting peptides has led to identification of novel candidate peptides. M12, a novel muscle-targeting peptide identified by phage display screening, showed the ability to direct targeted delivery of PMO to muscle in *mdx* mice when conjugated with PMO [62]. Compared to MSP-PMO, significantly higher level of dystrophin expression was obtained in body-wide peripheral muscles from *mdx* mice treated with M12-PMO under identical dosing conditions (Fig. 19.1). This is the first evidence showing that muscle-targeting peptide alone is sufficient to direct targeted delivery of PMO to muscle in DMD.

Fig. 19.1 (continued) of dystrophin expression in the indicated muscle groups from normal (*C57*), untreated *mdx* and *mdx* mice treated with M12-PMO (top panel) and MSP-PMO (bottom panel). 20% and 10% C57 represent 20% and 10% of the wild-type level, respectively. α-Actinin was used as a loading control

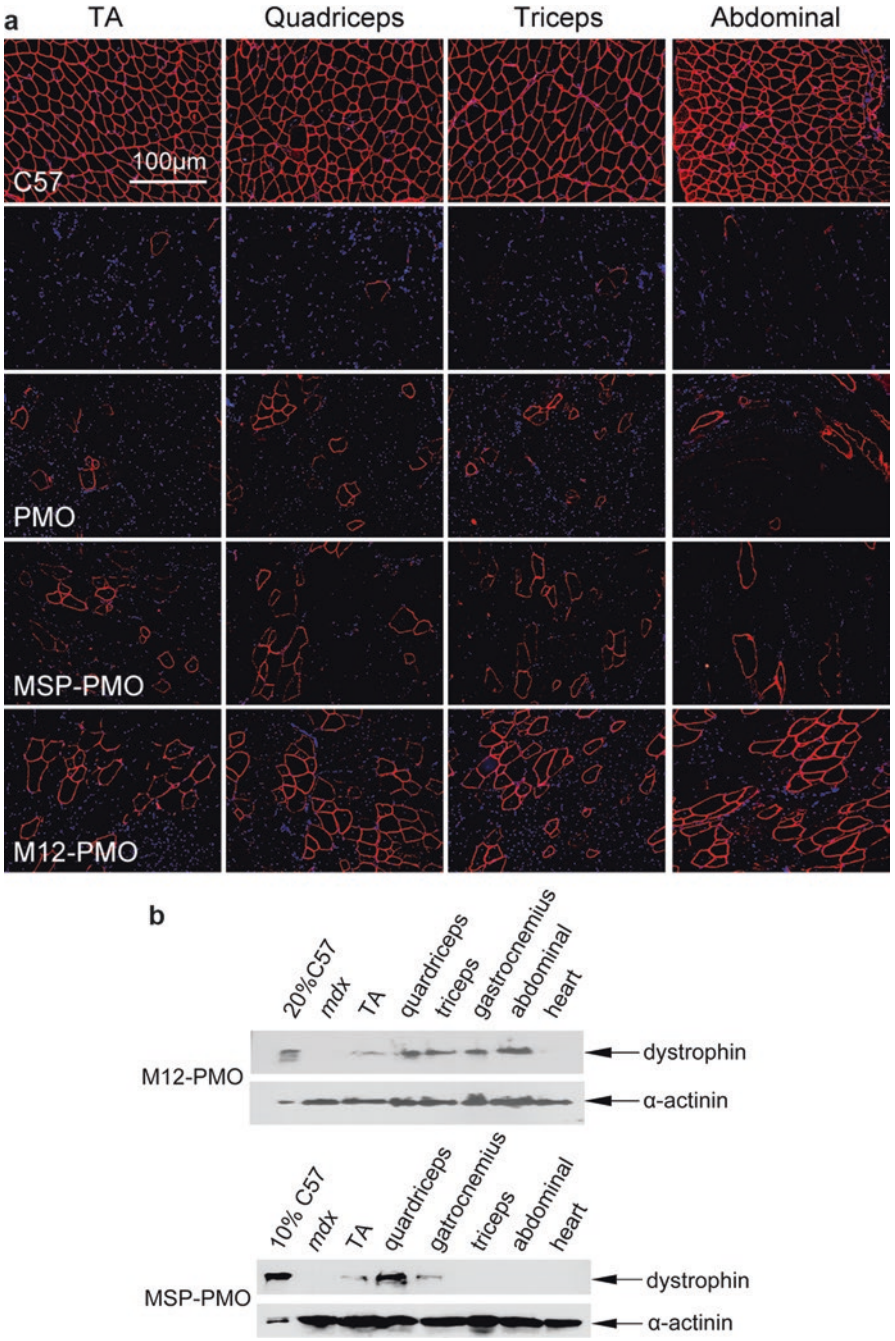

Fig. 19.1 Restoration of dystrophin expression after systemic administration of M12-PMO conjugates for 3 weeks at the dose of 25 mg/kg/week in *mdx* mice. Dystrophin expression following 3 weekly injections of M12-PMO conjugates at 25 mg/kg in adult *mdx* mice. (**a**) Immunofluorescence staining for dystrophin expression in *mdx* mice treated with the unmodified PMO, MSP-PMO and M12-PMO conjugates. Data from control normal *C57BL6* and untreated *mdx* mice are shown for comparison (scale bar = 100 μm). TA denotes the tibialis anterior muscle. (**b**) Western blot examination

Peptide conjugation presents the best avenue to improve delivery and efficiency of PMO. The increase in synthesis costs can easily offset with the lower doses required, and if targeted delivery is possible, toxicity will be significantly reduced. However, none of the studies so far have demonstrated effective delivery to the brain and heart; thus targeting further developments along these lines may yield the most clinical benefits.

19.5 Adjuvants Potentiate AON Activities

Strategies that can potentiate AON activities without the need for extensive testing that accompanies covalent modifications of the AONs would greatly accelerate clinical translation. Adjuvants for DMD are relatively under-explored with the initial attempt reported in 2003 [14]. Co-administration of pluronic copolymers F127 with 2′OMe AONs was shown to enhance its activities in the TA muscle of *mdx* mice [63]. However, subsequent systemic assessment resulted in very limited level of dystrophin expression in peripheral muscles from *mdx* mice after repeated administration of F127 with 2′OMe AONs at the dose of 100 mg/kg/week for 3 weeks [64]. Further modification was carried out on available pharmaceutical adjuvants such as modification on pluronic copolymers with PEI to form PEI-pluronic copolymers (PCMs). PCMs consisting of low molecular weight (Mw) PEI (LPEI) (Mw, 0.8–1.2 k) and a range of different Mws of pluronics were tried with hydrophilic-lipophilic balance (HLB). Examination on PCMs indicated that PCMs composed of pluronics with a Mw of 2–6 k and moderate HLB (7–23) increased PMO-mediated exon-skipping efficiency and dystrophin expression in the TA muscle of *mdx* mice [64]. An enhancement in the number of dystrophin-positive fibres was observed in peripheral muscles, but disappointingly, not in the heart from *mdx* mice systemically administered with PCMs and PMO. In contrast, polyelectrolytes (PEs) are polymers with ionizable groups, which results in ions on the polymer chain and counterions in polar solution when dissociated. A series of PEs were tested, and PE-3 and PE-4 enhanced PMO delivery and induced a fourfold higher level of exon skipping in *mdx* mice compared to PMO alone [65]. Besides PEs, a series of poly(esteramine)s (PEAs) constructed from LPEI and pluronics were evaluated for their ability to deliver AONs in *DMD* cells and in *mdx* mice, with PEA 02 showing a fourfold higher level of exon skipping than PMO formulated in Endo-Porter, a commercial delivery vehicle. Systemic investigation confirmed the enhancement effect of PEA on PMO delivery in body-wide peripheral muscles in *mdx* mice [66]. Thorough studies are warranted to verify the viability of these polymers as adjuvants for AON delivery in DMD.

Recently, high-throughput screening based on small-compound library has been actively pursued in DMD. Hu and coworkers developed a green fluorescent protein (GFP)-based reporter drug screening system and identified 6-thioguanine

(6TG) from a library consisting of 2000 bioactive compounds. Enhanced exon-skipping efficiency was observed when PMO was co-delivered with 6TG in vitro and in *mdx* mice intramuscularly, with approximately a twofold higher level of exon skipping compared to PMO alone in the TA muscle of *mdx* mice [67]. Unfortunately, the authors did not follow up with systemic administration; thus it is unknown if this compound can enhance exon skipping if administered systemically. Genevieve and coworkers made use of the same screening system with another drug library consisting of 300 compounds. A few candidate compounds were identified with dantrolene showing more appreciable clinical profiles and thus were chosen for detailed examination. Dantrolene is a compound already used for malignant hyperthermia and DMD for other purposes with few side effects reported. A synergistic effect was observed in most peripheral muscles except for triceps when dantrolene was co-administered with PMO into *mdx* mice systemically, but no improvement could be seen in the heart. Further mechanistic dissection revealed that dantrolene potentiates AONs by increasing exon-skipping frequency and the ryanodine receptor plays an important role for the functionality of dantrolene [31].

DMD was regarded as a metabolic disease before the identification of the causal dystrophin gene. Consequently, nutraceuticals (including coenzyme Q10, melatonin, green tea extract, soybeans and curcumin) have been extensively studied for DMD therapy [68]. Excitingly, Han and coworkers recently demonstrated a new concept that nutrients such as hexose can enhance the delivery of AONs to the energy-deficient muscles of *mdx* mice. Up to a tenfold higher level of dystrophin expression was achieved in body-wide peripheral muscles from *mdx* mice following co-administration of PMO in an excipient containing glucose and fructose (GF) at a 1:1 ratio, compared to PMO in saline (Fig. 19.2) [30]. GF enhanced the bioavailability of a range of oligonucleotides in muscle including AONs and siRNAs without altering their distribution patterns. Detailed mechanistic studies suggest that GF functions through cellular energy replenishment in energy-deficient muscles as evidenced by improved cellular uptake of AONs in mouse models of DMD and amyotrophic lateral sclerosis but not in normal wild-type *C57BL6* mice [69] (Fig. 19.3). Similarly, fructose alone also potentiated activities of different AONs in a context-dependent manner with the strongest synergistic effect observed with PMO. Intriguingly, although fructose potentiated the activity of PMO in the short-term use, in the long run, it failed to provide any dramatic beneficial effect on PMO uptake [59]. The mechanisms that orchestrate energy supply and enhanced PMO activity have not been fully elucidated, but evidence suggests that energy-deficient conditions in *mdx* mice and an energy-dependent pathway for PMO uptake might account for it. This finding has significant implications for AON-based exon-skipping therapy in DMD as GF has been extensively used in the clinic as infusion buffer and could be put to clinical use relatively quickly. Importantly, the enhancement effect of GF on PMO activity will significantly reduce the dose required and also ease the economical burden for patients with DMD [30].

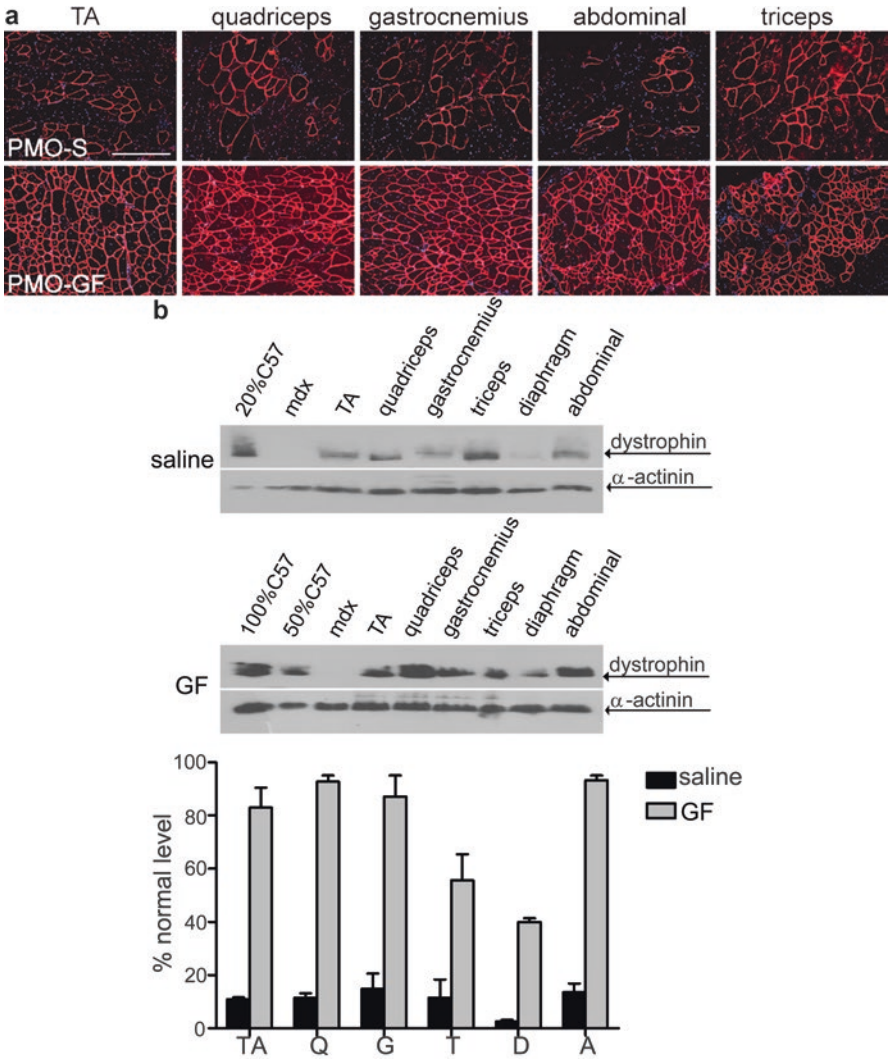

Fig. 19.2 Systemic efficacy of PMO-GF in *mdx* mice. Dystrophin restoration in *mdx* mice treated with PMO-GF at 50 mg/kg/week for 3 weeks followed by 50 mg/kg/month for 5 months intravenously. (**a**) Immunofluorescence staining for dystrophin expression in body-wide muscles from *mdx* mice treated with PMO-GF (scale bar = 200 μm). *TA* tibialis anterior. (**b**). Representative western blots show dystrophin restoration in *mdx* mice treated with PMO-S (saline) and PMO-GF (GF). 20%, 50% and 100% C57 represent 20%, 50% and 100% of the wild-type level, respectively. α-Actinin was used as a loading control. Bottom panel shows quantitative analysis of western blot results with Image J. *TA* tibialis anterior, *A* abdominal muscle, *D* diaphragm, *G* gastrocnemius, *Q* quadriceps, *T* triceps

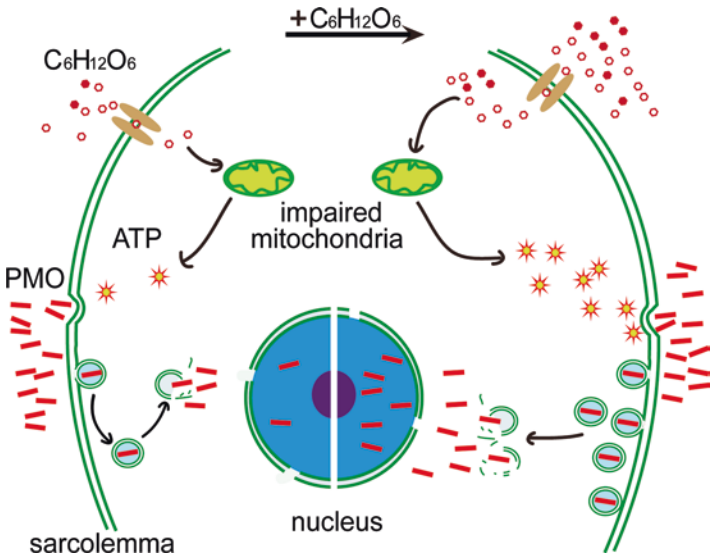

Fig. 19.3 Schematic illustration of the proposed mechanism underpinning GF-based PMO delivery. In the presence of sufficient GF ($C_6H_{12}O_6$), muscle cells can readily take up GF and thus facilitate impaired mitochondria to produce more ATP. The availability of ATP in the cytoplasm will promote PMO uptake in an energy-dependent manner

19.6 Summary and Future Direction

Overall, substantial progress has been achieved in the past decade for AON-mediated exon-skipping therapy, with the first-in-human clinical trial completed and first AON drug (*Exondys51*™) approved by FDA. *Exondys51*™ can be a good start-point, and more thorough investigations on *Exondys51*™ will provide a paradigm for other drugs under similar situations. But we have to bear in mind that the biggest challenge for *Exondys51*™ is the low systemic efficacy, particularly in the heart and brain. Currently, each approach developed for enhancing the activities of AONs faces different drawbacks, and thus a combinatorial therapy would be ideal and likely maximize the clinical benefits for exon-skipping therapy in DMD. Therefore, potent AONs developed can be tested with new clinically applicable adjuvants or novel muscle-targeting peptides. Similarly, AONs and muscle-targeting moieties can be loaded on nanoparticles simultaneously and then applied with adjuvants without covalent conjugation. Such combinations may ultimately result in a therapeutic AON product that combines low toxicity, high efficiency at a lower dose and cheap, accessible synthesis to improve the prognosis of the DMD sufferers in the clinic. Another highly promising delivery is to use exosomes. Exosomes are cell-derived nanovesicles which are taken up naturally by a large variety of cells and can be targeted. Exosomes may supplant the other delivery modes for AONs in the future.

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Chapter 20
AAV-Mediated Exon Skipping for Duchenne Muscular Dystrophy

Rachid Benchaouir and Aurelie Goyenvalle

Abstract Antisense-mediated exon skipping is one of the most promising therapeutic approaches for the treatment of Duchenne muscular dystrophy (DMD). In the past few years, this RNA-based strategy, mostly mediated by antisense oligonucleotides (AOs), has moved toward clinical evaluation, has demonstrated encouraging results, and has led the FDA to grant accelerated approval to one of these compounds recently. However significant clinical improvement in DMD patients has not been shown thus far, and AO-mediated exon skipping still faces major hurdles such as low efficacy in targeted tissues, poor cellular uptake, and relatively rapid clearance from circulation. These properties drive the need for repeated administrations in order to achieve a therapeutic response, with the negative consequence of accumulation in tissues and associated toxicity. To overcome these limitations, small nuclear RNAs (snRNAs) have been used to shuttle the antisense sequences, offering the advantage of a correct subcellular localization with pre-mRNAs and the potential for long-term correction when introduced into viral vectors such as adeno-associated virus (AAV) vectors. In this chapter, we review the development of the AAV-snRNA-mediated splicing modulation for DMD, focusing on the advantages offered by this technology over classical AOs as well as the challenges limiting their clinical application.

Keywords Exon skipping · Gene therapy · Duchenne muscular dystrophy · Small nuclear RNA · Splicing modulation · Viral vectors

R. Benchaouir
Université de Versailles St Quentin en Yvelines, U1179 INSERM/UVSQ,
UFR des Sciences de la Santé, Montigny-le-Bretonneux, France

LIA BAHN, Centre Scientifique de Monaco, Monaco, Monaco

SQY Therapeutics, UFR des Sciences de la Santé, Montigny-le-Bretonneux, France
e-mail: rachid.benchaouir@uvsq.fr

A. Goyenvalle (✉)
Université de Versailles St Quentin en Yvelines, U1179 INSERM/UVSQ,
UFR des Sciences de la Santé, Montigny-le-Bretonneux, France

LIA BAHN, Centre Scientifique de Monaco, Monaco, Monaco
e-mail: aurelie.goyenvalle@uvsq.fr

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20.1 Introduction

Splice-modulation therapy aiming at correcting genetic defects by molecular manipulation of the pre-messenger RNA (pre-mRNA) is a promising novel therapeutic approach for numerous genetic diseases. It has previously been reported that more than 15% of mutations in the Human Gene Mutation Database affect splice sites [1], but this number does not take into account mutations in other splicing elements such as splicing enhancers, silencers, or mutations affecting RNA structure known to affect splicing patterns. Current estimates suggest that as many as 50% of the human-disease-causing mutations affect splice site selection [2], which highlights the increasing number of disease candidates for these novel RNA-based strategies. Antisense-mediated splice modulation is mostly mediated by antisense oligonucleotides (AOs) and has demonstrated encouraging results for Duchenne muscular dystrophy (DMD) in particular. Synthetic AOs have indeed been used to skip exons of the dystrophin pre-mRNA in order to reframe transcripts and enable the synthesis of a slightly shorter but yet functional dystrophin protein. Two chemistries of AO targeting human dystrophin exon 51 have been evaluated in clinical trials, and one of these compounds (Eteplirsen, a phosphorodiamidate morpholino oligomer AO) has recently and surprisingly been granted an accelerated approval by the US Food and Drug Administration (FDA). However additional clinical trials have been requested to confirm the drug's clinical benefit and await further study to establish efficacy in DMD. There is widespread consensus that there is a critical need to improve current antisense tools and their delivery. This is particularly important for DMD where most muscles, including the diaphragm and heart, need to be targeted. Furthermore, repeated injections of AOs are required to maintain splice-switching effects, leading to their accumulation in tissues such as the kidney and liver and subsequent potential long-term toxicities [3].

An intuitive alternative to overcome these limitations would be a continuous production of antisense sequences in situ. This can be achieved by gene transfer of small nuclear RNAs (snRNAs) appropriately modified with specific antisense sequences. snRNAs comprise a small group of highly abundant, non-polyadenylated, noncoding transcripts that function in the nucleoplasm. They can be divided into two classes on the basis of common sequence features and protein cofactors: the Sm-class RNAs (comprised of U1, U2, U4, U4atac, U5, U7, U11, and U12) and the Lsm-class RNAs (comprised of U6 and U6atac) (for review, see [4]). Apart from U7 snRNP which functions in histone pre-mRNA 3′ processing, the other uridine-rich snRNPs form the core of the spliceosome and catalyze the removal of introns from pre-mRNA [5]. Sm class of snRNAs is transcribed by RNA Pol II, 5′ monomethylguanosine capped in the nucleus and exported to the cytoplasm for further processing. After the cytoplasmic formation of the so-called Sm core structure by the survival of motor neurons (SMN) protein complex around the conserved Sm-binding site of the spliceosomal snRNAs [6] and the hypermethylation of 5′ cap (trimethylguanosine) and 3′ trimming, the snRNP is translocated back into the nucleus for its activity.

The use of snRNA for splicing modulation offers many advantages such as the stability of the antisense sequence embedded into a snRNP particle, its specific subcellular colocalization with target pre-mRNAs, and the potential for permanent correction when introduced into viral vectors. In this chapter, we summarize the recent advances in viral vector-mediated antisense therapy using snRNAs for DMD. Following a description of the versatility of the snRNA approach, we discuss the strength and weakness of this strategy compared to synthetic oligonucleotides and conventional gene therapy.

20.2 The Emergence of the snRNA-Mediated Splicing Modulation Approach

Basic studies investigating the function of U7 snRNA have progressively led to the idea that snRNA derivatives could be used for specific antisense approaches. U7 snRNA is normally involved in the histone pre-mRNA processing through a complementary sequence to the histone downstream element (HDE) and a specific Sm core structure (Fig. 20.1). The U7 Sm-binding site recruits five Sm proteins that are also found in spliceosomal snRNPs and two specific proteins, Lsm10 and Lsm11 [7]. Studies from Daniel Schumperli's laboratory have shown that converting the noncanonical Sm-binding site of U7 snRNA (U7 Sm WT) into the consensus sequence derived from spliceosomal snRNPs (U7 SmOPT) resulted in assembly with Sm proteins D1 and D2 instead of Lsm10 and Lsm11 (Fig. 20.1). Moreover, the resulting U7 SmOPT snRNPs particles were found to accumulate more efficiently in the nucleus and could not process the histone pre-mRNA any longer [8, 9]. Based on these observations, the group suggested for the first time that U7 Sm OPT-derived RNAs equipped with antisense sequences targeting specific splice sites, with their exclusively nuclear location and their inability to cleave the target pre-mRNA, should be ideally suited to manipulate the splicing patterns of individual target genes. This hypothesis was first tested to correct the altered splicing of the β-globin gene in tissue culture model for β-thalassemia [10]. This approach was modeled on a strategy pioneered by Kole et al. where antisense oligonucleotides were used to target specific splice sites in pre-mRNAs and, hence, to redirect alternative splicing decisions (reviewed in [11]). The advantage of this type of approach was intuitive: while oligonucleotide-based redirection of splicing can be beneficial for many inherited diseases, the expression of antisense sequences from a snRNA-encoding gene such as a modified U7 SmOPT derivative could achieve more sustained or even permanent nuclear antisense effects. Initial work using the β-thalassemia model confirmed the feasibility of the U7 snRNA-mediated splicing modulation approach [10], and improved efficiency was obtained with U7 snRNA constructs carrying two tandem antisense sequences, targeting the two different splicing elements [12]. These so-called "double-target" constructs were thought to increase exon-skipping efficacy by maybe inducing a loop between the two base-pairing sites (Fig. 20.1).

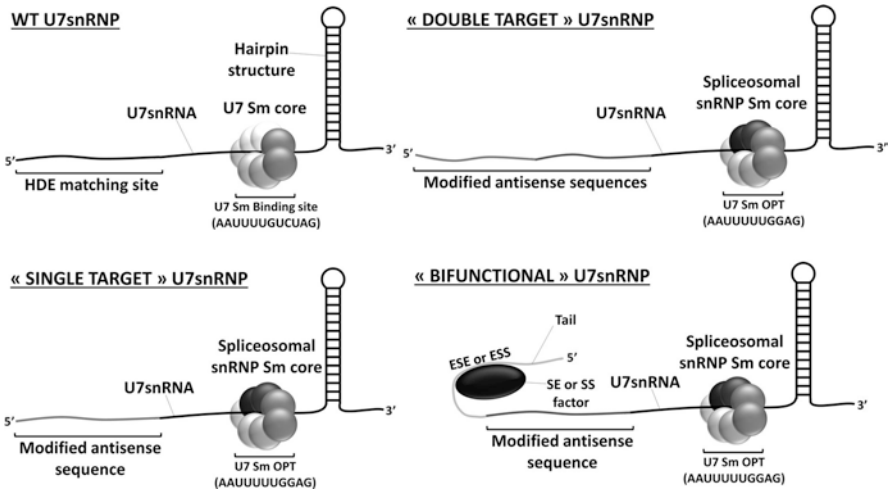

Fig. 20.1 Structure of the wild-type (WT) and modified U7 snRNA used to perform splicing modulations. WT U7 snRNA (top left) is a single-strand RNA molecule with a hairpin structure at the 3′ end and linked with a protein complex, the U7 Sm core. Altogether the U7 snRNA and the U7 Sm core form the U7 snRNA (ribonucleoprotein complex), playing a role in histone pre-mRNA processing. The Sm core of the U7 snRNA consists of seven proteins encircled around the snRNA-binding site and forming a torus structure. The 5′ part of the snRNA is complementary of the HDE (histone downstream element), sequence found in the 3′ end of the histone pre-mRNA. For splicing modulation, in particular in exon-skipping approaches, the U7 snRNA is genetically modified for a better subcellular localization (nucleus) and to avoid the cleavage of the histone pre-mRNA. To allow these properties, the U7 Sm-binding site is replaced by the consensus sequence derived from the spliceosomal snRNPs called the U7 Sm OPT. This modification leads to the replacement of two Sm proteins (represented in white in WT U7 snRNP figure), Lsm10 and Lsm 11, by D1 and D2 Sm proteins (schematized in dark gray in the three other modified structures). To specifically modify the splicing pattern of targeted pre-mRNAs, the HDE sequence is replaced by specific antisense sequences. The "single-target" snRNP (bottom left) contains only one specific complementary sequence, while the "double-target" snRNP (top right) is able to target two distinct regions. A 5′ end tail can be added to the U7 snRNA sequence to form a "bifunctional" U7 snRNA (bottom right). This structure carries exonic splicing enhancer (ESE) or silencer (ESS) sequence able to bind specific splicing enhancer (SE) or silencer (SS) factors to optimize the desired splicing modulation effect

Modified derivatives of the spliceosomal U1 snRNA have similarly been used to target nuclear splicing events. The attractiveness of U1 as a therapeutic target is due to the fact that U1 snRNA expression is sixfold higher than U7 snRNA expression per gene copy [13, 14] and because of its central role in splicing. Research however suggests that targeting U1 snRNA over the other snRNA constructs does not result in superior efficacy, perhaps due to the inability of the modified U1 to compete with the largely expressed wild-type U1 for specific binding sites [13]. Gorman and colleagues also investigated in this study the replacement of the U7 promoter by U1 promoter, which did not significantly increase the expression levels of snRNAs or the actual splicing modulation. These findings were consistent with previous work showing that the ~100-fold difference in steady-state levels between endogenous

U1 and U7 snRNAs could be accounted for by differences in functional gene copy number (~30-fold) and snRNP assembly (twofold to fourfold) [8]. Altogether, these pioneered studies established the proof of concept of the snRNP-mediated splicing modulation approach which has since then been investigated for many other disorders and DMD in particular.

20.3 Therapeutic Exon Skipping Mediated by snRNA for DMD

20.3.1 In Vitro Proof of Concept and Preclinical Studies on Mouse Models

Most mutations causing DMD disrupt the open reading frame, leading to aberrant translation and therefore to the absence of the essential muscle protein dystrophin. Interestingly, the allelic disease Becker muscular dystrophy (BMD), which results in a much milder phenotype, is mainly caused by mutations maintaining the open reading frame and allowing the production of a partially deleted but functional dystrophin [15]. This observation has led to one of the most promising therapeutic strategies for DMD which aims to convert an out-of-frame mutation into an in-frame mutation [15, 16]. This can be achieved using AOs that interfere with splice sites or regulatory elements within the exon and was first demonstrated by Pramono and colleagues in 1996 in lymphoblastoid cells and by Dunckley and colleagues in 1998 in cultured mouse cells in vitro [17, 18]. Since then, numerous in vivo studies have provided preclinical evidence for the therapeutic potential of this antisense strategy for DMD in several animal models. One model in particular, the *mdx* mouse (carrying a nonsense mutation in exon 23), is being widely used to test the efficacy of the exon-skipping approach using various oligonucleotide chemistries such as 2′OMethyl phosphorothiate (2′OMePS), phosphorodiamidate morpholino oligomers (PMO), locked nucleic acid (LNA), or tricyclo-DNA (tcDNA) [18–20]. However, despite previous encouraging clinical results obtained with some oligonucleotide chemistries (Drisapersen [14–19] and Eteplirsen [19–23]), the latest studies have shown significant limitations in terms of body-wide delivery, a particularly important point for pathologies such as DMD where most muscles, including the diaphragm and heart, need to be efficiently targeted. Moreover, in order to maintain high exon-skipping levels, repeated injections of AOs are required, leading to their progressive accumulation in tissues such as the kidney and liver and subsequent potential long-term toxicities [3]. To overcome these issues, several groups have attempted to achieve permanent in situ expression of antisense sequences using viral vectors to maintain therapeutic levels of dystrophin (Table 20.1).

Based on the pioneered work done on the β-globin gene, De Angelis and colleagues investigated the use of different snRNAs as antisense shuttles and compared U1 snRNA, U2 snRNA, and U7 snRNA derivatives targeting the dystrophin exon

Table 20.1 SnRNA-mediated splice-switching approaches for Duchenne muscular dystrophy

Authors and year	Model	U snRNA	Summary
In vitro studies			
De Angelis et al. 2002 [21]	DMD myoblasts	U1, U2, U7 snRNA	First demonstration of U snRNA-mediated exon-skipping approach for DMD in vitro (retroviral vector)
Brun et al. 2003 [22]	C2C12 and *mdx* myoblasts	U7 snRNA	Proof of principle of U7 snRNA-mediated exon 23 skipping in *mdx* myoblasts by transfection
Goyenvalle et al. 2009 [33]	DMD myoblasts and hDMD mouse	U7 snRNA	Development of bifunctional U7 snRNA for exon 51 skipping (lentiviral and AAV vectors)
Incitti et al. 2010 [60]	DMD myoblasts	U1 snRNA	Optimization of U1 snRNA constructs for exon 51 skipping (lentiviral vectors)
Goyenvalle et al. 2012 [42]	DMD myoblasts and hDMD mouse	U7 snRNA	Optimization of 11 U7 snRNA constructs for skipping of human exons and proof of principle of multiple exon skipping (lentiviral and AAV vectors)
In vivo studies			
Goyenvalle et al. 2004 [24]	*Mdx* mouse	U7 snRNA	First efficient rescue of dystrophin in vivo in *mdx* mouse using AAV-U7 snRNA
Denti et al. 2006 [23]	*Mdx* mouse	U1, U7 snRNA	Efficient exon 23 skipping in *mdx* mice after local AAV-U1 or U7 snRNA gene transfer
Denti et al. 2006 [25]	*Mdx* mouse	U1 snRNA	First body-wide gene therapy of *mdx* mouse model using AAV-U1 snRNA
Benchaouir et al. 2007 [61]	*Scid/mdx mouse*	U7 snRNA	Restoration of human dystrophin following transplantation of exon-skipping-engineered DMD patient stem cells into dystrophic mice (lentiviral vectors)
Denti et al. 2008 [26]	*Mdx* mouse	U1 snRNA	Persistent exon skipping and functional benefit observed 74 weeks after a single systemic administration of AAV-U1 snRNA
Bish et al. 2011 [62]	*GRMD* dog	U7 snRNA	Long-term restoration of cardiac dystrophin expression in GRMD model following AAV6-U7 snRNA treatment
Vulin et al. 2012 [29]	*GRMD* dog	U7 snRNA	Muscle function recovery in golden retriever muscular dystrophy after AAV1-U7 exon skipping
Goyenvalle et al. 2012 [31]	DKO mouse (dys-/utr-)	U7 snRNA	Rescue of severely affected dystrophin-/utrophin-deficient mice through scAAV-U7 snRNA-mediated exon skipping
Eckenfelder et al. 2012 [63]	C2C12 myoblasts and *mdx* mouse	U7 snRNA	The cellular processing capacity limits the amounts of chimeric U7 snRNA available for antisense delivery
Le Hir et al. 2013 [55]	DKO mouse and *GRMD* dog	U7 snRNA	AAV genome loss from dystrophic mouse muscles during AAV-U7 snRNA-mediated exon-skipping therapy

(continued)

Table 20.1 (continued)

Authors and year	Model	U snRNA	Summary
Le Guiner et al. 2014 [30]	*GRMD* dog	U7 snRNA	Forelimb treatment in a large cohort of dystrophic dogs supports delivery of a recombinant AAV for exon skipping in Duchenne patients
Peccate et al. 2016 [59]	*Mdx* mouse	U7 snRNA	Antisense pre-treatment increases gene therapy efficacy in dystrophic muscles
Gentil et al. 2016 [64]	*GRMD* dog	U7 snRNA	Dystrophin threshold level necessary for normalization of neuronal nitric oxide synthase, inducible nitric oxide synthase, and ryanodine receptor-calcium release channel type 1 nitrosylation in golden retriever muscular dystrophy dystrophinopathy

51 in vitro. Following transduction of DMD-derived cells with recombinant retroviral vectors, they demonstrated efficient skipping of exon 51 and partial rescue of dystrophin synthesis with U1 snRNA and U7 snRNA. Interestingly, the highest level of exon skipping was obtained with a U7 snRNA vector carrying two antisense sequences, which was called "double-target U7" [21]. This result was later confirmed by Brun and colleagues [22]. While these in vitro studies used stable transfection and retroviral transduction to express the chimeric snRNA continuously into the cells, snRNA systems have also been used in vivo to restore dystrophin expression in the *mdx* mouse model of DMD [23, 24]. The U7 snRNA engineered to target the exon 23 of the mouse dystrophin pre-mRNA was introduced in *mdx* muscles using adeno-associated virus (AAV) vectors which allow efficient gene transfer in skeletal muscle. A single administration of AAV-U7 resulted in persistent exon 23 skipping, leading to permanent rescue of dystrophin and muscle function [24]. Similar work using U1 derivatives also demonstrated efficient restoration of dystrophin in *mdx* muscles following AAV injections [23]. Systemic administration of AAV-snRNA was shown to induce body-wide restoration of dystrophin [25] and a lifelong beneficial effect in the *mdx* model [26] as well as in the severely affected dys-/utr- (dKO) mouse model [27].

20.3.2 Preclinical Work on Large Animal Model

The viral vector-mediated exon-skipping strategy has also been investigated in a larger animal model, the golden retriever muscular dystrophy (GRMD) dog, which presents a dystrophic phenotype very similar to DMD patients [28]. Interestingly, the GRMD mutation (a single-base change in the 3′ consensus splice site of intron 6 of the dystrophin gene) provokes inaccurate mRNA processing and exclusion of exon 7, which predicts a termination of the dystrophin reading frame within its N-terminal domain in exon 8. Due to exon phasing, both exons 6 and 8 need to be

skipped in order to restore the open reading frame of the GRMD dystrophin mRNA. Therefore, an AAV vector carrying two engineered U7 snRNA-targeting exons 6 and 8, respectively, was used to demonstrate efficient dystrophin rescue and muscle function recovery [29]. These results were later confirmed in a larger cohort of GRMD dogs following locoregional transvenous perfusion of the forelimb, supporting the concept of a future phase1/2 trial of locoregional delivery into the upper limbs of non-ambulatory DMD patients [30].

Restoration of cardiac dystrophin expression was also shown in the GRMD model following percutaneous transendocardial delivery of rAAV6 expressing a modified U7 [31]. This was accompanied by improved cardiac function as assessed by cardiac magnetic resonance imaging (MRI), suggesting this type of injection was a safe, effective method for restoration of dystrophin expression and improvement of cardiac function in the GRMD.

20.3.3 Translation to Human Dystrophin Exon Skipping and New Developments

Altogether these preclinical results have undoubtedly advanced the feasibility of exon skipping in different animal models, suggesting promising therapeutic outcomes based on AAV-snRNA. Consequently, different groups have tried to identify the most appropriate antisense sequences targeting the human dystrophin pre-mRNA that could induce the highest level of skip. For example, Incitti and colleagues have achieved skipping of exon 51 in human cells carrying deletions of exons 48–50 or 45–50 using U1 snRNA derivatives [38]. Appreciating that DMD is caused by mutations at different gene loci and that 70% are located between exons 45 and 55, our group has previously designed 11 U7 snRNAs targeting these different human dystrophin exons and demonstrated very encouraging results in DMD patient myoblasts and in a human DMD transgenic mouse model (*hDMD*) [39].

Since the optimization of the target sequences can be a long and expensive process for each snRNA derivatives, we also investigated the possibility of using a more universal system based on the previously described bifunctional AOs. It has indeed been demonstrated that tailed AOs carrying a splicing silencer sequence could induce splicing modulation even more efficiently than oligonucleotides acting through duplex formation only [32]. We therefore engineered U7 snRNA with splicing silencer motifs and demonstrated that bifunctional U7 snRNA constructs can achieve efficient exon 51 skipping in human myoblasts as well as in vivo in the *hDMD* mouse model [33] (Fig. 20.1).

Overall, these studies have not only demonstrated the feasibility of snRNA-mediated exon skipping but achieved impressive and long-lasting restoration of dystrophin, which represent crucial milestones for the clinical application of this strategy. Clinical evaluation of the AAV-snRNA-mediated exon-skipping approach is currently being planned by the French consortium sponsored by the Association Française contre les Myopathies (AFM).

20.4 Advantages and Challenges of the snRNA-Mediated Splicing Modulation

20.4.1 Advantages

The AAV-snRNA tandem confers several advantages to design a promising gene therapy approach for DMD. As previously mentioned, this pathology requires an efficient body-wide treatment to expect clinically relevant outcomes. The limited delivery and the need for repeated administration of synthetic AOs represent significant drawbacks of the classical exon-skipping strategy. The snRNA genes, including their natural promoters, are relatively short-sized expression cassettes (around 500 bp) which can easily be packaged in the recombinant AAV vector. These viral vectors offer advantageously a large panel of serotypes characterized by different tissue targeting, in particular, for the skeletal muscle as previously described [24]. The heart tropism of AAV vectors has also been well documented and is of particular interest since the cardiac physiopathology in DMD is one of the frequent causes of death [31, 34, 35]. Recently, a customized AAV variant has been positively tested for its capacity to transduce the central nervous system with high efficiency after intravenous injection [36]. The crossing of the blood-brain barrier, leading to potential correction of the DMD cognitive defects, provides a definite advantage of AAV compared to the synthetic AOs whose efficacy to enter the CNS remains very limited.

The robustness of the viral capsid also provides a protective environment for the snRNA molecules, allowing it to be delivered through the vascular system to the target cells without undergoing physical, chemical, or enzymatic degradation. Moreover, AAV vectors enable the introduction of several episomal copies of the snRNA transgene in the cell nuclei. These extrachromosomal copies allow a long-term transgene expression and avoid the periodic repeated administration required for synthetic AOs to achieve the same optimal levels of functional molecules. This characteristic is all the more advantageous when it applies to postmitotic tissues such as the principal target cells of the neuromuscular system (muscle fibers, cardiomyocytes, neurons). Additionally, embedding of the snRNA by cellular proteins to form the snRNP complexes provides their stability and more importantly their subcellular colocalization with the target pre-RNAs [9, 10]. These properties make the modulation of splicing by U7 snRNA very specific, and no toxic effect has ever been described [37]. Furthermore, the snRNA transgenes are transcribed from their natural promoter, which limits their level of expression from a quantitative point of view (endogenous levels) but also qualitatively (expression in cells having the correct transcriptional machinery). The latter advantage is used to prevent any side effects that may result from an overexpression of the protein of interest (dystrophin), which could occur in the case of gene transfer strategies using mini- or micro-dystrophins expressed from strong viral promoters. A T-cell immune response has previously been observed against dystrophin itself [38]. One of the hypotheses suggested was the production of the transgene by antigen-presenting cells that would

have been transduced by the AAV mini-dystrophin vectors. The advantage of AAV-snRNA system is its capacity to restore the protein of interest only in cells naturally expressing the mRNA, reducing the chances untoward T-cell activation through the transduction of antigen-presenting cells.

In view of the small size of snRNA constructs and their ease of cloning into AAV vectors, the idea of double or multiexon skipping has emerged to increase the number of eligible patients for gene therapy (Fig. 20.2). Indeed, the multiexon skipping of exons 45–55 would not only be applicable to a large proportion of DMD patients but would also be associated with a mild phenotype [39, 40]. While this strategy can be challenging using AOs [41], AAV vectors provide the advantage to introduce different antisense constructs (snRNA) into the same cell nucleus, making the viral approach much more appealing to achieve this strategy [42].

Implementation of a gene therapy approach for DMD by the use of AAV vectors requires modes of production compatible with clinical and industrial needs. Since at least 10^{15} viral genome particles are needed to treat a patient, new processes have supplanted the traditional research scale productions to reach these levels. Among these developments, we can mention the process using the baculovirus/insect cell

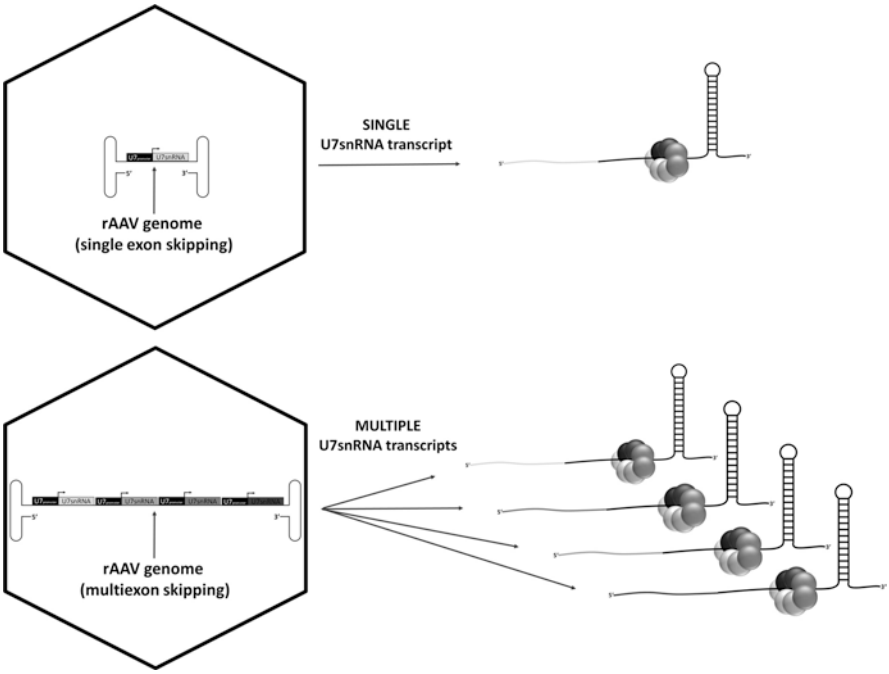

Fig. 20.2 Structure of the single-strand DNA genomes packaged in recombinant AAV (rAAV) capsids (hexagon). A single U7 snRNA molecule can be cloned and then transcribed from a single U7 snRNA gene under the control of its natural U7 promoter (top). The relatively short size of the U7 snRNA cassette authorizes the duplication of these constructions in a same and unique rAAV genome. This approach allows multiexon-skipping strategies through the expression of a different U7 snRNA from a single rAAV backbone (bottom)

system as one of the most promising methods since it has already been used in industry for the production of human recombinant proteins [43]. Efficient downstream processes are now available to be compatible with the good manufacturing practices guidelines. Other current developments are attempting to minimize the cost of processes which is also an important element in the perspective of moving this technology to industry and the clinic [44]. By comparison, the costs of AO synthesis are very high because some of these synthetic chemistries require multistage manufacture in production. Finally, it is important to note that many clinical trials are based on AAV approaches and that the first marketing approval for rAAV gene therapy has recently been delivered, calling for vector production improvements [45–47].

20.4.2 Limitations and Challenges of Viral Vector-Mediated Exon Skipping

Despite the advantages conferred by AAVs for the transfer of snRNAs, some limitations complicate their use as gene carriers. The major disadvantage concerns the incompatible relationship between the viral nature of the vectors and the immune system of the host. Two types of immune rejection are concerned. The pre-existing host immunity to viral capsids is the first barrier against AAV-mediated gene transfer [48]. Neutralizing antibodies are the main immune response that can thwart therapeutic efficacy, particularly for serotype 2 whose prevalence reaches nearly 70% of the human population [49]. To circumvent this seroprevalence, some have proposed the use of AAV variants that have a weak homology with classical serotypes [50]. Another alternative is to create chimeric AAV variants having the property to both escape antibody neutralization and improve tissue tropism [51]. Directed evolution (error-prone PCR, DNA shuffling) or rational capsid engineering is part of the most popular techniques for the development of these new vector generations with high therapeutic potential [52–54].

The second immunological barrier relates to the challenge of reinjecting the AAV vectors due to the vaccinating effect of the primary injection. Indeed, the degeneration/regeneration cycles that occur in dystrophin-deficient muscles lead to rapid loss of vector when suboptimal doses of recombinant AAV have been applied [29, 55]. For this reason, repeated injection of therapeutic vectors may lead to reinforcement of the initial rescue, allowing long-term phenotype stabilization. The problem of the vector readministration can be transiently circumvented by immune-modulation approaches. Application of transient immunosuppression has resulted in long-term expression of micro-dystrophin in naïve dystrophic dog models [56, 57]. It is possible that immunosuppression may also help readministration. Indeed, it was found that co-stimulation blockade enabled repeated AAV-1 injection in *mdx* mice [58]. However, immunosuppression poses problems of applicability when it concerns individuals already highly affected by their pathology, in particular DMD patients. As previously discussed, readministration strategies including different successive serotypes or hybrid vectors could represent an interesting alternative for a long-term muscle protection.

Alternatively, combined therapy using AOs and AAV vectors has been proposed to enhance the therapeutic efficacy of splicing modulation strategies. A recent study investigated the effect of a pre-treatment of muscle fibers with a single dose of the peptide-phosphorodiamidate morpholino (PPMO) antisense oligonucleotides. The PPMO pre-treatment, inducing temporary dystrophin expression at the sarcolemma, promoted the efficient maintenance of AAV genomes in dystrophic muscles and improved the AAV-U7 therapy benefits with an increase of the protein level sustained for at least 6 months [59].

20.5 Conclusion

SnRNA-mediated exon skipping is considerably enhanced by the use of AAV-derived vectors which enable optimized in vivo biodistribution. The numerous proofs of concepts reported in laboratory mouse models as well as in preclinical larger animal models (dogs) make it possible today to accelerate the passage of this approach toward the clinic. The low immunogenicity of AAV vectors, the diversity of serotypes and newly engineered particles available, and their capacity to be industrially processed, in addition to their recent first marketing approval, make the AAV a vector of choice for future splicing modulation-mediated gene therapy approaches. Immunological barriers should be solved by the implementation of alternative strategies consisting in transient blocking of the immune response effectors, by using variants allowing vector readministration, or by combining approaches with AAV and AOs molecules. SnRNAs represent very promising tools for the treatment of inherited diseases such as muscular dystrophies, and their upcoming clinical evaluation will be extremely informative for numerous other pathologies.

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Chapter 21
Alternate Translational Initiation of Dystrophin: A Novel Therapeutic Approach

Nicolas Wein and Kevin M. Flanigan

Abstract A founder allele in the *DMD* gene results in a syndrome ranging from minimally symptomatic Becker muscular dystrophy to asymptomatic hyperCKemia via expression of a highly functional N-terminal deleted version of the dystrophin protein (the ΔCH1 isoform). Translation of this protein results from utilization of a recently discovered internal ribosome entry site (IRES) within exon 5. The IRES is not active in the presence of a duplication of exon 2—the most common single-exon duplication—but is active in its absence. We have developed an AAV-encapsidated U7snRNA vector that targets and induces skipping of exon 2, resulting in either expression of a wild-type dystrophin or of the ΔCH1 isoform, either of which is therapeutic.

Keywords Duchenne muscular dystrophy · Actin-binding domain · Exon skipping · Internal ribosome entry site

21.1 Introduction

The *DMD* gene, comprising 79 exons, is one of the largest known genes, as it encompasses 2.22 Mb localized to chromosome Xp21. Three promoters have been described that drive expression of full-length 427 kDa dystrophin isoforms that predominate in different tissues, each differing only in the first exon: Dp427m

N. Wein
The Center for Gene Therapy, Nationwide Children's Hospital, Columbus, OH, USA

Department of Pediatrics, The Ohio State University, Columbus, OH, USA
e-mail: nicolas.wein@nationwidechildrens.org

K. M. Flanigan (✉)
The Center for Gene Therapy, Nationwide Children's Hospital, Columbus, OH, USA

Department of Pediatrics, The Ohio State University, Columbus, OH, USA

Department of Neurology, The Ohio State University, Columbus, OH, USA
e-mail: kevin.flanigan@nationwidechildrens.org

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(muscle) [1], Dp427c (cerebral) [2], and Dp427p (Purkinje cells) [3]. Altogether the locus produces at least eight different transcripts that result from the use of distinct and often tissue-specific promoters, by the mechanism of alternative splicing or by the use of different polyA tails.

The Dp427m isoform is of most relevance to the pathogenesis and treatment of muscular dystrophy. Dp427m is primarily expressed in the skeletal muscle and heart, where it forms a critical part of the dystrophin-associated glycoprotein (DAG) complex that connects the cytoskeleton of a muscle fiber to the surrounding extracellular matrix. Absence of this isoform leads to the more severe Duchenne muscular dystrophy (DMD), whereas expression of a partially functional version—detectable on Western blot as a diminished size or amount—leads to the milder Becker muscular dystrophy (BMD) [4]. Both are characterized by necrotic degeneration of muscle fibers, fatty replacement, and increased fibrosis, with the severity of pathology related to the severity of resulting muscle weakness. DMD is the more common form, affecting around 1 in 5200 boys [5], and historically led to loss of ambulation by age 12, although with the use of corticosteroids ambulation is often maintained until age 15 [6]. BMD is associated with a wide spectrum of clinical phenotypes, ranging from weakness similar to DMD but showing a delayed onset of symptoms and a slower rate of progression compared to DMD, to limb-girdle weakness presenting in late adolescence, to isolated quadriceps weakness and hyperCKemia (reviewed in [7]).

Deletions of one or more exons account for around 65% of all *DMD* mutations, exon duplications account for around 6–11%, and nonsense mutations account for around 15% [8, 9]. The effects of *DMD* mutations on phenotype can largely be predicted based on the reading frame rule, which proposes that mutations maintaining an open reading frame will lead to truncated proteins that will be at least partially functional and result in the less severe BMD phenotypes [7]. This is true for even large in-frame deletions, especially when it involves the rod domain of the protein. On the other hand, if the mutations lead to translational frameshifts, a premature termination codon occurs downstream in the new reading frame, resulting in no dystrophin protein. Based upon observations of the associated phenotypes, mutations that disrupt the reading frame but still allow the production of truncated dystrophin lacking specific domains provide important clues about the particular function and importance of such domains. For example, truncating mutations upstream of the cysteine-rich region have been shown to result in severe dystrophic phenotypes despite normal levels of dystrophin expression, thus highlighting the importance of the C-terminal part of dystrophin [10]. Despite predicting 90% of genotype-phenotype correlation, ~10% of mutations are found to escape the reading frame rule [11]. Such exceptions include nonsense-associated BMD, in which the predicted nonsense mutations affect splice suppressor or enhancer sequences, and lead to alternative splicing; in a context in which the flanking exons maintain an open reading frame (such as the entire regions between exons 23 and 42), the result may be relatively mild BMD rather than the DMD predicted by the nonsense allele [12].

21.2 The p.Trp3X Allele Results in Expression of a ΔCH1 Isoform

One such exception to the predicted reading frame rule leads to our current approach, which stems from the identification of the first North American founder allele in *DMD*, a predicted nonsense mutation with exon 1 (c.9G > A; p.Trp3X), which resulted in very mild Becker muscular dystrophy. It was first identified in several families in a cohort of 1100 dystrophinopathy patients [13], who along with other families were then shown to share a 3.7 million base pair region extending across exon 1, confirming that it was a founder allele in this population [14]. Despite the fact that a nonsense mutation should result in expression of no dystrophin, symptoms in those with the allele were minimal. Most patients had only myalgias with exertion; the most severely affected individual stopped walking at age 62, while others had no symptoms into their eighth decade [14].

We demonstrated that this mild phenotype was due to the expression of significant amounts of an isoform of dystrophin resulting from translational initiation from an AUG codon within exon 6 of *DMD* [13] (Fig. 21.1). The resulting isoform

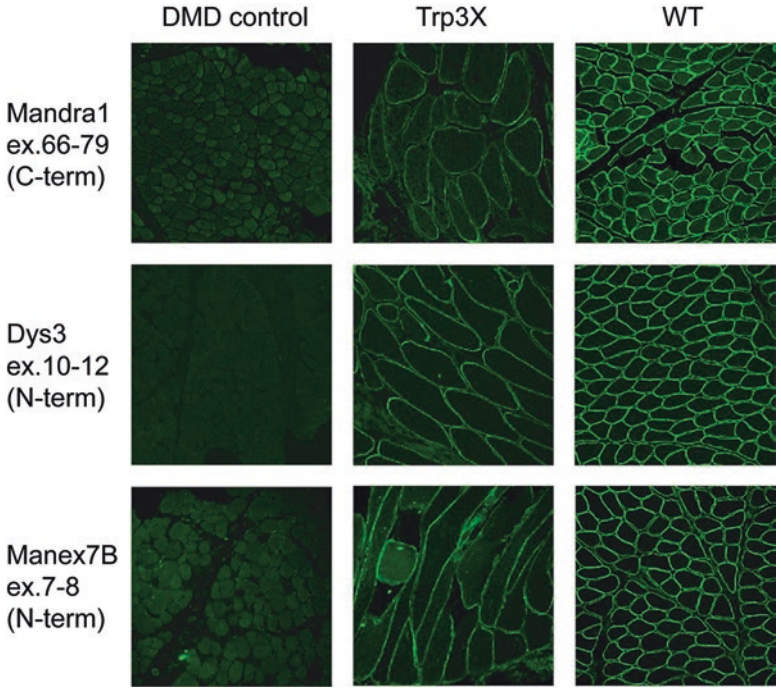

Fig. 21.1 Patients with nonsense mutations in exon 1 express an N-truncated dystrophin. Immunostaining of a muscle biopsy from a DMD patient, a Trp3X patient, and a healthy subject using three antibodies. Epitope mapping using exon-specific antibodies demonstrated that translation is initiated earlier than the exon 8 AUG codon

is approximately 412 kilodaltons (kD) in size but is expressed in significant amounts in muscle biopsies from p.Trp3X patients. Epitope mapping using exon-specific antibodies demonstrated that translation initiated earlier than the exon 8 AUG codon. This codon was invoked as an alternative initiation site to account for the variability seen in dystrophinopathy patients with deletions of exons 3–7, some of whom have typical DMD and others mild BMD [15, 16]. The absence of staining with the exon 1-specific antibody MANEX1A excluded readthrough of the putative nonsense mutation as an explanation [13]. We demonstrated instead that initiation occurs at one or both of two AUG codons within exon 6 (codons M124 and M128). The resultant protein, which we term ΔCH1, lacks the calponin homology domain 1 (CH1) that makes up the first half of the actin-binding domain 1 (ABD1) at the N-terminus of dystrophin.

21.3 The *DMD* Exon 5 Internal Ribosome Entry Site (IRES)

In our original description of the alternate translational initiation [13], we postulated that the mechanism responsible might be due to the presence of an IRES and that it might be a more generalizable feature of frame-interrupting mutations within the 5′ end of the gene. Eukaryotic translational initiation is commonly understood to occur in what is termed cap-dependent initiation, which begins with binding of the mRNA 5′-m7G cap to the cap-binding protein complex eIF4F. Cap-dependent translational initiation is considered to account for most translational initiation under physiologic conditions (for a detailed review [17]). However, several alternative translational mechanisms have been described, including upstream open reading frames, 3′ cap-independent translation elements, and internal ribosome entry sites (IRESs), which are RNA regulatory sequences that govern cap-independent translation initiation in eukaryotic cells when cap-dependent translation is compromised—i.e., during cell stress. IRESs are particularly well-defined in many viruses. For example, they drive internal initiation in all members of the *Picornaviridae* family, and among the earliest characterized was the encephalomyocarditis virus (EMCV) IRES. Eukaryotic cells use IRESs as well, and an increasing number of cellular IRESs have been identified. Many are found within the 5′ UTR of genes; however, examples exist of IRESs that are found downstream of the canonical initiation codon, resulting in the translation of proteins of diminished size. In some cases, these have been shown to be active in a pathophysiological context. For example, the presence of such an element in the *APC* coding sequence has been linked to a mild version of familial adenomatous polyposis coli in which patients with certain 5′ mutations still produce a partially functional protein through the use of a downstream initiation codon [18].

We demonstrated the presence of a dystrophin IRES by use of the dicistronic reporter construct pRDEF, which contains two luciferase genes: an upstream cistron consisting of a Renilla luciferase under the control of the SV40 promoter, signaling cap-dependent translation, and a downstream cistron consisting of firefly luciferase,

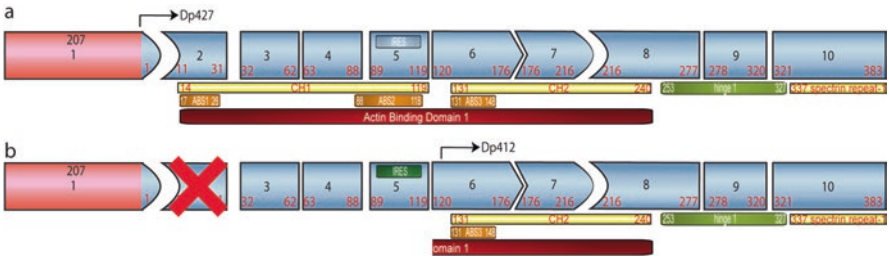

Fig. 21.2 Schematic representation of Dp427m and the IRES-driven isoform Dp412. The first ten exons of the human *DMD* gene are represented in blue. Red boxes correspond to the 5′ and 3′ UTR. Numbers above each exon indicate the length in base pairs. Red numbers correspond to the position of the first and last residue. Box below each exon corresponds to protein domains (NP_003997.1 putative domain represented by dotted box). The two start codons are represented by arrows

under the control of a sequence of interest [19, 20]. These cistrons are separated by a mutated nonfunctional defective EMCV (dEMCV) sequence that forms a secondary structure that has previously been demonstrated to prevent readthrough of the translation-competent ribosomal complex; downstream translation thus proceeds in a cap-independent fashion and can be normalized (as a transfection control) to the upstream reporter signal. Using this luciferase assay, we demonstrated that the presence of the *DMD* exon 5 coding sequence was sufficient to produce cap-independent translation, with activity of around 50% of the functional EMCV IRES, suggesting that the *DMD* IRES is relatively strong for a mammalian IRES [19]. As we excluded aberrant splicing or cryptic promoter activation, these data strongly support the presence of an IRES within *DMD* exon 5 (Fig. 21.2). Of particular interest in considering the therapeutic implication of this IRES, we demonstrated that it was glucocorticoid-responsive in a dose-dependent fashion in cell culture and importantly was active in patients with other 5′ frameshifting mutations who express the ΔCH1 isoform [21].

21.4 Therapeutic Exon 2 Skipping

Exon 2 duplications are the most common single-exon duplication associated with DMD, accounting for around 10% of all duplications [9]. We noted that duplications of exon 2 were nearly always associated with DMD, even though the duplication resulted in an altered reading frame and hence a premature termination codon in the second copy. At the same time, we noted that deletions of exon 2, which are similarly out of frame, had never been reported in either our large cohort or in the exhaustive dystrophinopathy mutational databases available [22, 23]. Assuming that such a mutation had never been ascertained because of IRES activity, we hypothesized that the IRES was active in the absence of exon 2 but not in the presence of a duplicated exon 2; consistent with this hypothesis (and the clinical

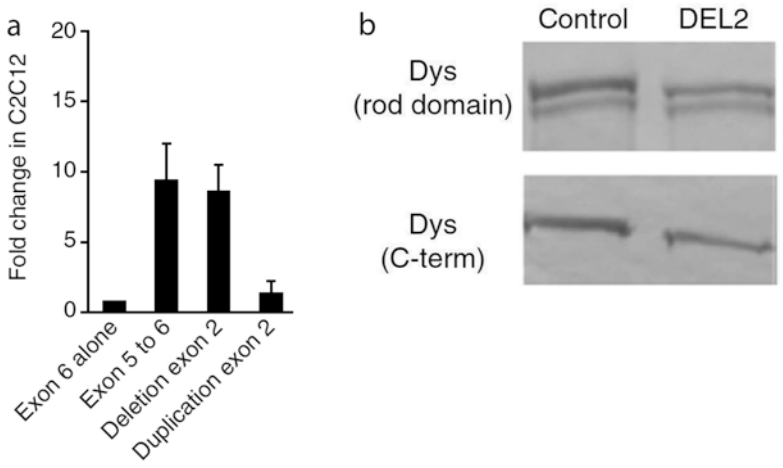

Fig. 21.3 Patient with a deletion of exon 2 expresses the IRES-driven isoform, but a patient with exon 2 duplication does not. (**a**) Dual luciferase assay demonstrating IRES activity in the presence of either a duplicated or deleted exon 2. Only absence of exon 2 allows IRES activity. (**b**) Western blot result obtained from muscle protein of a patient with a *DMD* exon 2 deletion (DEL2). A protein of smaller size is being expressed in this patient, which corresponds to the IRES-driven isoform [19]

observation), studies with the dual luciferase reporter showed this to be the case, and an essentially asymptomatic patient with a deletion of exon 2 was identified [19, 37] who expressed the same isoform (Fig. 21.3) [20]. The reason for the inactivity of the IRES in the presence of a duplicated exon 2 remains unclear, although we postulate that it may be due to secondary structure of the mRNA that precludes IRES recognition or function.

An obvious therapeutic implication of these results is that skipping of exon 2 would be expected to result in IRES activation. Furthermore, it is important to emphasize that for patients with DMD due to a duplication of exon 2, the presence of a functional IRES provides a wide margin of safety, as boys with exon 2 duplications cannot be "overtreated" to be made worse. Exon 2 skipping will result in either the wild-type transcript containing one copy of exon 2 or in the complete exclusion of exon 2, resulting in IRES activation and the expression of the ΔCH1 isoform which is highly protective. Finally, we note that among the 5′ exons, only exclusion of exon 2 results in an out-of-frame transcript. The effect of point mutations within the first 5′ exons varies, with some mutations (such as the exon 2 frameshift we reported in [20]) being associated with BMD leading to loss of ambulation in middle age. However, other 5′ mutations are more severe, such as point mutations within exons 3 and 4 resulting in a more severe BMD phenotype, with loss of ambulation in the third decade, an effect that may be due to the proximity of the premature termination codon to the IRES element, which might be predicted to affect efficiency of ribosomal reentry [24]. Similarly, some in-frame 5′ mutations (such as

a duplication of exons 3–4) result in moderately severe BMD, presumably due to protein instability. We might predict that in either such scenario, exon 2 skipping would result in increased IRES activity and increased protein translation, with a resultant amelioration of phenotype in patients.

Exon skipping is a promising therapeutic strategy for DMD. In its more common usage, as with the FDA-approved drug eteplirsen, the intent is to transform a severe DMD phenotype into a milder BMD phenotype by altering pre-mRNA splicing such that an out-of-frame deletion is changed to a larger but in-frame deletion. In the case of eteplirsen, the targeted exon is exon 51, found within the central rod domain, where in-frame deletions are relatively well-tolerated [25, 26]. Exon skipping can be produced by use of synthetically manufactured small RNA molecules, such as antisense oligonucleotides or phosphorodiamidate morpholino oligomers [27]. Alternatively, the antisense sequence can be carried by a small nuclear RNA (snRNA) and delivered using viral vectors such as adeno-associated virus (AAV). U7snRNA is normally involved in histone pre-mRNA 3′ end processing but can be converted into a versatile tool for splicing modulation [28–31]. The advantage of using U7 derivatives is that the antisense sequence is embedded into a small nuclear ribonucleoprotein (snRNP) complex, thereby protecting it from degradation and enforcing accumulation in the nucleus where splicing occurs. In contrast to the exon skipping approaches that are currently approved or in trials, which seek to restore an open reading frame, in the case of exon 2 skipping, we seek to induce either a wild-type transcript or an out-of-frame (del2) transcript.

In order to establish the potential clinical utility of exon 2 skipping in the activation of this IRES, we made a new mouse model of DMD carrying a duplication of the mouse *dmd* exon 2 [32]. This mouse (the Dup2 mouse) essentially mimics the phenotypic features of the standard *mdx* model (which contains a nonsense mutation in exon 23) [32] and provides a platform for testing skipping therapies. This model is complemented by studies in myofibroblasts, which are immortalized fibroblasts derived from duplication exon 2 patients and infected with a tetracycline-inducible MyoD construct, induction of which results in transdifferentiation into myoblasts that can be further differentiated into myotubes, allowing studies of *DMD* mRNA [29].

Our U7snRNA vector includes four copies of U7snRNA into a single AAV plasmid, which we term U7-ACCA. Two copies each contain sequence tails targeting either the exon 2 splice donor or acceptor sites, and each represents an individual cistron. Initial experiments using AAV1 delivery of the U7-ACCA system during in vitro differentiation to myofibroblasts resulted in significant expression of the IRES-initiated isoform by day 14. Delivery of AAV1.U7-ACCA to the tibialis anterior muscle of Dup 2 mice via intramuscular (IM) injection leads to highly efficient (~90%) exon 2 skipping at the mRNA level [30] (Fig. 21.4). Importantly, it leads to robust production of a protein that lacks the CH1 domain but nevertheless appropriately localizes to the sarcolemmal membrane, restores components of the dystrophin complex, and corrects the pathologic and physiologic defects of the TA muscle (Fig. 21.4) [30].

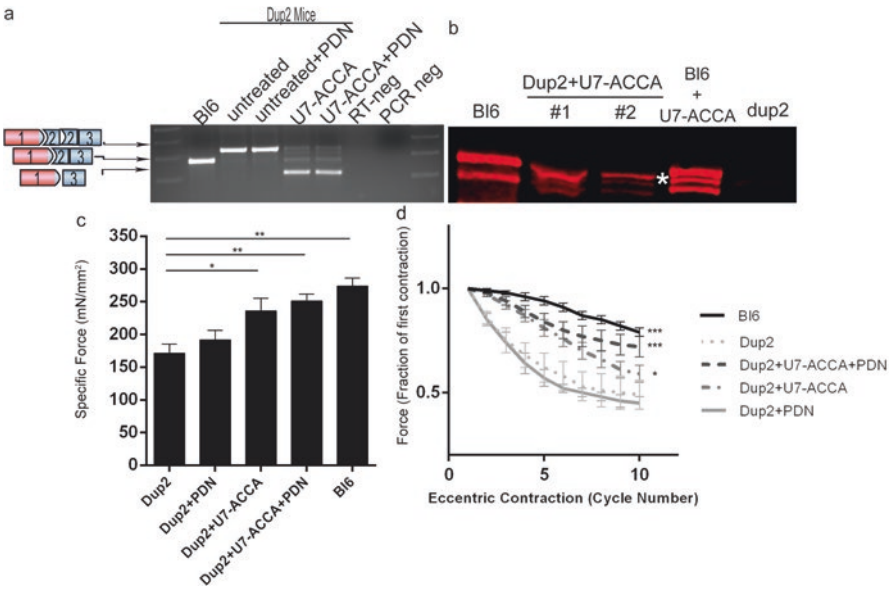

Fig. 21.4 Expression of the IRES-driven isoform following exon 2 skipping results in muscle improvement. (**a**) RT-PCR results indicate efficient exon 2 skipping in the treatment animals. (**b**) Western blot from treated animal demonstrates the presence of the IRES-driven isoform. This isoform can also be induced in Bl6 animals (isoform is indicated by a star). (**c**) and (**d**) represent specific force and eccentric contraction force assessments. These two tests demonstrated that muscle from treated animal gains force after treatment. *PDN* glucocorticoid, *Bl6* WT mouse, *Dup2* mouse carrying an exon 2 duplication, *U7-ACCA* AAV1-treated mice with a construct mediating exon 2 skipping [19]

21.5 Biologic and Therapeutic Implications of the ΔCH1 Isoform

We believe these results hold significant promise for patients with exon 2 duplications but also may be extendable to other mutations. Mutations prior to exon 6 comprise ~6% of dystrophinopathy patients but represent a population ignored by current mutation-specific approaches directed toward rod-domain mutations. We note that exon 2 deletion may prove beneficial in activating the IRES for many such mutations, a hypothesis currently under study in cell culture models.

Exon 2 skipping may be induced by antisense oligonucleotides, as we have demonstrated in cell culture [21]. However, the use of an AAV-mediated exon skipping approach may represent many advantages. The U7snRNP used in this study contains its own promoter, allowing continual expression of the antisense, which avoids the need for repeat injections. If readministration is required, one may use alternative AAV capsids. This system can in principle also be used for multi-exon skipping, as several copies of U7 targeting different exons can be cloned into a single AAV.

AAV9 as a delivery tool is particularly valued based on excellent muscle tropism, including the heart and diaphragm, which are responsible for the end-stage morbidity and mortality in DMD [33].

We believe that our results raise fundamental questions regarding the biology of the dystrophin protein. The identification of a highly functional N-deleted isoform is in contradiction with the current assumption that the entire ABD1 domain is required for actin binding of dystrophin. A key cellular role for Dp427m is the transmission of contraction force across the sarcolemma to extracellular structures by serving as bridge between the F-actin cytoskeleton and the muscle plasma membrane. Two regions within dystrophin are responsible for F-actin binding: ABD1, which is encoded by exons 2 through 8, and ABD2, which lies within the hinge repeat 10–17 [34, 35]. ABD1 consists of two calponin homology (CH) domains, CH1 and CH2. A central dogma in the dystrophin field is the requirement of an intact ABD1 in order to bind F-actin as a number of studies have shown a lack of stability of dystrophin in the setting of deletions within the ABD1 domain. However, most of these studies were performed with micro-dystrophin constructs lacking the ABD2 domain [36]. Such microproteins may bind actin and modify actin dynamics in a different manner compared to the full-length version [37–39]. Despite the canonical view that an intact ABD1 is required for dystrophin functionality, the human "experiment"—the presence of the founder allele in humans without significant symptoms and clearly no effect on reproductive fitness—argues that importance of an intact CH1 is not required or that ABD2 is sufficient for significant preservation of muscle function.

Another outstanding question remains regarding the evolutionary function of the IRES-driven isoform. The high degree of conservation of the IRES-containing exon 5 in 39 species indicates that this isoform may play a role outside a pathological context, although many cellular IRESs are active under stress conditions, suggesting a potential role of this isoform under conditions such as regeneration [6]. Of interest is the recent report of a novel embryonically expressed promoter and exon 1 that results in the expression of the same protein isoform, detectable with BMP-induced transition of induced pluripotent cells into the mesenchymal pathway [40]. We have confirmed that this promoter is not activated in U7snRNA-ACCA-treated muscle-derived mRNA (unpublished data), but these findings suggest that the Dp412/ΔCH1 isoform can be expressed by both transcriptional and translational regulatory mechanisms.

21.6 Summary

Although the native function and mechanisms of regulation of the ΔCH1 isoform remain to be clarified, the data from human expression clearly suggest a potential to benefit to patients. Together, the clinical and experimental data demonstrated that this protein—as the clinical course in patients makes clear—is highly functional and that deletion of a part of ABD1 can be well-tolerated. We anticipate that this

strategy can be extended beyond exon 2 duplication patients into those carrying missense mutations or in-frame deletion/duplications within exons 1–4—representing altogether up to 6% of patients. In the meantime, we are pursuing clinical development of this vector, including in-life toxicity studies, in support of an anticipated trial in subjects with duplications of exon 2.

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Chapter 22
Genome Editing for Duchenne Muscular Dystrophy

Christopher E. Nelson and Charles A. Gersbach

Abstract The recent genome editing revolution has been fueled by the discovery and adaptation of highly specific endonucleases including meganucleases, zinc finger nucleases (ZFNs), TALENs, and CRISPR/Cas9. These genome editing technologies permit user-defined genome modifications by creating double-strand DNA breaks and exploiting endogenous DNA repair pathways to introduce DNA sequence changes. Genome editing has entered multiple clinical trials in a range of diseases including HIV, cancer, and hemophilia, and several preclinical successes have been reported for treating models of neuromuscular diseases, including Duchenne muscular dystrophy (DMD). These studies include correction of numerous different mutations in patient-derived muscle cells and stem cells by a variety of genome editing strategies and endonuclease technologies. Preclinical studies have also shown efficacy of genome editing by restoring dystrophin protein expression and improving skeletal muscle physiology in animal models of DMD. This preclinical work highlights the potential for DNA repair therapy to treat DMD and other debilitating and fatal genetic diseases. Ongoing work seeks to address remaining issues including efficient delivery, addressing potential immune response or off-target interactions, and characterizing long-term safety and efficacy.

Keywords DNA repair · Genome editing · CRISPR/Cas9 · DMD · AAV Adenovirus

C. E. Nelson
Department of Biomedical Engineering, Duke University, Durham, NC, USA

Center for Genomic and Computational Biology, Duke University, Durham, NC, USA
e-mail: chris.nelson@duke.edu

C. A. Gersbach (✉)
Department of Biomedical Engineering, Duke University, Durham, NC, USA

Center for Genomic and Computational Biology, Duke University, Durham, NC, USA

Department of Orthopaedic Surgery, Duke University Medical Center, Durham, NC, USA
e-mail: Charles.gersbach@duke.edu

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22.1 Introduction

The genetic basis of Duchenne muscular dystrophy (DMD) was first identified in 1986 as a monogenic mutation in the Xp21 locus of the X chromosome [1, 2]. This mutation leads to the loss of functional dystrophin protein which presents clinically as progressive muscle wasting, loss of ambulation, and premature death [3]. Since this discovery, various gene therapy approaches have been pursued to treat this devastating disease. Recently, an exon skipping therapy, eteplirsen, was conditionally approved by the FDA for treating a subset of DMD patients amenable to exon 51 skipping (~13% of DMD patients, see Chap. 19). Gene-replacement therapy delivers an engineered and shortened dystrophin gene compatible with the size restrictions of adeno-associated virus (AAV) and is discussed in more detail in Chap. 18. Genome editing technologies have recently been developed that facilitate site-specific genome sequence modifications. These tools have enabled researchers to repair the native dystrophin gene in cultured human cells and in animal models of DMD. This chapter highlights the genome engineering approaches investigators have used to correct the *DMD* gene and the most recent preclinical advances and ongoing challenges.

22.1.1 Site-Specific Nucleases

The genome editing revolution has been fueled by the discovery and adaptation of site-specific endonucleases. These include meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR/Cas9 [4, 5]. These targeted nucleases are designed to form double-strand breaks (DSBs) in the desired genomic loci. Meganucleases, ZFNs, and TALENs rely on protein-DNA interactions for targeting specific DNA sequences [6–9]. In contrast, CRISPR/Cas9 is an RNA-guided endonuclease that is programed by RNA:DNA base pairing [10]. CRISPR/Cas9 targeting is limited by the proximity of a protospacer adjacent motif (PAM), a short sequence recognized by the Cas9 enzyme, to the desired target. For example, Cas9 derived from *Streptococcus pyogenes* (SpCas9) requires the presence of 5′-NGG-3′ at the 3′ end of the targeted sequence. However, the diversity of characterized CRISPR systems and associated PAM requirements are continually increasing, thus broadening the number of possible targets.

22.1.2 DNA Repair

After a DSB is formed in the genome, endogenous cell machinery repairs the break typically through one of two major pathways: homology-directed repair (HDR) or non-homologous end joining (NHEJ). Homologous recombination has been used to modify cultured cells and mouse embryos with correction efficiencies too low

for therapeutic use. However, in the presence of a DSB, homologous recombination efficiency is increased by several orders of magnitude [11]. By delivering a DNA repair template containing the intended sequence change flanked by sequences homologous to the nuclease target site, HDR can be used to exchange single nucleotides, repair larger mutations, or insert entire genes. However, HDR machinery is typically downregulated in post-mitotic cells including skeletal and cardiac muscle, leading to low efficiencies of genome editing by this mechanism in these tissues [12]. In the absence of a donor repair template, the NHEJ process is used to mend the break. The error-prone nature of NHEJ often results in small insertions or deletions (indels) at the target site [13]. The spectrum of indels that occur at a particular site is context-specific depending on the nuclease and the local sequence at the target loci. NHEJ can be used to disrupt target genes by introducing frameshifting indels [14]. Alternatively, dual DSBs can delete targeted genomic regions to remove mutated regions of a gene [15, 16]. A limitation of NHEJ for DNA repair therapy is that NHEJ does not precisely repair or replace absent genes. In addition to genome editing, zinc finger proteins, TALEs, and nuclease-deactivated CRISPR/Cas9 (dCas9) have been used as highly specific DNA-binding proteins and fused with a broad range of DNA-effector molecules that includes gene activation, gene repression, and epigenetic modification [17, 18]. dCas9 has also been fused with cytidine deaminases that specifically alter a single cytosine base pair at a desired site without making a DSB [19, 20]. An advantage of this base editing approach is that the DNA is not broken and greatly reduces the risk of off-target indel formation.

22.2 DNA Repair Therapy for DMD

22.2.1 Introduction to DMD Gene Repair Therapy

Patients with DMD are characterized by heterogeneous mutations including deletions, duplications, and small polymorphisms (Box 22.1). Natural history studies of Becker muscular dystrophy (BMD) patients, who harbor in-frame internal deletions of the dystrophin gene, have suggested that portions of the dystrophin protein are dispensable without substantial loss of function. In fact, large internal deletions with a preserved reading frame have been described in BMD patients with mild phenotypes [21]. Restoring the disrupted reading frame in DMD patients by internally shortening the dystrophin protein to create a BMD-like genotype is the premise behind exon skipping therapies in which nonessential exons are removed from the transcript. Micro-dystrophin gene-replacement therapy also benefits from the use of an internally shortened dystrophin protein that is amenable to packaging within AAV vectors (see Chap. 18). In contrast to micro-dystrophin gene therapy, genome editing uses engineered nucleases and the endogenous DNA repair pathways to restore dystrophin expression from the native *DMD* gene (Fig. 22.1).

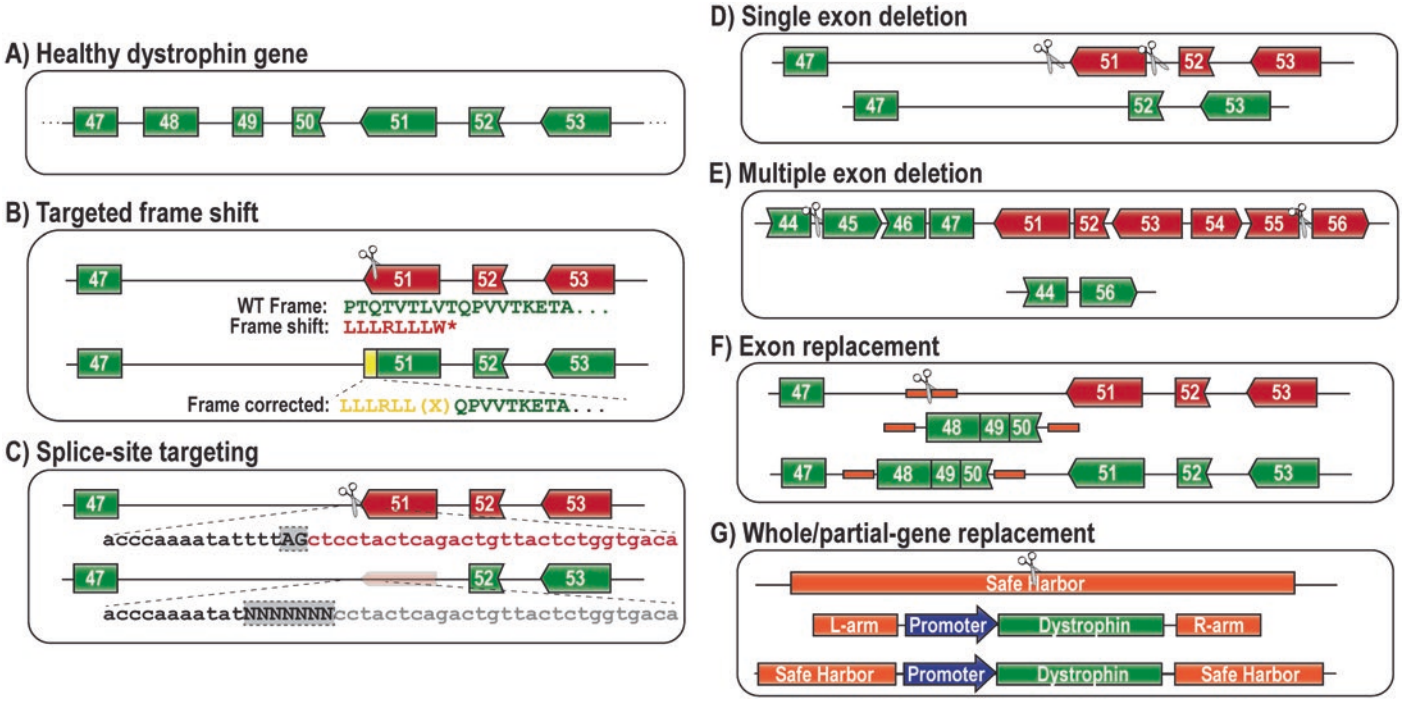

Fig. 22.1 Genome editing approaches for DMD. (**a**) Wild-type *DMD* gene showing exons 47–53 with in-frame exons labeled in green. (**b**) Deletion of exons 48–50 results in frameshifted exons 51 onward. Targeted indel formation within exon 51 upstream of the stop codon (*) restores the correct reading frame. (**c**) Disrupting splice motifs flanking the exon including the canonical 5′-AG splice acceptor will remove an exon from the transcript. (**d**) Multiplexed nucleases around exon 51 delete the exon from the genome and the transcript. (**e**) Multiplexed nuclease targeting larger regions extend applicability of genome editing to a larger patient base. (**f**) Replacement of one or more missing exons can restore the original transcript and protein. (**g**) Targeted integration into a safe-harbor locus can be used to integrate engineered dystrophin mini-genes or full-length genes. Green = in frame, red = out of frame, yellow = nonsense amino acids, orange = homologous regions, blue = promoter

Reported DNA repair strategies attempt to strike a balance between maximizing the efficiency of restoration of the dystrophin protein and maintaining as much of the original sequence of the native gene as possible.

Box 22.1 Large Deletions to Address Patient Heterogeneity

The heterogeneity of mutations in DMD complicates the design of a one-size-fits-all genome editing strategy. The primary strategy employed in cultured cells and in vivo for DMD gene editing has been deletions of one or more exons (Fig. 22.1d). Several studies have indicated the potential utility of genetic deletion of several exons to treat larger cohorts of patients including the regional hotspot of exons 45–55 [22, 23] or exons 43–55 [24]. Deletion of exons 45–55 is applicable to ~47% of patients based on patient transcript data collected from the *Universal Mutation Database* [25] on 3/1/2017 (Fig. 22.2), while some other reports from smaller patient databases indicated that this segment of the patient population was ~63% [25, 26]. Other noteworthy mutations include a high prevalence of exon 2 duplication [27] and a high indel percentage in exon 70. The exon 45–55 mutational hotspot can be seen in Fig. 22.2. When designing gene editing strategies, potential considerations include (1) the percent of patients amenable to the strategy, (2) the structure and function of the residual protein, and (3) gene deletion efficiency generally decreasing with the size of the deletion.

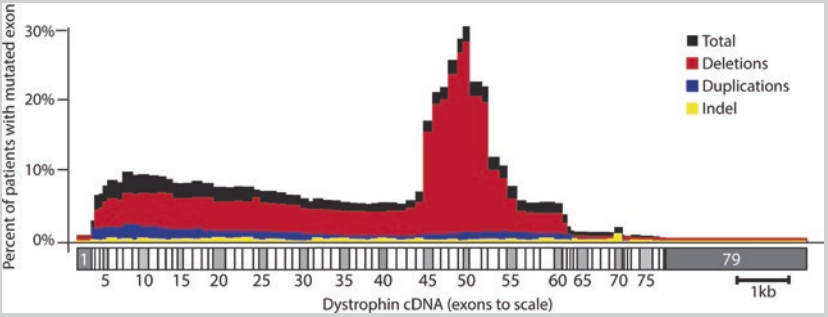

Fig. 22.2 Mutational hotspot region of exons 45–55. Mutations are annotated along the length of the transcript on the x-axis. The percent of patients with the indels (yellow), duplications (blue), or deletions (red) for each exon are shown. The data in the figure was derived from the *Universal Mutation Database* [25]

22.2.2 NHEJ-Based Methods

22.2.2.1 Frame Shift

Most DMD patients have large genetic deletions of one or more exons which disrupt the reading frame leading to a completely dysfunctional protein. One approach is to use a nuclease targeting the DNA between the splice acceptor of the first exon following the deletion and the premature stop codon that is typically introduced in this incorrect reading frame. Indel formation in this region will restore the reading frame in a fraction of these edited cells (Fig. 22.1b). Using this approach, meganucleases, TALENs, and CRISPR/Cas9 have been adapted to restore the reading frame in patient-derived IPSCs and myoblasts [28–31]. These reports showed reading frame restoration in a fraction of analyzed cells. In some reports, about one third of indels resulted in the desired frameshift. Clonal cell populations contain indels that restore the correct reading frame of dystrophin protein after differentiation. An advantage of this approach is that only a single nuclease is required and no repair template is necessary. Drawbacks include stochastic indel formation resulting in only a fraction of edited genes restoring the reading frame, the unknown effect of the novel amino acid sequence generated by the indels, and the limited sequence space available for

Table 22.1 Approaches for genome editing for DMD

Approach	Applicable mutations	Residual structure	Advantages/disadvantages	Reports
NHEJ frameshift	Customized for each exon	Internally shortened	– Small genomic regions available for targeting – One nuclease needed	[28–31]
NHEJ splice site	Customized for each exon	Internally shortened	– Small genomic regions available for targeting – One nuclease needed	[30, 31]
NHEJ deletion	Customized for larger segments of gene (Box 22.1)	Internally shortened	– Deletions may be large and less efficient to capture large numbers of patients – Two nucleases needed	[22, 23, 30–38]
HDR repair	Customized for each mutation	Normal	– HDR in muscle is inefficient	[30, 37, 39, 40]
HDR replace	~100% of patients	Truncated or full length	– HDR in muscle is inefficient	[41, 42]
Gene capture repair	Customized for each group of missing exons	Normal	Untested	Untested
Gene capture replace	~100% of patients	Truncated or full length	Untested	Untested
Gene regulation (e.g., utrophin)	~100% of patients	NA	– Does not restore dystrophin expression	[43–46]

designing nucleases and finding optimal target sites. To address some of these concerns, edited cells could be selected for ideal characteristics, expanded, and screened extensively for off-target modifications for cell therapy applications (Table 22.1).

22.2.2.2 Splice-Site Targeting

In an approach analogous to exon skipping, targeted indel formation within canonical splice motifs in the *DMD* gene can exclude target exons from the mature transcript and restore the proper reading frame (Fig. 22.1c). One study used TALENs or Cas9 to target the spice acceptor for exon 45 to correct patient-derived iPSCs [30], and another used Cas9 targeted to exon 51 or 53 in patient-derived myoblasts [31]. These reports showed dystrophin restoration in the patient-derived cells. However, these studies did not distinguish between frameshifting mutations and splice acceptor knockout. An advantage of this approach is only a single nuclease is needed and no correction template is required. One drawback is the unpredictable nature of splicing including activation of cryptic splice sites [47]. This can be addressed for cell therapy by screening corrected cells for the desired transcript and dystrophin restoration. Another drawback is the limited sequence space available for targeting near the canonical splice sites. For CRISPR/Cas9, this approach would be limited by the proximity of PAM sites to the splicing motif of interest. However, several CRISPR systems have been described with a broad spectrum of PAM requirements [48, 49], and the number of available systems is expected to continue to expand. To illustrate the potential of this approach, genome editing techniques have been successful in preclinical models of other diseases, including correcting splicing mutations as a therapy for tyrosinemia type I [50], spinal muscular atrophy [51], and β-thalassemia [52].

22.2.2.3 Genetic Deletions to Remove Exons

Genetic deletions can also be used to remove target exons from the genome and transcript by providing two nucleases flanking a target exon or group of exons (Fig. 22.1d). This is the most pursued method thus far with several studies using ZFNs, TALENs, and CRISPR/Cas9 for removal of target exons from the genome in patient-derived iPSCs and myoblasts [22, 23, 31–33, 43, 53]. Collectively, these reports showed restored dystrophin protein expression in corrected cells in culture, in vivo following transplantation into immunodeficient mice, and following plasmid electroporation in vivo.

Gene deletion has been demonstrated with CRISPR/Cas9 in the *mdx* mouse using adenoviral delivery [38] or adeno-associated virus (AAV) delivery [34–36] to remove exon 23, which carries a premature stop codon. AAV-delivered CRISPR showed restored dystrophin expression in local injections to ~5–10% of wild-type levels and improved skeletal muscle function with ~50% recovery of muscle strength relative to controls. Systemic AAV administration showed dystrophin restoration in

multiple skeletal and cardiac muscles [34–36]. A subsequent study demonstrated efficacy of a similar approach in an alternate mouse model (*mdx*^{4cv}) which carries a nonsense mutation in exon 53 [37]. Genetic correction was achieved by deletion of a 45 kb region spanning exons 52 and 53. Local administration led to abundant dystrophin restoration and improvements in muscle function. Systemic administration rescued dystrophin expression in multiple skeletal and cardiac muscles.

An advantage of a deletion approach is that indel formation is within introns and will not affect the reading frame. An additional advantage is that gene deletions can be extended to larger genetic regions to capture a larger patient base with a single treatment (Fig. 22.1e and Box 22.1). Disadvantages include the requirement for two nucleases to create the deletion, which may necessitate using multiple vectors for some strategies, and increased potential for off-target modifications.

22.2.2.4 Gene Capture

Gene capture techniques including homology-independent targeted integration (HITI), microhomology-mediated end joining (MMEJ), and obligate ligation-gated recombination (ObLiGaRe) have also been used to add large sections of DNA through NHEJ-mediated integration [54–57]. In these approaches, a donor vector containing nuclease target sites is provided, subsequently cleaved by the co-delivered nuclease, and incorporated into the genomic nuclease target site. This approach could be applied to restore missing exons or an engineered dystrophin cDNA. Although this has not been applied for DMD, one study reported DNA integration efficiencies in skeletal muscle with this approach of 3.4% in cardiac muscle and 10.0% in the quadriceps muscle [56].

22.2.3 HDR-Based Methods

HDR can be used to correct point mutations, replace absent exons to restore the wild-type dystrophin protein, or integrate a complete or truncated dystrophin cDNA into a safe-harbor locus (Fig. 22.1f, g) [41, 58–63]. Advantages of HDR gene correction include the potential to restore the entire structure of the dystrophin protein and applicability to a wide range of patients. A significant disadvantage of this approach is the lower efficiency of HDR in post-mitotic cells. For this reason, current HDR-based repair strategies are likely limited to cell therapy, where corrected cells can be selected and expanded, unless methods to increase HDR efficiencies in muscle are described. Importantly, methods to increase HDR efficiency are under widespread investigation for genome editing applications.

Meganucleases have been used to incorporate exons 45–52 into the dystrophin gene in vitro [39]. TALENs and CRISPR/Cas9 have been used to incorporate exon 44 in iPSCs [30]. For whole gene replacement, a microdystrophin expression cassette was integrated into the CCR5 locus in human myoblasts, which were then

transplanted and expressed dystrophin in vivo [41]. HDR has also been performed in mouse embryos to precisely correct the C to T transition in the mdx mouse [40]. This study also generated mosaic mice with gene correction ranging from 2 to 100%, allowing phenotypic comparisons at varying gene correction levels. However embryonic correction is not currently a feasible approach for human DMD patients. In addition to nucleases, triplex-forming molecules including peptide nucleic acids have been shown to facilitate HDR at target genomic loci [64].

HDR is active primarily during S/G2 phase which excludes nondividing cells [65] including skeletal and cardiac muscle. Bengtsson and co-authors recently described HDR in murine skeletal muscle, albeit at a low frequency [37]. Two CRISPR gRNAs targeting within exon 53 in the mdx^{4cv} mouse were provided with a repair template on an AAV donor vector to encourage precise repair of the premature stop codon located in exon 53. Deep sequencing showed 0.18% of extracted genomes had the successful HDR event. Although dystrophin was observed by staining arising from HDR or frame-corrected deletions, little functional benefit was observed compared to the larger deletion of the region with two gRNAs by an NHEJ-based mechanism [37]. HDR has been shown effective in vivo in other contexts including liver-directed repair [66] and hematopoietic stem cells in vivo [52], which have the advantages of targeting smaller numbers of cells and using methods of more efficient delivery to those tissues. Methods to encourage upregulating HDR machinery in skeletal muscle could be pursued to increase the efficiency of HDR in this tissue.

22.2.4 Other Genome Engineering Methods

Other genome engineering methods besides genome editing are emerging as strategies to treat DMD. One approach uses zinc finger proteins (ZFPs) or deactivated Cas9 (dCas9) fused to transcriptional activation domains to generate synthetic transcription factors that can be targeted to any gene promoter. This approach can be used to specifically increase expression of compensatory or modifier genes of the DMD phenotype. For example, ZFPs have been used to increase expression of utrophin, a protein with similar structure to dystrophin that is downregulated in adult muscle. In this work, utrophin upregulation improved the phenotype in *mdx* mice by partially compensating for dystrophin function [44, 45]. dCas9 has also been used to increase utrophin expression and laminin subunit alpha 1 [43, 46].

Using CRISPR-based base editing [19, 20], specific mutations can be targeted for repair, or exons can be excluded by modifying cytosines to disrupt splice motifs. Base editing has been applied to alter mRNA splicing [67], including the *DMD* transcript in patient derived iPSCs [68]. It is possible that this technique could be used to repair point mutations in DMD patients correctable by a C to T transition. The limitation of this approach is the proximity of available PAM sites to the desired C to T transition, but the continued development of new base editing tools is likely to expand the capabilities of this technology.

22.3 Preclinical Development Considerations

Considerations for preclinical development of genome editing technologies for neuromuscular disease include selection of appropriate animal models, safe and efficient delivery of genome editing tools, the potential for immunogenicity, and off-target nuclease activity. Advances in each of these areas will create more opportunity for DNA repair therapy for DMD and other diseases.

22.3.1 Animal Models

Numerous animal models exist for preclinical studies for DMD [69]. Many of these animal models may be applied for studying genome editing for DMD. Considerations for choice in animal model include the underlying genetics, the disease phenotype, presence of revertant fibers, and the condition of the immune system.

22.3.1.1 The mdx and mdx Variant Mouse Models

The most common DMD mouse model is the *mdx* mouse which has a C to T transition creating a premature stop codon in exon 23 terminating protein production [70, 71]. Multiple groups have used this mouse model for correction by genome editing by excising exon 23 in neonatal and adult mice [34–36, 38]. Exon 23 has also been precisely repaired by HDR in mouse embryos [40]. Another example is the mdx^{4cv} mouse which has a C to T transition in exon 53 creating a premature stop codon. Correction of this mutation by genome editing was demonstrated by creating a 45 kb deletion of exons 52 and 53 [37]. One advantage of the mdx^{4cv} mouse is a lower frequency of revertant fibers than the *mdx* mouse, which facilitates easier detection of dystrophin restored via genome editing. Additionally, the mdx^{4cv} mutation exists within the same mutational hotspot from the human gene (Fig. 22.2). Other *mdx* variants are available with various mutations and levels of background dystrophin expression from revertant fibers (see [69] supplemental Table S1). Dystrophin null mice can be crossed with immune compromised lines to evaluate dystrophin expression following human cell transplantation [22, 32].

22.3.1.2 Severe Phenotypes

A drawback of many of dystrophin knockout mouse models is the very mild phenotype in these animals relative to the human DMD phenotype. These models typically have only slight weakening of the skeletal muscle and cardiac deficiencies that take more than a year to develop. Multiple mouse models have been generated to more faithfully model the severity of DMD disease progression. Double knockout mice in which the absence of dystrophin is combined with loss of utrophin, α7-integrin,

MyoD, or other genes are available with a range of phenotypic severity [69]. A mouse mimicking the human deactivating mutation in cytidine monophosphate-sialic acid hydroxylase (Cmah) has also been used to better recapitulate the human DMD phenotype [72].

22.3.1.3 Genetically Humanized Mouse

A specific need for exon skipping and genome editing clinical development is the availability of an animal model harboring the human dystrophin sequence for generating molecules that specifically target human mutations. Because the mouse *Dmd* gene differs significantly from the human *DMD* gene, an hDMD/mdx mouse was created that contains the entire 2.5 Mb human dystrophin gene and was used for exon skipping studies [73]. Although this mouse contains a wild-type copy of the human gene, modifications of the humanized gene have been introduced to generate a DMD model that was also treated by genome editing [74, 75]. Other approaches could include humanizing small stretches of the mouse *DMD* gene to create a target for nucleases applicable to the human gene.

22.3.1.4 Larger Animal Models

Dogs are the more common large animal model for DMD preclinical studies that have a phenotype more representative of the human condition [76]. A recent report showed systemic dystrophin restoration and improvements in muscle histology in the deltaE50-MD dog model of DMD [77]. Other large animal models of DMD have recently been developed using genome editing including a rat [78], a pig [79], and a rhesus monkey model [80].

22.3.2 Delivery

The primary challenge for translation of genome editing for neuromuscular disease including DMD is the safe and efficient delivery of genome editing tools to skeletal muscles and cardiac tissue [81]. To date, AAV, adenovirus, electroporation, and cell therapy have shown variable levels of dystrophin restoration in mouse models of DMD following local or systemic delivery. Other delivery vehicles may be pursued that demonstrate effective muscle delivery in the future.

22.3.2.1 Adeno-Associated Virus (AAV)

AAV is a small, non-pathogenic, episomal virus that has been used for gene delivery in over more than 100 gene therapy clinical trials [82]. AAV has been used preclinically to deliver ZFNs to mouse models of hemophilia [83], a strategy which

has now advanced to clinical trials (NCT03061201/NCT02695160/NCT02702115/NCT03041324). AAV has also been used in several studies to deliver CRISPR/Cas9 to the mdx mouse [34–36]. Advantages of AAV include preferential tropism for skeletal and cardiac muscle, lack of any documented pathogenicity in humans, and a growing body of efficacy and safety in clinical trials. Potential drawbacks include the limited packaging size (~4.7 kb) which can prevent the efficient packaging of Cas9 and gRNA expression cassettes, as well as TALENs. An additional drawback is the potential for humoral and cellular response generated to delivered transgenes including Cas9 [84].

22.3.2.2 Adenovirus

Adenovirus has also been used to deliver genome editing components for cell therapy applications and in vivo. For cell therapy, adenovirus was used to deliver ZFNs [41] or Cas9 with gRNAs for selection-free editing of patient myoblasts [24, 31]. Adenovirus has also been used in local skeletal muscle injections in the *mdx* mouse to delete exon 23 and restore dystrophin protein expression [38]. This report also showed a significant decrease in Evans blue dye uptake at rest and after downhill treadmill runs. Advantages of adenoviral delivery include the large packaging size that accommodates all CRISPR gene-editing components or large dCas9-based fusion proteins. A disadvantage of using adenovirus includes a pronounced immune response to transduced cells [85]. More research is needed, as one report showed successful genome editing in the liver with adenoviral delivery of CRISPR components despite an immune response [86].

22.3.2.3 Lentivirus

Lentivirus has been used to deliver genome editing technologies in vitro and in vivo. Concerns regarding insertional mutagenesis and the inability to achieve systemic administration with lentivirus have limited development for DMD treatment. However, there is a clinical precedent for an integrating virus with a gammaretrovirus product that was recently approved in Europe for a stem cell therapy for adenosine deaminase (ADA) deficiency [87]. Integrase-deficient lentivirus (IDLV) is a potential option to avoid the risks of insertional mutagenesis [88, 89], and transient expression originating from IDLV may work well for ex vivo genome editing components. In addition, IDLV can function as a repair template for HDR [90]; this approach has been used to deliver a microdystrophin cassette [41] and also could be used to incorporate specific exons.

22.3.2.4 Other Delivery Vehicles

Non-viral delivery strategies have not been as widely reported for systemic skeletal and cardiac transfection in vivo owing to lower efficiency compared to viral transduction. Electroporation in vivo has been shown as a proof-of-principle for CRISPR

components in local skeletal muscle injections [33], but will be challenging in the clinical setting due to resulting inflammation and is not compatible with systemic delivery. A nanoparticle formulation has been used to deliver recombinant Cas9 protein, gRNA, and a homology repair template by intramuscular injection into the *mdx* mouse, leading to dystrophin restoration as observed by histological analysis [91]. Non-viral delivery has been pursued for genome editing in other applications, including delivery to the liver and ex vivo cell modification [52, 92]. New viral and non-viral vectors are being described and applied to genome editing and may find utility in skeletal and cardiac muscle in the future [81].

22.3.2.5 Cell Therapy

Another delivery method is to repopulate skeletal and cardiac muscle cells with corrected myogenic progenitor cells to restore dystrophin expression and muscle function. Encouraging results have been shown in local tissue repopulation with dystrophin-expressing myogenic progenitors, including dystrophin restoration and improved contractility [93]. Patient myoblasts have been corrected with genome editing technologies showing dystrophin expression in local transplantations in vivo [22, 32]. The Sleeping Beauty transposon was used to engineer iPSCs from a dystrophic mouse to express a micro-utrophin gene, leading to biochemical restoration of the dystrophin-glycoprotein complex and improved contractility after local transplantation in dystrophin/utrophin knockout mice [42]. However, a significant challenge remains in developing protocols for efficient systemic repopulation of skeletal and cardiac muscle as current methods lack the efficiency needed for DMD therapy [94]. Other muscle progenitors including CD133+ cells can be administered locally or systemically and contribute to muscle repair and dystrophin restoration [95–100]. One notable advantage of cell therapy is the ability to screen edited cells extensively for off-target modifications. There are also lower risks of immunogenicity of viral vectors and gene-editing components. Intracellular delivery barriers are also decreased ex vivo, and RNA or protein-based delivery are more feasible, which can decrease off-target activity [101].

22.3.3 Muscle Progenitors and Long-Term Efficacy

The permanence of correction will depend on the ability to target and correct the population of satellite cells that repopulate skeletal muscle. A previous report had indicated that AAV had limited ability to target satellite cells based on expression of reporter genes [102]. However, a recent report showed successful gene editing in isolated and expanded satellite cells after a single administration of AAV9 carrying CRISPR components [35]. These two reports can be reconciled if transgene expression from satellite cells is lost quickly after transduction, but genome marking by gene editing is retained. In fact, sustained dystrophin expression was observed 18 months after a single administration of AAV-CRISPR [103]. Nevertheless the

limits of satellite cell editing are still unknown, and it is not yet clear to what extent edited satellite cells are participating in muscle regeneration. Future work will likely focus on long-term maintenance of dystrophin expression and improved phenotype.

22.3.4 Immunogenicity and Off-Target Activity

Immune responses can be generated to foreign genome editing components including Cas9, gRNAs, ZFPs, TALE proteins, or meganucleases. In a similar fashion to siRNA, gRNAs could potentially activate toll-like receptors. A few reports have documented immune response to CRISPR components including humoral and cellular responses. Adenoviral delivery of CRISPR/Cas9 to the liver generated Cas9-specific antibodies [86] and AAV delivery of CRISPR/Cas9 led to Cas9-specific antibodies and T-cells [84, 104]. More research is needed to determine routes to avoid immune activation including limiting gene expression to muscle cells with muscle-specific promoters [37], using self-inactivating vectors [105], applying transient immune suppression, or inducing tolerance.

In contrast to ex vivo gene editing, where edited cells can be screened extensively for off-target modifications, in vivo genome editing has the potential for every cell in the body to be edited at on- or off-target genes. Unintended on-target modifications may occur including large deletions [106], genomic rearrangements, or integration of the DNA genome of the viral vector [104]. To examine off-target modifications, extensive screening is now possible with next-generation sequencing techniques including in vitro screening (Guide-seq [107]) and ex vivo genome digestions (Digenome-seq [108]). Methods to determine the biological consequence of potential off-target modifications will be needed to determine safety. Other methods to reduce off-target modifications can be pursued including high-fidelity Cas9 molecules [109, 110] or alternate CRISPR systems with reported higher specificity, such as Cas12a [111]. Finally, muscle-specific promoters limit expression of gene-editing constructs to skeletal and cardiac muscle limiting off-target modifications in other tissues [37].

22.4 Conclusions

DNA repair has been successfully applied to correct the genetic basis of DMD with multiple reports of phenotype improvement in murine models of DMD. Future work will be needed to evaluate long-term functional improvement, cardiac physiology, safety, and efficacy. Also, comparisons will need to be made with other methods to restore dystrophin expression, including exon skipping and microdystrophin gene therapy. Clinical development will also be informed by the results from other ongoing genome editing clinical trials. If the challenges can be addressed, genome editing has the potential to become an exciting new class of therapies for DMD and other debilitating neuromuscular disorders.

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Chapter 23
Sarcolipin Knockdown Therapy for Duchenne Muscular Dystrophy

Satvik Mareedu, Shalini Dwivedi, Nandita Niranjan, and Gopal J. Babu

Abstract Duchenne muscular dystrophy (DMD) is an X-linked genetic disorder caused by mutations in the *DMD* gene which encodes dystrophin, a sarcolemmal protein. Although loss of dystrophin is the primary cause of this disease, activation of several secondary mechanisms contributes to the disease progression. The dysregulation of Ca^{2+} cycling and elevation of intracellular Ca^{2+} concentration is suggested to play a central role in disease pathogenesis in DMD. The sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) activity, which accounts for >70% cytoplasmic Ca^{2+} removal during muscle contraction, is significantly decreased and majorly contributes for the intracellular Ca^{2+} overload in DMD. We have shown that abnormally high-level expression of sarcolipin (SLN), an inhibitor of SERCA pump, is the cause of SERCA dysfunction in dystrophic muscles. Here we review the recent findings from genetically modified mouse models which demonstrated that reducing SLN expression is sufficient to improve the SERCA function and mitigate the severe muscular dystrophy phenotype in mouse models of DMD. The concept of SLN knockdown in mitigating DMD should be helpful to identify new drugs as well as generating novel gene therapy-based approaches for the treatment of DMD.

Keywords Sarcolipin · SR Ca^{2+} ATPase · Duchenne muscular dystrophy · Mouse models · AAV · Gene therapy

23.1 Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive muscle-wasting disorder which affects one in 5000 live births [1–3]. DMD is caused by mutations in the *DMD* gene which encodes dystrophin [4, 5], a protein that contributes to membrane stability during muscle contraction [6, 7]. Lack of dystrophin causes muscle

S. Mareedu · S. Dwivedi · N. Niranjan · G. J. Babu (✉)
Department of Cell Biology and Molecular Medicine, New Jersey Medical School, Rutgers, The State University of New Jersey, Newark, NJ, USA
e-mail: sm1629@gsbs.rutgers.edu; Dwivedi-shalinimys.20@gmail.com; Niranjan-nn277@njms.rutgers.edu; babugo@njms.rutgers.edu

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to undergo stretch-induced damage, loss of sarcolemmal integrity, and membrane rupture. These changes lead to progressive muscle wasting with repeated muscle damage and inadequate repair [8, 9]. The progressive nature of this disease leads to restrictive pulmonary function, diaphragm dysfunction, and cardiomyopathy [10–13]. Despite the availability of extensive clinical management practice, currently there is no curative treatment for this devastating disease. Many of the therapeutic strategies followed in the preclinical studies and clinical trials were based on the concept of either replacing or skipping the mutated exon(s) in *DMD* gene. These strategies include exon skipping, gene replacement, stem cell replacement therapy, membrane stabilization and/or upregulation of compensatory proteins, and reduction of the inflammation [14–17]. Even though therapeutic potential of mini- or micro-dystrophin administrations to compensate the loss of dystrophin was efficient and reached phase I trials, there were many limitations such as inefficient protein expression and development of neutralizing antibodies against the vector and the gene transduced [18–20]. On the other side, usage of a corticosteroid that could potentially delay disease progression by retarding the collateral consequences was recently approved by the United States Food and Drug Administration (FDA) [21]. However, steroids couldn't alleviate the pathology, and long-term corticosteroid therapy is also associated with a compromised immune system. Recent developments in antisense oligonucleotide therapy provide a novel approach to target-specific gene mutations. The recently FDA-approved drug, Exondys 51, specifically targets Exon 51 mutation, and Translarna, the European Medicines Agency-approved drug, specifically targets nonsense mutation in the dystrophin gene. However, these drugs are restricted in their generic usage. Furthermore, these drugs also face major challenges such as targeting the heart and respiratory tissues and adverse side effects. Therefore, identification of new therapeutic targets based on disease mechanism is necessary to complement the existing strategies for the effective treatment of this lethal disease.

23.2 Abnormal Ca^{2+} Cycling in Dystrophic Muscles

Abnormal elevation of cytosolic Ca^{2+} concentration is a hallmark of DMD and contributes to the pathophysiology and progression of the disease [22–25]. Several mechanisms could contribute to the elevation of cytoplasmic Ca^{2+} concentration in dystrophic muscles. The lack of dystrophin makes the sarcolemma more susceptible to rupture. Transient micro-ruptures recruit proteolytically activated Ca^{2+} leak channels around the area of ruptures during the resealing process and trigger localized elevation of Ca^{2+} ions [22, 26]. In dystrophic muscles, the Ca^{2+} flux occurs through Ca^{2+}-permeable growth factor-regulated channels and transient receptor potential channels [27–30]. The increased Na^{+} overload due to the enhanced Na^{+}/H^{+} exchanger type I activity in dystrophic muscle has been suggested to indirectly cause Ca^{2+} overload [31, 32].

Defects in sarco/endoplasmic reticulum (SR) Ca^{2+} cycling majorly contributes to the cytosolic Ca^{2+} overload and muscle pathogenesis in DMD. Although some studies have reported no change in the total SR Ca^{2+} content [33], many studies have found reductions in the rate of Ca^{2+} transients [34, 35] and reductions in SR Ca^{2+} release [36, 37] in dystrophic muscle fibers. It has been suggested that SR Ca^{2+} leak via defective ryanodine receptor 1 (RyR1) and decreased levels of its modulator, FKBP12, can contribute to muscle weakness in DMD [38]. We have recently shown that the SR Ca^{2+} uptake was significantly decreased in the diaphragm, slow- and fast-twitch skeletal muscles [39], and ventricles [40] of dystrophin mutant (*mdx*) and dystrophin/utrophin double mutant (*mdx:utr−/−*) mouse models of DMD. Goonasekera et al. [41] have shown that the SR Ca^{2+} ATPase (SERCA) activity, SR Ca^{2+} content, and Ca^{2+} transient amplitude were significantly reduced in the skeletal muscles of *mdx* mice. Apart from the role in regulating muscle relaxation, SERCA plays an important role in maintaining the cytosolic Ca^{2+} levels. In muscle, SERCA activity accounts for 70–90% of cytosolic Ca^{2+} removal [42]. Thus the decreased SERCA activity could significantly contribute to the cytoplasmic Ca^{2+} overload in DMD. Consistent with this notion, overexpression of SERCA1 in the skeletal muscles of *mdx* mice mitigated dystrophic phenotypes and contraction-induced muscle damage [41, 43]. Similarly, stabilization of the SERCA pump via heat-shock protein 72 overexpression has been shown to mitigate the muscular dystrophy in *mdx:utr−/−* mice [44]. However, the mechanism(s) causing SERCA dysfunction in dystrophic muscle is not fully understood.

23.3 Sarcolipin Inhibits SERCA Pump and Causes Cytosolic Ca^{2+} Overload in DMD

In muscle, SERCA function is modulated by a family of small molecular weight membrane proteins: phospholamban (PLN), sarcolipin (SLN), and myoregulin (MLN) [45–48]. In rodents, MLN is primarily expressed in skeletal muscles [47, 48], whereas SLN is predominantly expressed in the tongue, diaphragm, and slow-skeletal muscles but not expressed in the fast-twitch skeletal muscles [48, 49]. On the other hand, in larger mammals, SLN is expressed in all skeletal muscle tissues [49]. In the heart, PLN expression is high in the ventricles and low in the atria [48], while SLN expression is high in atria and very low in the ventricles of both rodents and larger mammals [49]. Recent mouse genome screening studies have identified endoregulin (ELN) and another-regulin (ALN), which resembled other muscle-specific SERCA regulators [48]. Together these studies suggest the complex interplay of these regulators in affecting SERCA function in various muscles.

Among the various regulators, pathophysiological relevance of PLN and SLN in SERCA regulation was extensively studied [50]. In relevance to DMD, decreased expression of SLN and increased expression of PLN at the mRNA levels were reported in the tongue muscle and increased PLN protein expression in the masseter

muscle of *mdx* mice [51]. In our studies, we did not find PLN protein expression in dystrophic diaphragm, quadriceps, and soleus muscles [39]. The PLN levels were also unaltered in atria and in the ventricles of *mdx* and *mdx:utr−/−* mice compared to that of wild-type (WT) control mice [40]. On the other hand, SLN protein expression was abnormally high in the diaphragm, slow- and fast-twitch skeletal muscles [39], and atria and ventricles [40] of *mdx* and *mdx:utr−/−* mice. These findings corroborate with a report on the upregulation of SLN mRNA in the muscles of *mdx* mice [52]. Similar to mouse models, SLN protein levels were elevated in the muscles of a canine model of DMD and in the skeletal muscle and ventricles of DMD patients [40]. Together these findings suggested that SLN upregulation is a common molecular change in dystrophic muscles of human and animal models of DMD. Studies using transgenic and knockout mouse models have demonstrated that changes in SLN levels can affect the SERCA function in cardiac and skeletal muscles [53–56]. We therefore hypothesized that in dystrophin-deficient skeletal and cardiac muscles, high-level expression of SLN can chronically inhibit SERCA function and cause cytosolic Ca^{2+} overload. These changes could lead to a plethora of downstream effects like activation of Ca^{2+}-dependent proteases, improper muscle regeneration, mitochondrial dysfunction, muscle weakness, and cardiomyopathy (Fig. 23.1). Accordingly, reduction or ablation of SLN expression is anticipated to improve the SERCA function and mitigate DMD.

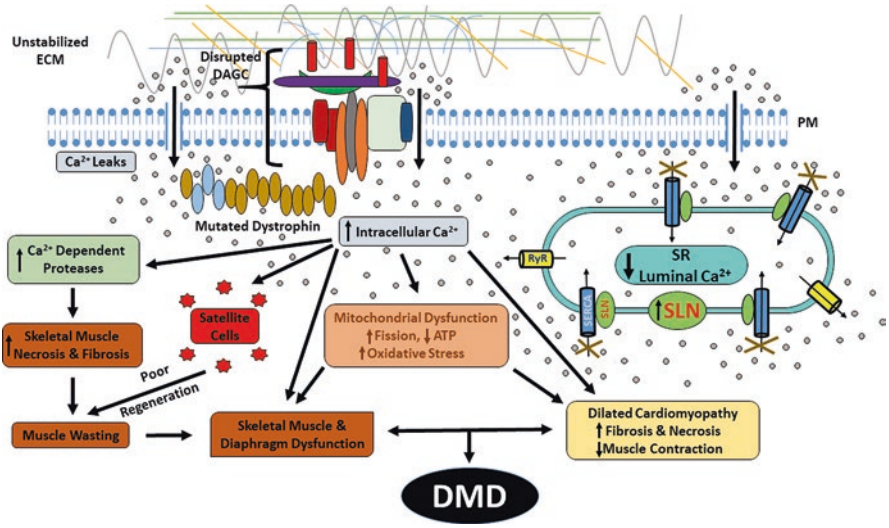

Fig. 23.1 Schematic diagram emphasizing the role of SLN upregulation in abnormal Ca^{2+} cycling and muscle pathogenesis in DMD. Abnormally high-level expression of SLN can chronically inhibit SERCA function and cause cytosolic Ca^{2+} overload in the dystrophin-deficient skeletal muscle and heart. These changes could lead to a plethora of downstream effects like activation of Ca^{2+}-dependent proteases, improper muscle regeneration, mitochondrial dysfunction, diaphragm and skeletal muscle dysfunction, and cardiomyopathy, resulting in DMD

23.4 Ablation of SLN Expression Mitigates DMD in Mouse Models

To test the above hypothesis, we took the genetic approach and ablated SLN gene in *mdx* and *mdx:utr−/−* mice. The *mdx* and *mdx:utr−/−* [40] pups deficient for SLN were alive and delivered normally in a Mendelian ratio and were undistinguished from the wild-type (WT) control mice. The survival of the SLN-deficient DMD mice was also significantly improved [40]. These findings suggest that germline deletion of SLN expression has no effect on the growth and development of dystrophic mice. Further these findings implicated the beneficial effect of SLN reduction in mitigating the DMD phenotype.

The haploinsufficiency or total loss of SLN gene improved the SERCA function as evidenced by the increased rate of Ca^{2+}-dependent Ca^{2+} uptake in dystrophic muscles of both *mdx* (Fig. 23.2a) and *mdx:utr−/−* mice [40]. Furthermore, the

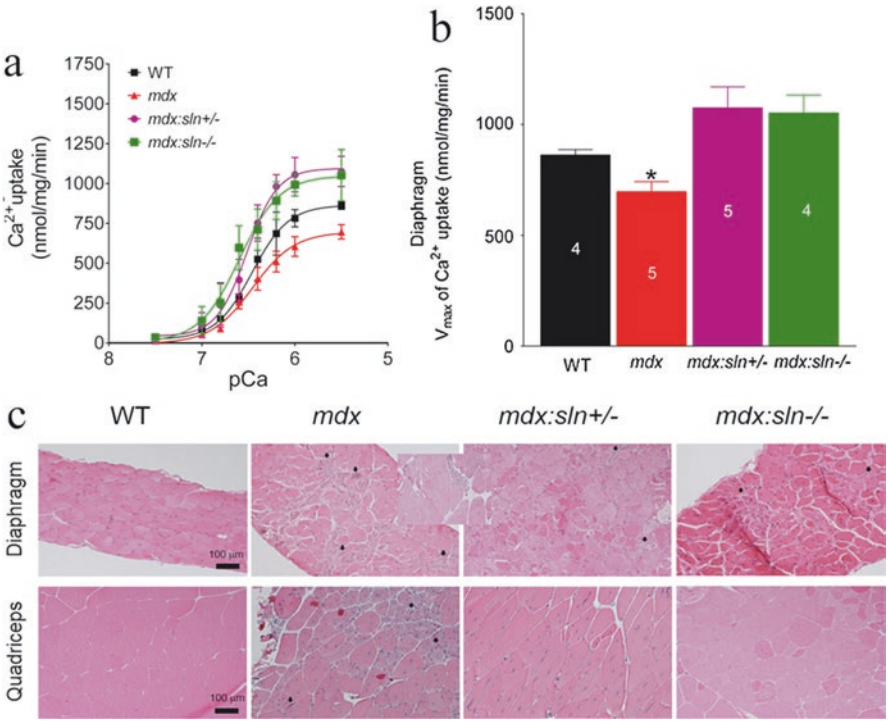

Fig. 23.2 Ablation of SLN improved SERCA function and mitigates muscle pathology in *mdx* mice. (**a**) The Ca^{2+}-dependent SR Ca^{2+} uptake and (**b**) V_{max} of Ca^{2+} uptake which are significantly decreased in the diaphragm of *mdx* mice are restored in the *mdx:sln+/−* and *mdx:sln−/−* mice. (**c**) H&E staining of the diaphragm and quadriceps show decreased mononuclear invasion (indicated by arrows) in the *mdx:sln+/−* and *mdx:sln−/−* mice compared to *mdx* littermates. Scale bar is 100 μm

maximum velocity (V_{max}) of SR Ca^{2+} uptake was significantly increased in the SLN-deficient dystrophic muscles of both *mdx* (Fig. 23.2b) and *mdx:utr−/−* [40] mice. Unlike other SERCA inhibitors, SLN levels can uniquely affect the V_{max} of Ca^{2+} uptake [54, 57]. The improved rate and V_{max} of SR Ca^{2+} uptake in SLN deficient dystrophic muscles therefore suggest that SLN upregulation could be the major cause of SERCA dysfunction in DMD.

Reduction or ablation of SLN expression also attenuated the activation of Ca^{2+}-dependent protease, calpain, in the dystrophic muscle [40]. Histopathological analysis of tissues from SLN deficient *mdx* and *mdx:utr−/−* mice revealed that reduction in SLN expression is sufficient to mitigate the severe muscular dystrophy phenotype. Hematoxylin and eosin (H&E) staining of the diaphragm and quadriceps from 4 to 5-month-old *mdx* mice demonstrated central nucleation, necrotic areas, and extensive mononuclear invasion and disorganized muscle fibers, whereas in the diaphragm and quadriceps of 4–5-month-old *mdx:sln+/−* and *mdx:sln−/−* mice, although central nucleation was evident, the muscle fibers were well organized, and necrotic areas were diminished (Fig. 23.2c). Similar improvements were also found in the *mdx:utr−/−* mice mutant for one SLN allele (*mdx:utr−/−:sln+/−*) and *mdx:utr−/−* deficient for SLN (*mdx:utr−/−:sln−/−*) [40]. In addition, the fibrosis was significantly reduced in these muscles [40]. Furthermore, ablation of SLN expression improved the muscle regeneration process as well as prevented the fiber-type transition in dystrophic muscles [40].

In addition to these structural improvements, beneficial effects also occur at the molecular levels. Reduction or complete loss of SLN restored the SERCA isoform expression as well as normalized CSQ levels in dystrophic muscles [40]. Structural mitigation also reflected in functional improvements in DMD mice deficient for SLN. Ablation of SLN improved the forelimb muscle strength in the *mdx:utr−/−* mice. Studies using isolated muscle preparations have demonstrated that reduction in SLN expression is sufficient to improve the functional properties of dystrophic diaphragm and skeletal muscles in *mdx:utr−/−* mice [40]. Taken together these studies have shown that reducing SLN expression is sufficient to mitigate the pathophysiological changes in DMD mice.

23.5 Amelioration of Cardiomyopathy in DMD Mice

Our recent studies have demonstrated that SLN protein expression also significantly increased in the ventricles of DMD patients and in mouse models [40]. These findings suggest that SLN upregulation is a common secondary change in skeletal and cardiac muscles of DMD. Thus, SLN ablation is anticipated to ameliorate the dystrophic cardiomyopathy in DMD mice. Consistent with this notion, germline reduction or ablation of SLN expression improved the cardiac function and ameliorated the cardiomyopathy in *mdx:utr−/−* mice [40]. Indeed, these improvements were greater than the skeletal muscle recovery and suggesting that SLN ablation is beneficial to cardiac function in DMD mice.

23.6 Sarcolipin Gene Therapy in Mouse Models

The proof of concept experiments using the germline gene knockout mice have led to test the gene therapy studies in DMD mice. As a first step toward this goal, SLN expression was knocked down in 1-month-old *mdx:utr−/−* mice using adeno-associated virus 9 (AAV9) expressing short-hairpin RNA specific for SLN (AAV9.sh*SLN*) and studied whether reducing SLN expression during postnatal period mitigates DMD and associated cardiomyopathy [40]. The AAV9-mediated SLN gene therapy in *mdx:utr−/−* mice showed that intravenous delivery of AAV9.sh*SLN* led to significant reduction in SLN expression in both skeletal and cardiac muscles. Furthermore our findings demonstrated that AAV treatment resulted in (1) improved SERCA function, (2) reduced diaphragm and muscle pathology, (3) enhanced muscle mechanics, and (4) prevention of cardiomyopathy in *mdx:utr−/−* mice. These findings enabled the evaluation of AAV-mediated SLN gene knockdown therapy as a potentially effective clinical therapy for the treatment of DMD.

23.7 Future Directions

In conclusion, our studies using mouse models provided initial evidence that SLN could be a potential therapeutic target for the treatment of DMD. However, data from larger animal models are still needed to validate the therapeutic potential of targeting SLN in treating DMD before going for clinical trials. Furthermore, the beneficial effects of reducing SLN expression will not address the primary issue of loss of dystrophin in DMD. Therefore, to enhance the treatment efficiency, the combination therapies including but not limited to expressing mini- or micro-dystrophin along with SLN gene silencing must be explored. Finally, it is necessary to understand the biology of SLN and the molecular mechanisms associated with its activation in dystrophic muscles. Currently, all this information are missing and are equally important to validate SLN targeting as a safe therapy for the treatment of DMD.

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Chapter 24
Gene Therapy for Central Nervous System in Duchenne Muscular Dystrophy

Cyrille Vaillend, Faouzi Zarrouki, and Ophélie Vacca

Abstract The development of molecular therapies enabling compensation of brain alterations in Duchenne muscular dystrophy is a major objective given the high level of functional impairment associated with intellectual disability and neuropsychiatric disorders in this syndrome. Functional and preclinical studies in mice lacking distinct brain dystrophins identified an accurate set of phenotypes, from the molecular to neurophysiological and behavioral levels, which can be used as markers of efficacy for brain gene therapy. Pioneer studies in this past decade provided encouraging results, demonstrating that both *dmd*-gene splice-switching correction and replacement strategies hold realistic prospects to rescue expression and function of the brain full-length (Dp427) or short C-terminal (Dp71) dystrophins responsible for variable degrees of cognitive impairment in DMD. Strategies that could correct or alleviate both muscle and brain dysfunctions entail selection of molecular tools able to cross the blood-brain barrier following systemic delivery, to largely spread in neural tissues, and to selectively target the neural cell types (neurons, astrocytes) that require rescued expression of distinct brain dystrophins. Recent breakthroughs show that this can be achieved by engineering naked antisense oligonucleotides with specific chemistries and/or adeno-associated virus vectors with selective capsid properties, thus raising new hopes to bring gene therapy closer to whole-body delivery and full treatment of DMD symptoms.

Keywords Duchenne muscular dystrophy · DMD · Dystrophin · Central nervous system · Adeno-associated virus · AAV · Dp427 · Dp71

C. Vaillend (✉) · O. Vacca
Paris-Saclay Institute of Neuroscience (Neuro-PSI), UMR 9197,
Université Paris Sud, CNRS, Université Paris Saclay, Orsay, France
e-mail: cyrille.vaillend@u-psud.fr; ophelie.vacca@inserm.fr

F. Zarrouki
Paris-Saclay Institute of Neuroscience (Neuro-PSI), UMR 9197,
Université Paris Sud, CNRS, Université Paris Saclay, Orsay, France

Université de Versailles Saint-Quentin-en-Yvelines, INSERM U1179,
Université Paris Saclay, Montigny-le-Bretonneux, France
e-mail: faouzi.zarrouki@uvsq.fr

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24.1 Needs and Obstacles to Treat Brain Dysfunction in Duchenne Muscular Dystrophy

Evaluation and characterization of cognitive and brain functioning in Duchenne muscular dystrophy (DMD) have been partly overlooked for many years. Nevertheless, it is undisputable that this multifactorial handicap comes along with significant cognitive and behavioral disturbances associated with important healthcare, educational, and quality-of-life concerns. The presence of intellectual disability, as well as the underdiagnosed comorbidity with autism spectrum, attention-deficit hyperactivity, and obsessive-compulsive disorders, is a major concern given the high level of functional impairment and compromised academic achievement associated with these conditions [1–4]. Developing therapeutic and accompanying strategies to alleviate non-motor aspects of DMD is even more critical since medical advances have extended the lifespan of patients considerably.

The finding that dystrophin is normally expressed not only in muscle but also in brain structures involved in cognitive functions led to the hypothesis that the cognitive impairment is a primary brain defect resulting from the same mutations that cause myopathy [5]. Hence the assumption that brain dysfunction in DMD could be treated using gene correction or replacement strategies comparable to those designed for muscle therapy. The past decades of research have indeed witnessed acceleration in our understanding of the dystrophin-dependent physiological mechanisms responsible for muscular dystrophy, leading to the development of innovative tools for molecular therapies and the start of early-phase clinical trials. However, much less is known on the efficacy of these approaches to alleviate the cognitive deficits associated with this syndrome. One major issue is the high clinical heterogeneity, likely due to the presence of several internal promoters within the dystrophin gene, giving rise in the brain to a range of dystrophin proteins expressed in different cell types and involved in distinct brain mechanisms. The variety of mutation patterns in patients may thus result in distinct phenotypic profiles, and a robust natural history taking into account the complex genomic organization of the *dmd* gene seems essential to correctly design therapeutic approaches [6]. Phenotypic variability has also been addressed by means of multidisciplinary studies in mouse models expressing a selective loss of different dystrophins. This enabled characterization of an accurate set of mutation-specific phenotypes that can be used to validate the efficiency of brain therapies in preclinical studies [7]. Treatment strategies entail development of separate molecular tools in order to rescue expression of specific dystrophin-gene products and to target specific cell types in mature nervous tissues. Another main technical challenge is to improve the capacity of such molecular tools to cross the blood-brain barrier, either through systemic or local delivery. The capacity to target both muscle and brain alterations can be considered as a key factor to narrow the selection of the molecular tools that could bring gene therapy closer to a full compensation of functional impairment in DMD patients.

24.2 Cognitive Dysfunction in Duchenne Muscular Dystrophy

Cognitive dysfunction in DMD is documented in several single-case reports, clinical follow-up, and meta-analyses of patient cohorts [1, 8, 9]. It is associated with a significant reduction of adaptive-skill and schooling performance but is independent from progression of the muscular handicap. The distribution of full-scale intelligence quotient (IQ) scores shows a leftward shift of one standard deviation compared to scores measured in siblings, normal population, and children with other muscular pathologies, with about one third of DMD patients displaying IQ scores below 70, which defines intellectual disability (mental retardation). However, global IQ scores do not necessarily provide an accurate picture of cognitive functioning, and several studies showed the presence of memory processing and attention deficits regardless of whether the patients displayed high or low IQs [10]. Moreover, the literature is often contradictory regarding the exact nature of the DMD neuropsychological profile, which encompasses a variety of alterations in language skills, learning and memory, executive functions, and visuospatial and fine-motor skills, with lower performance in both verbal and non-verbal tests and variable comorbid diagnosis of neuropsychiatric disorders. Evaluation accuracy has progressively improved by accounting for age, physical status, familial factors, and sample sizes and by the use of meta-analytical statistical methods and longitudinal studies [11–13]. Nevertheless, conclusions of many studies remain elusive or incomplete when patients with different mutation profiles are intermingled within cohorts, because mutations preventing expression of distinct dystrophin-gene products likely induce separate cognitive defects. As a result, the deficits exhibited by small subpopulations of patients may be underestimated, while those expressed in larger subgroups may lead to overgeneralization.

24.3 Genotype-Phenotype Relationships

Early reports showed that deletions of exon 52 [14] and mutations affecting the carboxyl terminus of dystrophin [15] were associated with intellectual disability. Studies in larger cohorts of DMD patients further supported the hypothesis that mutation location determines cognitive disability, but not motor outcomes [16, 17], and that heterogeneity of cognitive deficits mainly relies on the distinct phenotypes exhibited by patients with proximal versus distal mutations in the *dmd* gene (Fig. 24.1). It is now well admitted that mutations preventing expression of the full-length brain dystrophin (Dp427) may lead to mild cognitive and behavioral disturbances with an IQ most frequently in the normal range, while a cumulative lack of Dp140 and Dp71 is associated with higher incidence of intellectual disability. Dp71 loss is a clear aggravating factor systematically resulting in severe intellectual disability (IQ < 55) [18].

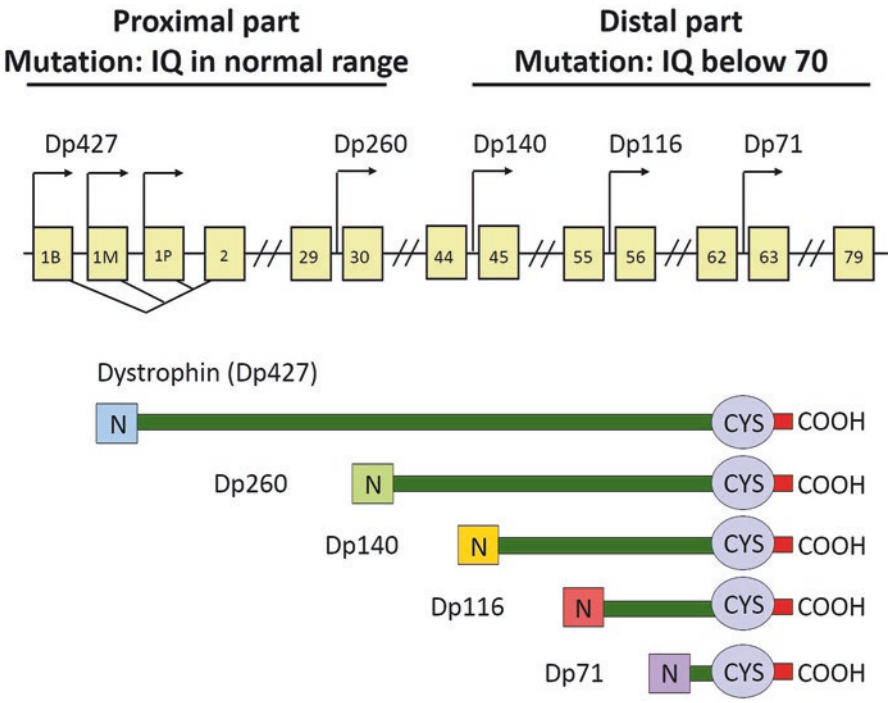

Fig. 24.1 The human dystrophin gene and corresponding protein products. Top drawing shows exon numbers (yellow boxes) and intronic regions (black line) flanking transcription start sites of distinct internal promoters (arrows). Alternatively spliced first exons give rise to distinct full-length forms of dystrophin (1B, brain; 1M, muscle; 1P, Purkinje neurons of the cerebellum), whereas the shorter dystrophin-gene products derive from distinct internal promoters, as indicated. Dp427 is found in principal neurons of the brain and cerebellum, Dp260 is a retinal dystrophin, Dp116 is expressed in peripheral nerves, Dp140 is mainly expressed in fetal brain, and Dp71 is expressed in glial cells of the adult brain. Dp140 and Dp71 are the main brain dystrophins which mutations were associated with intellectual disability. The main protein structural domains are shown, including the specific NH2-terminus domain (N), the central rod domain (green bar), the cysteine-rich domain (CYS, blue), and the COOH-terminus (red). *Adapted from* Perronnet and Vaillend [7] *with permission*

Considerable progress has been made in recent years to achieve reliable detection of mutations and comprehensive analysis and/or inference of the expression profiles of the distinct brain dystrophins in patients' cohorts, which is critical for diagnosis, prognosis, and development of adapted therapeutic approaches. Fine combinations of clinical, cognitive, molecular, and protein data in large cohort studies recently enabled emergence of consistent genotype-phenotype relationships that converge with mouse-model studies to the hypothesis that each brain dystrophin contributes to the cognitive and behavioral deficits in DMD [6, 19]. These studies showing association of specific mutations sites with distinct cognitive and neurobehavioral profiles enable more rigorous comparisons with the results obtained in mouse models in which a selective loss of Dp427 or Dp71 was associated with specific

brain dysfunctions [7, 20, 21]. Translational research refined in light of clinical data will likely help to achieve such a fine-level characterization of the core endophenotypes leading to intellectual disability and neuropsychiatric disorders in DMD. Identification of common phenotypes in patients and mice is critical for preclinical studies, and mouse models are valuable tools to characterize the cellular and molecular mechanisms underlying brain dysfunction and to define reliable markers of treatment efficacy.

24.4 Phenotypes of Mouse Models Lacking Dystrophins

24.4.1 The mdx Mouse Lacking Full-Length Dystrophin (Dp427)

The *mdx* mouse holds a nonsense mutation in exon 23 of the dystrophin gene which results in the absence of the full-length dystrophin (Dp427) in both muscle and brain tissues. This genetic model is suitable to evaluate the core brain and cognitive alterations that are common to all DMD patients, even though aggravation of the phenotype and presence of intellectual disability is generally associated with mutations in more distal parts of the gene leading to cumulative loss of other brain dystrophins. In the *mdx* model, the absence of muscle dystrophin causes a degeneration of skeletal muscle fibers, but muscle wasting is delayed compared with the human condition, likely due to higher efficiency of chronic regeneration cycles, and *mdx* mice only show strong motor impairment after more than 1 year of age [22]. Nevertheless, appropriate behavioral evaluation in young-adult *mdx* mice enables identification of enhanced fatigability, reduced muscle resistance, and impaired motor coordination, which can be quantified using the rotarod, inverted grid, and grip tests to discriminate and follow the effect of a therapy on muscle function [23–27]. Because this constitutes a potential risk of biased reliability in cognitive tests, it is important to select behavioral tests and parameters with low sensitivity to motor dysfunction. Conversely, central dysfunctions might also contribute to altered brain control of motor functions, due to the role of the cerebellum in motor coordination [24] or that of the amygdala in the emotional control of locomotor activity [28]. While *mdx* mice may display normal spontaneous locomotor activity in some experimental conditions [29, 30], they may also show enhanced fearfulness in responses to mild stressors such as standard manual restraint [31]. This particular phenotype, attributed to altered amygdala function, is associated with a drastic reduction in motor activity characterized by long periods of immobility (freezing). It has to be taken into account in preclinical studies at several levels: First, fear-related immobility due to experimental stress may lead to underestimation of treatment effects on muscular dystrophy, as quantification of treatment effectiveness often depends on improved locomotion in *mdx* mice. Second, this behavioral phenotype can be used as a pertinent marker of therapy effectiveness on brain functions.

Because brain Dp427 is normally expressed in several brain structures involved in cognition and emotional behavior, such as the hippocampus, amygdala, cerebellum, and associative cortical areas, the *mdx* mouse may display a variety of cognitive deficits depending on these different structures. Fine-level behavioral analyses have been performed during the past 20 years in order to specify the nature and severity of these deficits and to select the most severe and replicable phenotypes that could be used as relevant markers of treatment efficacy in preclinical studies. Beyond the emotional disturbances described above, presence of specific cognitive deficits has been highlighted: While learning performance of *mdx* mice is preserved in some tasks, delayed acquisition has been demonstrated in specific conditioning paradigms involving cue-outcome associative learning. Moreover, deficits in long-term memory and/or cognitive flexibility were identified in both spatial and nonspatial tasks, even when initial learning was unaffected, while short-term memory was largely unimpaired [21, 29, 32–34]. The deficits in hippocampal-dependent and amygdala-dependent long-term memories suggested that Dp427 loss could specifically impair memory consolidation processes. In support of this hypothesis, *mdx* mice display alterations in synapse ultrastructural organization and hippocampal synaptic plasticity, which are considered as specific neurophysiological features and mechanisms required for learning-induced remodeling of neuronal networks during memory consolidation [35–37].

The molecular mechanisms underlying behavioral disturbances in *mdx* mice are still unclear. Brain Dp427 is normally expressed in postsynaptic densities of central inhibitory synapses in principal neurons. Its loss induces aberrant molecular, structural, and physiological changes in synapses and compensatory changes in interneuron density [38], which likely contribute to the altered synaptic plasticity and memory deficits in this model [24, 31, 36, 39–42]. It is believed that Dp427 is a key component of the molecular scaffold that regulates clustering and/or properties of postsynaptic $GABA_A$ receptors in inhibitory synapses [43]. The loss of Dp427 would thus lead to impaired GABAergic inhibition and consequent enhancement of plasticity at glutamatergic synapses [36, 42, 44]. A drastic decrease in the number of α-subunit-containing $GABA_A$ receptor clusters (30–70% decrease among studies) has been reported in the various brain structures that normally express Dp427, including the amygdala, hippocampus, and cerebellum [31]. However, because the total amount of $GABA_A$ receptors is not significantly decreased, it is believed that Dp427 is dispensable for anchoring of these receptors but rather involved in their stabilization in large clusters at the synapse. The loss of dystrophin would thus result in an apparent loss of large synaptic clusters, due to the lateral diffusion of unstable receptors to extrasynaptic sites [28, 45].

24.4.2 The Dp71-Null Mouse

This transgenic mouse is a unique model to unravel the specific brain dysfunctions and behavioral phenotypes due to Dp71 loss, which are thought to aggravate the cognitive impairment and lead to the most severe cases of intellectual disability.

A selective absence of Dp71 is not typically observed in DMD patients, for whom distal mutations normally lead to cumulative loss of all dystrophins. However, a recent case report revealed that a rare but selective dysfunction of Dp71 can result in intellectual disability without muscular dystrophy [46], which further supports the importance of the Dp71-null mouse to decipher the mechanisms of intellectual disability associated with mutations in the *dmd* gene. The Dp71-null mouse does not have muscular dystrophy, as Dp71 is not expressed in skeletal muscles, but it displays cognitive deficits in visuospatial navigation and recognition tasks involving hippocampal-dependent long-term memory [47], which is reminiscent of some deficits reported in DMD patients with mutations upstream of exon 63 [19]. These deficits have been associated with enhanced hippocampal excitatory neurotransmission, suggesting that hyperexcitability of neuronal networks could be a basis of the brain dysfunctions due to Dp71 loss. A range of synaptic alterations have been described in this model, including postsynaptic disorganization of glutamatergic synapses, reduced synapse plasticity, and changes in synapse ultrastructure [37, 47], thus providing additional cellular and neurophysiological markers of brain dysfunction in this model.

The molecular basis of synaptic and cognitive dysfunctions in Dp71-null mice is still unclear. Dp71 is the main *dmd*-gene product expressed in the central nervous system (CNS), where it likely endorses multiple functions due to expression in both neuronal and glial subdomains [48]. One recent and most exciting finding of medical importance for DMD is the role that Dp71 appears to play at the glial-vascular interface in clustering and/or stabilizing potassium (Kir4.1) and water (aquaporin 4, or AQP4) channels. Dp71 expression is clearly enriched in perivascular-astrocyte endfeet throughout the brain, and it is believed that its loss may significantly alter potassium and water homeostasis as well as vascular permeability, with putative effects on neuronal excitability [49]. Considerable advances have been made in understanding the role of Dp71 in retinal glial cells and its involvement in blood-retinal barrier function. Despite structural and functional differences between the retina and brain, it is believed that Dp71 may play a common role in glial-vascular mechanisms throughout the CNS [50–53]. In the neural retina, macroglial cells (Müller glial cells and astrocytes) express Dp71 at the inner limiting membrane (ILM) and around blood vessels [54, 55]. The altered expression and/or distribution of AQP4 and Kir4.1 in the retina of Dp71-null mice appears to have functional implications in both astrocyte and vascular network development [55], as well as in retinal osmoregulation and vascular permeability [56]. This parallels the observation of retinal vascular abnormalities with diagnosis of retinal ischemia and proliferative retinal vasculopathy in some DMD patients [57–59]. Furthermore, the deletion of Dp71 was associated with retinal vascular inflammation, vascular lesions with increased leukocyte adhesion, and capillary degeneration, suggesting a role for Dp71-dependent mechanisms in retinal vascular inflammation diseases [60]. Impaired polarization of Kir4.1 channels in Müller glial cells was proposed to underlie a slight reduction in b-wave amplitudes of the scotopic electroretinogram [61]. Thus, a range of molecular and cellular alterations related to the disruption of Dp71-dependent neuronal/glial/vascular interactions are being identified, which could be used as markers of treatment efficacy in future studies aimed at rescuing Dp71 function.

24.5 Exon-Skipping Strategies to Rescue Brain Full-Length Dystrophin

24.5.1 Intracerebral Administration of Adeno-associated Virus (AAV) to Mediate Skipping of Exon 23 in mdx Mice

During the past decade, the development of molecular tools enabling rescue of dystrophin (Dp427) in the *mdx* mouse by exon skipping has shown a rapid evolution. The first successful rescue of brain Dp427 function was achieved using U7 small nuclear RNAs modified to encode antisense sequences expressed from recombinant AAV (rAAV) vectors [40]. As the structure of brain Dp427 is similar to that of muscle Dp427, the AAV 2/1-U7 system was used. AAV 2/1-U7 has proven efficient to induce skipping of the mutated exon 23 and to rescue expression of a functional dystrophin-like product in muscle tissues of *mdx* mice to about 50–80% of normal dystrophin expression levels from 4 to 13 weeks after injection [62]. In this pioneer study, intra-hippocampal administration of the AAV 2/1-U7 system also led to the skipping of exon 23 in the pre-mRNA of Dp427 in this brain structure, thus restoring an open reading frame and expression of a truncated brain dystrophin (Fig. 24.2a).

Interestingly, dystrophin expression was also recovered in adjacent, afferent, cortical areas such as the entorhinal cortex, likely due to efficient retrograde transport of rAAV2/1 vectors. Four months after a single stereotaxic injection of rAAV2/1-U7 in the hippocampus of *mdx* mice, dystrophin rescue was only partial (estimated at 15–25% of WT levels) and much lower than the levels obtained in muscle tissues. However, an immunofluorescence study of treated hippocampal cryo-sections revealed typical punctate dystrophin immunoreactivity (Fig. 24.2b, c) and colocalization with $GABA_A$ receptors, indicating that the rescued dystrophin was correctly localized at inhibitory synapses of hippocampal pyramidal neurons. Surprisingly, the partial rescue of brain Dp427 expression was sufficient to obtain a complete recovery of $GABA_A$ receptor clusters (Fig. 24.2d), as both the size and number of clusters were normalized by the treatment. This demonstrated the possibility to reach therapeutic threshold in the brain with this approach and to reverse the molecular synaptic defects in *mdx* mice. A follow-up study [63] showed that 25% dystrophin rescue is also sufficient to normalize synaptic plasticity in the hippocampus of the *mdx* mouse 2 months after intra-hippocampal injection (Fig. 24.2e, f). These studies showing that AAV-mediated exon skipping in the brain could rescue Dp427 expression and reverse synaptic dysfunctions in *mdx* mice without overt toxic effects opened the way to vectorized antisense-mediated therapy for the neuronal and cognitive defects in DMD. However, the spread of AAV transduction area was limited to ~1 mm away from injection sites, suggesting that multiple injection sites and/or the use of distinct delivery routes, different AAV serotypes, or naked antisense oligonucleotides (AONs) would be required to achieve transduction of the larger network of brain structures underlying cognitive deficits in *mdx* mice.

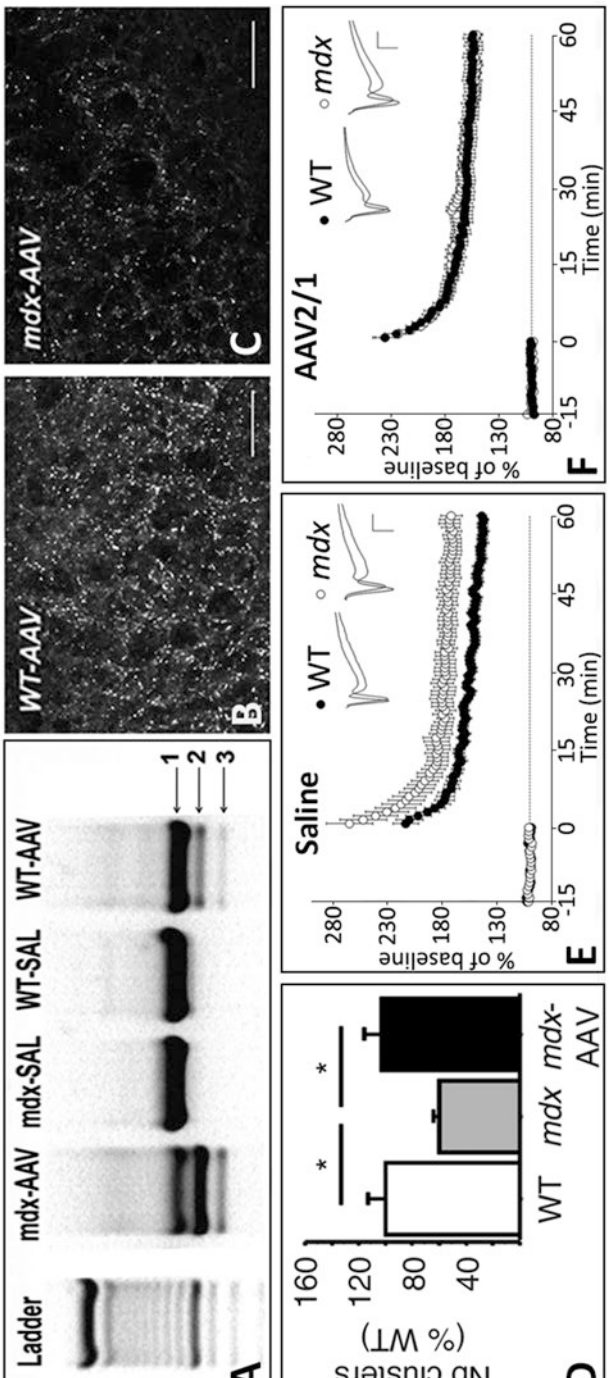

Fig. 24.2 AAV-mediated rescue of brain Dp427. (**a**) Expression of Dp427 transcripts analyzed by nested RT-PCR 4 months after intra-hippocampal injections of the AAV2/1-U7 system: Only homoduplexes of the 900-bp native (non-skipped) dystrophin mRNA (1) were detected in hippocampal extracts from saline-injected WT and *mdx* mice, whereas AAV-treated mice also expressed a 688-bp skipped dystrophin mRNA, as reflected by heteroduplexes containing both skipped and non-skipped mRNAs (2) and homoduplexes only containing the short mRNA lacking exon 23 (3). (**b**, **c**) Confocal images of dystrophin immunoreactivity in hippocampal pyramidal neurons in WT-AAV (**b**) and *mdx*-AAV mice (**c**), characterized by punctate staining reflecting synaptic relocalization (scale bar, 20 µm). (**d**) The number of $GABA_A$ receptors in dendritic layer of hippocampal neurons (% of WT) was reduced in untreated *mdx* mice but normalized to WT levels in treated *mdx-AAV* mice. *$p < 0.05$. (**e**, **f**) Abnormally enhanced LTP in *mdx* mice (**e**) was normalized to WT levels following AAV2/1-U7-mediated exon skipping (**f**). Calibration bars of representative traces: 5 ms, 0.5 mV. *Adapted with permission from* Vaillend et al. [40] *and* Dallerac et al. [63]

24.5.2 Intracerebral Administration of Antisense Oligonucleotides (AONs) to Mediate Skipping of Exon 23 in mdx Mice

The potential of naked AONs to correct brain dysfunction in *mdx* mice was first addressed using phosphorodiamidate morpholino oligomers (PMO) [31], which are currently under clinical trials for treatment of muscular dystrophy. Such morpholino oligomers have better diffusion properties than AAV vectors [64], do not induce immunological responses, and may display longer half-life in the CNS than in other tissues. In this study, intracerebroventricular infusion of antisense morpholino during 1 week allowed a restoration of ~25% of brain dystrophin 35 and 50 days after administration. Dystrophin rescue was effective in several distant brain structures, thus demonstrating that PMO has a good diffusion in the CNS following intraventricular delivery. This was accompanied by a substantial reduction of the abnormal stress-induced fear responses of *mdx* mice, which demonstrated the important role played by Dp427 in controlling amygdala-dependent emotional behavior. Analysis of the kinetics of Dp427 rescue revealed that the dystrophin protein was not yet expressed 3–4 weeks after treatment and no longer detected at delays >12 weeks. Importantly, significant reductions of fear responses were only effective when the dystrophin protein was readily detectable 5–7 weeks postinjection. This temporal window of dystrophin protein re-expression following AON administration likely is a critical factor of success in brain gene therapy for DMD, suggesting that chronic therapy will be required to maintain stable compensation of brain dysfunction.

24.5.3 Systemic Administration of New Classes of AONs to Mediate Skipping of Exon 23 in mdx Mice

In order to satisfy the requirements for systemic treatment by exon skipping, there was a need to develop new AON chemistries that could target both peripheral and central tissues affected by dystrophin loss, i.e., having the capacity to cross the blood-brain barrier (BBB). In spite of the advances made in AON chemistry and design, the efficiency of systemic administration was limited due to poor tissue uptake and inability to cross the BBB, and brain dystrophin rescue had only been achieved by means of intracerebral administration, as described above. A new class of AONs made of tricyclo-DNA (Tc-DNA AONs) was recently characterized that showed unique pharmacological properties and uptake by many tissues including the brain after systemic administration, making this new tool very attractive for whole-body treatment strategies in DMD [27]. *Mdx* mice injected intravenously in the retro-orbital sinus for 12 weeks with 200 mg/kg/week Tc-DNA AONs showed strong expression of exon 23-skipped dystrophin mRNA (up to ~30% of WT levels) and Dp427 protein (up to ~50%) in various muscles including the heart and diaphragm, which was five- to sixfold higher than expression induced by high doses of 2′OMe and PMO AONs. Detection of the skipped mRNA in brain tissues, including cortical,

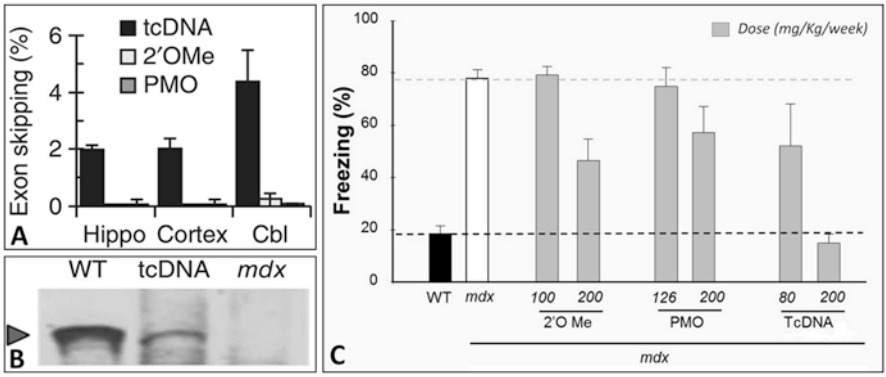

Fig. 24.3 Brain Dp427 rescue by systemic administration of naked Tc-DNA AONs. (**a**) Quantification of exon 23-skipped Dp427 mRNA in the hippocampus (Hippo), cortex, and cerebellum (Cbl) following IV injection of 200 mg/kg/week Tc-DNA, 2′OMe, and PMO for 12 weeks. Only Tc-DNA AONs crossed the BBB. (**b**) Western blot showing detection of the Dp427 protein (arrowhead) in the cerebellum from TcDNA-treated *mdx* mice compared with WT and untreated *mdx* mice. 120 μg of total protein were loaded for the tcDNA and control *mdx* samples and 20 μg for the WT control. (**c**) Restraint-induced unconditioned fear responses expressed as the percentage of tonic immobility (freezing) in 5 min. Only Tc-DNA AONs (200 mg/kg/week) enabled normalization of the freezing behavior in *mdx* mice. *Adapted with permission from* Goyenvalle et al. [27]

hippocampal, and cerebellar areas, was only found in mice treated with Tc-DNA AONs, thus demonstrating unprecedented capacity to cross the BBB (Fig. 24.3a).

Although the brain expression levels of exon 23-skipped mRNA were relatively low (on average below 6%), the dystrophin protein was readily detectable in western blots (Fig. 24.3b) and in immunofluorescence analysis of brain cryosections as typical punctate staining suggesting synaptic relocalization. Most importantly, this partial rescue of brain Dp427 expression enabled full restoration of a normal emotional behavior in *mdx* mice treated with Tc-DNA AONs (Fig. 24.3c), which did not display the enhanced fearfulness observed in untreated mice or in mice treated with 2′OMe and PMO AONs. Thus, systemic administration of Tc-DNA AONs enabled re-expression of a functional Dp427-like protein in both muscle and non-muscle tissues. This demonstrates the potential of Tc-DNA AONs for future therapy aimed at treating both the predominant musculo-cardiorespiratory defects and the cognitive impairment of DMD patients.

24.6 New Prospects for AAV-Mediated Dp71 Replacement Therapy

Molecular tools and gene therapy approaches specifically targeting the Dp71 protein have long been lacking, which precluded interventions aimed at modulating and/or rescuing Dp71 expression in mouse models of DMD. Recently, a new AAV vector,

the ShH10, engineered in vitro by directed evolution to specifically target glial cells, has been shown to transduce almost exclusively Müller glial cells (MGCs) in both rat [65] and mouse retina (Fig. 24.4a) [66]. In mouse retina, transduction of MGCs was more efficient in Dp71-null mice than in wild-type mice (Fig. 24.4b, c), suggesting high suitability for rescue strategies in this transgenic model.

The complete murine Dp71 sequence from exon 63 to exon 79 without splicing was then cloned under control of a strong ubiquitous CBA promoter, to develop the first tool for Dp71 replacement strategies in CNS (Fig. 24.4d). The ShH10 vector expressing Dp71 was highly efficient following intravitreal injection to induce re-expression of Dp71 in MGCs of Dp71-null mice [67]. Indeed, this treatment

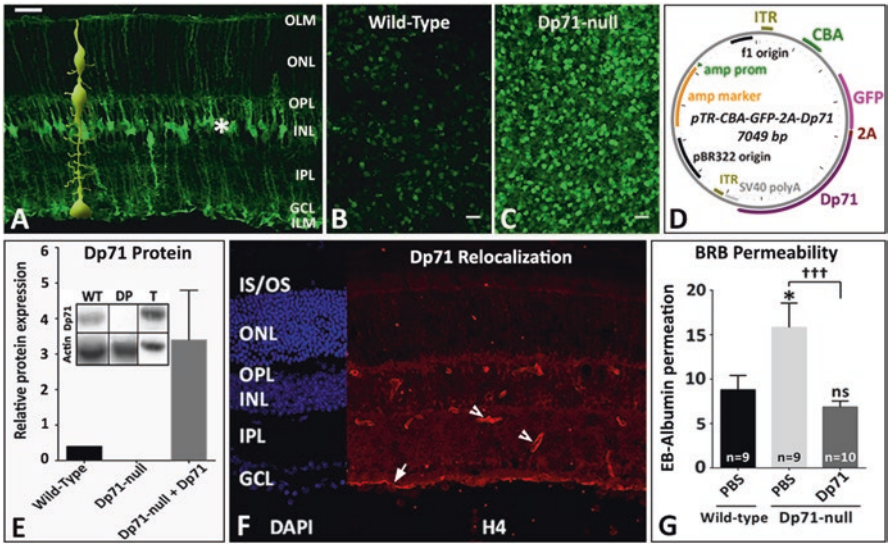

Fig. 24.4 AAV-mediated Dp71 rescue. (**a**) Retinal cryosection showing GFP expression (green) selectively in Müller glial cells (MGCs), thus demonstrating the glial specificity of the ShH10 capsid. The superimposed drawing represents a MGC crossing the retina from outer limiting membrane (OLM) to ILM. The white asterisk shows the z level at which confocal images in (**b**) and (**c**) were taken. (**b**, **c**) GFP immunoreactivity in MGC cell bodies of WT (**b**) and Dp71-null (**c**) retinas showing a much better transduction in Dp71-null mice. (**d**) Dp71 expression plasmid containing a CBA promoter, a GFP reporter gene linked to Dp71 coding sequence with the viral 2A peptide, and two ITR for encapsidation in the ShH10 vector. (**e**) Western blots showing expression of Dp71 in WT but not Dp71-null mice (DP) and re-expression of Dp71 in treated Dp71-null mice (T) 2 months after intravitreal injection of the ShH10-GFP-2A-Dp71 vector. Histograms show quantitative data highlighting the sevenfold overexpression in Dp71-null mice. (**f**) Retinal cryosection of Dp71-null retina labeled with anti-dystrophins antibody (H4, red) showing relocalization of Dp71 around vessels (white open arrows) and at the ILM (white arrow). (**g**) BRB permeability quantified by the Evans blue (EB) method, showing increased EB-albumin leakage in retinal tissue due to BRB breakdown in Dp71-null compared to WT mice (PBS) and rescue of BRB function following Dp71 overexpression (Mann-Whitney test; $*p < 0.05$, $\dagger p < 0.001$; $n \geq 9$). *Adapted with permission from* Vacca et al. [66] (*Panels* **a**–**c**) *and* Vacca et al. [67] (*Panels* **e**–**g**)

induced an overexpression of Dp71 (Fig. 24.4e) and a relocalization of Dp71 (Fig. 24.4f) and of critical components of the Dp71-associated protein complex at the MGC endfeet, such as AQP4 and Kir4.1 channels. Functionally, this led to the restoration of blood-retinal barrier (BRB) function (Fig. 24.4g) and enabled efficient reabsorption of an experimentally induced retinal edema. Although a putative application for brain gene delivery remains to be tested, these results open new routes for the development of molecular tools that could specifically rescue Dp71 expression in CNS glial cells and give hope for compensation of the most severe cognitive deficits associated with the DMD syndrome.

24.7 Treating the Nervous System Versus Treating Other Organs

24.7.1 Developmental Stage and Brain Plasticity

Alterations of critical mechanisms taking place during vulnerable periods of brain development, such as neurogenesis, cell migration, and neuronal connectivity, are thought to underlie irreversible neurodevelopmental disorders. This suggests that the brain dysfunctions associated with intellectual disability and other neurological disorders may not be accessible to pharmacological or genetic correction when treatments start in postnatal periods. Strikingly, however, a number of studies in mouse models indicate that reversing the underlying molecular mechanisms can overcome or at least alleviate cognitive deficits, even if treatments are started in adulthood and do not necessarily rescue all brain structural abnormalities. One likely hypothesis is that the same genes involved in brain development also play a critical role during learning-induced activity-dependent remodeling of neuronal networks, i.e., during adult brain plasticity, and/or that particular treatment strategies may reactivate some features of developmental plasticity that facilitate recovery [68, 69]. In DMD, brain imaging studies and autopsies yielded uneven conclusions, suggesting that macroscopic brain abnormalities are not features of all patients [70, 71]. Studies in *mdx* mice unveiled that brain alterations due to dystrophin loss are mainly located at the cellular level and are associated with substantial rearrangements of synapse density and ultrastructure [35–37]. Yet, rescuing stable expression of Dp427 in the adult brain of *mdx* mice resulted in successful normalization of $GABA_A$ receptor clustering, hippocampal synaptic plasticity, and behavioral features [27, 40, 63]. This suggests that at least part of the behavioral deficits in this model are reversible because they result from alterations of Dp427-dependent mechanisms involved in the modulation of synaptic plasticity by GABA receptor in the adult brain.

24.7.2 The Vascular Barrier

The design of specific AON chemistries and AAV vectors able to cross the CNS barriers, such as the vascular barrier, appears as an important challenge for development of therapies for whole-body treatment in DMD. Because of the difficulty to package large dystrophin coding sequences in an AAV vector, correction using small vectorized or naked AONs mediating skipping of the mutated exon constitutes the most promising strategy for Dp427 rescue. Some naked AONs showing good therapeutic effects for muscle treatment in preclinical studies may eventually pass the blood-cerebrospinal fluid barrier [31]. However, most of them cannot cross the vascular barrier following systemic administration, with the exception of the recently engineered Tc-DNA AON, likely due to unique properties associated with the tricyclo-DNA chemistry (i.e., higher resistance to RNase, strong hydrophobicity, and capacity to form nanoparticles in solutions) [27]. Strikingly, only the Tc-DNA AON demonstrated capacity for functional correction of neurobehavioral deficits in *mdx* mice in a comparative study.

For AAV vectors, systemic delivery routes have to be tested to target CNS, because invasive intracerebral injections resulting in a small transduced area and putative cerebral damages are less desirable options for human gene therapy. Some natural or designed AAV vectors have shown the capacity to cross the blood-brain barrier (BBB) after intravenous delivery even in adult animals and may therefore offer a good opportunity for noninvasive brain delivery in DMD [72]. However, this has not been tested yet to rescue brain dystrophins. Moreover, recent reports indicate that a lack of brain dystrophins and/or associated proteins may increase the capacity of some circulating molecules to cross the BBB. Indeed, a blood barrier breakdown with fluid leakage into the neural tissue was shown in Dp427-deficient *mdx* mice [73, 74], Dp71-null mice [56, 60], α-dystrobrevin knockout mice [75], and laminin-α2-deficient mice [76]. Although the vascular barrier in these models is abnormally permeable to high molecular weight proteins such as serum albumin, a putative permeability to AAV particles has not been demonstrated to date. In the retina of Dp71-null mice, which exhibit a blood-retinal barrier (BRB) breakdown [56], no virus leakage has been observed into the bloodstream when AAV particles were injected into the vitreous. This is an important result regarding AAV-mediated retinal gene therapy, as intravitreal administration may thus only target retinal territories even if the BRB is compromised. Importantly, the inner limiting membrane (ILM) of the retina also constitutes a physical barrier for penetration of AAV particles following intravitreal injection [77], even though this delivery route is safer than subretinal injections that may cause retinal detachment. To date, all clinical trials seeking to treat retinopathies using AAVs were performed by subretinal injections [78], and the development of therapeutic tools able to cross the ILM is a critical challenge. Strikingly, Dp71 loss alters the expression and/or localization of major glial components of the ILM such as β-dystroglycan and laminin [66], leading to increased ILM permeability to AAV particles (Fig. 24.4b, c). Future studies in Dp71-null mice will determine whether such alterations of extracellular matrix integrity could also facilitate AAV-mediated brain gene therapy following systemic administration.

24.7.3 Targeting Specific Neural Cell Types

Even though both naked AONs and AAV vectors are promising tools to target all organs, even the brain and its impenetrable BBB, another critical challenge for brain gene therapy in DMD is to target precisely and efficiently the desired cell types to avoid off-target responses. This may not be an issue for antisense-mediated exon-skipping approaches, as splicing of the dystrophin pre-mRNA will only occur in the cells that would normally express dystrophin. With vectorized transgenes, such as for Dp71 rescue experiments, off-target responses might occur, as AAV in the brain may transduce a range of different cell populations. Although distinct AAV serotypes may show specific cell-type preferences, they have the general ability to transduce all major cell types in the brain, including neuronal, microglial, and macroglial cell subtypes, oligodendrocytes, and endothelial cells [79]. Driving expression of the transgene from a specific promoter of the targeted cell population can circumvent this problem [72, 80]. Recent advances in AAV capsid engineering have resulted in the generation of new AAV vectors with improved properties such as increased transduction efficiency, targeted transduction of naturally inaccessible cell types, reduced immunogenicity, and very low off-target responses [81, 82]. These important achievements are expected to improve and pave the way for new clinical applications based on AAV gene therapy.

24.7.4 Therapeutic Threshold

Earlier exploitation of AAV technology has shown that even modest changes in a brain protein expression level may have significant functional impact in animal models of genetic diseases. For example, in a mouse model of spinocerebellar ataxia type 1, a genetic dominant neurodegenerative disease, AAV-shRNA-based therapy enabled improvement of motor coordination by repressing expression of mutant ataxin-1 in cerebellar neurons by less than 10%, likely because the shRNA was allele-specific and did not alter the wild-type copy needed for normal cell function [83]. In recessive diseases characterized by lack of expression or loss of function of a protein, partial re-expression may also induce significant functional improvements, suggesting that only a small quantity of protein is required to ensure cellular function. Thus, in mice expressing low dystrophin levels due to skewed X-inactivation [84] or after muscle gene therapy in dystrophin-deficient DMD models [85, 86], only a partial expression of dystrophin was sufficient to improve muscle function. In muscle gene therapy, however, a minimum threshold of dystrophin-expressing fibers is likely required to achieve efficient therapeutic effect. Most clearly in brain gene therapy studies in *mdx* mice, rescuing brain Dp427 expression to 15–25% of WT levels with the AAV2/1-U7 system enabled full normalization of both molecular ($GABA_A$ receptor clusters) and neurophysiological (synaptic plasticity) brain defects. Likewise, rescuing less than 10% of brain Dp427 with systemic delivery of

Tc-DNA AONs normalized the stress-induced abnormal enhancement of fear responses [27, 40, 63]. Thus, less than 25% re-expression of brain Dp427 appears to be sufficient to correct brain and behavioral functions.

In contrast, the first attempt to rescue Dp71 expression and function in the retina of Dp71-null mice through AAV-mediated gene delivery was associated with a sevenfold overexpression of Dp71 (Fig. 24.4e). The main effect of this treatment was to relocalize critical channels involved in retinal ion homeostasis and rescue BRB function without any overt detrimental side effect [67]. The high efficiency of the ShH10 capsid and strong ubiquitous CBA promoter may likely explain why Dp71 was overexpressed compared to wild-type Dp71 expression levels. To induce a more physiological restoration, smaller amounts of viral particles could be tested for dose-response effects, and the use of a glial-specific promoter to drive Dp71 glial expression may also help to reach more reasonable expression levels [87]. These investigations could inform us about the therapeutic threshold of the Dp71 restoration, in other words at what amount of Dp71 the BRB permeability can be restored.

24.8 Summary and Future Directions

The current results from CNS-targeted gene delivery in mouse preclinical studies are encouraging, showing that both splice-switching correction and replacement strategies hold realistic prospects to rescue expression and function of the brain full-length/large and short C-terminal dystrophins, respectively. Functional and preclinical studies undertaken in this laboratory helped establishing an accurate set of biomarkers and phenotypes in DMD mouse models, from the molecular to neurophysiological and behavioral levels, which can be used to determine if molecular therapies developed to treat DMD may also correct brain, cognitive, and neurobehavioral defects in this disease. More studies are needed, however, to precisely characterize the multifarious roles played by the different brain dystrophins and to identify specific targets relevant to distinct patients' mutation profiles. Perspectives of bench to bedside gene therapy translation also poses the challenge to engineer specific chemistries for naked AON and novel properties for AAV vectors, in order to minimize neurotoxicity, to target selective neural cell types, and to ensure penetration of CNS barrier and deep spread in neural tissues following systemic delivery. This will help to circumvent current hurdles and bring gene therapy closer to whole-body delivery and full treatment of the DMD syndrome.

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Chapter 25
Therapeutic Approaches for Dysferlinopathy in Animal Models

William Lostal and Isabelle Richard

Abstract Dysferlin deficiencies are responsible for muscular dystrophies with different presentations with the most frequent being limb-girdle muscular dystrophy type 2B and Miyoshi myopathy. The dysferlin (*DYSF)* gene is expressed through a 6.2 kb coding sequence, a size that impedes classical gene transfer approach using adeno-associated vectors (AAVs). There is no treatment for dysferlin deficiencies, but in the last decade, a large spectrum of strategies has been evaluated in different setups. In this review, we present the different models that are available for preclinical studies as well as the different therapeutic approaches evaluated so far. Among the latter, the most extensively studied strategies are AAV-mediated transfer of either full-length or truncated dysferlin using two or one AAV, RNA-based strategies such as exon skipping and trans-splicing, and overexpression of a protein proposed to compensate the absence of dysferlin. Gene editing and cell therapy have also been set off.

Keywords Dysferlin · In vivo · Treatment · Mouse · Skeletal muscle

25.1 Introduction

Dysferlinopathies are a clinically heterogeneous group of muscular dystrophies due to mutations in dysferlin [1–3]. The most frequent presentations are the limb-girdle muscular dystrophy type 2B (LGMD2B) [1, 3, 4] and the Miyoshi myopathy (MM) [3, 4]. Additional minor phenotypes were also reported: distal myopathy with anterior tibial onset (DMAT) [5], proximo-distal dysferlinopathy [6], pseudometabolic myopathy, or isolated hyperCKemia [7]. The age of onset is variable from childhood to the fourth decade [8, 9], and patients usually lose their ability to walk 10–30 years after [8]. There is no treatment today for these diseases.

W. Lostal · I. Richard (✉)
Généthon INSERM, U951, INTEGRARE Research Unit, University Paris–Saclay, Evry, France
e-mail: lostal@genethon.fr; richard@genethon.fr

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The human dysferlin is a 237-kDa tail-anchored type II transmembrane protein. It has 13 domains including 7 C2 domains and 6 specific domains: FerI, FerA, FerB, a double DysF internal Nter, a double DysF internal Cter, and a C-terminal transmembrane domain (TM) [10] (*see* Fig. 25.1a). It was shown that dysferlin plays a major role in muscle membrane repair [11], especially due to the C-ter part [12]. The gene is expressed as a 6.2-kb cDNA, composed of 55 exons (*see* Fig. 25.1a) and predominantly present in skeletal muscle and in peripheral blood monocytes [13]. Importantly, transgenic overexpression of dysferlin in the sole skeletal muscle of A/J mice demonstrated a complete rescue of the muscular dystrophy phenotype, indicating that correcting the deficit in muscle is sufficient [14]. In addition, Roche et al. showed that macrophage infiltration in impaired muscle is a consequence and not a cause of the myofiber damage [15].

There is no treatment for dysferlinopathies to date, but a large number of different approaches based on genetic intervention are being investigated in dysferlin-deficient animals. The variety of proposed approaches is in line with the large size of its coding sequence, which imposes adaptations or alternatives of the classic approach of gene transfer. Indeed, most of the strategies that were tested in experimental studies in Duchenne muscular dystrophy, another example of a disease with a large gene, have also been investigated in dysferlin-deficient models. These approaches include AAV-mediated transfer of either a full-length or partial dysferlin, RNA-based strategies such as exon skipping or trans-splicing, the overexpression of an alternative protein proposed to compensate at least some of the function of dysferlin, or more specific approaches such as gene editing, cell therapy, or naked DNA transfer. This chapter highlights the available dysferlin-deficient animal models that are useful for determining outputs and evaluating treatments and the different engineered approaches evaluated so far to correct the deficiency in dysferlin (*see* Fig. 25.1b).

25.2 Available Dysferlin-Deficient Animal Models

Three mouse models deficient in dysferlin have been described: two are naturally occurring animal models, SJL and A/J mice [16], and the last one is an engineered knockout (KO) [11]. All these strains are available in the Jackson laboratory (www.jax.org).

The SJL genome presents a splice site mutation, leading to the removal of exon 45 corresponding to the C2E protein domain in dysferlin [17–19]. Since this corresponds to an in-frame deletion, a residual protein can still be present in the muscle.

Fig. 25.1 (continued) for the 3′ vector, with an overlapped sequence up to 1475 bp. Two different sets of overlapped vectors have been tested experimentally and showed a high efficiency after systemic delivery (# from Ex24 to 30 [36] and $ from the middle of Ex24 to the middle of Ex31 [38]). (**d**) The minidysferlin vectors that were tested experimentally were depicted. * = this vector was used in a rAAV vector in dysferlin-deficient mice [39]. ** = constructs evaluated C2D, C2E, and C2F removal [24]. (**e**) RNA-mediated strategies based on exon skipping and trans-splicing. Nota bene: for Philippi et al. evaluated four different PTM targeting introns 30, 31, 35, or 36

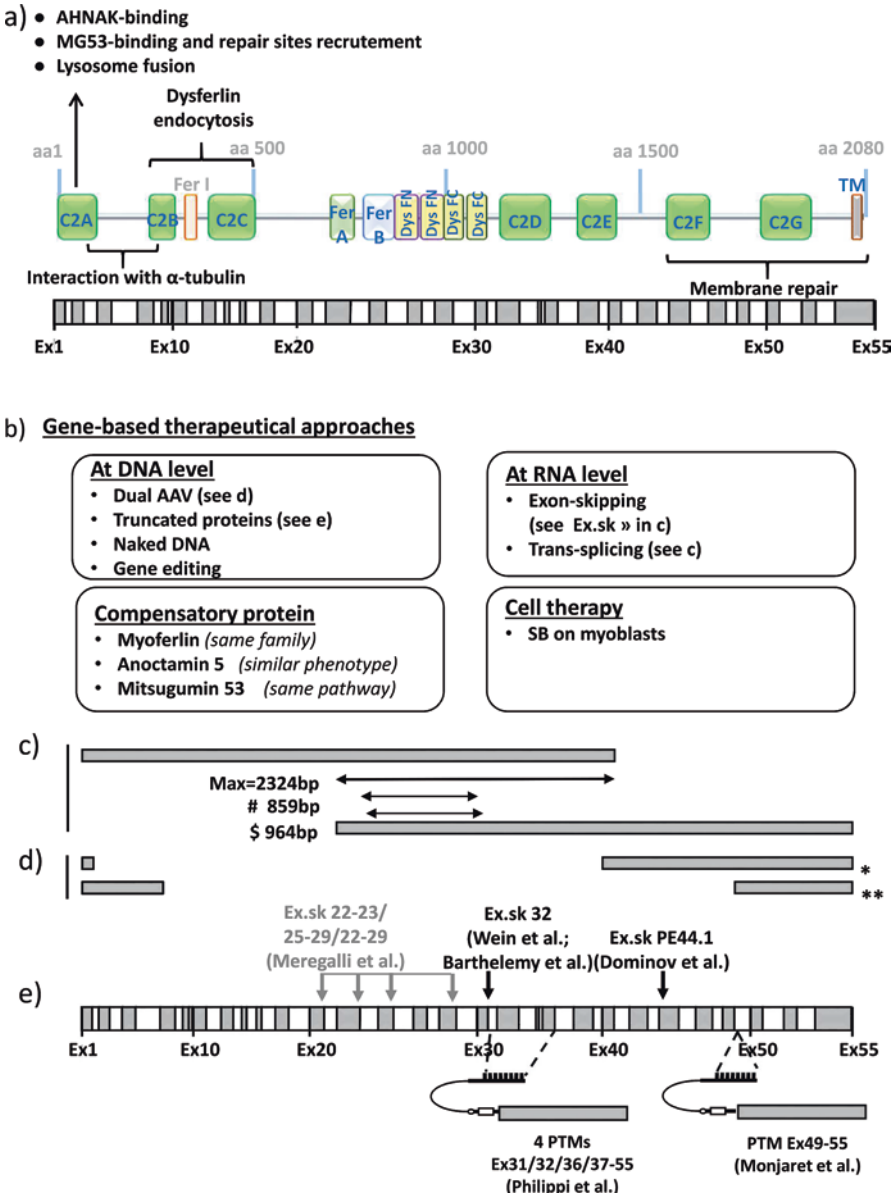

Fig. 25.1 Schematic illustration of the dysferlin gene structure and dysferlinopathy therapy. (**a**) Scheme of the 237 kDa human dysferlin protein including functions and interactions of distinct regions (adapted from [49]). Dysferlin is composed of 13 domains including 7 C2 domains and 6 specific domains: FerI, FerA, FerB, a double DysF internal Nter, a double DysF internal Cter, and a C-terminal transmembrane domain (TM). The corresponding exons are depicted below. (**b**) Gene-based therapeutic approaches presented in this review. (**c**) For dual AAV overlapped vectors, in an optimal design (removing all unnecessary sequences and considering the minimal size of regulatory sequences), the maximal dysferlin sequence in the 5′ vector will be 4047 bp and 4520 bp

The mutation leads to a dystrophic phenotype including centronucleated fibers (CNF), size variation, inflammatory infiltrates, and eventually muscle fat replacement. A progressive loss of muscle mass and strength was observed. The A/J mice present an insertion of a unique early transposon (ETn), a retransposon in intron 4 of the *DYSF* gene [19], leading to a complete absence of the dysferlin protein. This mutation is associated with a late-onset progressive muscular dystrophy. A/J was questioned as an adequate model for dysferlinopathies because it carries a null allele for the complement element C5 and the fact that abnormality of the complement pathway was proposed to play a role in the pathophysiology of dysferlin deficiency [20, 21].

A backcross onto the C57BL/6 background was performed for the A/J strain, allowing the possibility to use strain-matched control and eliminating the additional mutation in C5. The resulting model is called B6.A/J-*Dysf* ^{prmd} (aka BLA/J) [22] and has been widely characterized and used in different studies [23–25]. The first dystrophic features appear at 2 months of age, with CNF and inflammation areas. This model presents differences in the locomotor activity and membrane repair process [22]. Interestingly, the phenotype resembles more the LGMD2B phenotype than the Miyoshi myopathy with the gluteus and the psoas as the most affected muscles. This model was also crossed in two different immune-deficient backgrounds: scid (*Prkdc*^{scid}) and NRG (NOD.Cj-*Rag1*^{tm1Mom} *Il2rg*^{tm1Wjl}) [26, 27].

The engineered KO called *Dysf*^{tm1Kcam} was generated using a targeting vector to replace a 12-kb region carrying exon 51–54 in the 3′ part of dysferlin gene [11]. However, it was recently defined that the cassette inserted itself in exon 48 [28]. This model showed a slowly progressive muscular dystrophy with presence of centronucleated and necrotic fibers as soon as 2 months of age.

Besides mouse models, dysferlin-deficient zebrafish were generated using morpholino [29, 30]. A reduced level of expression was observed in the different morphants, leading to a disorganization and impairment of the zebrafish muscle. Such model can be very useful in pharmacological screenings, for example. Because a large animal model can present an interest in preclinical studies for evaluation of biodistribution, for example, the Jain Foundation (https://www.jain-foundation.org/) in collaboration with the Comparative Neuromuscular Laboratory (CNL) of San Diego, USA, undertook a screening of biopsies in order to identify a canine or a feline model for LGMD2B, without any success as of today.

25.3 AAV-Mediated Transfer of Partial or Full-Length Dysferlin

25.3.1 AAV as Vector for Transfer

Recombinant vectors based on the adeno-associated virus (AAV) are valuable and widely used tools to introduce genes in vivo, in particular in a gene therapy perspective. This vector has been used in more than 100 clinical trials worldwide with

promising results obtained in genetic diseases affecting the retina, skeletal muscle, and liver among others. AAV is a small replication defective, non-enveloped virus belonging to the genus *Dependoparvovirus*. It is composed of an icosahedral proteic capsid and a ~4.7-kb genome comprising two inverted terminal repeats (ITR) and two open reading frames (ORF), rep and cap, that can be replaced by a recombinant cassette to generate a vector. The packaging size of AAV limits the applications of its use to genes with a size lower than 5 kb. It can nevertheless be diverted to transfer large disease genes. As exemplified in the case of dystrophin, one possibility is to reconstitute a larger ORF with two or three AAVs through the use of homologous recombination [31, 32]. The second possibility is to transfer truncated versions of the gene that would have conserved at least part of its function [33, 34].

25.3.2 Large Gene Delivery Strategies

In this strategy, fragments of the desired large ORF are inserted in independent AAVs that are then injected at the same time. The reconstitution is achieved by the cell machinery, thanks to the recombinogenic properties of sequences carried by the cassettes (i.e., ITR, overlapping fragment, recombinogenic sequence that can be associated with splicing of the excedent sequences). For the transfer of the full-length dysferlin, several approaches have been tested over the past decade. The first study based on concatemerization and splicing was a proof of concept for dysferlin with spectacular dysferlin expression and correction of the pathological signs after intramuscular (IM) injection and restoration of dysferlin functions in dysferlin-deficient mouse model [22]. The positive results obtained in a second study based on the use of a single vector where the full-length cDNA was incorporated, while initially reported as showing a high capacity of the specific serotype, highlighted that it is possible to reconstitute dysferlin from fragmented and randomly packaged AAV [35]. Then, we published in 2015 a comparative study of four commonly large gene AAV approaches (concatemerization/splicing, overlapping vectors, hybrid and full-length fragmented AAV) [36]. Details of the packaged dysferlin fragments are presented in Fig. 25.1c. The overlap strategy was demonstrated as the more efficient approach to deliver dysferlin into the skeletal muscle. The level of protein was 0.5- to 2-fold compared to wild-type (wt) level after systemic delivery in upper and lower limbs muscle; and the expression was associated with correction of histopathology and functional capacities of dysferlin-deficient mouse. More recently, a preclinical study in mice and nonhuman primates (NHP) evaluating such vectors confirmed this efficiency with no toxicity or immune response [37, 38], paving the path to the clinical trial.

A phase I trial (NCT02710500) is ongoing, with estimated completion date in March 2018. It is a double-blind, randomized controlled study with direct intramuscular injection of dual AAV gene vector (rAAVrh.74.MHCK7.DYSF.DV) to the extensor digitorum brevis muscle (EDB). Two doses will be evaluated, with three patients in each cohort.

25.3.3 Truncated Proteins

In that case, the ORF inserted in the vector codes for a smaller version of the protein deleted of certain domains but which has conserved at least a partial capacity to correct the consequences of the genetic defects. The demonstration of the potential of minidysferlin was obtained, thanks to the observation that a patient presenting a mild phenotype was expressing a very short form of dysferlin with a deletion from exons 2–40 [39]. A rAAV minidysferlin based on this ~73-kDa protein was evaluated in dysferlin-deficient mice. It led to membrane repair restoration [39] but no full restoration of the phenotype of deficient animals [40], indicating that the addition sequences in dysferlin are required for a therapeutic effect.

These observations led to the need to define the best combination of dysferlin domains to transfer. A recent publication evaluated a nano-dysferlin construct with a removal of three C2 domains (C2D, C2E, C2F) based on 3D structural modeling and C2 domain redundancy [24]. After AAV injection in muscle of young BLA/J animals, the authors observed improvement of muscle integrity and reduction of damaged fibers as detected by Evans blue permeability after 3 weeks of expression. More impressively, the nano-dysferlin after systemic injection and 8 months of expression, albeit with only 10% of dysferlin-positive fibers, led to improved muscle tissue with fewer Evans blue dye (EBD) fibers and an increase of locomotor activity on rearing capacity.

Besides these proof-of-principle studies modifying dysferlin, these findings showed that identifying and understanding the role of each of dysferlin domains is a prerequisite to determine truncated isoforms for a potential clinic perspective. All the described constructs are presented in Fig. 25.1d.

25.4 RNA-Based Strategies

Besides the strategies based on gene transfer, a number of approaches relying on modulating pre-mRNA splicing have been tested in cellular and animal models deficient in dysferlin. In the exon-skipping approach, the exon or region carrying a frameshift mutation is deleted using antisense sequences to restore the reading frame, leading to a truncated form of protein that can still be functional. A second option that was explored is the use of the trans-splicing system, where a mutated part of the endogenous mRNA sequence is replaced by a normal sequence, during the maturation of the pre-messenger, by forcing splicing toward a pre-trans-splicing molecule (PTM). The evaluated strategies are described in Fig. 25.1e.

25.4.1 Exon Skipping

Thanks to the demonstration of modulatory aspect of the protein [39], the exon-skipping strategy was thought as potential strategy for dysferlinopathies. However, the domains of dysferlin are usually encoded by several exons, suggesting that multiple exon skipping should be considered taking into account structural information. This is supported by the severe clinical presentation associated with large deletions, such as covering exons 14–18 or 15–18 [41]. It seems therefore that this strategy will be limited to very specific situations. For the different studies performed so far, the selection of the exon to be targeted was based on clinical or genetic observations. First, the observation of a mildly affected patient with an in-frame skipping of exon 32 [42] led Wein et al. to target this exon. The authors successfully skipped exon 32 on patient cells [43] with functional restoration of membrane repair capacity [44]. In the second case, the mutation consisted of a deep intronic mutation, creating a strong splice donor consensus sequence and secondarily a pseudoexon identified as PE44.1 [45]. An antisense oligonucleotide (AON) was used in mutated patient cells to target potential exonic splicing enhancers in PE44.1, leading to its skipping out of the PE44.1 and an increase of normal dysferlin expression [45]. In a third example, the authors designed a frame restoration strategy to target exons 22–23, 25–29, and 22–29 by AON on CD133+ stem cells isolated from patients. A low efficiency in myoblasts was observed but did not lead to detection of dysferlin-positive fibers, after in vivo intramuscular injection of these modified cells in scid/blA/J mice [46].

25.4.2 Trans-splicing

Trans-splicing strategies can be performed to replace either the 5′ part or the 3′ part of the defected mRNA. For dysferlin, all trans-splicing strategies reported have utilized therapeutic RNAs to replace the 3′ part of the transcript to be repaired. A first study used a pre-trans-splicing molecule (PTM) carrying a complementary region to intron 48, an acceptor splice site, and the cDNA region covering exons 49–55 [47]. We showed in vitro efficacy by detection of a corrected mRNA. In addition, we provided the first demonstration of DYSF trans-splicing reprogramming in vivo after intramuscular injection in WT animals. However, we were also able to demonstrate a major drawback of the technique: generation of undesirable translated products of RNA-trans-splicing molecules from putative start codon in PTM. A second study evaluated four different PTMs carrying sequences corresponding to exons 31–55, 32–55, 36–55, or 37–55. Two of them (Ex32–55 and Ex36–55) were evaluated in vivo in WT and BLA/J animals [48]. By targeting specific intronic regions with weakly defined 3′ splice site, they successfully restored the trans-spliced mRNA in LGMD2B myoblasts and, for the first time, expression of the dysferlin protein with up to 35% of positive fibers after local injection in muscle of dysferlin-deficient mice.

25.5 Use of Proteins that Can Compensate the Absence of Dysferlin

Because dysferlin belongs to the large ferlin family, a group of six single-pass transmembrane proteins with a short C-terminal extracellular domain and multiple cytosolic C2 domains makes it possible to propose that some members of the family, if expressed in the right cells at the right time, could compensate for at least some of the functions of dysferlin. The ferlin proteins closest to dysferlin are otoferlin and myoferlin. They share a high domain homology (at least 6 C2 domains, FerB, which is a specific ferlin domain) [49]. Since myoferlin is highly expressed in developing skeletal muscle where it regulates fusion and muscle regeneration [50, 51], whereas otoferlin is mainly expressed in the inner ear and involved in SNARE-mediated exocytosis [52], myoferlin was selected for a compensatory study [40]. We generated a mouse strain overexpressing myoferlin and crossed it with the dysferlin-deficient strain, BLA/J. We observed a rescue of the membrane fusion defect but with no correction at histology level, suggesting that the pathogenicity of dysferlin deficiency is not only due to membrane repair capacity, but there is a unique function of dysferlin important for muscle homeostasis.

A complementary approach was also performed using anoctamin 5 (ANO5). This protein, also called TMEM16E, belongs to the anoctamin protein family that includes calcium-activated chloride channels. Recessive mutations in ANO5 lead to both a proximal limb-girdle muscular dystrophy (LGMD2L) and a distal Miyoshi-like phenotype (MMD3) [53]. This protein was selected because of the similarity of the dual clinical presentations seen in both ANO5 and DYSF deficiencies. In addition, low level of anoctamin 5 was observed in BLA/J animals compared to WT. A gene transfer strategy using rAAV vector to deliver Ano5 was evaluated [54]. While no toxic effect was observed related to the expression of the transgene, no therapeutic effect was observed in dysferlin-deficient mouse.

A third approach involved Mitsugumin53 (MG53), also called TRIM72 (Tripartite motif containing protein 72), known to play an important role in membrane repair and to interact with dysferlin [55]. In a first study, MG53 gene delivery was performed on γ-sarcoglycan-deficient hamster model [56], presenting a defect in membrane repair capacity [57]. After systemic delivery, the authors showed an increase of membrane repair resealing, ameliorated pathology, and improved muscle. Based on these observations, a recombinant human MG53 protein treatment was evaluated on dysferlin-deficient mouse [58], leading to a less permissive muscle fiber to EBD and a better sarcolemmal membrane integrity of dysferlin-deficient muscle fibers in mice. These studies demonstrated MG53 treatment as a possible compensatory approach, for membrane repair defect.

25.6 Additional Approaches

Besides the approaches presented above, additional studies may be more anecdotal for now but with some potential as demonstrated (*see* Fig. 25.1b).

25.6.1 Full-Length Delivery of Naked DNA

A recent paper described the use of naked DNA as a potential simple approach to transfer the full-length dysferlin cDNA [27]. The authors performed a locoregional delivery of dysferlin plasmid into hind limbs in the immunodeficient Bla/J/NRG mice. In co-injection with follistatin plasmid, significant reduction of EBD uptake was observed showing the restored membrane repair function up to 3 months postinjection.

25.6.2 Cell Therapy

A first ex vivo cell therapy using the full-length dysferlin has been reported. In this strategy, the authors used Sleeping Beauty (SB), a system widely used for stable gene transfer [59]. This approach was based on the use of a plasmid carrying the transgene flanked by transposon sequences and the transposase SB coding sequence. SB activity excises the transposon and integrates it into the target genome. The authors developed a SB dysferlin system to correct dysferlin-deficient mouse myoblasts and engrafted them in immunodeficient mouse [60]. They showed a successful in situ engraftment, with numerous dysferlin-positive fibers in scid/blA/J muscle after 6 weeks.

25.6.3 Gene Editing

With the wake of the CRISPR/Cas9 tsunami, it is obvious to think about edition of the large dysferlin gene. Only one study has reported efforts in that sense using patients iPSc carrying a nonsense mutation (c.5713C > T;p.R1905X). A TALEN nuclease was associated with a donor sequence for inserting the full-length WT human dysferlin in the H11 safe harbor locus of chromosome 22 [61]. The authors also evaluated gene editing using CRISPR/Cas9 on the same cells but with limited efficiency.

25.7 Additional Considerations and Conclusions

Obviously, the most advanced approach to date is the full-length gene replacement using the dual AAV since presently at the level of clinical trial. This translation into the clinics is supported by a tremendous effort on the natural history of dysferlinopathies ([62]; COS study is currently ongoing under the umbrella of the Jain Foundation https://www.jain-foundation.org/) and enables the establishment of patient registries. It is, indeed, mandatory to define sensitive outcome measures for

therapeutic evaluation. A central question is also the level of dysferlin required for a therapeutic benefit. We showed that 1–4% of expressed dysferlin after systemic injection of AAV is enough to improve significantly the phenotype of Bla/J mice [22], suggesting that a minimal dose of vector could be sufficient.

For other approaches, additional studies are required. For example, it would be necessary to understand better the function and structure of the different domains of dysferlin for a rational design of minidysferlin. It can be predicted that we will see more experimental works in the future on correction of deep intronic mutations through exon skipping and on gene editing. The combination of the large number of introns and the evolution of genetic diagnosis through genome sequencing will probably uncover more mutations leading to inclusion of intronic sequences. The genetic information will also help to identify areas of hotspots that may be targeted by gene editing. Besides the approaches presented in this review, which focuses mainly on gene-based strategies, additional efforts are being pursued as well on pharmacological therapies.

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Chapter 26
Muscle Cell Membrane Repair and Therapeutic Implications

Renzhi Han

Abstract Plasma membrane forms the physical barrier that separates the cellular interior from the exterior environment, and its integrity is essential for cell survival and function. Mammalian cells have evolved efficient membrane repair mechanisms that are activated to reseal injured plasma membrane and maintain cell viability. Many of the membrane repair proteins have first been identified in skeletal muscle, where defects in the genes encoding these proteins often lead to myopathies. Dysferlin is a muscle-specific protein implicated in mediating Ca^{2+}-activated membrane-membrane fusion to facilitate membrane repair. Genetic mutations in dysferlin gene are linked to several forms of muscular dystrophy. Likewise, anoctamin 5 (Ano5), synaptotagmin VII (Syt7), and TRPML1 have been found to play roles in muscle membrane repair, and their genetic defects have been shown to cause various forms of myopathies. Other proteins such as MG53 and annexins were found to interact with dysferlin and modulate the membrane repair process and other membrane tracking events in muscle. Given the importance of membrane integrity in human health and disease in general, the membrane repair proteins have become promising targets for therapeutic development that are aimed to boost the intrinsic membrane repair function of the cells.

Keywords Anoctamin 5 · Dysferlin · Gene therapy · Membrane repair · MG53 Muscular dystrophy · TMEM16E

26.1 Muscle Membrane Repair Machinery

As the physical barrier to encapsulate the cellular contents in a closed space, the plasma membrane is essential for cell survival and function. Any insult to the plasma membrane integrity would be disastrous if left unrepaired. Since the membrane

R. Han (✉)
Department of Surgery, Davis Heart and Lung Research Institute, The Ohio State University Wexner Medical Center, Columbus, OH, USA
e-mail: renzhi.han@osumc.edu

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damage occurs physiologically and pathologically all the time [1], the cells have evolved efficient mechanisms to rapidly reseal injured membrane. The importance of such an emergency response is underscored by the work in the last two decades that have revealed several key membrane repair components are linked to human myopathies. These include dysferlin [2–4], synaptotagmin VII (Syt7) [5], transient receptor potential cation channel (TRPML1, also known as mucolipin-1 [MCOLN1]) [6], and anoctamin 5 (Ano5, also known as TMEM16E) [7–9]. Recent studies have identified additional proteins involved in the membrane repair process, such as MG53 [10], annexins (A1, A2, A5, and A6) [11–16], calpain [17–20], and ESCRT III [21, 22]. These studies together have now painted a clearer picture of the membrane repair machinery and how they work for membrane repair.

26.1.1 Dysferlin and Synaptotagmin VII

Genetic mutations in the dysferlin gene were identified in patients with limb girdle muscular dystrophy type 2B (LGMD2B) [3], Miyoshi myopathy (MM) [2], or distal anterior compartment myopathy [23]. Dysferlin is a 230-kDa protein that is highly expressed in striated muscles and located at the sarcolemma and transverse tubules (T-tubules) [3]. As a member of the ferlin family, dysferlin contains a single transmembrane domain at its carboxyl-terminus and multiple C2 domains (Fig. 26.1a), which are about 130 amino acids in length with Ca^{2+}-sensitive phospholipid- binding activities. In addition, dysferlin also carries Ferlin-specific motifs, FerI, FerA, and FerB, although the functions of these structures have not yet been defined.

The C2 domains in dysferlin are highly similar to those in synaptotagmins (Syts) [24, 4, 25, 26, 19] and can mediate the dimerization of dysferlin [27]. *Syt*s generally contain two C2 domains (Fig. 26.1a) and play important roles in Ca^{2+}-triggered vesicle fusion at the synapse for neurotransmission [28]. Interestingly, the *Syt7*-null mice develop autoimmune myositis with defective membrane resealing [5]. The amino-terminal C2 domain (C2A) in dysferlin mediates Ca^{2+}-sensitive phospholipid binding, thus likely involved in vesicle fusion within the cell [26, 29]. For all these and the pathological features of patient skeletal muscles with dysferlin gene mutations, it was hypothesized that dysferlin plays a role in membrane repair of skeletal muscle by regulating Ca^{2+}-mediated vesicle fusion. Bansal et al. [4] designed a series of experiments to test this hypothesis in the skeletal muscle of a mouse model with dysferlin gene disruption using laser-induced membrane injury/repair. Isolated myofibers were injured at a small region of the plasma membrane with a beam of laser irradiation in the presence of a membrane-impermeable lipophilic dye FM1-43 to monitor the membrane repair process [4]. FM1-43 is nonfluorescent in saline but exhibits increased fluorescence intensity upon binding phospholipids. Dysferlin-null myofibers allowed more entry of FM1-43 dye after injury than control cells, indicating that dysferlin-null myofibers have slower membrane resealing kinetics [4, 30]. Control myofibers damaged in the absence of Ca^{2+} displayed similar repair defects, thus supporting the role of dysferlin in Ca^{2+}-regulated

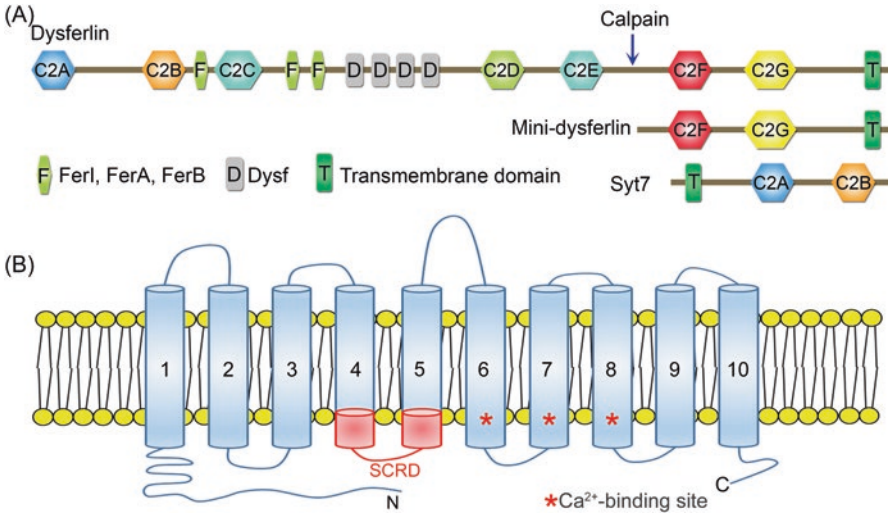

Fig. 26.1 (**a**) Schematic of dysferlin, calpain-released mini-dysferlin, and Syt7. Dysferlin contains multiple C2 domains (C2A-C2G), which confers Ca^{2+}-dependent phospholipid binding, and a carboxyl-terminal transmembrane domain. Dysferlin also has Fer domains (FerI, FerA, and FerB) and DysF domains with their functions undetermined. Calpain cleaves dysferlin between C2E and C2F to release a syt-like carboxyl-terminal fragment (mini-dysferlin) with only the last two C2 domains and the transmembrane domain. Syt7 carries an amino-terminal transmembrane domain and two tandem C2 domains. (**b**) Structural characteristics of Ano5. Ano5 carries ten transmembrane helices with a large amino-terminal region and a relatively short carboxyl-terminal region facing the cytosol. The second intracellular loop between TM4 and TM5 forms a functional lipid-scrambling domain (SCRD). Several Ca^{2+}-binding sites are present in TM 6–8 (red stars)

membrane repair response. Moreover, by flushing the control myofibers through a syringe to mechanically damage the cell membrane, Bansal et al. observed dysferlin-enriched membrane "patch" formation at the damage site [4], consistent with the "patch" hypothesis for membrane repair. It is believed that dysferlin functions as a Ca^{2+} sensor to promote vesicle-membrane fusion via its C2 domains like Syts and form a resealing membrane patch.

Interestingly, Cooper and her colleagues found that dysferlin is proteolytically cleaved by ubiquitous calpains, which were previously shown to be involved in membrane repair [17, 31, 18, 32], to release a 72-kDa, Syt-like carboxyl-terminal fragment containing the last two C2 domains and the transmembrane domain [19, 20]. This 72-kDa mini-dysferlin is specifically recruited to the injury site, highlighting the similarity between dysferlin and Syts in mediating Ca^{2+}-regulated vesicle fusion. Different from the hypothesis that dysferlin mediates Ca^{2+}-regulated vesicle fusion for membrane repair, a recent study showed that dysferlin is involved in phosphatidylserine (PS) accumulation at the damage site [33], which may be sensed by macrophages to remove the repair patch and restore the cell membrane integrity. Therefore, dysferlin may participate in different steps during the membrane resealing response.

26.1.2 Anoctamin 5 (TMEM16E)

Anoctamin 5 (Ano5, or TMEM16E) belongs to the anoctamin protein family, consisting of ten homologous members [34]. Anoctamin proteins are predicted to contain eight transmembrane domains. But the crystal structure of a TMEM16 family member from the fungus *Nectria haematococca* was found to contain ten transmembrane domains [35] (Fig. 26.1b). The anoctamin proteins have been shown to function as Ca^{2+}-activated chloride channels (CaCCs), phospholipid scramblases, or both [34].

Recessive mutations in *ANO5* result in LGMD2L and Miyoshi myopathy 3 (MMD-3) [7], while dominant mutations lead to gnathodiaphyseal dysplasia, a disorder of bone dystrophy [36, 37]. The clinical manifestations in LGMD2L and MMD-3 resemble those in dysferlinopathies [7]. The Ano5 protein is localized to the ER and intracellular vesicles, and its expression is upregulated during muscle differentiation similar to dysferlin [38, 39]. Interestingly, even before the gene was identified in an LGMD2L patient, the fibroblasts derived from this patient were shown to have a membrane repair defect [9]. Recently, several lines of mouse models with *Ano5* gene disruption were found to have distinct pathological presentations. Two of these lines with complete disruption of Ano5 showed no obvious muscular dystrophy at up to 80 weeks of age [40, 41]. However, the third line of *Ano5*-knockout (KO) mice carrying a gene trap cassette in the 8th intron showed some features of muscular dystrophy, intracellular aggregates, and defective membrane repair [42]. It is not clear what exactly underlies the different presentation of pathology in these animals. But clearly, complete loss of Ano5 protein in mice does not seem to cause obvious muscle pathology as seen in the LGMD2L and MMD3 patients. This may be due to the presence of a more effective compensatory mechanism in mice than in humans. Another possibility is that the truncated Ano5 peptides expressed from the mutations result in membrane repair defect and muscle necrosis.

As a membrane protein located primarily at the ER/SR and intracellular vesicles, it is unclear how Ano5 may be involved in cell membrane repair. Adeno-associated virus (AAV)-mediated *Ano5* gene transfer into *dysferlin*-null mice showed that the presence of Ano5 did not significantly improve the membrane repair capacities of *dysferlin*-null myofibers or the muscle histopathology in these animals [43], suggesting that Ano5 plays a distinct role from dysferlin in the membrane repair process. Ano5 carries a functional lipid scramblase domain (Fig. 26.1b), suggesting that Ano5 may regulate lipid translocation across membranes. But how the lipid scramblase activity is linked to membrane repair requires future investigations.

26.1.3 TRPML1

TRPML1 (also known as mucolipin 1, MCOLN1) belongs to the mucolipin subfamily of transient receptor potential (TRP) channels, which include two other members, TRPML2 (MCOLN2) and TRPM3 (MCOLN3) [44]. Like other TRP

channels, TRPML proteins consist of six putative transmembrane domains with the amino- and carboxyl-termini facing the cytosol [45] (Fig. 26.2a). TRPML1 is ubiquitously expressed in almost every tissue and cell type, while TRPML2 and TRPML3 are restricted in particular cell types [45]. TRPML channels are localized predominately on the membranes of late endosomes and lysosomes [45, 46]. Loss-of-function mutations in TRPML1 result in mucolipidosis type IV, a severe lysosomal storage disorder that is characterized by neurodegeneration and psychomotor disabilities [47–49].

TRPML1 is a nonselective cation channel that releases Ca^{2+} from the endolysosomal lumen. Phosphatidylinositol 3,5-bisphosphate [$PI(3,5)P_2$], which is localized on endosomes and lysosomes, is known to be an endogenous activator of TRPML1 channel [50]. In contrast, $PI(4,5)P_2$ and sphingomyelins (SMs) in the plasma

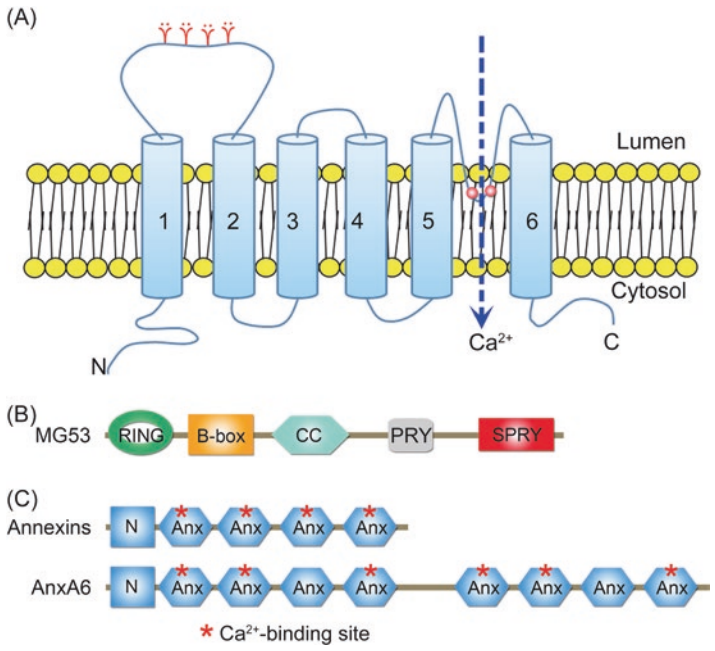

Fig. 26.2 (**a**) Structural aspects of TRPML1. TRPML1 consists of six transmembrane domains with the amino-terminal and carboxyl-terminal tails facing the cytosol. The first luminal loop is uniquely large and contains four N-glycosylation sites. Two di-leucine motifs are located at each tail to mediate the localization of TRPML1 to late endosomes and lysosomes. Two negatively charged amino acid residues (D471 and D472) within the last luminal loop are potential pore-forming determinants. (**b, c**) Schematic of MG53 (**b**) and annexin proteins (**c**). MG53 contains an amino-terminal tripartite motif, a RING finger domain conferring ubiquitination activity, a Zn^{2+}-binding B-box specific for TRIM family proteins thought to mediate protein-protein interactions, a coiled-coiled domain that mediates hetero- and homo-oligomerization, and a carboxyl-terminal PRY-SPRY domain. The annexin proteins contain an amino-terminal variable and four annexin repeats (Anx). AnxA6 is an atypical annexin containing eight annexin repeats. The annexin repeats coordinate Ca^{2+} binding despite the lack of EF hands

membrane inhibit TRPML1 [51, 52]. Genetic disruption of TRPML1 in mice recapitulates many aspects of mucolipidosis type IV disease [53]. Interestingly, the TRPML1-KO mice also display membrane repair defect in skeletal muscle and develop a muscular dystrophy phenotype [6]. Several lines of evidence support a direct role of TRPML1 in lysosomal exocytosis, which has been implicated in membrane repair. A gain-of-function mutation in TRPML1 results in enhanced lysosomal exocytosis in cells [54]. Acute stimulation of TRPML1 with a synthetic small molecule activator ML-SA1 induces cell surface expression of the lysosomal-associated membrane protein 1 (Lamp1) and lysosomal enzyme release in control but not TRPML1-KO macrophages [55]. Moreover, lysosomal exocytosis upon membrane injury is impaired when TRPML1 is genetically disrupted or pharmacologically inhibited [6]. The question remains as for how TRPML1 is activated by membrane damage. TRPML1 is unlikely to be directly activated by Ca^{2+} [50] but could be indirectly activated by a Ca^{2+}-dependent signal (e.g., lipid reorganization through a Ca^{2+}-regulated lipid scramblase; see Ano5 above). In addition, oxidative levels are known to be increased at damage sites [10], and it is possible that intracellular reactive species, which are known to activate several members of the TRP channels [56], may activate TRPML1 when the membrane is injured.

26.1.4 Mitsugumin 53 (Trim72)

Mitsugumin 53 (MG53, also known as TRIM72) is a member of the large family of E3 ubiquitin ligases, tripartite motif (TRIM) proteins [57]. MG53 contains an amino-terminal TRIM domain and a carboxyl-terminal PRY-SPRY domain (Fig. 26.2b), which commonly exists in most of TRIM family proteins. MG53 is abundantly expressed in striated muscles and to a lower level in the epithelia of lung and kidney [10, 58, 59]. Although MG53 does not contain transmembrane domains, it is associated with intracellular vesicles and the sarcolemma in striated muscles likely through its binding to PS, which is enriched in these membranes [10, 60].

Substantial evidence exists to support a central role of MG53 in membrane repair. Cai et al. first observed that MG53-containing vesicles translocate toward the sites of membrane damage following mechanical or laser-induced injury via an oxidation-dependent manner [10]. Disruption of MG53 leads to defective membrane repair and increases cell death with such membrane insults. Ultrastructural analysis using electron microscopy revealed a lack of vesicle accumulation at sites of injury in MG53-null muscle fibers. In response to increased oxidation, as it occurs during acute damage of the cell membrane, MG53 forms oligomer complexes [10]. The cysteine 242 residue of MG53 plays an essential role in its oxidation-mediated oligomerization and membrane repair [10]. Modification of cysteine residues with alkylating reagents prevents the nucleation process of MG53-mediated membrane repair [61]. Zn^{2+} binding to the RING and B-box motifs of MG53 is also essential for the assembly of membrane repair machinery [62, 63]. MG53 was also found to colocalize with annexin A5, another PS-binding protein involved in membrane repair [10]. Moreover, MG53 interacts with dysferlin through the C2A domain

in a Ca^{2+}-sensitive manner [64, 65]. These data suggest that MG53 senses the entry of oxidized milieu to form oligomeric complexes and facilitate vesicle translocation to the damage sites, where it also coordinates with other membrane repair proteins (e.g., dysferlin and annexins) to create a membrane resealing patch.

26.1.5 Annexins

The annexin proteins are able to bind phospholipids and actin in a Ca^{2+}-dependent manner. The annexin repeat domains contain Ca^{2+}- binding sites (Fig. 26.2c). Annexins preferentially bind PS, phosphatidylinositols (PtdIns), and cholesterol [66]. There are 12 annexin genes in humans, with each showing distinct tissue distribution. Different Ca^{2+} affinity allows each annexin protein to respond to changes in intracellular Ca^{2+} levels under unique spatiotemporal conditions [67].

The annexins contain a conserved carboxyl-terminal core domain formed by multiple annexin repeats and a variable amino-terminal head, which differs in length and sequence compositions among the family members. Both the amino- and carboxyl-terminal regions can bind lipid membrane in a Ca^{2+}-dependent manner [68]. Because of their membrane- binding capacity, annexins have broad roles in regulating membrane trafficking and actin organization.

Several annexins have been shown to directly regulate membrane repair. Annexins (Anx) A1, A2, A5, and A6 translocate to the sites of muscle membrane damage in zebrafish muscle [69]. This translocation occurs in a sequential manner with AnxA6 arriving first. AnxA6 is also observed to move to the damage site in mouse skeletal muscle [14]. AnxA6 is unique among the annexin family members, as it contains two core domains (eight annexin repeats), while all other annexins contain only one core domain (four annexin repeats) [70]. Like other annexins, AnxA6 is capable of membrane binding through both the amino- and carboxyl-terminal annexin core domains, thus facilitating coalescence of two opposing membranes, a step required for vesicle-membrane fusion during membrane repair [71]. Although AnxA6-null mice do not exhibit any overt phenotype [72], a genetic variant in AnxA6 was found to modify the disease severity in a mouse model of muscular dystrophy, *Sgcg*-KO mouse that lacks γ-sarcoglycan [14].

Additionally, Marg et al. (2012) observed that AnxA1 migrates to the membrane damage site in cultured human muscle cells [73]. Such a translocation of annexins in response to membrane injury does not seem to be muscle specific, as Jaiswal et al. found AnxA1 and AnxA2 at the membrane injury site in human cancer cell lines, MCF and HeLa cells [74]. Both AnxA1 and AnxA2 were found to directly bind dysferlin [12, 69] and contribute to membrane repair.

AnxA5, the smallest annexin, assembles to oligomers in a Ca^{2+} and PS-dependent manner [75], and accumulates at the membrane damage site [13]. In neuroblastoma cells, AnxA5 was shown to assemble into complexes that also contain AnxA1 and AnxA2 in a time-dependent manner at the plasma membrane upon increased Ca^{2+} levels [76]. AnxA5-null perivascular cells showed increased dye uptake upon membrane injury [13], suggesting that AnxA5 is involved in the membrane resealing

process. Preventing AnxA5 from forming two-dimensional membrane-associated arrays also resulted in defective membrane repair [13]. Interestingly, the introduction of extracellular recombinant AnxA5 prior to laser-induced membrane damage was sufficient to improve the membrane repair capacity of AnxA5-null cells to near wild-type levels [13], suggesting that AnxA5 can act from the exterior of the cells, similar to MG53 [77]. These studies suggest that AnxA5 is a membrane repair protein, which can work from both the interior and exterior of the cell.

26.1.6 Other Membrane Repair Proteins

Besides the membrane patching hypothesis, there is also substantial evidence to support the removal of a damaged membrane for repair via endocytosis [78, 79, 16] and/or extracellular budding [16, 22, 21]. It was observed that uncoated, irregularly shaped endosomes appeared intracellularly within a few minutes after cell injury [78]. These endosomes are morphologically similar to vesicles formed at the periphery of cells exposed to bacterial sphingomyelinase [78], suggesting a mechanism by which Ca^{2+}-triggered exocytosis of lysosomes might promote lesion removal by endocytosis. Lysosomal acid sphingomyelinase (ASM) is released to the cell surface in response to Ca^{2+} influx in wounded cells [80] and removes the phosphorylcholine head group of sphingomyelin to generate ceramide-enriched microdomains [81], which drive membrane invagination for endocytosis. ASM inhibitors block the formation of the plasma membrane-associated ceramide microdomains triggered by Ca^{2+} influx in cells treated with pore-forming toxin streptolysin O [82] and attenuate membrane repair [79].

More recently, removal of the damaged membrane has been shown to occur through membrane budding mediated by the endosomal sorting complex required for transport (ESCRT) complex [22, 21]. It was found that ESCRT III components are required for the repair of small (<100 nm) but not large plasma membrane lesions [22]. Membrane injury-triggered Ca^{2+} elevation results in assembly of ESCRT III and accessory proteins at the site of injury, initiated by apoptosis-linked gene (ALG)-2, a Ca^{2+}-binding protein [21]. ALG-2 facilitates the accumulation of ALG-2-interacting protein X (ALIX), ESCRT III, and Vps4 complex at the injured cell membrane, which in turn results in cleavage and shedding of the damaged portion of the cell membrane [21].

26.2 Therapeutic Development to Boost Membrane Repair

Maintaining the plasma membrane integrity is crucial for cell survival and thus alleviation or prevention of diseases. Skeletal muscle membranes, in particular, are constantly under mechanical stress, damaged and repaired during physiological activities. Disturbance of the membrane repair process in skeletal muscle often leads to muscular diseases. Identification of the membrane repair machinery and their underlying mechanisms offers new opportunities to target the membrane repair

process for the treatment of these diseases and potentially others as well. A good example is MG53, which has emerged as a promising therapeutic target for a number of diseases.

An extensive body of work by Ma and colleagues has demonstrated the critical role of MG53 for membrane repair in a variety of cell types. AAV-mediated delivery of MG53 in the δ-sarcoglycan-deficient hamsters improved membrane repair and ameliorated muscular dystrophy and heart failure in this animal model [83], highlighting the potential of MG53 gene therapy for muscular dystrophy. Moreover, application of recombinant MG53 (rhMG53) to the exterior of the muscle fibers is rapidly attracted to the site of injury, effectively preventing the influx of membrane-impermeable dye [77]. In addition, MG53 is a soluble protein and can be produced from bacterial cells. These features make MG53 an ideal candidate for protein therapy. Indeed, intravenous administration of rhMG53 in a short-term study was shown to improve the membrane integrity of myofibers as evidenced by reduced dye uptake after eccentric running in the *mdx* mice and also reduce muscle necrosis [77]. Similar protective effects of rhMG53 are seen in myocardial infarction [84], acute lung injury [59], and acute kidney injury [58] in rodent and large animal models. These findings support the concept of targeting cell membrane repair in regenerative medicine and highlight MG53 as a potential therapy for restoration of membrane integrity in a broad range of human diseases.

Although being an attractive therapeutic target for membrane repair, MG53 has also been found to mediate a number of other actions in the body. For example, it was recently shown that MG53-mediated skeletal muscle insulin resistance is the initiating and major factor of the pathogenic process of global metabolic disorders [85, 86]. Increased levels of MG53 were detected in the skeletal muscle of multiple animal models of insulin resistance and obese humans [85]. Metabolic disorders induced by over-nutrition are profoundly alleviated by MG53 disruption [85, 86], and transgenic overexpression of MG53 in mice promotes insulin resistance and other metabolic disorders [85]. Moreover, cardiac-specific transgenic expression of MG53 leads to diabetic cardiomyopathy by upregulation of peroxisome proliferation-activated receptor alpha and impairment of insulin signaling [87]. Finally, MG53 has been found to play a negative role in IGF-1-induced myogenesis [86, 88, 89]. Future clinical applications of MG53 will need to carefully evaluate these potential side effects. It is noteworthy that many of the adverse effects of MG53 seem to be associated with relatively chronic processes, while the protective effects of MG53 to promote membrane repair are via its acute action. Therefore, it is possible to maximize the beneficial effects of MG53 while minimizing its negative impact by controlling the treatment timeframe.

26.3 Conclusions and Future Directions

The genetic and cellular studies in mouse models and other organisms have produced a better understanding of the membrane repair process. Many of the key proteins involved in membrane repair have been identified, particularly in skeletal muscle likely due to the high levels of mechanical stress during their physiological

activities. The basic membrane repair machinery seems to be shared by many different types of cells and tissues. Ca^{2+}-regulated exocytosis, endocytosis, and extracellular membrane budding may work in concert to effectively reseal the damaged membrane. Targeting the membrane repair process will likely yield promising therapeutics for many human diseases.

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Chapter 27
Dystroglycanopathy Gene Therapy: Unlocking the Potential of Genetic Engineering

Charles H. Vannoy, Anthony Blaeser, and Qi L. Lu

Abstract Muscular dystrophy-dystroglycanopathies (MDDGs) are neuromuscular disorders associated with aberrant *O*-glycosylation of α-dystroglycan—an extracellular peripheral membrane glycoprotein central to the dystrophin-glycoprotein complex. The majority of these disorders are caused by loss-of-function mutations in a multitude of genes that disrupt the posttranslational modification of α-dystroglycan, affecting its ability to function as a receptor for extracellular matrix proteins containing laminin globular domains. As a result, clinical manifestations of MDDGs are highly variable, exhibiting a wide spectrum of clinical phenotypes including mild to severe defects in the development of the muscles, brain, and/or eyes. Over the last couple of decades, significant progress has been made in the elucidation of *O*-mannosyl glycan structures on α-dystroglycan and characterization of the underlying mechanisms of MDDGs, which has prompted concerted efforts toward the development and evaluation of potential clinical treatment options. Current genetic engineering efforts designed to treat MDDGs employ adeno-associated virus (AAV)-mediated delivery of expression vectors for gene replacement/supplementation and antisense oligonucleotide (AON) splice-modulation therapy to suppress exon trapping. Future therapeutic strategies are focused on the optimization of these current technologies and exploration of newer technologies such as genome editing. In this chapter, we address the disruption of functional α-dystroglycan as it relates to various clinical manifestations and highlight the potential genetic engineering strategies for treating MDDGs with an emphasis on preclinical data. We also discuss the problems that must be solved before effective treatment options are readily available.

Keywords Dystroglycan · Dystroglycanopathy · Gene therapy · Glycosylation Muscular dystrophy · Therapeutic strategies

C. H. Vannoy · A. Blaeser · Q. L. Lu (✉)
McColl-Lockwood Laboratory for Muscular Dystrophy Research, Carolinas Medical Center, Atrium Health, Charlotte, NC, USA
e-mail: charles.vannoy@atriumhealth.org; anthony.blaeser@atriumhealth.org; qi.lu@atriumhealth.org

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27.1 Introduction

Dystroglycan is a complex protein involved in physiological processes responsible for maintaining skeletal muscle membrane integrity and functional development of the central nervous system [1–3]. This protein is encoded by a solitary gene (*DAG1*) that maps to chromosome 3p21.31 in humans [4]. During transcription, a 5.8-kb mRNA transcript is translated into a single polypeptide and then posttranslationally cleaved to yield two mature protein chains: a cell-surface α subunit (156 kDa) and a transmembrane β subunit (43 kDa) [5–8]. Both proteins are ubiquitously expressed, and each protein has distinct surface characteristics that determine how they interact with other molecules [6]. β-Dystroglycan associates with dystrophin via its cytoplasmic carboxy-terminal region, which in turn binds to the filamentous actin (F-actin) cytoskeleton [9]. The amino-terminal region of β-dystroglycan couples with the carboxy-terminal region of α-dystroglycan, securing this soluble subunit to the outer surface of the cell. α-Dystroglycan is extensively glycosylated and acts as a cellular receptor for extracellular matrix proteins containing laminin globular domains [10–12]. As a result, dystroglycan is a central component of a multimeric protein complex that creates a physical linkage between the cytoskeleton and the basement membrane. Given the complex nature of α-dystroglycan and its direct correlation with muscular dystrophy-dystroglycanopathies (MDDGs), this chapter will focus on the biochemical composition and subsequent disruption of α-dystroglycan as it relates to various clinical manifestations and potential treatment options.

An in-depth look at the structural aspects of α-dystroglycan at the molecular level helps to facilitate a better understanding of the physiological and pathological functions of the protein. The core α-dystroglycan protein is predicted to be approximately 72 kDa in size, yet the native α-dystroglycan protein migrates as a diffuse band anywhere from 120 to 250 kDa, depending on tissue type [4–6]. Previous studies have determined that a majority of the protein molecular weight is due to a diverse carbohydrate composition that is heterogeneous in various tissues and cell types, including both *N*- and *O*-linked glycans [5, 13–15]. In the case of *O*-linked glycosylation, glycans are attached via an *O*-glycosidic linkage to the free hydroxyl group of specific amino acids (e.g., serine (Ser), threonine (Thr), or tyrosine (Tyr)). More specifically, α-dystroglycan contains a clustered Ser/Thr-rich domain in the central region of the protein, which affords numerous sites for *O*-linked glycosylation [16, 17]. A variety of sugar residues can be attached to these Ser/Thr sites [18], yet it is the *O*-mannosylation that is of particular interest with regard to dystroglycanopathies. In Fig. 27.1, we depict three *O*-mannosyl glycans identified on α-dystroglycan (designated as M1, M2, and M3 by Yoshida-Moriguchi et al. [19]).

Over the past couple of decades, many researchers have contributed to the elucidation of a biosynthetic pathway for these *O*-mannosyl glycans on α-dystroglycan, identifying at least 18 associated genes (listed in Table 27.1). Before the addition of mannose to the Ser/Thr residues takes place, various genes are involved in mannose and dolichol metabolisms, which include *DPM1/2/3* [24, 25, 31], *DOLK* [34], and *GMPPB* [38]. Once mannose has been properly modified into an acceptable sugar

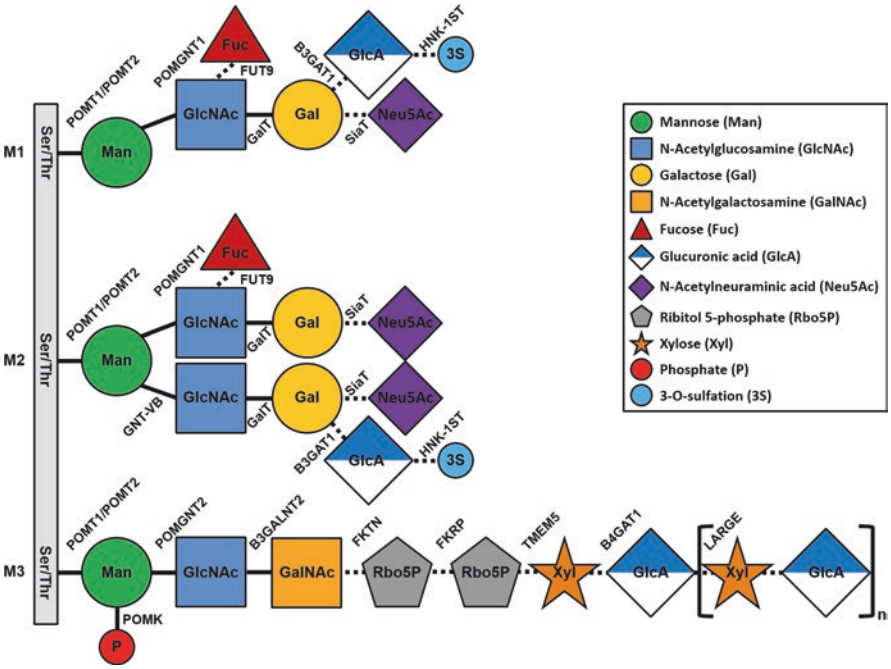

Fig. 27.1 Schematic representation of *O*-mannosyl glycans associated with α-dystroglycan. The glycan structures are designated as M1, M2, and M3 with the core sugar sequence connected by solid lines. Additional sugar modifications to each respective core glycan are connected by dotted lines. Genes/glycosyltransferases involved in the biosynthetic pathway are identified at the site of action. Sugar residues are initially attached to serine (Ser) and/or threonine (Thr) amino acids. Symbolic representation of monosaccharides and other small molecules is described in the side panel. *Fuc* fucose, *Gal* galactose, *GalNAc N*-acetylgalactosamine, *GlcA* glucuronic acid, *GlcNAc N*-acetylglucosamine, *Man* mannose, *Neu5Ac N*-acetylneuraminic acid, *P* phosphate, *Rbo5P* ribitol-5-phosphate, *Xyl* xylose, *3S* 3-*O*-sulfation

donor (dolichol-phosphate-mannose), the formation of the glycan begins with the transfer of mannose to Ser/Thr residues via *POMT1*/*POMT2* in the lumen of the endoplasmic reticulum [41]. At this point, there is some divergence in elongation, which creates three distinct glycans. A majority of the *O*-mannosyl sites are extended in the Golgi complex via a β-1, 2 linkage with *N*-acetylglucosamine (GlcNAc) by POMGNT1 [56], which can then be further branched by an additional GlcNAc at the β-1,6 position and/or elaborated by fucose (Fuc) or galactose (Gal) [106–109]. These terminal Gal residues are then further modified with sulfated glucuronic acid (GlcA) and/or neuraminic acid (Neu5Ac) to generate the M1 and M2 glycans [55, 106, 110]. On the other hand, a small number of *O*-mannosyl sites, considered exclusive to α-dystroglycan, undergo subsequent elongation in the ER with the β-1,4 addition of GlcNAc by *POMGNT2*, the β-1,3 addition of *N*-acetylgalactosamine (GalNAc) to GlcNAc by *B3GALNT2*, and phosphorylation of the *O*-linked mannose at the

Table 27.1 Overview of genes associated with MDDG

Encoding gene	OMIM[a]	Molecular function[b]	Phenotype (OMIM)[c]	Reference
DAG1	128239	Encodes for α- and β-dystroglycan	MDDGA9 (616538) MDDGC9 (613818)	[6, 20–23]
DPM1	603503	Dolichyl-phosphate mannosyltransferase	CDG1E (608799)	[24–30]
DPM2	603564	Dolichyl-phosphate mannosyltransferase	CDG1U (615042)	[25, 31, 32]
DPM3	605951	Dolichyl-phosphate mannosyltransferase	CDG1O (612937)	[25, 33]
DOLK	610746	Dolichol kinase	CDG1M (610768)	[34–37]
GMPPB	615320	GDP-Man pyrophosphorylase	MDDGA14 (615350) MDDGB14 (615351) MDDGC14 (615352)	[38–40]
POMT1	607423	Forms a complex with POMT2 known as POMT that adds the first Man sugar to the Ser/Thr in the peptide chain of the *O*-mannosyl glycan	MDDGA1 (236670) MDDGB1 (613155) MDDGC1 (609308)	[41–50]
POMT2	607439	Forms a complex with POMT1 known as POMT that adds the first Man sugar to the Ser/Thr in the peptide chain of the *O*-mannosyl glycan	MDDGA2 (613150) MDDGB2 (613156) MDDGC2 (613158)	[41, 42, 49–54]
POMGNT1	606822	Transfers GlcNAc in a β-1,2 linkage to *O*-linked Man	MDDGA3 (253280) MDDGB3 (613151) MDDGC3 (613157) RP76 (617123)	[42, 49, 50, 55–63]
POMGNT2 (formerly GTDC2)	614828	Transfers GlcNAc in a β-1,4 linkage to *O*-linked Man	MDDGA8 (614830)	[19, 64]
POMK (formerly SGK196)	615247	Phosphorylates the 6-position of *O*-linked Man	MDDGA12 (615249) MDDGC12 (616094)	[19, 65–67]
B3GALNT2	610194	Transfers GalNAc in a β-1,3 linkage to GlcNAc on the *O*-mannosyl glycan	MDDGA11 (615181)	[19, 68, 69]
ISPD	614631	CDP-Rbo synthase	MDDGA7 (614643) MDDGC7 (616052)	[70–74]
FKTN	607440	Rbo5P transferase that uses CDP-Rbo	CMD1X (611615) MDDGA4 (253800) MDDGB4 (613152) MDDGC4 (611588)	[42, 50, 70, 75–88]
FKRP	606596	Rbo5P transferase that uses CDP-Rbo	MDDGA5 (613153) MDDGB5 (606612) MDDGC5 (607155)	[50, 70, 80, 89–91]
TMEM5	605862	Transfers Xyl in a β-1,4 linkage to Rbo5P on the *O*-mannosyl glycan	MDDGA10 (615041)	[65, 71, 92]

(continued)

Table 27.1 (continued)

Encoding gene	OMIM[a]	Molecular function[b]	Phenotype (OMIM)[c]	Reference
B4GAT1 (formerly B3GNT1)	605517	Transfers GlcA in a β-1,4 linkage to Xyl on the *O*-mannosyl glycan	MDDGA13 (615287)	[93–96]
LARGE	603590	Transfers repeating units of [-3-Xyl-α-1,3-GlcA-β-1-] to GlcA on the *O*-mannosyl glycan	MDDGA6 (613154) MDDGB6 (608840)	[50, 97–105]

[a]Online Mendelian Inheritance in Man (OMIM) is a continuously updated catalog of human genes and genetic disorders and traits, with a particular focus on the gene-phenotype relationship
[b]*CDP-Rbo* cytidine diphosphate ribitol, *GalNAc N*-acetylgalactosamine, *GDP* guanosine diphosphate, *GlcA* glucuronic acid, *GlcNAc N*-acetylglucosamine, *Man* mannose, *POMT* protein *O*-mannosyltransferase, *Rbo5P* ribitol-5-phosphate, *Ser/Thr* serine/threonine, *Xyl* xylose
[c]*CDG* congenital disorder of glycosylation, *CMD* cardiomyopathy—dilated, *MDDGA* muscular dystrophy-dystroglycanopathy (congenital with brain and eye anomalies), *MDDGB* muscular dystrophy-dystroglycanopathy (congenital with mental retardation), *MDDGC* muscular dystrophy-dystroglycanopathy (limb-girdle), *RP* retinitis pigmentosa

6-position by *POMK* to generate the core M3 glycan [19, 64]. The continued extension of the M3 glycan proceeds to the Golgi where two ribitol 5-phosphate (Rbo5P) molecules are added on to the GlcNAc, the first by *fukutin* and the second by *FKRP* [70]. *TMEM5* and *B4GAT1* continue the sugar chain elongation process by adding a xylose (Xyl) residue to the terminal Rbo5P via a β-1,4 linkage and a GlcA via a β-1,4 linkage to Xyl, respectively [92–94]. Finally, the process culminates with repeating units of [-GlcA-β-1,3-Xyl-α-1,3-] added onto the terminal GlcA residue via *LARGE* [97]. These repeating disaccharide units are responsible for the functional binding of α-dystroglycan to extracellular matrix proteins containing laminin globular domains [12]. The authors refer the reader to other reviews for a more comprehensive summary detailing the posttranslational modifications of α-dystroglycan [111–114].

27.2 Clinical Manifestation of Muscular Dystrophy-Dystroglycanopathy (MDDG)

Abnormal synthesis of the peptide chain or *O*-mannosyl glycans on α-dystroglycan leads to disruption of the dystrophin-glycoprotein complex and subsequently results in various forms of dystroglycanopathies, which can be divided into two classes. Primary MDDGs are caused by mutations in the *DAG1* gene and are extremely rare [20–22], whereas secondary MDDGs are caused by mutations in the other 17 identified genes that are responsible for coding the proteins or enzymes involved in the *O*-glycosylation pathway of α-dystroglycan without directly affecting the expression of α-dystroglycan. MDDGs present a wide range of disease phenotypes that are broadly divided into three types (A, B, and C) based on clinical and pathological severity.

On the most severe end of the clinical spectrum, MDDGA disorders, previously designated as Walker-Warburg syndrome (WWS) or muscle-eye-brain disease (MEB), exhibit significant defects in the central nervous system including distinctive structural changes in the brain (lissencephaly type II or cerebellar cysts) and eye (retinal hypoplasia) in addition to the typical myopathic changes [42, 98, 115, 116]. These disorders frequently result in early lethality while surviving patients often suffer varying degrees of mental retardation. MDDGB disorders are less severe than MDDGA, showing evidence of cognitive impairment but a structurally normal brain on imaging [117]. Typical findings on brain MRI reveal that patients are afflicted with milder central nervous system abnormalities that include microcephaly or minor white matter changes. MDDGC disorders, previously designated as limb-girdle muscular dystrophy, are the most common form of the disease and present with predominantly milder myopathic phenotypes that may or may not involve the central nervous system and/or eye abnormalities [89]. Initial symptoms are mostly muscle weakness as a result of progressive muscle degeneration followed by infiltration and accumulation of fibrotic and adipose tissues. This process affects the majority of skeletal muscles with a variable degree of severity, affecting the diaphragm muscle most aggressively. Histopathological changes in cardiac muscles are limited, but dilated cardiomyopathy is a frequent observation in clinics, especially in MDDGC5 patients [118–120].

The range of clinical severities can be attributed to several factors including the site of mutations and levels of gene expression, both of which affect the functionality of the genes. Variation in the genetic background is also considered a potential disease modifier. Since the direct cause of the diseases may be associated with the abnormal *O*-mannosyl glycosylation of α-dystroglycan, the levels of residual *O*-mannosyl glycans in diseased muscles, representing the degree of functionality of the pathogenic gene, are likely the key factor in determining the disease severity. However, this correlation, although strongly supported by data from animal model studies, has not yet been firmly established from clinical data [121]. This discrepancy is largely due to the fact that muscle biopsy samples from patients are very limited in size and do not represent overall levels of glycosylated α-dystroglycan in body-wide muscles. In general, the glycosylation pattern of α-dystroglycan is variable between tissue types and developmental stages [122]. This variation is also affected by varying degrees of muscle regeneration [123, 124]. Furthermore, the gene mutation and severity of skeletal myopathy is not predictive of other prominent complications such as respiratory and cardiac failure [119, 120].

As a consequence, a clear genotype-phenotype correlation has yet to be established in the clinic. Further investigations that can reveal additional knowledge will be required to fully understand genotype-phenotype relationships and the natural history of dystroglycanopathies. These factors, along with the variable age of onset, the presence of central nervous system and/or eye abnormalities, as well as the severity of cardiomyopathies along the spectrum, complicate the development of treatment options. Therefore, it is crucial that these issues are factored into the design of genetic engineering strategies.

27.3 Genetic Therapy for Dystroglycanopathies

27.3.1 Gene Therapy

Since most types of MDDGs arise from defects that are associated with single-gene mutations, gene therapy is considered to be a viable therapeutic approach that does not require correction of the mutant allele. The concept of gene therapy is fairly straightforward, and several different approaches are currently being investigated, including (1) replacement of the defective gene that causes disease with a normal version of the gene, (2) introducing a "surrogate" gene to help ameliorate the disease caused by the defective gene, or (3) inactivating or "knocking out" the defective gene. It would seem that the latter of these strategies is impractical for MDDGs because all known genes in the biomimetic pathway of α-dystroglycan are essential for proper function. However, the two primary approaches are quite feasible and will be discussed in detail in this section.

Gene replacement therapy with a normal copy of the gene is clearly the ideal therapeutic approach for loss-of-function mutations in MDDGs, the results of which have been investigated in several animal models. One of the first gene therapy studies revealed that *LARGE* gene transfer was able to restore α-dystroglycan function in the skeletal muscles and ameliorate the dystrophic phenotype of $LARGE^{MYD}$ mice, a model with a mutation in the same gene as and clinical phenotype similar to the MDDGA6 [125]. This study provided evidence that LARGE expression plays a critical role in the glycosylation of α-dystroglycan. In addition, the LARGE-modulated glycosylation had a direct effect on the morphological and pathological phenotype of the muscles. In another study, recombinant *FKTN* was injected into knock-in mice with a retrotransposal *FKTN* insertion, a phenomenon evident in a majority of FCMD (Fukuyama-type congenital muscular dystrophy) cases, and subsequently analyzed for glycosylation and laminin-binding activity [126]. Results indicated that *FKTN* gene transfer reduced the amount of hypoglycosylated α-dystroglycan and increased levels of the normal-sized α-dystroglycan species. However, the mouse model used in this study retained a modicum of intact α-dystroglycan, which was sufficient in preventing the development of histopathological features commonly associated with MDDG. As a result, no therapeutic consequence could be evaluated. In a continuation of their *FKTN* studies, Kanagawa et al. focused on rescuing the intrinsic defects of satellite cells as an alternative therapy in two distinct *FKTN* conditional knockout mice that exhibited phenotypes reminiscent of MDDG [127]. AAV-mediated delivery of *FKTN* cDNA restored fukutin expression in satellite cells with improvement in their regenerative potential, suggesting that fukutin is important for muscle regeneration. Around the same time, therapeutic approaches aimed at replacing mutated FKRP with normal protein to restore the link between the extracellular matrix and the cell receptors were developed in an *FKRP*-deficient mouse model that contains a proline-to-leucine missense mutation at position 448 ($FKRP^{P448L}$) and is clinically relevant to MDDGC5,

also known as LGMD2I [128]. The therapeutic potential of AAV-mediated *FKRP* gene therapy was evidenced by the restoration of functionally glycosylated α-dystroglycan in skeletal and cardiac muscles. The transgene expression led to a significant improvement in muscle pathology and function. Additionally, overexpression of exogenous FKRP protein did not induce secondary expression of functional glycosylation of α-dystroglycan in non-muscle tissues, such as the liver and kidney. A similar study was conducted by Qiao et al., which used an AAV-mediated *FKRP* gene therapy in an *FKRP*-deficient mouse model that contains a common amino acid change from leucine to isoleucine at position 276 ($FKRP^{L276I}$) and mimics the milder, late-onset phenotype of LGMD2I [129]. Systemic delivery of the human *FKRP* gene into $FKRP^{L276I}$ mice at multiple ages rendered body-wide FKRP protein expression and restored glycosylation of α-dystroglycan in both skeletal and cardiac muscles. *FKRP* gene therapy ameliorated dystrophic pathology and cardiomyopathy such as muscle degeneration, fibrosis, and myofiber membrane leakage, resulting in restoration of skeletal muscle and heart contractile functions. To further expound on *FKRP* gene therapy studies, Vannoy et al. reported an age-dependent response to *FKRP* gene replacement therapy that addressed the effectiveness of the treatment at different stages of disease progression [130]. Specifically, the researchers administered AAV vectors incorporating a codon-optimized *FKRP* gene intravenously to $FKRP^{P448L}$ mutant mice at various age groups spanning the course of the disease. In the early stages of the disease progression, the treatment was highly effective. At the middle stages of disease progression, the treatment was capable of halting disease progression with some beneficial improvements to pathology and function. However, at the later stages, the treatment seemed to halt disease progression but had minimal pathological benefit to the mice. Overall, the study suggests that restoration of *FKRP* gene function in an *FKRP*-deficient mouse model can essentially halt disease progression at whatever stage it has reached but has limited ability to reverse secondary pathologies including fibrosis. Gene replacement studies involving *LARGE*, *FKTN*, and *FKRP* help to lay the groundwork for the preclinical evaluation of these therapeutic strategies related to dystroglycanopathies. These studies give evidence to suggest that small quantities of exogenously delivered genes that express normal enzymatic activity might be sufficient to restore enzymatic function or that partial restoration of α-dystroglycan glycosylation may be effective in reducing disease severity in dystroglycanopathies. Additionally, results helped support the notion that each of these proteins/glycosyltransferases is essential in the *O*-mannosylation of α-dystroglycan.

Given the involvement of a multitude of genes in MDDG disorders, the development of gene replacement therapies to target every individual gene is a considerable obstacle that would require a significant amount of time, energy, and funding. One potential solution is the application of a functionally dominant glycosyltransferase to rescue multi-gene defects. Multiple research groups have suggested that LARGE overexpression can compensate for the lack of function of several other MDDG-related genes, including *FKRP*, *FKTN*, *POMT1*, and *POMGnT1*, which account for a majority of the clinically diagnosed dystroglycanopathies. Barresi et al. were the

first to show that overexpression of LARGE led to a recovery of α-dystroglycan function as a receptor in cell cultures derived from individuals diagnosed with FCMD, MEB, and WWS [125]. A few years later, the first in vivo study provided evidence that *LARGE* gene transfer can bypass the glycosylation defects of α-dystroglycan in MDDG models other than LARGE^{myd} mice [126]. Several other reports subsequently showed that the overexpression of LARGE induced hyperglycosylation of α-dystroglycan with a high affinity for extracellular ligands in other MDDG mouse models such as *POMGnT1* knockout and *FKRP*-deficient mice [131, 132]. In the *POMGnT1* knockout model, AAV-mediated *LARGE* treatment yielded partial restoration of α-dystroglycan glycosylation and ligand-binding activity. The dystrophic phenotype in skeletal muscles was ameliorated as revealed by significantly reduced fibrosis, necrosis, and numbers of centrally located nuclei with an overall improved motor function. Similar results were demonstrated in the *FKRP*-deficient mouse model where ectopic expression of *LARGE* led to the restoration of α-dystroglycan glycosylation and laminin-binding activity. However, this study also showed that overexpression of FKRP was unable to correct the glycosylation defects and improve pathology in muscles of the LARGE^{myd} mice. These results suggest that modulation of *LARGE* activity can be a versatile treatment for dystroglycanopathies, regardless of the causative gene.

Conversely, given the demonstration that overexpression of *LARGE* can modify the core glycans by attachment of the [-GlcA-β-1,3-Xyl-α-1,3-] disaccharide to various complex *N*- and *O*-glycans on non-dystroglycan proteins or by continued elongation of the repeating units [133, 134], other studies have cast doubt about the potential efficacy of *LARGE* gene therapy to unrelated dystroglycanopathies [135, 136]. For example, one group reported that transgenic expression of *LARGE* exacerbates the muscle pathology of *FKRP*-deficient mice [135]. The overexpression of LARGE produced no pathology but led to a loss of force in response to eccentric exercise in the older normal mice. This result indicates a functional impairment, albeit limited, introduced by *LARGE* overexpression over time, although the loss was not observed in the younger mice. Other studies suggest that the detrimental effect of *LARGE* overexpression might be related to the *LARGE*-mediated suppression of muscle regeneration via downregulation of insulin-like growth factor 1 and that excess glycosylation may disturb cellular homeostasis [136, 137]. Furthermore, it is now understood that *LARGE*-mediated rescue of disease phenotypes requires at least partial function of some of the MDDG-associated genes such as *FKRP*, *FKTN*, *POMT1*, and *POMGnT1*. So, even though the exogenous expression of LARGE would thus appear to exhibit a very broad therapeutic window, this strategy would require extremely careful modulation to avoid unwarranted side effects. Clearly, more comprehensive investigations are required to understand the potential therapeutic windows for *LARGE* gene therapy and its applicability to non-LARGE-related MDDGs [138].

Gene therapies for dystroglycanopathies are not limited to replacement of the defective gene but also include alternative strategies that utilize surrogate genes not directly involved in the biosynthetic pathway of *O*-mannosyl glycans on α-dystroglycan. For example, Thomas et al. [139] utilized the *B4GALNT2* (formerly

GALGT2) gene that, when overexpressed, inhibits the development of muscle pathology associated with various forms of muscular dystrophy [140–142]. Mechanistically, *B4GALNT2* overexpression induces glycosylation of α-dystroglycan with the cytotoxic T cell glycan (GalNAcβ1,4-[Neu5Ac/Gcα2,3]-Galβ1,4-GlcNAcβ-) concomitantly promoting dystrophin or surrogate gene/protein expression. Treatment of FKRP^{P448L} mutant mice with AAV-mediated *B4GALNT2* inhibited downstream pathological events, despite a lack of functional *O*-mannosyl glycans on α-dystroglycan. Efforts to rescue dystrophic phenotypes with a surrogate gene have also been made by Vannoy et al. [143], which used a miniature form of agrin (mAgrin). Results in vitro demonstrated that overexpression of mAgrin was able to enhance laminin binding to primary myoblasts and fibroblasts from an FKRP^{P448L} model and that this enhancement is diminished when mAgrin is in molar excess relative to laminin. However, in vivo results failed to demonstrate a histological or functional improvement in the dystrophic pathology when mAgrin was delivered into FKRP^{P448L} mice. These results likely reflect an insufficient binding affinity of mAgrin to hypoglycosylated α-dystroglycan and the possibility of abnormal binding and disruption due to a molar excess of mAgrin.

27.3.2 Antisense Oligonucleotide Therapy

An alternative to gene replacement therapy is gene manipulation using short single-stranded pieces of chemically modified nucleotides, also known as antisense oligonucleotides (AONs). AONs are designed to be complementary to specific mRNA targeted gene and thus can be used as therapeutic agents that block the disease processes by altering the synthesis of a particular protein. This approach has been used previously to alter splicing events in other disease states, including Duchenne muscular dystrophy [144–146].

MDDGA4 (alternatively referred to as FCMD) disorders are caused by ancestral insertion of an SINE-VNTR-*Alu* (SVA) retrotransposon within the final coding exon (exon 10). This insertion activates a rare alternative "donor" site, which is normally inaccessible and creates a new splice "acceptor site" in the retrotransposon sequence. This "exon trapping" event results in the incorrect splicing of the *FKTN* mRNA transcript and generates an alternative fukutin protein with a modified carboxy-terminus. As a result, the aberrant form of fukutin is displaced from the Golgi apparatus to the endoplasmic reticulum, which subsequently disrupts the glycosylation pathway of α-dystroglycan. Taniguchi-Ikeda et al. have been able to correct the disorder by utilizing specifically designed AONs to suppress this exon trapping event [75]. In this approach, the AONs bind to the fukutin transcript before it is spliced, thereby favorably restoring its normal splicing pattern and expression of fukutin. In mice carrying the retrotransposon insertion, introduction of these AONs partially restored functional fukutin protein in skeletal muscle tissue with an observed recovery of normal levels of glycosylated α-dystroglycan. Additionally, administration of AONs also had the intended effect of blocking the deleterious splicing event in lymphoblasts and myotubes derived from patients with FCMD.

As predicted, the rescue of normal fukutin protein expression restored the link between α-dystroglycan and extracellular matrix proteins. This study demonstrates the capability of using antisense-mediated splicing modulation in molecular therapy to rescue fukutin function and potentially creates a treatment option for FCMD.

27.3.3 Genome Editing

Another potential therapy for MDDGs utilizing genetic engineering is genome editing—the process by which engineered nucleases work like molecular scissors to insert, delete, or replace DNA in the genome of a living organism. In theory, genome editing is advantageous over gene replacement therapy because there is no need to include extra genetic material (i.e., complete coding sequences and regulatory sequences) when only single nucleotide polymorphisms or small proportions of the gene need to be altered, which represent the majority of MDDGs. Over the past few decades, various mechanisms have been identified and modified for use in genome editing, which includes meganucleases, zinc finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs). However, these methods have been quickly eclipsed by a more sophisticated technology based on CRISPR (clustered regularly interspaced short palindromic repeat)/Cas (CRISPR-associated) systems, which can achieve target specificity through small synthetic guide RNAs that can be easily interchangeable for targeting new sites in the genome of interest. A simpler version of this system, commonly referred to as CRISPR/Cas9, was developed for genome editing purposes [147] and has been applied with varying degrees of success to numerous cell lines as well as in the generation of animal models of human diseases. With regard to dystroglycanopathies, one research group studied the effect of CRISPR/Cas9-mediated *TMEM5* gene deletion on core M3 glycan processing [92]. Generation of TMEM5-deficient cells disrupted the posttranslational modification of α-dystroglycan, resulting in abnormal glycosylation patterns. Ultimately, this helped to identify the enzymatic function of TMEM5 as a xylosyltransferase that forms the Xylβ1-4Rbo5P linkage on *O*-mannosyl glycan.

Use of the CRISPR/Cas9 system approach for the correction of disorders related to MDDGs remains in the early development stage, thus significant challenges and obstacles still exist before the approach can be clinically relevant. However, these technologies are developing at a rapid pace, and the continued advancement of this novel, programmable genome editing platform brings it one step closer to being a viable genetic engineering tool that could potentially revolutionize the field.

27.4 Summary and Future Direction

Significant progress has been achieved in the genetic and clinical characterization of various MDDG disorders caused by aberrant glycosylation of α-dystroglycan. Through numerous extensive studies, researchers have been able to elucidate the

molecular structure of multiple *O*-mannosyl glycans found on α-dystroglycan and identify at least 18 causative genes involved in the biosynthetic pathway. The identification of each newly associated gene helps to facilitate a deeper understanding of the mechanisms that underlie each one of these clinical disorders, while simultaneously creating a more complex system. Given this complexity, it is perhaps obvious that MDDG disorders involving various types of mutations would require distinct therapeutic strategies. A number of successful preclinical studies focused on the treatment of MDDGs have been reported, including gene therapies utilizing *LARGE*, *FKTN*, and *FKRP* and splice-blocking AON therapy for FCMD. Additionally, the emergence of genome editing technologies—ZNFs, TALENs, and CRISPR/Cas9—offers an alternative method with the potential for targeted and precise correction of genes associated with MDDG. However, none of these therapeutic strategies is without limitations or associated complications. For gene replacement therapy, sustained or long-term tissue-specific gene expression has yet to be evaluated in the clinical setting. Off-target effects and issues related to specificity, efficiency, and translatability of in vivo delivery methods present serious impediments for the gene editing technology moving forward. Furthermore, all of these genetic engineering approaches only address the underlying issue and do not take into account the secondary pathologies associated with the genetic defect, which may cause potentially irreversible damage.

So far, the majority of studies related to MDDGs have only been conducted in animal models or cell lines, but efforts to accelerate these technologies into a clinical trial setting are progressively increasing. Future advancement of this field will depend on the continued development of safe and efficient delivery strategies capable of achieving body-wide gene transduction, timely intervention, and appropriate functional assessment of each therapy. This, coupled with a better understanding of the physiopathological mechanisms of each disorder, will undoubtedly lead to successful clinical trials in the future.

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Chapter 28
RNAi Therapy for Dominant Muscular Dystrophies and Other Myopathies

Scott Q. Harper

Abstract Historically, the muscle gene therapy field has been primarily focused on replacing defective or missing genes underlying recessive disorders and has matured to the point where several gene replacement strategies have now been tested or are underway in human clinical trials. Unfortunately, gene replacement strategies are not indicated for treating dominant diseases, where reduction or elimination of an abnormal allele would be needed, and as a result, gene therapies for dominant muscular dystrophies have lagged behind. Importantly, the emergence of RNA interference (RNAi) as a gene-silencing tool provided a means to begin closing this development gap. In the first edition of this chapter of *Muscle Gene Therapy*, we discussed the prospects of combining RNAi and gene therapy to treat dominant muscle diseases, but proof of concept for its practical usage had not been demonstrated at the time. Here, in this second edition, we update our current understanding of the mechanisms underlying RNAi, compile several preclinical examples of RNAi-based gene therapies for muscle diseases, and discuss current prospects for translating these strategies toward the clinic.

Keywords RNA interference · RNAi · Dominant myopathy · Gene silencing · microRNA · miRNA · Short hairpin RNA · shRNAs · Small inhibitory RNA · siRNAs

28.1 Prevalence of Dominant Myopathies

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 <200,000 people in the USA or 1 in 2000 Europeans [1, 2]. X-linked

S. Q. Harper (✉)
Department of Pediatrics, The Ohio State University, Columbus, OH, USA

Center for Gene Therapy, The Research Institute at Nationwide Children's Hospital, Columbus, OH, USA
e-mail: Scott.Harper@nationwidechildrens.org

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recessive DMD has been historically considered the most common (1/3500 with recent updates defining incidence more accurately at 1/5000 newborn males) [3], followed by the dominant disorders myotonic dystrophy type 1 (DM1; 1/8000 to 1 in 15,000) [1, 4] and facioscapulohumeral muscular dystrophy (FSHD; 1 in 8333–20,000) [5–7]. In addition to FSHD and myotonic dystrophy, other dominant muscular dystrophies and myopathies arise from mutations in at least 42 other known genes (Table 28.1). The relative abundance of dominant muscle disease genes and the fact that two of the top three most prevalent muscular dystrophies are dominantly inherited supports that this class of disorders is important. Thus, therapeutic approaches for suppressing dominant disease genes should be explored more broadly, and RNAi-based gene therapies are a leading candidate strategy to accomplish such a goal.

Table 28.1 Mutations causing dominant muscular dystrophies and other myopathies

Gene	Clinical disorder	OMIM	Epidemiology
ACTA1	Congenital myopathy with Fiber type disproportion	255,310	
ACTA1	Congenital myopathy with excess of thin myofilaments	161,800	
ACTA1	Congenital myopathy, with cores	161,800	
ACTA1	Nemaline myopathy-3	161,800	
ACTA1	Scapulohumeroperoneal myopathy	102,610	
APOE	Sporadic inclusion body myositis	147,421	
APP	Sporadic inclusion body myositis	147,421	
BAG3	Dilated cardiomyopathy 1HH	613,881	
BAG3	Myofibrillar myopathy type 6	603,883	
CAV3	Distal myopathy	607,801	
CAV3	Distal myopathy, Tateyama type	614,321	<1 in 1000,000
CAV3	Familial hypertrophic cardiomyopathy	192,600	
CAV3	LGMD1C	601,253	192,600
CAV3	Long QT syndrome 9	611,818	
CAV3	Rippling muscle disease	606,072	
CHRNA1	Congential slow-channel myasthenic syndrome	601,462	
CHRNA2	Congential slow-channel myasthenic syndrome	601,462	
CHRNA3	Congential slow-channel myasthenic syndrome	601,462	
CHRNA4	Congential slow-channel myasthenic syndrome	601,462	

(continued)

Table 28.1 (continued)

Gene	Clinical disorder	OMIM	Epidemiology
Chrom 19p13	Vacuolar neuromyopathy	601,846	
CLCN1	Thomsen myotonia congenita	160,800	
CRYAB	Dilated cardiomyopathy, 1II	615,184	
CRYAB	Myofibrillar myopathy type 2	608,810	
DES	LGMD1E (now MFM1)	602,067/now 601,419	
DES	Myofibrillar myopathy type 1	601,419	
DES	Scapuloperoneal syndrome, neurogenic, Kaeser type	181,400	
DMPK	Myotonic dystrophy type 1	160,900	
DNAJB6	LGMD1E (formerly LGMD1D)	603,511	
DNM2	Myotubular (or centronuclear) myopathy	160,150	
DNMT3B	FSHD	Unassigned	
DUX4	FSHD	158,900	
FHL1	Reducing body myopathy, X-linked 1a	300,717	
FHL1	Scapuloperoneal amyotrophy	300,695	
FLNC	Cardiomyopathy, familial restrictive 5	617,047	
FLNC	Distal myopathy type 4	614,065	
FLNC	Myofibrillar myopathy type 5	609,524	
GSK3B	Sporadic inclusion body myositis	147,421	
HNRNPDL	LGMD1G	609,115	
KBTBD13	Nemaline myopathy type 6	609,273	
LDB3	Cardiomyopathy, dilated, 1C	601,493	
LDB3	Cardiomyopathy, hypertrophic, 24	601,493	
LDB3	Left ventricular noncompaction	601,493	
LDB3	Myofibrillar myopathy 4	609,452	
LMNA	Congenital muscular dystrophy	613,205	
LMNA	Dilated cardiomyopathy 1A	115,200	
LMNA	Emery-dreifuss muscular dystrophy type 2	181,350	
LMNA	LGMD1B	159,001	
MTMR14	Myotubular (or centronuclear) myopathy	160,150	
MYF6	Myotubular (or centronuclear) myopathy	159,991	
MYH2	Proximal myopathy and ophthalmoplegia	605,637	
MYH7	Dilated cardiomyopathy 1S	613,426	
MYH7	Hypertrophic cardiomyopathy 1	192,600	

(continued)

Table 28.1 (continued)

Gene	Clinical disorder	OMIM	Epidemiology
MYH7	Laing distal myopathy	160,500	
MYH7	Left ventricular noncompaction 5	613,426	
MYH7	Myosin storage myopathy	608,358	
MYH7	Scapuloperoneal syndrome, myopathic type	181,430	
MYOT	LGMD1A	604,103	
MYOT	Myofibrillar myopathy 3	609,200	
MYOT	Spheroid body myopathy	182,920	
PABPN1	Oculopharyngeal muscular dystrophy	164,300	
PSEN1	Dilated cardiomyopathy 1 U	613,694	
PSEN1	Sporadic inclusion body myositis	147,421	
RYR1	Central core disease	117,000	
SCN4A	Hyperkalemic periodic paralysis, type 2	170,500	
SCN4A	Hypokalemic periodic paralysis, type 2	613,345	Baltic Sea countires: Sweden, Finland, England; 1 in 1000 in Sweden; 1 in 300,000 in England; 1 in 5000 in Finland
SCN4A	Myotonia congenita, atypical	603,967	
SCN4A	Paramyotonia congenita	168,300	
SMCHD1	FSHD type 2	158,901	
TAU	Sporadic inclusion body myositis	147,421	
TIA1	Welander distal myopathy	604,454	
TNPO3	LGMD1F	608,423	
TPM2	Arthrogryposis multiplex congenita, distal, type 1	108,120	
TPM2	Arthrogryposis, distal, type 2B	610,680	
TPM2	Cap myopathy-2 (CAPM2)	609,285	
TPM2	Nemaline myopathy type 4	609,285	
TPM3	Cap myopathy-1 (CAPM1)	609,284	
TPM3	Myopathy, congenital, with fiber-type disproportion	255,310	
TPM3	Nemaline myopathy type 1	609,284	
TTN	Hypertrophic cardiomyopathy, familial type 9	613,765	
TTN	Tibial muscular dystrophy (Udd distal myopathy)	600,334	
ZNF9	Myotonic dystrophy type 2	602,668	

At least 42 different genes are associated with dominant muscular dystrophies and other myopathies. This list includes Alzheimer's disease-related genes, TAU, APP, PSEN1, GSK3B, and APOE that are overexpressed in sporadic inclusion body myositis (IBM)

28.2 RNA Interference

RNA interference (RNAi) is a cellular mechanism to control gene expression prior to translation [8]. RNAi is mediated by small (~21–25 nucleotide, nt) noncoding RNAs (microRNAs or miRNAs) as well as several proteins involved in miRNA processing and gene silencing [9–11]. A key feature of RNAi is sequence specificity: miRNAs share nucleotide sequence homology and base pair with 3′ untranslated (UTR) regions of cognate mRNAs [12]. These base pairing interactions allow miRNAs to act as guides that 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 [10, 11, 13–16]. 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 microRNA biogenesis when developing RNAi as a therapeutic tool.

28.3 RNAi Pathway

Rationally designed RNAi molecules are based on the structure and, in some cases, the nucleotide sequence of natural miRNAs (Figs. 28.1 and 28.2) [17–19]. Like other coding and noncoding transcripts in the cell, primary miRNA (pri-miRNA) precursors vary in size and how they are transcribed: most miRNAs are RNA polymerase II (pol II) transcripts, while others may be transcribed by RNA polymerase III (pol III), and expression may be tissue-specific [19–22]. 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 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 [23–27]. DGCR8 recognizes and binds an important miRNA structural feature—a characteristic junction between the miRNA double-stranded stem and flanking single-stranded sequences [27]. 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 nucleotide, nt) hairpin pre-miRNA containing a 2 nt 3′ overhang [26, 27]. The nuclear export factor, exportin-5 (Exp5), in complex with

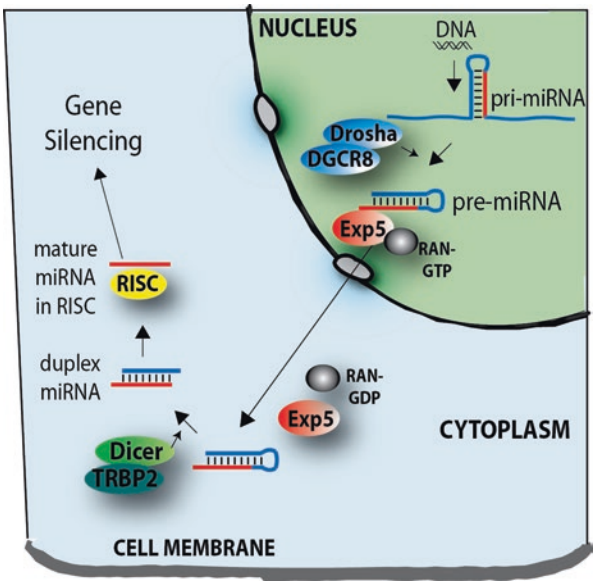

Fig. 28.1 MicroRNA biogenesis pathway. See text for details. Designed therapeutic microRNA shuttles, shRNAs, and siRNAs mimic pri-, pre-, and mature-miRNAs, respectively. Upon delivery to cells, exogenous inhibitory RNAs therefore enter the microRNA biogenesis pathway at different points, but all elicit gene-silencing effects

RAN-GTP, binds this overhang and then shuttles the pre-miRNA to the cytoplasm [28]. There, GTP hydrolysis provides energy to dissociate the complex, thereby releasing the pre-miRNA for association with the enzyme Dicer and its cofactor TAR RNA-binding protein (TRBP). The Dicer/TRBP complex recognizes the Drosha-generated 3′ nt overhang and initiates another staggered cleavage event ~22 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 [29–33]. 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. Since a Dicer cut is ~21 nts 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 [34, 35]. Indeed, some miRNAs are bifunctional, and both strands can direct gene silencing [35]. 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.

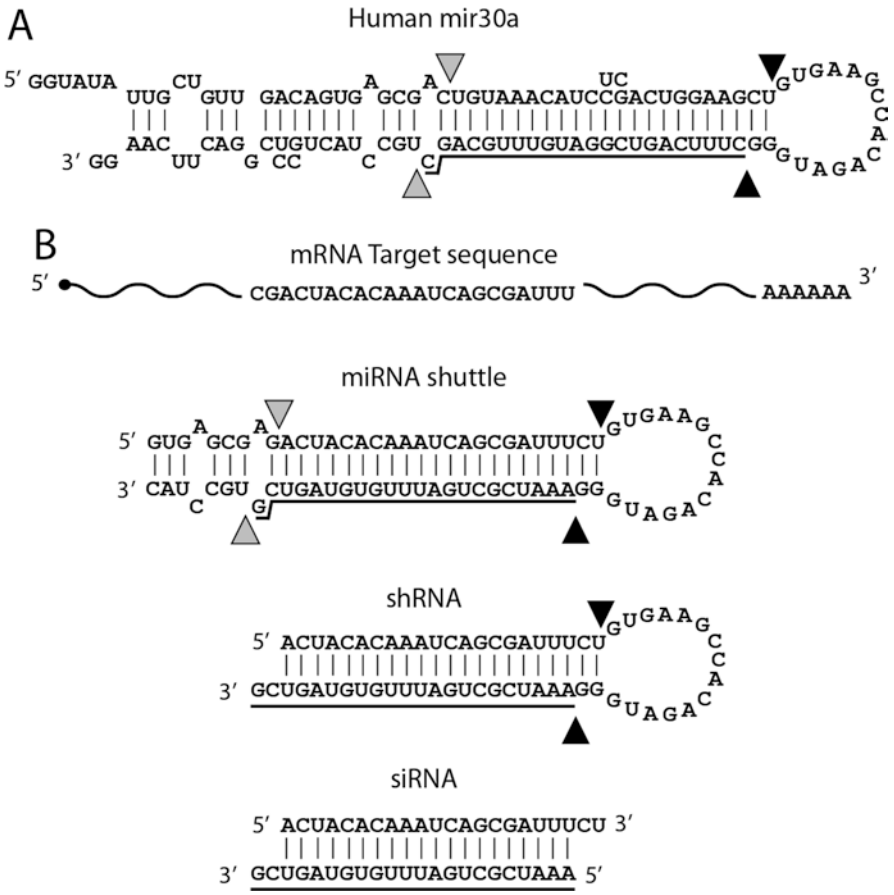

Fig. 28.2 Natural microRNA sequences and structures are used to design therapeutic inhibitory RNAs. (**a**) Human mir-30a. Gray and black triangles point to Drosha and Dicer nuclease sites, respectively. Note the staggered cuts leaving 2 nucleotide 3′ overhangs. Underlined sequence indicates the mature 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

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 change little or not at all. In some instances, only 7 nt of complementarity between the guide

strand and a target mRNA (miRNA nts 2–8; called the seed match) may be required to elicit gene-silencing effects [36]. 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.

28.4 RNAi Triggers for RNAi Therapeutics

Small inhibitory RNAs (called RNAi triggers) 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. 28.1). There are three major classes of designed inhibitory RNAs (Fig. 28.2). (1) Small inhibitory RNAs (siRNAs) are in vitro synthesized, dsRNAs that are structurally identical to miRNA duplexes [10]. When delivered to cells, all siRNAs bypass the transcription and nuclear processing steps of the miRNA pathway. Some designed siRNAs are processed by Dicer [37], 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 microRNA sequences [38]. 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 [17, 18]. 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 microRNA is used to deliver an artificial siRNA. MiRNA shuttle transcripts are produced intracellularly and utilize all processing steps required for natural miRNA biogenesis. In addition to these three main systems, *mirtrons* are another interesting system worth mentioning. These hairpin RNAs bypass the Drosha step and rely upon RNA splicing to generate the pre-miRNA, which then becomes a substrate for Exp-5 and Dicer [39].

Each of the three main 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, including muscle [40–49]. 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 approximately two helical turns downstream, which ultimately determines the sequence of the mature guide strand molecule (Fig. 28.2). 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 [50, 51]. Another difference between shRNAs and miRNAs is potential for nonspecific toxicity; miRNAs may be safer than shRNAs in vivo [18]. 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 [40–42, 52]. However, a few 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 [42, 53]. This observed toxicity seems to be related to shRNA-induced saturation of endogenous microRNA biogenesis pathways, thereby interfering with natural microRNA function [18, 42, 53, 54]. Importantly, lowering the dose of vector-expressed shRNAs in the liver, or using a less-powerful microRNA shuttle system in the brain, mitigated these toxic effects [42, 53]. 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, cancer, and—since the publication of the first edition of *Muscle Gene Therapy*—models of muscular dystrophy [40–49, 52, 55–57].

28.5 Necessity for Disease Allele-Specific Silencing

With the exception of extremely rare cases of X-linked dominant FHL1 mutations in males [58], patients with dominant disorders possess one mutant and one normal copy of their specific myopathy-related gene. Since normal copies of disease genes often encode essential proteins, normal allele haploinsufficiency may contribute to myopathic phenotypes as well. Thus, therapy development for many dominant muscular dystrophies may require specific silencing of the dominant allele while avoiding the normal allele. 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 type 1 (NEM1; OMIM #609284) can arise from autosomal dominant or recessive TPM3 mutations [59–67]. Patients with dominant NEM1 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 [67, 68]. 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 with dominant disease. In this example, it would be advantageous to restrict gene knockdown to the affected allele while leaving the normal allele unperturbed (Fig. 28.3).

Since 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 one base pair? In short, the answer is yes, but it can be difficult. As previously discussed, perfect sequence complementarity between an inhibitory RNA and target mRNA causes message degradation; imperfect base pairing leads to translational inhibition [69]. 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 necessarily reflect reduced potency; microRNAs 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 [36, 70–72]. 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. If allele-specificity cannot be achieved, alternative strategies can be pursued, including non-allele-specific knockdown of both mutant and wild-type alleles coupled with replacement of an RNAi-resistant wild-type cDNA (Fig. 28.3).

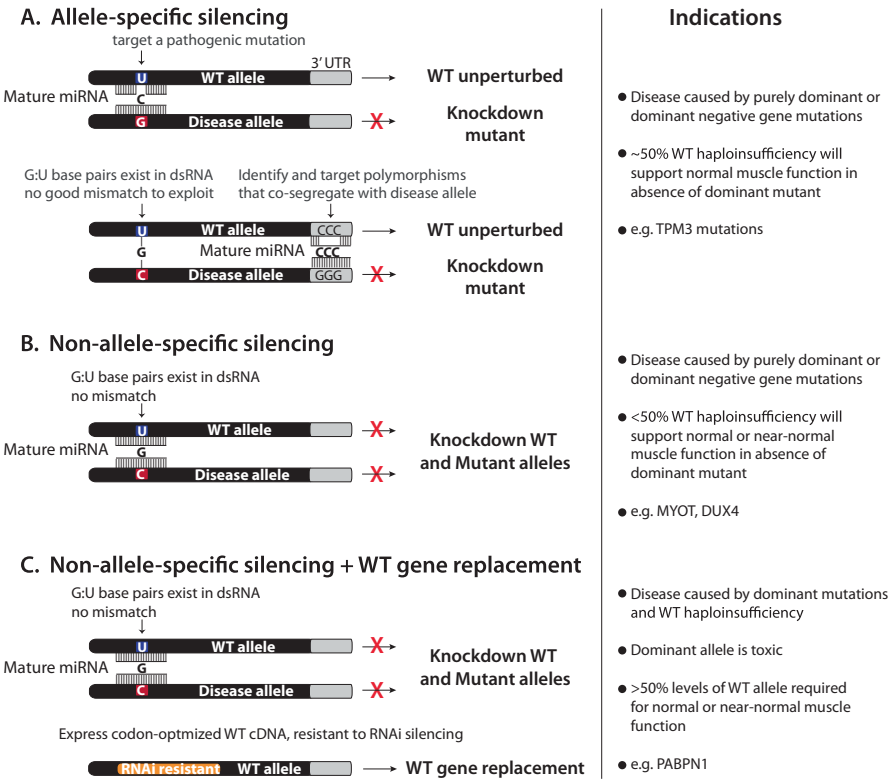

Fig. 28.3 Gene-silencing strategies to treat dominant myopathies**.** For each figure, two alleles of a hypothetical gene are shown: a wild-type (WT) and mutant. Grey bars denote 3′ UTRs. (**a**) Two strategies can be undertaken to direct disease allele-specific silencing with RNAi. The example at the *top* shows a mature microRNA sitting atop a stretch of sequencing containing a single base disease mutation. In this example, the wild-type allele contains a U, while the mutant has a G at the same position. A microRNA could be designed with perfect complementarity to the mutant allele but have a central mismatch with the wild-type. In some cases, this may permit specific silencing of only the mutant transcript while leaving the normal copy unperturbed. The bottom of *panel A* shows a second set of WT and mutant gene alleles, where the single base change associated with disease changes from a U (in WT) to C (in mutant). Importantly, G:U base pairs occur naturally in double-stranded RNA molecules, so designing a microRNA to discriminate between the two allele at the mutation site would be difficult. An alternative strategy would rely upon identifying silent polymorphisms that co-segregate with the mutant allele and then designing microRNAs that target those polymorphic regions. (**b**) Non-allele-specific gene silencing. (**c**) Non-allele-specific gene silencing combined with replacement of an RNAi-resistant, codon-optimized, wild-type cDNA. This strategy was employed in [47]

A second approach may involve identifying polymorphisms that may co-segregate with the mutant allele and then designing inhibitory RNAs to those proxy regions of a disease-causing transcript [73]. Finally, allele-specific silencing may not be necessary in some cases (Fig. 28.3).

28.6 Progress in Preclinical Development of RNAi Therapy for Dominant Muscular Dystrophies and Other Myopathies

Among the first applications of RNAi therapy using AAV vectors were preclinical strategies to suppress genes involved in neurodegenerative disease and hepatitis virus infection in the liver [40, 41, 74]. When the first edition of *Muscle Gene Therapy* was published, no such studies had yet been published targeting dominant muscle disease genes. Importantly, progress has been made in the field, and there are now six studies published between 2011 and 2017 demonstrating proof of principle for RNAi-based gene therapy of FSHD, limb-girdle muscular dystrophy type 1A (LGMD1A), DM1, and oculopharyngeal muscular dystrophy (OPMD) (Table 28.2) [44–49]. Each of these studies was performed in mice, and, importantly, each demonstrated the ability to improve different molecular, histopathological, and functional aspects associated with the various myopathies in animal models using both intramuscular and intravascular delivery routes (Table 28.2). Perhaps equally as important is that no obvious deleterious effects were observed due to the RNAi treatment, supporting translation of these strategies in the near future. Of note, while the first five published studies specifically targeted dominant disease alleles using RNAi, the OPMD study utilized a combination non-allele-specific gene knockdown plus gene replacement strategy (Fig. 28.3c), as expression of both mutant *PABPN1* and haploinsufficiency of the WT gene contributed to OPMD pathology in mice [47]. The specific strategy utilized will vary depending upon the pathogenic mechanisms underlying each disease.

28.7 Summary

RNAi therapeutics is an emerging field. Several preclinical studies demonstrated its immense potential for treating dominant neurodegenerative diseases, chronic viral infection, and cancer. We are now able to add dominant muscular dystrophies to the list of diseases for which RNAi-based gene therapy may be a prospective treatment. Importantly, there is now clinical precedence for applying AAV-based RNAi, with a recent clinical trial using AAV and shRNAs targeting hepatitis B virus in the liver of humans with chronic hepatitis [57]. This work sets the stage for translation of this approach more broadly, to attack a range of diseases. Although currently in its infancy as a technology, the potential for disease allele specificity may someday allow RNAi therapeutics to be an important tool for personalized medicine.

Table 28.2 Preclinical studies of RNAi-based gene therapy for dominant muscular dystrophies, published between 2011 and 2017

PMID	Year	Disease	Gene target	Vector Features	Model	Delivery route	Outcomes
21,730,972	2011	Facioscapulohumeral muscular dystrophy (FSHD)	Over-expressed WT human FRG1	ssAAV6 U6 promoter Designed miRNA	FRG1-high mouse	Intramuscular/P1–P2 5×10^{10} total dose	Improved muscle mass, grip strength, histopathology
21,829,175	2011	Facioscapulohumeral muscular dystrophy (FSHD)	Over-expressed WT human FRG1	ssAAV6 U6 promoter Designed miRNA	FRG1-high mouse	Intravascular/6 weeks 5×10^{12} total dose	Improved histopathology and treadmill running
22,508,491	2012	Facioscapulohumeral muscular dystrophy (FSHD)	Over-expressed WT human DUX4	ssAAV6 U6 promoter Designed miRNA	AAV. DUX4 mouse	Intramuscular/8 weeks 3×10^{10} total dose	Improved grip strength, histopathology
24,781,192	2014	Limb girdle muscular dystrophy type 1A (LGMD1A)	Mutant human MYOT	ssAAV6 U6 promoter Designed miRNA	MYOT T57I mouse	Intramuscular/P1–P2 5×10^{10} total dose	Improved muscle mass, specific force, histopathology; reduced intramuscular protein aggregation
26,082,468	2015	Myotonic dystrophy (DM1)	Hybrid gene: Human skeletal actin (HSA) containing DM1-associated expanded CTG repeat	ssAAV6 U6 promoter Designed miRNA	HSA-LR mouse	Intravascular/4 weeks 4×10^{12} total dose	Improvements in DM1-related splicing defects, myotonia; reduction in nuclear RNA foci

(continued)

Table 28.2 (continued)

PMID	Year	Disease	Gene target	Vector Features	Model	Delivery route	Outcomes
28,361,972	2017	Oculopharyngeal muscular dystrophy (OPMD)	*Gene knock down*: Mutant bovine PABPN1; WT mouse PABPN1 *Gene replacement*: Human codon-optmimized RNAi-resistant WT PABPN1 (optPABPN1)	*Gene knockdown*: scAAV8 expressing three different shRNAs from three different RNA pol III promoters (U61, U69, H1) *Gene replacement*: ssAAV9 expressing human codon-optmimized RNAi-resistant WT PABPN1 from muscle-specific SPc5-12 promoter	A17 mouse	Intramuscular/10–12 weeks 2.5×10^{10} scAAV8 shRNAs 1.3×10^{11} ssAAV9 optPABPN1	Improved fibrosis, histopathology, muscle force; normalization of transcriptional changes associated with mutant PABPN1 expression

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Chapter 29
Gene Therapy for Facioscapulohumeral Muscular Dystrophy (FSHD)

Daniel G. Miller

Abstract Facioscapulohumeral muscular dystrophy (FSHD) is a relatively common myopathy affecting 1/8500–15,000 individuals. FSHD is caused by inappropriate expression of the transcription factor double homeobox protein 4 (DUX4) so gene therapies must either prevent expression of DUX4 or interrupt the pathogenic downstream effectors of DUX4. The autosomal dominant inheritance pattern and the fact that the primary pathology is limited to multinucleate muscle fibers make gene therapies a challenging prospect for this important dystrophy without a treatment. Genetic correction of even a large percentage of myonuclei may not be sufficient to produce a phenotypic change in the setting of multinucleate myofibers when the disease is caused by a dominant-negative mechanism. In this chapter, I outline what is known about the molecular pathology of FSHD and discuss several gene therapy approaches to interrupting the cycle of DUX4 expression and muscle cell death.

Keywords Facioscapulohumeral muscular dystrophy · FSHD · Double homeobox protein 4 · DUX4 · Autosomal dominant · SMCHD1 · Gene therapy

29.1 Genetics of Facioscapulohumeral Muscular Dystrophy (FSHD)

FSHD is an adult-onset myopathy with incidence of 1 in 15,000 and prevalence of 1 in 8300 individuals [1]. FSHD occurs secondary to epigenetic changes resulting most commonly from a reduction in the number of 3.3 kb D4Z4 units arrayed on chromosome 4 to less than 11 units [2–6] (Fig. 29.1a). Approximately 5% of FSHD-affected individuals have a mutation producing SMCHD1 haploinsufficiency resulting in an alteration of the chromatin structure of the D4Z4 macrosatellite array on

D. G. Miller (✉)
Departments of Pediatrics and Genome Sciences, Division of Genetic Medicine, University of Washington, Seattle, WA, USA
e-mail: dgmiller@uw.edu

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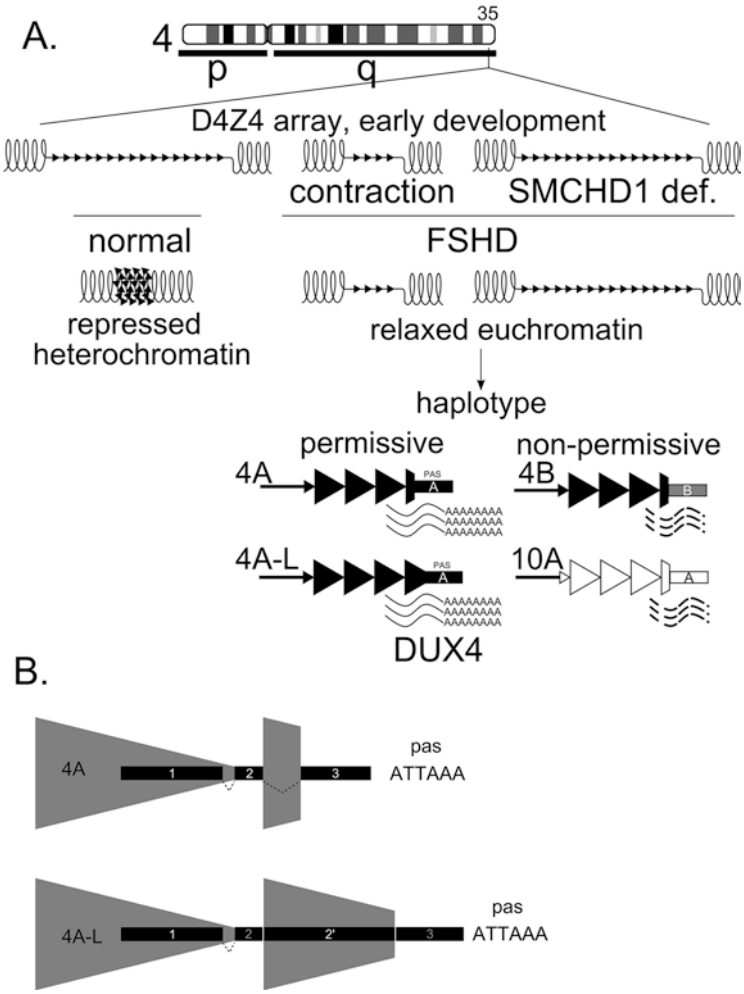

Fig. 29.1 Genetics and epigenetics of FSHD. (**a**) Diagram of human chromosome 4 showing short (p) and long (q) arms with the position of the FSHD-causing D4Z4 array indicated by a line and the band number 35. Beneath the chromosome is a blowup of the chromatin consequence of various genetic alterations that produce FSHD. Normal D4Z4 arrays have a length greater than 10 D4Z4 units and become compacted as heterochromatin during development. FSHD-prone arrays are either shortened by intrachromosomal recombination and contraction of the number of D4Z4 units to less than 11 or intermediate length arrays (11–25 units) remain as euchromatin due to mutations that cause haploinsufficiency of SMCHD1. In the context of a euchromatinized array, a permissive haplotype is also necessary for the production of DUX4 and consequent muscle pathology. 4A and 4A-L haplotypes are the most common and are present in ~50% of North Americans. The critical change is the presence of a polyadenylation signal (PAS) in a sequence that becomes exon 3 distal telomeric to the D4Z4 array. (**b**). Diagram of the exon structure of the most distal full D4Z4 unit and the last partial D4Z4 unit in permissive 4A and 4A-L arrays. Introns are indicated by dashed "splicing" lines

chromosome 4 [7]. The contraction- or SMCHD1-induced epigenetic profile results in the possibility of transcription of the normally repressed DUX4 retrogene [8]. Additional events [9] result in DUX4 transcription in <5% of cultured myocyte nuclei [8–11]. When array contractions occur on permissive haplotypes containing a functional polyadenylation signal present in ~50% of the population [12, 13], DUX4 protein is made and results in myopathology that manifests as muscle weakness (Fig. 29.1). The small percentage of DUX4-expressing cells suggests that even in the diseased state, there is significant (>95%) suppression of DUX4 transcription, and FSHD appears to be driven by the occasional nucleus that overcomes suppressive mechanisms and begins to produce DUX4 [9]. Once produced, DUX4 spreads from the cytoplasm to adjacent nuclei within the same myofiber [11, 14]. Thus, genetic strategies that alter the cytoplasmic makeup of the myofiber (RNAi, etc.) may be more effective at preventing DUX4-mediated pathology than strategies that rely on altering the DNA of individual nuclei. These unique disease characteristics along with the location of diseased myonuclei in muscle fiber syncytia make gene therapy approaches challenging for this prevalent muscular dystrophy without a current treatment.

29.2 Platforms for Testing Gene Therapy Approaches

The DUX4 retrogene is unique to the primate lineage [15–17] so the mRNA structure and chromosomal context surrounding the D4Z4 macrosatellite array are unique to humans. Likewise, most DUX4 DNA binding sites are unique to the human genome [18–22], suggesting that the cellular consequence of DUX4 expression varies from species to species. Human myocytes from biopsies of FSHD-affected muscles die within 24 h of endogenous DUX4 activation making them a useful model for assessing chromatin and contextual gene-expression effects of therapies [11]. However, arrayed “retro-orthologs” exist in other species including mouse [16, 17], and FSHD-like pathology has been modeled by forced expression of human DUX4 in human or mouse [23, 24] cells where cell death occurs 12–24 h after DUX4 induction [23]. Mouse [19, 25, 26] and zebrafish [27] models have also been constructed allowing the use of these model systems to assess in vivo delivery of therapies designed to disrupt the function of mature DUX4 mRNAs [28].

29.2.1 Mouse Models of FSHD as Gene Therapy Testing Platforms

Mouse models showing FSHD-like muscle pathology constitutively produce low levels of human DUX4 from an X-linked [25, 26] or ROSA-inserted transgene that can be further induced with doxycycline via a tet-responsive promoter driving

DUX4 or Cre-recombinase-mediated excision of stop codons between promoter and DUX4 at the ROSA locus [60]. Forced overexpression of human DUX4 using adeno-associated virus (AAV) vectors has also been shown to create myopathology in mice and serves as a testing platform for therapies targeting DUX4 transcripts [25, 29, 30]. These models will be useful for studying in vivo administration of therapies designed to reduce DUX4 transcript levels or block DUX4 translation since the human mRNA structure has been retained in the engineered mice and expression vectors.

29.2.2 Cell Culture Models of FSHD as Gene Therapy Testing Platforms

Strategies designed to alter transcriptional activity of DUX4 promoter sequences within D4Z4 require the arrayed context of the macrosatellite repeat, perhaps the subtelomeric location of the array on chromosome 4, and other epigenetic mediators such as small RNAs that direct chromatin formation at D4Z4 arrays. Therefore, gene therapy strategies that affect epigenetic properties of D4Z4 will need to be tested in human cells and then taken directly to human clinical trials after safety and toxicity studies have been conducted in animals. Studying human DUX4 in human muscle tissue or in cultured human myocytes can also be difficult due to low, infrequent DUX4 expression [8–11]. DUX4 mRNA can be detected in muscle biopsies from FSHD-affected individuals [31], but protein staining has been difficult to demonstrate. Alternatively, DUX4 expression can be observed in cultured human myocytes, but DUX4 protein from endogenous DUX4 genes in their arrayed context on chromosome 4 is present in 0.5–4.3% of cultured differentiated FSHD myocytes with variability seen between individuals [8, 11]. More sensitive biomarkers will be essential for human clinical trials [31–33].

29.2.3 Zebrafish Models of FSHD as a Gene Therapy Testing Platforms

Zebrafish have been engineered to contain human DUX4-expressing sequences [27], and zebrafish muscles show DUX4-associated pathology [27, 29, 31]. While this organism is perhaps best utilized for genetic and small molecule screens, rapid development and readily observable muscle phenotypes make it an important model organism and allow quick testing of gene therapy strategies in the context of organized muscle tissue in vivo.

29.3 Gene Therapy Approaches

Gene therapy for a dominantly inherited myopathy requires a different approach than the more classic application of gene addition therapies for recessively inherited conditions. FSHD is caused by the aberrant production of a toxic protein rather than the lack of sufficient protein levels typical of recessively inherited conditions. Muscle disease adds a layer of complexity because muscle tissue is made up of myofiber syncytia where multiple nuclei share a common cytoplasm so modification of a fraction of nuclei may not be sufficient to overcome the toxicity produced by a few DUX4-expressing nuclei. With these caveats in mind, we explore several strategies that may be feasible despite these added complexities.

29.3.1 Destabilization of the DUX4-Coding mRNA

Gene therapy targets for FSHD treatment are limited by our partial understanding of the disease process. Currently, very little is known about how DUX4 expression results in cell death, making the prevention of DUX4 transcription, translation, or localization, the primary targets for gene therapy strategies. The DUX4 mRNA produced from permissive chromosome 4 haplotypes has a unique 5′ untranslated region allowing for some specificity in targeting strategies [8, 31].

29.3.1.1 RNAi-Based Approaches

One approach to reducing DUX4 mRNA levels in muscle fibers is to express an inhibitory RNA from a viral vector that mediates destruction of DUX4 mRNA through the RNA-induced silencing complex and the associated pathways. This approach has the added benefit of cytoplasmic activity so mRNAs can be degraded regardless of the nucleus of origin. The fact that myofibers are postmitotic also makes RNA interference an attractive approach because inhibitory RNAs will not be diluted with each cell division. Finally, muscle diseases have been a primary focus of AAV-mediated gene therapy, and AAV vectors are ideal vehicles for RNAi delivery [34, 35]. AAV serotypes that selectively target muscle exist, and efficient transduction of muscle tissue has been demonstrated in a variety of small and large animal models [36–38]. Human clinical trials for Duchenne Muscular Dystrophy are currently underway and use AAV vector delivery of microdystrophin transgenes as a technique for gene replacement.

In addition to expression in early development [62, 63], DUX4 is normally expressed in testes [8] and thymus [39] tissue, but these transcripts are spliced differently to contain 3′ exons distal to the D4Z4 array [8]. DUX4 mRNAs produced pathologically from permissive chromosome 4 haplotypes have a unique 3′ end due

to the termination of exon 3 at a polyadenylation signal present only on permissive haplotypes [2] (Fig. 29.1). Thus, the unique sequence of exon 3 and splice junctions necessary for its incorporation can be used to develop specific reagents for DUX4 mRNA reduction.

The expression of multiple RNAi molecules targeting several different regions of the DUX4 gene would likely be an effective strategy for DUX4 mRNA reduction. Using this approach multiple gene therapy vectors or a single multi-cistronic vector could be constructed that contains micro-RNA expression cassettes for targeting multiple regions of the DUX4 gene. This approach has the added benefit of redundancy assuring continued transcript destruction even if mutations develop that prevent the activity of one or several miRNAs. Additional safety can be achieved by using AAV serotypes that preferentially transduce muscle and muscle-specific promoters for transcription of DUX4 miRNAs.

29.3.1.2 Exon-Skipping Strategies to Prevent Polyadenylation

Forced exon skipping has been a popular strategy investigated for the treatment of Duchenne muscular dystrophy (DMD) [40–42]. The approach involves the production and delivery of small modified oligonucleotides that mask splice donor and/or acceptor sites within the gene [43] forcing frameshift mutations in mutated dystrophin exons to be spliced out resulting in a shortened yet functional dystrophin molecule. Here we propose to utilize similar techniques to skip the distal 3′ exon containing the cryptic polyadenylation signal in permissive D4Z4 arrays (Fig. 29.1b). Removal of the polyadenylation signal for the DUX4 transcript should reduce the stability of pathogenic RNAs arising from euchromatinized permissive D4Z4 arrays. This approach also has the benefit of being relatively specific for pathogenic transcripts because the most distal 3′ exon is uniquely expressed in individuals with FSHD.

29.3.2 Gene-Editing Approach to Mutate DUX4

FSHD is caused by aberrant expression of DUX4 so gene-editing strategies that mutate DUX4 or prevent protein production should be therapeutic. DUX4 expression occurs in one in >50 nuclei in human FSHD myocyte cultures, and this infrequent expression is sufficient to result in myotube destruction [11] so the thresholds for a therapeutic benefit are unknown for an approach that attempts DUX4 mutation. Since DUX4 expression appears to be stochastic, preventing DUX4 expression in a subset of nuclei may be sufficient to reduce the probability of DUX4 production to a point where an overall change in myofiber survival is realized despite correction rates below 100%. Quantitative studies to determine disease thresholds in cell culture, animal models, or mosaic individuals will be important to determine modification efficiencies that would predict a phenotypic change in FSHD-affected individuals.

The CRISPR/Cas9 system for creating targeted double-stranded DNA breaks in the genome has received recent public and scientific attention. The most enticing potential of this technology is the ability to "edit" the genome in precise directed ways. Gene editing generally means providing both a targeted break mediated by a guide RNA sequence and the Cas9 nuclease and a template for repair so that small changes can be incorporated. DNA double-stranded breaks produced in the absence of a repair template are quickly repaired by an alternate pathway called nonhomologous end joining (NHEJ), a process where DNA ends are bound by repair proteins, polished by the addition or removal of nucleotides, and pasted back together without reference to the reading frame of the gene. While this reaction produces undesirable outcomes for gene-editing applications, NHEJ is more efficient than template-directed repair and often results in a frameshift at the site of the break. Thus a significant application of CRISPR/Cas9 approach is the production of mutations at specific genomic sites (Fig. 29.2a).

DUX4 mutagenesis could be targeted to several critical sites within sequences unique to the most distal D4Z4 unit in the array. Targeting the distal unit minimizes competition with the 100s of DUX4 copies present on both alleles of chromosome 10, the nonpathogenic chromosome 4 allele, and scattered in the telomeric region of multiple acrocentric chromosomes [44]. The most obvious target is the polyadenylation signal since a functional polyadenylation site determines whether a D4Z4 array haplotype is permissive for causing FSHD, and polymorphisms in this sequence are central to the production of DUX4 [2]. Changing the sequence to a nonpermissive haplotype or mutating the sequence altogether should neutralize the pathogenicity of a shortened D4Z4 array as evidenced by multiple asymptomatic individuals with short nonpermissive euchromatinized arrays [13]. The splice donor and acceptor sites leading to inclusion of the polyadenylation sequence are also important targets where production of DUX4 could be disrupted. In particular the splice acceptor at the intron/exon 3 junction is unique to 4qA-type permissive alleles accounting for the vast majority of permissive haplotypes (Fig. 29.1b).

Mutations could also be introduced into the coding sequence of DUX4 by inserting a premature stop codon or other frameshift mutation. Utilizing CRISPR-/Cas9-mediated DNA cleavage for this approach has the added danger of generating DNA breaks in permissive nonpathogenic long D4Z4 arrays. Such off-target breaks would likely induce intrachromosomal rearrangements that generate short DUX4-producing permissive arrays. Therefore, mutation of DUX4-coding sequences with CRISPR/Cas9 should be limited to the most distal copy of DUX4 within the array. With current technology it is difficult to imagine how the coding region of DUX4 in the most distal copy could be targeted while excluding the 100s of other copies presented at other locations.

29.3.3 Inhibition of DUX4-mRNA Expression

Once DUX4 protein is produced, the effects are amplified by the spread of molecules to adjacent nuclei within myotube syncytia where transcription at multiple DUX4 target sites is activated. Therefore, preventing expression of DUX4 mRNA

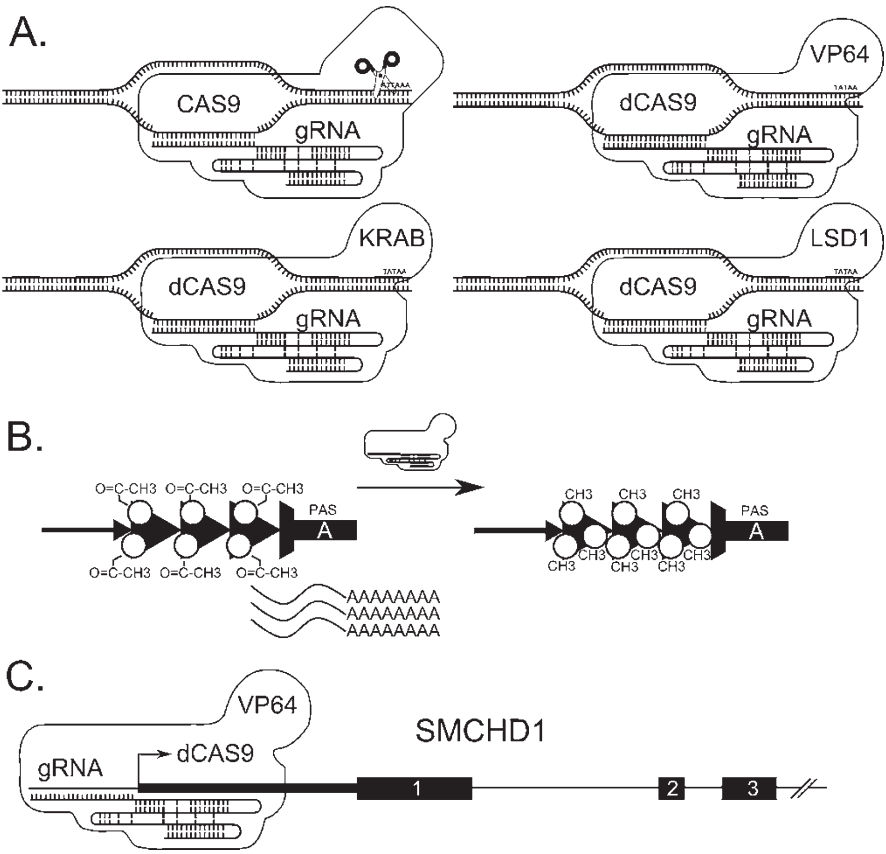

Fig. 29.2 CRISPR/Cas9 approaches to FSHD therapies. (**a**) Diagram of various CRISPR/Cas9 and dCas9 constructs designed to alter transcriptional activity of DUX4. VP64, herpes simplex virus transcriptional activation domain. KRAB, *Kruppel*-associated box transcriptional silencing domain. LSD1, lysine-specific demethylase 1 domain. (**b**) Diagram of a short permissive D4Z4 array with 3 units. Small circles are used to depict histone complexes and their associated chemical modifications (acetyl O=C-CH3) and methyl (CH3). *PAS* polyadenylation signal. A stands for an A-type haplotype that generally indicates a permissive allele containing a polyadenylation signal when located on chromosome 4. (**c**) Diagram of the SMCHD1 promoter region and first three exons of the gene. Also shown is the association of a guide RNA (gRNA)-directed dCas9-VP64 fusion protein used to activate transcription of the SMCHD1 gene

may be a more effective therapy than promoting the destruction of DUX4-coding mRNAs. Transcriptional inhibition has the added benefit of reducing the possibility that transcripts that escape destruction are translated and contribute to the production of DUX4.

29.3.3.1 Inhibition of DUX4 Transcriptional Initiation

Genetic approaches for transcriptional repression have not been investigated as thoroughly as approaches for transcriptional activation largely because recessively inherited disease has been the predominant focus of gene therapy over the past 20–30 years. Recently, an increased understanding of cancer genetics and the role of epigenetics in the propagation of cancer have attracted the attention of scientists in other fields [45]. Targeted transcriptional inhibition could involve disruption of the formation of the transcription initiation complex within the D4Z4 promoter by mutation of enhancer binding sites [46] or core transcriptional initiation sequences such as the TATA box (TACAA at the DUX4 promoter). Although it is clear that DUX4 production requires exon 3 from the most terminal portion of the D4Z4 array, it is not clear whether DUX4 transcription can be initiated at multiple upstream D4Z4 units and spliced into the single distal exon 3 sequence for termination. Thus, inhibition of transcription initiation may be difficult both due to multiple initiation sites within D4Z4 arrays and the lack of sequence specificity in promoters associated with permissive shortened D4Z4 arrays. Tools for this approach might include the CRISPR/Cas9 mutagenic strategies described in Sect. 29.3.2 or steric interference of transcription by designing proteins that specifically bind and block the assembly of the transcription initiation complex.

29.3.3.2 siRNA Expression to Induce Locus-Specific Epigenetic Changes

Production of noncoding RNAs from D4Z4 that direct the assembly of inhibitory chromatin is believed to be central to DUX4 transcriptional repression [31, 47]. Both long [48] and short [31, 61] noncoding RNAs have been identified, and transfection of several sequences results in transcriptional repression of DUX4. Production of shRNAs in muscle fibers in an attempt to force the chromatin structure of shortened D4Z4 arrays into an inhibitory conformation could be a viable strategy for DUX4 transcriptional repression [47] (Fig. 29.2b). AAV vectors have been shown to be effective for delivery of shRNA expression cassettes to mature muscle fibers in mice and large animals [30, 49].

29.3.3.3 dCas9-Targeted Transcriptional Silencing

A transcriptional silencing approach gaining traction involves the use of a mutated form of Cas9 (dCas9) that allows association of cleavage-deficient Cas9 with specific gRNA-directed sequence targets. Fusion of dCas9 with protein domains that facilitate transcriptional repression such as the Kruppel-associated box (KRAB) domain allows targeted transcriptional repression at specific promoters (Fig. 29.2a). The KRAB domain generally works by recruitment of other corepressors and direct inhibition of RNA polymerase so it would appear that these complexes must remain associated with D4Z4 to maintain suppression [50–52].

29.3.3.4 dCas9-Targeted Histone Modifications

Association of chromatin-modifying domains such as LSD1 with dCas9 results in the removal of H3K4me2 histone modifications resulting in reduced transcription and chromatin changes that may persist after the complex has disassociated from the promoter [53]. Persistent expression of these constructs in myofibers may result in successive silencing of active loci enhancing the efficiency of the approach. Small versions of Cas9 isolated from *S. aureus* (SaCas9) [54] or *C. jejuni* (CjCas9) [55] can be used so that these constructs can be expressed from AAV vectors again allowing efficient and somewhat targeted delivery to adult muscles.

29.3.4 Alteration of SMCHD1 Expression Levels

An alternative approach to specific chromatin modification at D4Z4 is to alter SMCHD1 expression. FSHD2-causing SMCHD1 mutations result in haploinsufficiency of SMCHD1, and reduced SMCHD1 levels prevent efficient chromatin-mediated repression of intermediate length (~11 to 30 units) D4Z4 arrays (Fig. 29.1a). Furthermore, rare individuals with both a permissive short D4Z4 array (<11 units) and SMCHD1 haploinsufficiency have more severe phenotypes than D4Z4 genetic alteration alone indicating that SMCHD1 levels affect disease severity in individuals with a type I mechanism as well. It stands to reason that increasing SMCHD1 levels in the setting of FSHD1 may also ameliorate the disease and produce a milder phenotype.

29.3.4.1 Augmented Transcription of SMCHD1

SMCHD1 is a large protein with a cDNA of 8672 bps precluding it from efficient expression from most retroviral vector systems and putting it well beyond the 4.5 kb packaging capacity of AAV. Although retroviral vectors based on both spumaviridae [56] and lentiviridae can package RNAs larger than 8.6 kb, the production of high-titer vector preparations for efficient in vivo gene therapy is perhaps overly optimistic, and efficient in vivo transduction of muscle fibers has not been achieved with retroviral vectors. Therefore, SMCHD1-mediated gene therapies for FSHD need to be realized by augmented transcription of the endogenous sequence or stabilization of the SMCHD1 mRNA or protein. Additional precautions need to be taken with this approach because increased expression of SMCHD1 in myoblasts inhibits their fusion and differentiation in culture (Miller DG, unpublished results) so modeling this approach in the context of muscle repair and/or developmental muscle growth will be important prior to testing in humans.

29.3.4.2 dCas9-Targeted Transcriptional Induction of SMCHD1

Targeted delivery of transcriptional enhancers could result in increased SMCHD1 transcription. dCas9 fusions to transcriptional enhancers such as VP64 have been shown to facilitate targeted activation of a number of genomic targets in cell culture [57] (Fig. 29.2b, c). As with transcriptional inhibition approaches, specific guide RNA-directed binding of a mutated Cas9 (dCas9) is utilized to deliver the activation domain of a transcriptional enhancer such as VP64. Using this approach transcriptional activation of eight different genes was shown with increases ranging from 2- to 250-fold [57]. Although the authors showed significant specificity of gene activation, VP64 is a strong viral transcriptional enhancer from herpes simplex virus, and unregulated delivery of such transcriptional activators should immediately raise concerns of off-target effects that have the potential for oncogenesis. Therefore, this approach should either incorporate a mechanism for deactivation of the dCas9-VP64 fusion or removal of cells containing the expression vector by use of "suicide genes" such as HSV-TK.

29.3.4.3 Targeted Stabilization of SMCHD1 mRNA

Protein activity within the cell is partly a function of the intracellular concentration of the protein. Protein concentration is dependent on the rate of transcription, the half-life of the mRNA, and the half-life of the protein. mRNA half-life is partly determined by sequences present in the 5′ and 3′ untranslated regions so these sequences could be changed to facilitate mRNA stability. The 3′ untranslated regions of many mRNAs contain destabilizing sequences that upon removal increase mRNA half-life. These can include AU-rich elements (AUREs), stem-loop secondary structures, intron response elements (IREs), and sequences that facilitate long range folding [58]. Removal (using CRISPR/Cas9 mutagenesis) or disruption of secondary structures could result in increased half-life and increased SMCHD1 expression. Alternatively, some mRNAs contain sequences that promote the binding of proteins or miRNAs that increase mRNA stability, and the incorporation of these sequences in the DNA sequence encoding the 3′ UTR of SMCHD1 using gene editing technologies could potentially have therapeutic effects for FSHD.

29.4 Summary

FSHD has an epigenetic mechanism where genetic mutations (D4Z4 array contraction or SMCHD1 mutations) result in a change in chromatin conformation at D4Z4 arrays on chromosome 4. These conformational changes ultimately result in inappropriate DUX4 expression in muscles when they occur on arrays with permissive haplotypes. The DUX4 transcription factor targets hundreds of genes in the human

genome but it remains unclear which pathways are responsible for the cell death that occurs ~24 h after DUX4 activation. The prevention of DUX4 transcription and the destruction of DUX4 mRNA once it is produced are primary targets for gene therapy approaches to FSHD.

Important considerations for FSHD gene therapy include the dominant pattern of inheritance, the fact that muscle fibers are syncytia containing multiple nuclei and observations that a single DUX4-expressing nucleus is sufficient to result in the death of a multinucleate myotube in cell culture. Thus gene therapy approaches that abrogate the effects of DUX4 expression must either act on cytoplasmic targets obviating the need to alter every nucleus within a myofiber or have an extremely high efficiency. Despite the epigenetic changes resulting from inherited or spontaneous mutations, most myonuclei do not express DUX4, and FSHD appears to progress due to stochastic expression of DUX4 in myonuclei throughout the lifetime of the individual. An attractive gene therapy approach is to reduce the likelihood that a nucleus will begin expressing DUX4 and reduce the odds that DUX4 will ever be expressed in any particular fiber. This logic would suggest that it may be possible to modify a fraction of nuclei and produce a change in the FSHD phenotype. One study compared percent mosaicism in blood lymphocytes and age of onset of FSHD symptoms and found an inverse correlation [59]. A careful comparison of D4Z4 array length mosaicism in the muscle with clinical severity might be more informative and could shed light on thresholds for phenotypic change that will be necessary to achieve success with gene therapy.

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Chapter 30
Gene Therapy and Gene Editing for Myotonic Dystrophy

Marinee Chuah, Yoke Chin Chai, Sumitava Dastidar, and Thierry VandenDriessche

Abstract Myotonic dystrophy is one of the most common dominant neuromuscular disorders that results in muscle dysfunction. Myotonic dystrophy type 1 (DM1) or Steinert's disease is caused by an expanded CTG repeats in the 3′ untranslated region of the *dystrophia myotonica protein kinase* (*DMPK*) gene, whereas myotonic dystrophy type (DM2) is caused by expanded CCTG repeats in the first intron of the *CCHC-type zinc finger*, *nucleic acid-binding protein* (*CNBP*) gene. The clinical manifestations worsen with each generation (anticipation) consistent with an expansion of the repeats. The tri- or tetranucleotide repeat expansion results in gain-of-function pathogenic RNAs which are retained in the nuclei and sequester RNA-binding proteins such as MBNL and CUGBP that interfere with splicing. Unfortunately, there is currently no cure available for these dominant neuromuscular diseases. Nevertheless, some promising therapeutic strategies have been developed that are aimed at directly tackling the genetic cause of the disease. In particular, antisense oligonucleotide technologies, gene therapy, and gene editing or small molecules are being explored. Recently, a phase 1/2a clinical trial has been completed that is based on the premise of promoting RNase-H-mediated degradation of the expanded CUG transcripts using antisense oligonucleotides. In this review, we summarize the current progress on different cellular and animal models as well as various therapeutic strategies for DM with specific emphasis on gene therapy and gene editing approaches using TALENs and CRISPR/Cas9. Lastly, translational challenges and future promising therapeutic avenues are discussed.

M. Chuah (✉) · T. VandenDriessche (✉)
Department of Gene Therapy & Regenerative Medicine, Faculty of Medicine & Pharmacy, Free University of Brussels (VUB), Brussels, Belgium

Center for Molecular & Vascular Biology, Department of Cardiovascular Sciences, University of Leuven, Leuven, Belgium
e-mail: marinee.chuah@vub.be; thierry.vandendriessche@vub.be

Y. C. Chai · S. Dastidar
Department of Gene Therapy & Regenerative Medicine, Faculty of Medicine & Pharmacy, Free University of Brussels (VUB), Brussels, Belgium
e-mail: yoke.chin.chai@vub.be; Sumitava.Dastidar@vub.be

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30.1 Prevalence and Pathogenesis of Myotonic Dystrophy (DM)

Myotonic dystrophy (DM) is an autosomal dominant muscle disorder caused by mutations in two different genomic loci, which result in two forms of genetically distinct diseases—DM type 1 (DM1) and type 2 (DM2). It is one of the most common neuromuscular disorders with a prevalence ranges from 5 to 10 patients per 100,000 individuals [1, 2]. In some restricted areas, such as the Lac-St-Jean region (Quebec, Canada), a higher prevalence (up to 1 per 600 individuals) has been reported due to a founder effect [3]. At the genetic level, DM1 (also known as Steinert's disease, MIM #160900) is caused by an unstable expanded trinucleotide CTG repeats in the 3′ untranslated region (UTR) of the *dystrophia myotonica protein kinase* (*DMPK*) gene on chromosome 19q13.3 [4–6]. The number of CTG repeats expanded between 80 and several thousand units in DM1 patients [2, 4], in contrast to only between 5 and 37 units in healthy individuals. The expanded CTG repeats are pathogenic, and its length increases during the patient's lifetime with intra- or intertissue variability [7]. Additionally, the repeat size also increases with successive generations, which ultimately gives rise to severe disease phenotypes, a phenomenon defined as anticipation. In particular, the severe congenital form of DM1 is associated with large CTG expansions (>1000 triplet repeats) [8], though other epigenetic mechanisms also play a role [9]. This represents a unique characteristic which provides a molecular basis for the pathogenesis of DM1 [10, 11]. The pathogenesis of DM2 (also referred as proximal myotonic myopathy, PROMM, MIM #602668) is different from DM1. It is caused by the expansion of extremely unstable tetranucleotide CCTG repeats in intron 1 of the *CCHC-type zinc finger, nucleic acid-binding protein* (*CNBP*; also known as *ZNF9*) gene on chromosome 3q21 [12]. The CCTG repeat size ranges from 75 to 11,000 units in patients as compared to <30 units in healthy individuals [12, 13]. Studies in nonhuman primates suggest that this unprecedented large variability of CCTG repeats expansion originates from an AluSx element insertion into an early primate genome [14] and is responsible for variable clinical manifestations. This complicates disease diagnosis. Interestingly, by using high-resolution nuclear magnetic resonance (NMR) spectroscopy, a recent study revealed a mini-dumbbell structure potentially formed by two CCTG repeats at single or multiple sites of the expanded CCTG repeats during DNA replication. This facilitates the occurrence of large CCTG repeats expansion in DM2 [15]. Although DM2 patients represent <10% of DM patients overall, it was recently found that DM2 exhibits similar or higher prevalence than DM1 in some countries, such as in Finland and Germany, due to undiagnosed patients with symptoms frequently occurring in the elderly population [16].

Clinically, DM1 and DM2 are multisystem disorders characterized by myotonia, progressive muscle weakness and wasting, heart conduction defects, cognitive impairments, endocrine disorders, insulin resistance, cataracts, and gastrointestinal manifestations [17, 18]. In men, there may be early balding and risk of sterility. Albeit bearing distinctive genetic mutations, DM1 and DM2 patients share many of these clinical symptoms. Nevertheless, the symptoms are usually more severe in DM1 than in DM2 [2]. Depending on the severity of the symptoms, the patient's life expectancy is reduced correspondingly, especially when confronting irreversible respiratory and/or cardiac failures [17]. Despite these similarities, the two DM forms are not identical with regard to the affected muscle types, disease onset [19], and the type of muscle fiber atrophy [20]. Additionally, the pathogenic mechanism for muscle atrophy in DM1 is triggered by the large CTG repeats via the p16 stress pathway (but not in DM2 [21]). This accounts for the premature senescence or lower proliferation capacity of the satellite cell-derived myoblasts [22]. DM1 patients are categorized into late-onset, adult-onset, juvenile-onset, childhood-onset, and congenital subgroups based on the age of onset and clinical severity [2, 23], whereas the clinical subgroups of DM2 are less well defined. The congenital form of DM1 is more severe and associated with fetal hypotonia, sleep disorders, and respiratory distress at birth, whereas no congenital form is associated with DM2. However, the surviving children with congenital DM1 present with motor and mental retardation. Recently, this motor and mental retardation was revealed to be associated with a reduced connectivity in a large brain network and the loss of compensatory mechanisms that correlated with isolated impairment in visuospatial reasoning in patients' daily life [24]. Furthermore, an in vitro study using pluripotent human embryonic-derived neuronal stem cells model revealed a reduced proliferative capacity, and an increase in autophagy linked to mTOR signaling pathway alterations further corroborated a mechanism by which DM1 mutation leads to cognitive impairments [25]. Unfortunately, clinical management in congenital and childhood DM1 remains supportive and requires regular surveillance and treatment of the disease manifestations, as well as genetic counseling, family planning, and undertaking by the affected family members [26].

At the molecular level, the pathogenic expanded repeats harbored in both *DMPK* and *CNBP* genes are transcribed, resulting in mutant RNAs which are retained in the nuclei as discrete ribonuclear foci/aggregates [12, 27, 28]. Both DM1 and DM2 share a common molecular mechanism of toxic RNA gain-of-function due to a similar characteristic between the expanded CUG- and CCUG-transcripts (C/CUG^{exp}-RNA) (Fig. 30.1). Early studies reported that the expression of ribonuclear foci was the key determinant for the development of DM1 phenotypes in the transgenic *HSA* mice (bearing either 220 or 300 CTG repeats in the 3′ UTR of *human skeletal muscle alpha-actin* (*HSA*) or the human *DMPK* gene, respectively) [29, 30]. Later on, members of the muscleblind-like (MBNL) protein family (including MBNL1, MBNL2, and MBNL3) were found to have high binding affinity to the CUG expanded repeats. This binding results in its sequestration in the C/CUG^{exp}-RNA nuclear aggregates which in turn causes functional loss of the MBNL proteins [31–34]. This is corroborated by the appearance of several cardinal features of DM in

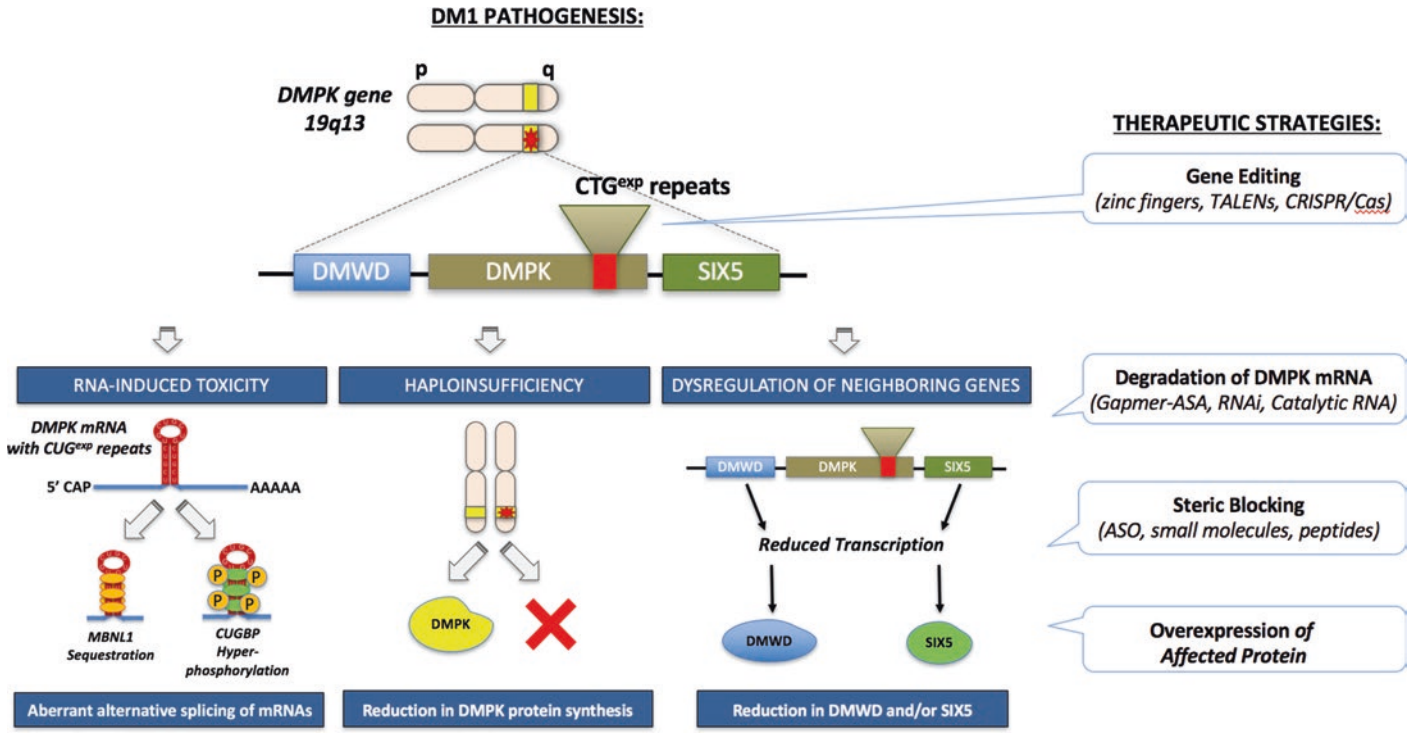

Fig. 30.1 DM1 pathogenesis and potential therapeutic strategies. The unstable CTG^{exp} repeat region gives rise to toxic expanded RNAs (CUG^{exp}). The expanded CUG^{exp} repeats form a stable hairpin structure that sequesters RNA-binding proteins such as MBNL1 and causes the stabilization of CUGBP via hyperphosphorylation. Therefore, the functional loss of MBNL1 and increase of available CUGBP cause imbalances in cellular mechanisms and pathways. The unstable CTG^{exp} repeats also result in a reduction of the DMPK protein synthesis (due to haploinsufficiency) as well as a reduction in expression of the neighboring DMWD and/or SIX5 genes, leading to DM1 phenotypes. Potential therapeutic strategies currently involved are listed on the right

studies involving mice models carrying *Mbnl* deficiencies such as myotonia (in *Mbnl1*$^{-/-}$ mice) [35], cognitive impairment (in *Mbnl*2$^{-/-}$ mice) [36, 37], and heart and muscle defects (in *Mbnl1:Mbnl2* double knockout mice) [38]. Indeed, MBNL proteins play many crucial roles in RNA homeostasis during fetal and postnatal development, in particular on the regulation of alternative splicing during gene expression [39, 40]. Consequently, the loss of functional MBNL in adult tissues causes deregulation of alternative splicing or missplicing of a subset of pre-mRNAs and therefore leads to re-expression of a fetal splicing pattern of specific transcripts [41, 42]. Interestingly, knockdown of MBNL proteins greatly reduced ribonuclear foci in DM1 fibroblasts [43], suggesting a role of MBNL proteins in ribonuclear foci formation. In fact, nuclear retention of the C/CUG^{exp}-RNA nuclear aggregates was promoted by the MBNL1, which subsequently resulted in repression of aberrant protein expression from the expanded repeats [44]. Recently, Gourdon et al. reported the detection of abundant ribonuclear foci formed by the sense DMPK RNA in human DM1 fetal tissues, which was clearly co-localized with MBNL1 and MBNL2 proteins. Additionally, they also found that DMPK sense and antisense transcripts were expressed in the heart, muscle, and brain of the transgenic mice from embryonic to fetal stages. These findings suggested that the congenital form of DM1 (the most severe form of the disease) and postnatal DM1 share a common pathogenic mechanism including the formation of toxic nuclear foci at the early stages during development [45]. Moreover, the mutated DMPK gene may also affect other vital developmental pathways associated with DM1 pathogenesis. For instance, the wild-type DMPK is required by the serum response factor *(SRF)* to co-regulate normal skeletal and cardiac tissue growth and maturation [46], and to maintain smooth muscle contractility (manifested as DM1-associated gastrointestinal hypomotility) [47]. Furthermore, hypermethylation of a differentially methylated region (DMR) due to CTG expansion in DMPK was uncovered recently to be linked to DM1 pathogenesis by causing haplo-insufficiency of a neighboring gene SIX5 in DM1-derived human embryonic stem cell lines. In fact, depletion or deletion of SIX5 in mice has been reported to directly contribute to premature cataracts [48, 49], cardiac conduction defects, and testicular atrophy [50, 51]. Meanwhile, DM1- and DM2-associated cataracts were also reported to be attributed to the activation of the innate immune response and interferon signaling by the accumulated double-stranded RNA in DM cells, indicating a complex mechanism of DM pathogenesis [52].

DM is also considered a spliceopathy as missplicing events have been found in affected tissues of DM1 patients [53]. Indeed, the loss of functional MBNL1 alone was reported to be associated with more than 40 splicing changes in affected muscles of DM1 patients, and some of them were found to be associated with DM symptoms. For instance, myotonia was associated with the missplicing of chloride voltage-gated channel 1 (*CLCN1*) pre-mRNA [54, 55], muscle weakness with bridging integrator 1 (*BIN1*) and a voltage-dependent calcium channel (*Cav1.1*) pre-mRNAs [56, 57], muscle fiber disorganization with *DMD* exon 78 [58], insulin resistance with insulin receptor (*INSR*) pre-mRNA [59], and brain lesions with microtubule-associated *Tau* isoforms aggregation [60]. Additionally, sequestra-

tion of MBNL also has a significant impact on other biochemical and physiological functions of these RNA-binding proteins including mRNA stability, transportation, and localization, as well as miRNA processing [61]. Moreover, the expression of CUG^{exp}-RNA deregulates the activity of another RNA-binding protein, CELF1 (*CUGBP*, *Elav-like family member 1*, also known as CUGBP1). Hyperphosphorylation of CELF1 through an inappropriate activation of the protein kinase C (PKC) pathway in DM1 cells has been reported to stabilize CELF, which in turn results in alternative splicing deregulation and translation defects [62, 63]. Furthermore, alterations of additional disease modifiers, such as p68, multifunctional RNA-binding protein Staufen1 (Stau1), NKX2.5, SMART/HDAC1-associated repressor protein (SHARP), and MEF2c, have also been linked to DM1 pathogenesis. Several studies have been undertaken to reveal their contribution to the progressive degenerative process of DM1 [64–68]. It has been recently demonstrated that Stau1 may have a broad impact as a splicing regulator in DM1 myoblasts. In this study, Stau1 rescued the alternative splicing profile of *INSR* and *CLC1* pre-mRNAs but also shifted the splicing patterns away from wild-type conditions when overexpressed [69]. All of these findings suggested that besides modifying the alternative splicing of a subset of pre-mRNAs, the expression of CUG^{exp}-RNAs also has a profound negative impact on other mechanisms that leads to adverse changes at transcriptional, posttranscriptional, and translational levels [70, 71]. For DM2 pathogenesis, a genome-wide analysis on a small cohort of DM2 patients resulted in the identification of a panel of alternative spliced exons in 218 genes. These genes were associated with the deregulation of development, cell survival, metabolism, calcium signaling, and contractility through numerous pathways and networks important for muscle physiopathology. These events suggested potential roles of the identified spliced variants in DM2 pathogenesis [72]. Nevertheless, by performing exon-array profiling and RT-PCR validation on DM patients as compared to other neuromuscular disorder patients (including Duchenne, Becker, and tibial muscular dystrophy), Bachinski et al. concluded that there was no evidence on qualitative splicing differences between DM1 and DM2 [73].

Though some of the complications of the disease (e.g., cataracts) can be treated, there is unfortunately no cure for DM1 or DM2. Therefore, developing therapeutic approaches that target the primary cause of the disease should theoretically provide better treatment outcomes and—by default—normalize all of the aforementioned secondary consequences of the disease. Current therapeutic approaches focus primarily at the RNA and protein levels and have moved beyond the initial proof-of-concept stage into translational studies and even a phase 1/2 clinical trial. Correcting the cause of the disease at the DNA level is now emerging due to breakthrough developments of potential novel genome engineering tools, such as single-stranded oligonucleotides and zinc-finger nucleases [74–76], TALENs [77–79], and CRISPR/Cas9 [80, 81]. In the following sections, we will discuss the various state-of-the-art preclinical and clinical therapeutic approaches, as well as various in vitro cellular and clinically relevant animal models to validate and consolidate an effective therapeutic platform for DM1 or DM2.

30.2 Cellular and Animal Models for DM Therapies

In vitro and in vivo DM1 and DM2 models are not only useful for deciphering the underlying molecular pathophysiological mechanisms, but they are instrumental toward validating safe and effective therapeutic strategies. DM1 primary cells [59, 82], immortalized muscle cell lines [64, 83, 84], and embryonic stem (ES) cell lines have been described. Several cell lines have been developed containing DMPK alleles with varying lengths of CTG repeats and/or exhibit different molecular changes [9, 67, 85–87]. Immortalized human DM2 myoblast cell lines were compared with immortalized human DM1 myoblast cell lines [88], revealing differences in fusion capacity. Upon myogenic differentiation, alternative splicing defects were observed in differentiated DM1 which were absent in the DM2 muscle cell lines. The emergence of inducible pluripotent stem cell (iPS) technology has also provided unprecedented opportunities to unravel the pathogenic mechanism of DM1 and DM2 in vitro. We and others have generated patient-specific iPS, myogenic progenitor cells, or differentiated myotubes. Through the generation of iPS cell lines from DM1-patient fibroblasts, a correlation between the repeat lengths and cellular expansion rate was apparent [89].

Several complementary transgenic DM1 mouse models have been generated that have been described in detail elsewhere [90]. A few of the key models will be outlined here. The DM300/SXL mouse model was generated through the insertion of 45 kb of the DM1 locus in order to express the human *DMPK* gene with 300 CTG repeats [30]. These mice display DM1-associated phenotypes, including high mortality, growth retardation, muscle defects, and cognitive impairments [85, 91, 92]. Interestingly, the length of the CTG can be expanded to more than 1500 repeats in DMSXL mice due to intergenerational instability [93]. It is noteworthy that the promoter of the human *DMPK* transgene has an almost ubiquitous expression and similar expression pattern to that of the murine *Dmpk* gene. However, the level of expression of human *DMPK* is low in skeletal muscles and high in the brain as compared to murine *Dmpk* in adult DMSXL mice. Nonetheless, nuclear CUG^{exp}-RNA aggregates are detected in several tissues including the muscle and brain. The human skeletal alpha-actin long repeat (HSA-LR) mouse model bears a 220 CTG repeat in a non-*DMPK* gene (i.e., 3′ UTR of the *human skeletal alpha-actin* (*HSA*) gene), in which its expression is restricted to skeletal muscles only [29]. As a functional outcome, the HSA-LR mice display numerous CUG^{exp}-RNA nuclear aggregates, robust DM-specific molecular defects (e.g., alternative splicing deregulation), and myotonia. Lastly, the EpA960 mouse model is an inducible and tissue-specific transgenic mouse model that expresses 960 interrupted CTG repeats within the *DMPK* 3′ UTR [94]. These interrupted CTG repeats are expressed highly in both cardiac and skeletal muscle which causes a number of molecular changes including splicing deregulation and CELF1 upregulation. Consequently, this leads to severe cardiac phenotypes and muscle wasting within a few weeks [63, 94].

DM2-HSAtg mice expressing an intronic $(CCTG)_{121}$ expansion were generated based on analogy to HAS-LR mice [95] (R. Krahe, unpublished data). This DM2-HSAtg mice exhibited ribonuclear inclusions, *CELF1* upregulation, and DM2 muscle pathogenic phenotypes but no missplicing. Alternatively, DM2 mouse models were developed based on the inactivation of the alternative *CNBP/ZNF9* pathway. *Zfn9*$^{+/-}$ mice displayed myotonia, muscle wasting, cardiac conduction defect, and cataracts but without repeat expansion and missplicing [96]. However, the clinical relevance of the Cnbp-deficient mice model was challenged by contradictory studies reporting on the effects of CCTG expansion on CNBP/ZNF9 expression in DM2 patients [97, 98]. The development of improved transgenic DM2 animal models that replicate most if not all of the known pathophysiology of the cognate human disorder may foster the development and preclinical validation of new therapeutic approaches.

30.3 Therapeutic Approaches for DM1 and DM2

Several therapeutic approaches have been developed to rectify the toxic RNA gain-of-function mechanism of DM1. These strategies include the use of antisense synthetic oligonucleotides (ASO), the use of pharmacologic compounds such as small molecules or peptides, and the use of gene therapy or gene editing approaches. This could be accomplished either by degradation of the CUG^{exp}-RNA or steric blocking to unfold the secondary structure of the CUG^{exp}-RNA. Alternatively, the undesirable binding of MBLN proteins to the CUG^{exp} was inhibited. Finally, editing the pathogenic alleles at the DNA level itself by genome engineering is also being considered. In the following section, we summarize the different therapeutic approaches in respect to their molecular targets, therapeutic efficacies, and their modes of administration.

30.3.1 Degradation of the Pathogenic RNA Expanded Transcripts

30.3.1.1 Gapmer-Antisense Oligonucleotides (Gapmer-ASOs)

The working principle of antisense oligonucleotides (ASOs) [99] is based on its specific binding to targeted RNA through complementary nucleotide base pairing. Several chemical modifications have been developed to improve their RNA/DNA binding properties and their tissue penetration while reducing their sensitivity to nucleases [100]. In fact, the use of gapmer-ASOs has recently been proved to have strong therapeutic potential by exploiting an RNase-H mechanism that activates degradation of the cognate target RNA transcripts. In general, gapmer-ASOs are made up of 8–10 DNA nucleotides surrounded by chemically modified nucleotides at both

5′ and 3′ ends. In the formed ASO/RNA heteroduplex, the presence of non-modified nucleotides represents a "gap" which allows the RNAse-H1 to recognize and subsequently degrade the DNA/RNA duplex. This molecular recognition results in cleavage of the target RNA counterpart followed by exonuclease degradation of the RNA fragments. CAG-gapmer-ASOs have been specifically designed to target CUG repeats in the DM1 CUG^{exp}-RNA. The CAG-gapmer-ASOs have been modified with locked nucleic acid (LNA) or 2′-*O*-methoxyethyl (2These CAG-gapmer-ASOs triggereMOE). These CAG-gapmer-ASOs triggered efficient RNase-H-mediated degradation of the CUG^{exp}-RNA in cellular models of DM1 [38]. It is particularly encouraging that intramuscular delivery of these ASOs leads to 50% reduction of CUG^{exp}-RNA levels in the EpA960 mouse model, consistent with a reduction in ribonuclear foci and a partial rescue of splicing defects. In parallel, systemic administration of MOE gapmer-ASOs targeting CUG^{exp}-independent sequences within the mutant transcript has also been demonstrated to lead to an efficient degradation of CUG^{exp}-RNAs. Consequently, relatively robust correction of both molecular and functional phenotypes in HSA-LR mice could be achieved [101]. Furthermore, 2′,4′-constrained ethyl (cEt)-modified ASOs were developed by Seth et al., and they reported a significantly enhanced in vivo potency of cEt-modified ASOs as compared to MOE gapmer-ASOs with a favorable safety profile [102, 103]. Interestingly, by systemic administration, this group demonstrated that cEt-modified ASOs showed potent activity against DMPK that was specific to the skeletal and cardiac muscle in wild-type mice, human DMPK transgenic mice, and nonhuman primates [104]. Recently, short synthetic ASO composed exclusively of 8–10 subunits of LNA (designated as all-LNAs) was developed and found to bind efficiently to long CUG^{exp} of the mutated *DMPK* transcripts in cells derived from DM1 patients and in skeletal muscle of DM1 mouse model [105]. As a result, ribonuclei foci formation was significantly reduced along with a correction of the abnormal alternative splicing of MBNL exons due to prevention of MBNLs sequestration by the all-LNAs. Additionally, all-LNAs exhibited high molecular stability, a relatively low immunogenicity, and relatively long-acting activity (that lasted a minimum of 14 days in HSA-LR mice), at least when administered at relatively high doses intramuscularly. Therefore, developing ASO-based drugs that are highly complementary to the pathogenic CUG^{exp} repeats may represent a promising strategy to treat DM1. Interestingly, some studies show that the beneficial effects of gapmer-ASOs in postmitotic skeletal muscle tissue were sustained for a relatively long period of time (up to 1 year) after treatment was discontinued.

30.3.1.2 Antisense RNA

In the field of gene therapy, antisense RNA (asRNA) has generally been adopted [106], as a means to "silence" pathogenic gene expression in order to alleviate the disease symptoms. In the case of DM1, asRNA has been designed to bind to a complementary sequence upstream of the expanded repeats in the 3′ UTR of

the *DMPK* transcript. This strategy was shown to significantly reduce the level of *DMPK* mRNA, consequently restoring some cellular functions in DM1 muscle cells [107]. Nevertheless, a 50% reduction of wild-type *DMPK* mRNAs was also observed since the asRNA recognized a nucleotide sequence that was common between the pathogenic and wild-type *DMPK mRNA*. To overcome the lack of specificity of this asRNA-based approach on silencing pathogenic *DMPK* transcripts, Francois and colleagues developed a modified human U7 small nuclear RNA (hU7-snRNA) that could selectively silence only the mutated DMPK mRNAs in DM1 cells [108]. Specifically, this modified hU7-snRNA was conjugated to a (CAG)n antisense sequence and packaged into lentiviral vectors. Upon delivery, the constructs enabled nuclear localization of the antisense sequence, subsequently leading to specific targeting of the expanded CUG repeat tract. Remarkably, this strategy resulted in specific degradation of CUG^{exp}-RNAs with almost no effect on the wild-type *DMPK* mRNAs in DM1 muscle cells. The precise mechanism of this silencing is not fully understood. Nevertheless, it is particularly encouraging that this silencing has enabled correction of some typical DM1 features, consistent with the reversion of splicing misregulation, disappearance of ribonuclei foci, and even an improvement in myogenic differentiation of the diseased DM1 muscle cells. These in vitro studies provided strong scientific evidence on the feasibility of developing antisense-based gene therapy approaches for DM1. It is tempting to speculate that the incorporation of this CUG^{exp}-RNA-targeted hU7-snRNA expression cassette in AAV vector could allow for sustained expression, body-wide transduction of skeletal muscle, and possibly even cardiac tissue, in DM1. This could obviate some of the limitations of ASOs and other strategies that have only transient effects and do not effectively reach the afflicted muscle with comparable high efficiency as in the case of AAV. This would still need to be validated in preclinical models.

30.3.1.3 RNA Interference

RNA interference (RNAi) has been exploited to silence nuclear-retained *DMPK* transcripts in DM1. Lentiviral vectors have been designed that expressed short hairpin RNA (shRNA) complementary to *DMPK* mRNA sequences (either in the coding or 3′ UTR region but excluding the CUG repeats) [109]. This resulted in a significant reduction of the intended target, namely, the nuclear-retained pathogenic CUG^{exp} transcripts in DM1 cells. However, the normal cytoplasmic *DMPK* transcripts were also targeted since the design of the shRNA did not allow the pathogenic and wild-type mRNAs to be discriminated. An alternative strategy based on small interfering RNA (siRNA) oligonucleotides was also explored to degrade nuclear CUG^{exp}-RNA transcripts through RNA interference. These siRNA oligonucleotides were designed to specifically target CUG repeats. For instance, a recent study showed that introduction of CAG/CUG siRNA duplex in skeletal muscles of HSA-LR mice by in vivo electroporation resulted in a significant reduction of expanded CUG^{exp}-transcripts [110]. However, the specificity of these siRNA was

not restricted to the DMPK CUG^{exp} since other transcripts containing such CUG repeats were also targeted. Chamberlain and co-workers demonstrated recently that systemic delivery of miRNA-based RNAi using a muscle-tropic AAV6 vector could efficiently mitigate DM1 phenotypes of terminally differentiated myofibers in HAS-LR mice [111]. This efficient AAV-mediated delivery of RNAi may provide a long-term therapy for DM1.

30.3.1.4 Ribozymes

Ribozymes are catalytic RNAs that have been used as therapeutic agents to repair RNA transcripts or to degrade RNA by cleaving the RNA targets [112, 113]. For gene therapy of DM1, scientists have reported on the use of group I intron ribozyme to target and reduce CUG repeats in the 3′ UTR of human *DMPK* transcripts. Consequently, the 12 CUG repeats were replaced by 5 repeats, both in an in vitro assay and in cultured human fibroblasts [114]. In another study, a lentiviral vector expressing the nuclear-retained hammerhead ribozyme was used to induce endonucleolytic cleavage of the *DMPK* 3′ UTR sequence [115]. This resulted in a significant reduction of pathogenic DMPK CUG^{exp}-transcripts in DM1 myoblasts, consistent with a reduction of the number of CUG^{exp}-RNA nuclear foci and the partial restoration of alternative missplicing of insulin receptor (INSR) transcripts. However, the intrinsic design of the ribozyme did not allow discrimination between wild-type and pathogenic DMPK mRNA to the extent that the wild-type mRNA was also cleaved. Since ribozymes typically have a relatively short half-life, it will be important to design strategies to overcome this and/or to express higher levels. It will also be necessary to validate their therapeutic potential in vivo, for example, following de novo expression in the afflicted muscle using AAV vectors.

30.3.1.5 RNA Endonuclease

Artificial site-specific RNA endonuclease (ASRE) are customized synthetic endoribonucleases that demonstrated high specificity and efficiency in cleaving RNA targets [116]. They have also been engineered to treat DM1 by directing the endonuclease against the CUG^{exp} repeats in the DMPK RNA [117]. This DM1-ASRE contains a specific RNA recognition domain specific to the $(CUG)_8$ sequence and conjugated to an RNA endonuclease domain resulting in efficient cleavage of the CUG^{exp}-RNA. Expression of this ASRE in DM1 cells resulted in a reduction of the number of nuclear foci as well as the restoration of splicing profiles of the *bridging integrator 1* (*BIN1*), *cardiac troponin T* (*cTnT*), and *insulin receptor* (*INSR*) transcripts. Although ASRE-based strategies could provide an alternate gene therapy approach for DM1, their potential off-target effects would need to be carefully assessed and optimized in order to address some of the prevailing safety concerns.

30.3.2 Steric Blocking

30.3.2.1 ASOs

ASOs (typically sized between 15 and 30 nucleotides) have also been used to mask specific regulatory nucleotide sequences by modulating the splicing machinery and forcing inclusion or exclusion of a specific exon [118, 119]. These regulatory sequences typically include splice acceptor or donor sites, exonic splicing enhancers, and intronic splicing enhancers and allow for "exon skipping." Wheeler and colleagues reported on the restoration of the splicing profile of the *Clcn1* channel and subsequent reversion of myotonia by ASO-mediated exon skipping in an HSA-LR mice model [120]. ASOs can be rendered relatively nuclease-resistant in order to improve their efficacy. Such a CAG_{25} "morpholino" (PMO) has been designed to target the CUG^{exp} tract. In vivo electroporation into the muscle of HSA-LR mice resulted in a reversion of quintessential DM1-associated phenotypes, including splicing deregulation and myotonia [121]. Interestingly, these ASOs were specifically engineered to sterically block the MBNL1 factor from the pathogenic nuclear CUG^{exp}-RNA aggregates. Steric blockade of CUG^{exp} repeats by the CAG25-PMOs killed two birds with one stone: it could overcome the nuclear sequestration of MBLN1 restoring its intracellular distribution and functional activity but, at the same time, also decreased the pathogenic CUG^{exp}-RNA levels by increasing nuclear export and cytoplasmic degradation. Similarly, fully modified 2′*O*-Methyl (2′OMe) phosphorothioate (CAG)7-ASO (PS58) could degrade CUG^{exp}-RNAs in DM1 muscle cells [122]. In fact, 50–60% reduction of CUG^{exp}-RNA levels in muscles was obtained by this strategy when (CAG)7-ASO was injected intramuscularly in DM300 and HSA-LR mice. This finding suggested a partial normalization of alternative splicing defects by the (CAG)7-ASO. Subsequently, (CAG)n-ASO was further optimized in function of the molecular length, and the studies showed that CAG sequences shorter than 20 nucleotides were effective in correcting DM molecular defects in vitro and in vivo [84, 123]. In this respect, both the type of chemical modifications and the length of the ASO influenced efficacy.

Despite their promise, there are still a number of key issues that would need to be further optimized. It is ultimately not realistic to treat DM1 patients by local injection of ASOs. In addition, since the effect is expected to be transient, these injections would need to be repeated on a regular basis which complicates clinical translation. Moreover, in vivo biodistribution and cell/tissue penetration of ASOs are not optimal. This is compounded by the fact that morpholinos exhibit a relatively poor tissue penetration in DM1 mice [124]. Nevertheless, it is encouraging that tissue uptake of these ASOs upon systemic delivery by IV injection into the HSA-LR mice was greatly improved by coupling a cell-penetrating peptide (CPP) to the morpholino. Consequently, relatively efficient MBNL1 redistribution and correction of abnormal splicing were achieved in the skeletal muscle, even alleviating some of the quintessential disease manifestations like myotonia. However, comprehensive toxicity studies are required to further address the safety of this approach, particularly since some CPP-conjugated ASOs could potentially contribute to cellular toxicity [125].

30.3.2.2 Small Molecules/Peptides

The interaction between MBNL1 and the pathogenic CUG^{exp}-RNA is a crucial step in the pathophysiological process that contributes to DM1. Consequently, disrupting this interaction using small molecules or peptides may overturn some of the adverse effects of this interaction. Using comprehensive library screens, attempts were made to identify molecules that are either competitive antagonists of MBNL1 on the binding site of the C/CUG repeats [126–129], effective blockers that prevent binding of MBNL1 to C/CUG repeats [86, 130–134], specific effectors that perturb the formation of RNA foci in cellular models [83], or therapeutic agents that correct some of the specific deleterious phenotypes in animal models [135]. Though some potentially interesting leads were identified, it is beyond the scope of this gene therapy review to provide a comprehensive discussion of these more conventional pharmaceutical approaches. To our knowledge, none of these compounds have been tested in clinical trials yet, and it remains to be seen how their efficacy and safety profile compare to that of the most promising gene therapy approaches described herein.

30.3.3 MBLN1 Overexpression

A central mechanism in DM1 pathogenesis is the loss of function of the MBNL1 due to its sequestration to the CUG^{exp}-RNA complex in the cell nucleus. Several studies have demonstrated that MBNL1 loss-of-function accounted for more than 80% of the alternative splicing changes in the HSA-LR mouse model of DM1 [35, 42]. Consequently, overexpressing MBLN1 may potentially mitigate some of the pathophysiological consequences of the CUG^{exp}-RNA in DM1. This was supported by transgenic mouse studies, revealing that the DM1-associated phenotypes could be reversed after crossing HSA-LR mice with transgenic mice that were specifically designed to overexpress MBNL1 in the muscle [136]. This justified developing an AAV-based strategy to treat DM1 by overexpressing MBLN1 after gene therapy in DM1 mouse models. AAV vectors encoding MBLN1 were therefore injected intramuscularly in HSA-LR mice [137]. Though only a relatively modest twofold increase of MBNL1 protein level was apparent, it was associated with a reversion of some of the DM1 phenotypes in the injected target muscle. This would need to be corroborated by systemic AAV-MBLN1 administration in the hope to achieve efficient MBLN1 overexpression in the skeletal muscle and heart.

30.3.4 Gene Editing

Genome editing using TALENs, ZFNs, homing endonucleases, and CRISPR/Cas9 represents a powerful approach for the correction of various disease mutations [138]. This strategy is highly suitable for treating DM1 as removal of the CTG^{exp} repeat tract or insertion of regulatory sequence in the *DMPK* gene will abolish the

transcription of the pathogenic CTG^{exp}. Hence, all of the downstream pathogenic events associated with DM1 would be rectified. A recent study reported in vitro correction of DM1 phenotypes in neural stem cells (NSCs) generated from DM1-iPS cells via TALEN-mediated homologous recombination (HR) [79]. This is an interesting proof-of-concept study demonstrating the potential of TALENs to produce DM1-corrected progenitor cells. We have recently explored the use of CRISPR/Cas9 to completely excise the expanded trinucleotide CTG repeats (CTGexp) in the DMPK gene in order to generate DM1-corrected myogenic cells derived from DM1 patient-specific iPS cells [139]. Relatively robust phenotypic correction was observed after gene editing in the CRISPR/Cas9-corrected DM1-HIDEMs, consistent with the disappearance of ribonuclear foci, one of the hallmarks of DM1. This paralleled the reversion of the abnormal splicing patterns in DM1. Similarly, Wieringa and co-workers have shown that CRISPR/Cas9 enabled complete and precise removal of the CTGexp alleles in myoblasts from DM1 mice and patients. This resulted in normalization of the myogenic capacity, nucleocytoplasmic distribution, and abnormal RNP-binding behavior of transcripts from the edited DMPK gene [80].

Though the overall efficiency of gene editing is currently not as high as with more conventional gene addition strategies, incremental changes in technology may eventually bridge this gap. It will be important to conduct the necessary in vivo studies using these various gene editing platforms to establish safety and efficacy in the appropriate preclinical models described above. Finally, comprehensive genome-wide analysis of off-target effects would be required to formally rule out any off-target effects.

30.4 Translational Challenges and Concluding Remarks

Since the genetic etiology of DM1 and DM2 has been identified, a lot of progress has been made at attempting to block the pathogenic effects of the cognate mRNAs containing the nucleotide repeat expansions. Some of these emerging therapeutic approaches were validated in vitro in cellular models of DM1 or DM2, whereas others are already matured toward preclinical validation in in vivo models that mimic the cognate human disease. Though the available animal models are not perfect in the sense that they do not replicate all of the key pathogenic features of DM1 or DM2, they are complementary and provide useful tools to assess efficacy and safety of the different therapeutic modalities that are currently under development. It is particularly encouraging that the first safety clinical trials have started in DM1 patients with ASOs and that no adverse effects were reported. However, further studies in patients with DM1 (or DM2) are needed to convincingly demonstrate efficacy based on validated functional endpoints or surrogate markers. Nevertheless, several challenges would still need to be addressed. The consequences of modulating or inactivating the wild-type alleles are, at present, not fully understood. The selected therapeutic approaches should therefore ideally only target the pathogenic RNA. However, this is not always the case as the wild-type transcript can

sometimes also be targeted, due to the lack of specificity of the targeting modality. Similarly, gene editing approaches typically do not discriminate between the wild-type and pathogenic allele since the DNA sequences flanking the pathogenic repeat expansion are usually identical. Nevertheless, the presence of single nucleotide polymorphisms (SNPs) could potentially be exploited to discriminate between the two alleles and develop a more refined and specific targeting strategy. It is also important to ensure that only the desired locus is targeted, while off-target effects in other genomic loci should be minimized. The gene editing technology is evolving rapidly, and it is likely that the next-generation editing tools will become increasingly more specific. For instance, the latest-generation Cas9 proteins (e.g., HF-Cas9) already show reduced off-target effects, compared to the early-generation versions [140]. There are also some important challenges remaining regarding the efficiency of delivering the therapeutics to the desired target tissues since DM1 and DM2 are a multisystem disorder affecting many tissues and organs. This is compounded by the need to obtain sustained therapeutic effects while minimizing the risk for potential toxicities. Though oligonucleotide-based approaches will require repeated administration to sustain long-term benefits to the patient, AAV vector-based approaches will likely allow prolonged expression of the therapeutic gene, at least in the skeletal muscle. Though, multi-year expression has been achieved in different tissues after AAV-based gene therapy in clinical trials, it is currently not known if expression will be lifelong and if vector re-administration would be required. It is important to continue to explore this diverse array of gene therapy and gene editing strategies in parallel in preclinical studies and to move the most promising strategies forward into the clinic. This ultimately offers the best hope to those patients and their families that are blighted by these dominant genetic diseases.

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Chapter 31
Gene Therapy for Oculopharyngeal Muscular Dystrophy

Alberto Malerba, Fanny Roth, Vanessa Strings, Pradeep Harish, David Suhy, Capucine Trollet, and George Dickson

Abstract Oculopharyngeal muscular dystrophy (OPMD) is a rare, late-onset, autosomal-dominant disease affecting 1:100,000 individuals in Europe. OPMD is due to mutation in the N-terminal domain of exon 1 of the polyA-binding protein nuclear 1 (PABPN1). Patients with the disease express an expanded PABPN1 (expPABPN1) ranging from 11 to 18 alanines instead of the normal 10. OPMD is mainly characterized by ptosis and dysphagia, although muscles of the lower limbs can also be affected late in life. Currently, OPMD patients are referred to surgeons for a cricopharyngeal myotomy or corrective surgery to extraocular muscles to ease ptosis. Pharmacological treatments are not commercially available, but several compounds are in preclinical and clinical stages of development. A gene therapy approach designed to inhibit the expression of expPABPN1 is an appealing strategy. However, due to the type of mutation, genetic strategies to knock down expPABPN1 invariably affect the expression of wild-type PABPN1 with potential negative consequences for the treated muscles. We recently demonstrated that a dual gene therapy approach designed to inhibit mutant and wild-type endogenous PABPN1 by shRNA, in combination with expression of an RNAi-resistant sequence-optimized recombinant PABPN1 gene, substantially rescues the pathology in the A17 mouse model of OPMD. This is currently the only preclinical gene therapy study for OPMD. In this chapter, we describe this approach in a general context of other possible treatments

A. Malerba · P. Harish · G. Dickson (✉)
Centres of Gene and Cell Therapy and Biomedical Sciences, School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey, UK
e-mail: Alberto.Malerba@rhul.ac.uk; Pradeep.Harish@rhul.ac.uk; g.dickson@rhul.ac.uk

F. Roth · C. Trollet
Sorbonne Universités, UPMC Univ Paris 06, UM76, INSERM U974, Institut de Myologie, Paris, France
e-mail: f.roth@institut-myologie.org; capucine.trollet@upmc.fr

V. Strings · D. Suhy
Benitec Biopharma, Hayward, CA, USA
e-mail: VStrings@benitec.com; DSuhy@benitec.com

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for OPMD, and we discuss the likely future developments that may allow the translation of such an approach towards a therapeutic treatment for OPMD in humans.

Keywords Gene therapy · Oculopharyngeal muscular dystrophy · Intranuclear inclusions · PABPN1 · OPMD

31.1 Pathogenesis and Molecular Genetics

Oculopharyngeal muscular dystrophy (OPMD) (MIM #164300) is an autosomal-dominant inherited disease that is clinically characterized by ptosis of the eyelids and dysphagia. Furthermore, at later stages of the disease, proximal limb weakness may also occur. OPMD is a late-onset disease with symptoms usually appearing in the fifth decade of life. Since the description in 1966 in a cluster of patients from Quebec, many cases have been described. OPMD patients have now been found in more than 33 countries around the world [1–12]. In Europe, the estimated prevalence of disease is 1:100,000, but the largest OPMD clusters are in the French-Canadian population in the province of Quebec [13, 14], where the estimated prevalence is 1:1000 and amongst the Bukhara Jews in Israel where this disease affects 1:600 people [15]. The peculiar distribution is undoubtedly due to founder effects as highlighted by the initial work of the Canadian neurologist André Barbeau in 1966 [16].

The degenerative dystrophy and progressive onset of fibrosis in the pharyngeal muscles create difficulties for the patient in swallowing the food bolus. This issue, together with a decreased relaxation of the cricopharyngeal muscle (the main muscle of the upper oesophageal sphincter located between the pharynx and the oesophagus), can delay the transfer of the bolus through the upper oesophageal sphincter and possibly occlude the trachea resulting in increased risk of choking and pulmonary infection. Extraocular muscles may become gradually affected, but complete external ophthalmoplegia is rare [14]. Weakness and atrophy occur in limb-girdle muscles, with symmetric and non-selective muscle involvement. Because muscles are mainly affected in OPMD, the disease is considered a primary muscle disorder. However, it has been described that the nervous system might also be affected, for example, the peripheral nerves (such as pharyngeal nerves [17, 18]) or the central nervous system with correlated functional consequences [19, 20]. The presence of cognitive impairments in patients is still a largely unexplored aspect of the disease that should be more extensively studied in the future with specific tests as performed by Dubbioso et al. [19].

OPMD is usually transmitted as an autosomal-dominant trait with complete penetrance. In 1995, the locus was mapped to the chromosome 14q11.2–q13 containing the polyadenylate-binding protein nuclear 1 gene (*PABPN1*). Brais et al. [21] was the first to identify the molecular defects responsible for OPMD. The normal *PABPN1* gene (NG_008239) has a repeat of six trinucleotides (GCG) ((GCG)6) coding for an alanine stretch at the 5′end of exon 1. The existence of a (GCA)3 (GCG) coding sequence adjacent to the (GCG)6 repeat leads to a wild-type PABPN1 protein with a 10 alanine residue stretch, whereas the mutated *PABPN1* (called

Table 31.1 Allelic frequency of the expanded alleles of PABPN1 described in two studies [21, 23]

	Allelic frequency (%)	
	Brais et al. [21]	Richard et al. [23]
(GCN)11	N/A	9
(GCN)12	5	12
(GCN)13	40	48
(GCN)14	26	14.5
(GCN)15	21	15
(GCN)16	7	1
(GCN)17	1	0.5
(GCN)18	0	0

For clarity, only the frequencies of expanded alleles are presented

expanded PABPN1 or expPABPN1) has (GCN)11–18 repeats corresponding to a stretch of 11–18 alanine residues in the N-terminal domain [21–23] (Table 31.1). Since four different codons—GCA, GCT, GCC and GCG—encode the amino acid alanine, GCN, where N represents any A/C/G/T nucleotide, is a generic designation for these four possible codons. Homozygous patients are characterized by a more severe phenotype and earlier onset of symptoms (approximately 18 years earlier [24]). Furthermore, rare cases of recessive transmission have been described where patients have a homozygous mutation in both alleles due to one extra (GCN) triplet added to each allele (named (GCN)11). These patients have a milder phenotype compared to the other affected patients as well as a later onset of the disease.

Because of the rarity of the disease, it has until recently been difficult to sample enough OPMD patients to establish phenotype–genotype correlations, particularly for the 'rarest' genotypes including homozygotes and GCN11 subjects. Previous clinical studies in cohorts of 17–86 patients [12, 25–31] have suggested that the variability in the age of onset and severity of weakness may depend on the size of the (GCN)n mutations. This finding has recently been confirmed on a large French cohort of 354 OPMD patients: despite a high dispersion of age within each genotype (ranging from 1 to 11 years of deviation), the mean age at diagnosis and the severity of the clinical symptoms correlate to the number of (GCN) repeats [23].

31.2 Polyadenylate-Binding Protein Nuclear 1 (PABPN1) and Intranuclear Inclusions

PABPN1 encodes an ubiquitously expressed polyadenylation factor involved in many biological processes [32]. PABPN1 has firstly been described for its role in the stimulation of the polyA polymerase (PAP) and the control of the poly(A) tail length on RNA transcripts [33–36]. Over the last few decades, several other roles of the protein have been described including a role of the protein to regulate the use of alternative polyadenylation (APA) sites [37, 38], which in turn affects mRNA levels and stability. PABPN1 is also involved in the long non-coding RNA (lncRNA) [39]

and small nucleolar RNA (snoRNA) [40] processing and in the nuclear surveillance that leads to hyperadenylation and decay of RNA [41]. A role for PABPN1 in splicing regulation has also been demonstrated [42, 43]. While most of PABPN1 functions have been identified using induction of a loss of function of PABPN1, only few studies so far have analysed these functions in OPMD patients. Although the global analysis of the poly(A) tail length of RNA samples from OPMD patients did not reveal any significant changes [44], specific defects in poly(A) tail length regulation of nuclear-encoded mitochondrial mRNAs were found [45]. Given that poly(A) tails play an essential role in mRNA stability, these defects result in accelerated decay of these mRNAs. APA was found deregulated in an OPMD mouse model [37] but so far has not been confirmed in OPMD patients.

OPMD is also considered an aggregopathy as expPABPN1 is misfolded and prone to form nuclear insoluble aggregates, and intranuclear inclusions (INI) are the main histopathological hallmark of the disease [46, 47]. However, similar to other neurodegenerative diseases [48, 49], it is still unclear whether INIs have a pathologic function or a protective role in OPMD as a consequence of a cellular defence mechanism [50–52]. INIs contain several proteins including heat-shock proteins, splicing factors, poly(A) RNA and both normal and expanded PABPN1 [44, 53].

31.3 Experimental Approaches to Therapy

The monogenic nature of OPMD presents the disease as an ideal candidate for gene therapy-based strategies. However, other experimental approaches across the cell therapy, pharmacological and surgical spectra, have also been shown to reduce disease symptoms in clinical trials and animal models.

In the absence of a curative treatment, most surgical approaches have been palliative. Surgical techniques used to alleviate ptosis include blepharoplasty to remove excessive skin and the orbicularis muscle from upper eyelids, external advancement on the levator palpebrae superioris (wherein the muscle is tightened), the resection and tightening of the Muller's muscle (unaffected muscle controlled by the autonomic nervous system) or in severe cases a frontalis sling can be performed where the frontalis muscle is coupled directly to the eyelid, allowing opening the eyes by raising the forehead [54–57]. The disease pathophysiology results in persistent spasm/failure of relaxation of the cricopharyngeus muscle, which narrows the upper oesophageal pathway. The application of cricopharyngeal myotomy has been widely adopted as the primary procedure administered to dysphagic patients in the case of OPMD. However, the nature of the disease presents various complications: the late onset of the disease may render the patient either medically unsuitable or unwilling to undertake the procedure; furthermore, the high risk of symptom recurrence and the nature surgery can only be performed once presents no long-term solution to the symptoms. The recently reported cricopharyngeal dilatation is currently under investigation as a relatively non-invasive repeatable procedure to alleviate dysphagic symptoms [58].

Myoblast transplantation has been considered as an appealing therapeutic strategy for muscular dystrophies. Myoblasts are indeed well-characterized muscle progenitors, for which isolation and expansion are feasible in vitro and were the first cell candidates to be tested for cell therapy in the muscle. OPMD, which only has a small subset of muscles initially impacted, is an ideal muscle disease to be approached with autologous myoblast transplantation. Perie et al. [59] proposed and advanced an autologous myoblast transplantation therapeutic strategy into a successful Phase I/II clinical trial, in a process which could be performed in addition to and simultaneously with the cricopharyngeal myotomy. Myoblasts derived from the neighbouring clinically unaffected sternocleidomastoid muscles were implanted into the affected pharyngeal muscles. The transplantation was reported to be well tolerated, with a greater than 15% improvements in clinical trial readouts (such as the 80 ml drinking test) being observed and quality of life questionnaires. The authors also reported that the extent of amelioration of disease symptoms directly correlated with the amount of myoblasts implanted into the muscles.

Disrupting the formation of nuclear aggregates, thereby freeing the trapped RNAs and proteins including normal PABPN1, has also been considered a possible therapeutic approach. Small-molecule drugs that reduce nuclear aggregates have been well-studied in both animal models of OPMD and clinical trials conducted. Specifically, the disaccharide compound trehalose has been shown to be particularly effective in reducing the formation of INIs and also permitting protein clearance through increased autophagy. In a recent clinical trial, the drug was reported to be well tolerated, and the authors demonstrated a greater than 35% increase in the 80 ml drinking test, with improvements also reported in muscle power, functional tests and quality of life [60]. Other strategies employ the use of chemical and molecular chaperones [61], chemotherapeutic agents such as heat-shock protein inducers (ZnSO4, 8-hydroxyquinoline) or anti-inflammatory agents (ibuprofen and indomethacin) [62] and have been investigated in various cell models of OPMD. Anti-prion agents (6-aminophenanthridine and guanabenz) have known antagonistic actions against prion protein-associated amyloid fibre formation and have also been shown to alleviate symptoms in cell and animal models of OPMD [63].

31.4 DNA-Directed RNA Interference (ddRNAi) for Genetic and Acquired Disorders

Although a myotomy or the transplantation of autologous myoblasts may temporarily provide some benefit to relieving the clinical manifestation of OPMD in the patient, the underlying root cause of the disease, the genetic mutation in PABPN1, has not been rectified. Inevitably, the disease symptoms may eventually reappear. Thus, a gene therapy-based approach could provide the only viable mechanism for a long-term treatment of this disease.

Traditionally, in the field of gene therapy, the correction of genetic disorders has been rooted in the principle of expressing corrective genes to produce normal proteins in cells or tissues where there was a defective copy of that same gene. Such approaches have been applied to autosomal recessive disorders where products like Glybera work by replacing a copy of the lipoprotein lipase (LPL) gene in muscle cells of patients lacking the ability to produce the functional enzyme [64]. Haemophilia is another disease where gene therapy-based approaches have been used to introduce factor IX [65]. In addition to providing a mechanism to restore function by introducing a healthy copy of the gene, a gene therapy approach often confers the ability to use tissue-specific viral capsids as well as permit long-term sustained expression of the delivered gene. Autosomal-dominant diseases, where only one mutant allele contributes to the disease pathology, can be significantly more challenging to treat. Well-known examples of autosomal-dominant diseases include Huntington's disease, transthyretin-related hereditary amyloidosis as well as retinitis pigmentosa. OPMD is also classified as an autosomal-dominant disease. As the mutant allele produces a protein product which is either toxic or detrimental to the disease phenotype, gene therapy treatments are geared towards preventing the expression of the diseased gene. Several genetics-based therapeutic modalities can be used to directly downregulate expression of the mutant protein including antisense oligonucleotides, ribozymes, genome editing as well as RNA interference (RNAi). In genome editing, enzymes with the ability to cleave nucleic acids can be directed to specific target genes through modalities like zinc finger nucleases, meganucleases or systems such as the CRISPR/Cas9, in order to create site-specific double-stranded breaks rendering the mutant gene susceptible to disruption. Likewise, the application of small interfering RNA (siRNA) approaches to inhibit gene expression via RNAi has been considered for a number of these diseases [66, 67]. Eventually, like other oligonucleotide approaches, siRNAs are metabolized with the cells, and the compound must be readministered to maintain long-term therapeutic benefit. For diseases arising from mutations within patient's genes, this likely will require a lifetime of receiving these medications on a regular schedule. Alternatively, one can elicit RNAi from a vector-based approach. This is termed ddRNAi. In this process, a DNA template that encodes for short hairpin RNAs (shRNA) is introduced into the nucleus of transduced cells and uses the endogenous transcriptional machinery of the cells to produce a constantly replenishing pool of shRNA [68]. Once produced in the nucleus, the shRNAs are exported into the cytoplasm and the looped sequences are cleaved, the resultant siRNAs that are produced enter into the same cellular machinery utilized by chemically synthesized siRNA. In cases in which the shRNA expression cassettes are introduced into the target tissues within the context of the recombinant viral vectors, transduction can result in months or years of durable, steady-state levels of therapeutic shRNA expression in order to suppress the mRNA produced from mutant alleles. A wide variety of mutations can lead to autosomal-dominant disorders including trinucleotide repeat disorders that lead to the insertional mutations that may cause a frameshift in the protein or alternatively lead to an expansion of a single amino acid in the context of an otherwise normal protein. PolyQ expansion disorders in which a CAG sequence

repeats lead to multiple additions of a glutamine residue are commonly associated with Huntington's disease and certain types of spinocerebellar ataxia [69]. Because RNAi recognizes and cleaves the target mRNA via sequence specificity, the nature of these mutations often makes it difficult to design an RNAi approach that can selectively target the mutant allele and leave the wild-type allele untouched [70]. Although a significant amount of progress has been made to develop allele-specific approaches [71, 72] to be able to selectively target the disease-causing gene while leaving the expression of the wild-type gene intact, there are a large number of different mutations as well as different types of mutations (point mutants, insertions, deletions, etc.) that may lead to the diseased protein. For instance, over 150 different mutations have been noted in subjects diagnosed with retinitis pigmentosa [73]. Because many of these genetic diseases are classified as rare or orphan indications, development of allelic specific approaches can significantly limit the number of patients that can be treated. More recently, Farrar and colleagues have described a tact in which they have used a dual-vector system where two adeno-associated virus (AAV) vectors have been employed in what could be termed a 'silence and replace'-based approach to treat dominant retinitis pigmentosa [74]. One AAV was designed to express shRNA designed to target and knock down both the mutant and wild-type alleles of the rhodopsin gene (RHO). By targeting both diseased and wild-type alleles, the approach to suppression of the mutant phenotypes can be broadly applied across all of the disease mutants. Yet, rhodopsin must ultimately be restored as it is a critical protein involved in transducing light signals into the electrical signals that are interpreted by the brain as vision. Thus, a second AAV vector comprised of the wild-type sequences of the RHO gene is co-administered to restore normal rhodopsin function. In order to prevent suppression of RHO provided in trans from the second AAV vector, one can take advantage of codon degeneracy to produce a wild-type protein at the amino acid level from a genetic sequence that is insensitive to the shRNA being produced [75]. The use of recombinant viruses for the efficient delivery of genetic elements comes with limitations, including a finite packaging capacity of the recombinant genome. Typically, the delivered construct must accommodate therapeutic sequences as well as the transcriptional regulatory elements that drive their expression. Thus, if the size of the replacement gene is sufficiently large, it can restrict the ability to add additional regulatory elements on the same recombinant expression constructs. In the aforementioned silence- and replace-based approach for treating retinitis pigmentosa, the size of the RHO gene necessitates the use of two vectors. Furthermore, because many of the genome editing techniques require co-expression of foreign proteins to achieve their effects, there is little room left in the molecular design of these drugs to combine both gene silencing and gene replacement. Thus, therapeutic administration of two different gene therapy products would require the manufacture of two independent viral vectors, each needing to pass independent CMC scrutiny before it can be administered into humans. RNA interference has a distinct advantage in this regard in that the transcriptional cassettes required to produce shRNA are comparatively small, as little as 350 nucleotides, and the inhibition takes advantage of existing cellular machinery of the RNA-induced silencing complex; co-expression of foreign proteins to achieve gene

silencing is not required. Depending on the size of the corresponding codon-optimized gene to be expressed, there is ample capacity to co-express the healthy gene on the same cassette as the shRNA. Thus, in an optimal setting from a clinical and commercial perspective, the therapeutic vector to treat the autosomal-dominant disorder would be composed of sequences encoding for both the 'silence' and 'replace' functions.

31.5 Gene Therapy for OPMD

Here, we describe the design of an AAV-based 'silence and replace' strategy for the treatment of OPMD which combines the robust knockdown of the expPABPN1 coupled with the co-expression of a codon-optimized, wild-type PABPN1 [76]. This is the only preclinical study of gene therapy for OPMD published thus far. In this study, gene therapy was performed in the most common murine model of OPMD, the A17 mouse model. This mouse was generated in the FvB background by over-expressing a bovine-expanded (17 alanine residues) PABPN1 (expPABPN1) [52, 77]. expPABPN1 is placed under control of the human alpha-actin muscle-specific promoter (HSA1) which drives gene overexpression in only skeletal muscle. Both endogenous murine PABPN1 alleles are functional and express normal murine PABPN1. Therefore, the mouse phenotype is due to the overexpression of exp-PABPN1 over the normal protein. Heterozygous mice recapitulate most of the features of human OPMD patients such as progressive muscle weakness and atrophy, fibrosis deposition and a substantial (and progressively increasing with age) presence of nuclei containing INIs [45, 52, 77]. As mentioned previously, the primary target of a gene therapy approach is the downregulation of expPABPN1 with the aim of reducing aggregates freeing the normal PABPN1 associated with the INIs. However, the transcript with the additional 7 GCG triplets at the 5′end of exp-PABPN1 gene cannot be specifically targeted. DNA-directed RNA interference (ddRNAi) is a strategy that has the ability to downregulate a specific target mRNA. In this gene therapy application, ddRNAi has been used to knockdown all endogenous PABPN1. In particular, three shRNA sequences (designated as sh-1, sh-2 and sh-3) driven by U61, U69 and H1 polymerase-III promoters, respectively, were designed to target PABPN1 mRNA in regions of conserved identity between mouse, bovine and human species (sh-1 and sh-3) or specifically bovine and human PABPN1 (sh-2). These shRNA were assembled into a tricistronic expression cassette (named shRNA3X) to provide strong knockdown of PABPN1 transcript levels. shRNA3X was packaged in adeno-associated viral vector (AAV-shRNA3X). Because PABPN1 is such an important protein for cell survival and it is associated to multiple crucial biological pathways, the expression of functional PABPN1 is required in muscle cells. Therefore, a second AAV vector was used to deliver sequence-optimized human PABPN1 (named AAV-optPABPN1) in tandem. The use of a sequence-optimized normal PABPN1 is crucial as the redundancy of the genetic code is exploited to largely modify the nucleic acid sequence of PABPN1 (i.e. 230 out of

921 nucleotides mismatch, corresponding to 25% difference) which makes the primary nucleic acid sequence resistant to the ddRNAi-induced cleavage.

After preliminary in vitro studies to screen effective siRNAs against PABPN1 and to verify that the sequence-optimized PABPN1 was resistant to the cleavage by the shRNA3X, AAV-shRNA3X, AAV-optPABPN1 or a combination of the 2 AAVs were injected in tibialis anterior (TA) muscles of 10–12-week-old male A17 mice. Muscles were analysed 18 weeks after injection in order to observe the effect of the gene therapy treatment in a medium/long term. This study provided crucial information about the need of normal PABPN1 in muscle cells. First, while intranuclear aggregates in shRNA3X-treated muscles are greatly reduced, muscles do undergo a massive process of tissue degeneration/regeneration meaning that at least a small amount of normal PABPN1 is needed for muscle cell survival. Secondly, AAV-mediated expression of a sequence-optimized human PABPN1 alone was not sufficient to ameliorate the disease, although we cannot exclude that these results were affected by the mouse model we used that is clearly a pro-gain-of-toxic function model. Only the combined treatment to suppress the endogenous PABPN1 and express the normal protein abrogated the INI and the related toxic gain of function and simultaneously provided the needed normal PABPN1 to significantly reduce the loss of function and correct the pathological phenotype. Histology of muscles treated with the combined vectors showed the almost complete abrogation of KCl-resistant intranuclear inclusions, while the muscle architecture was preserved and

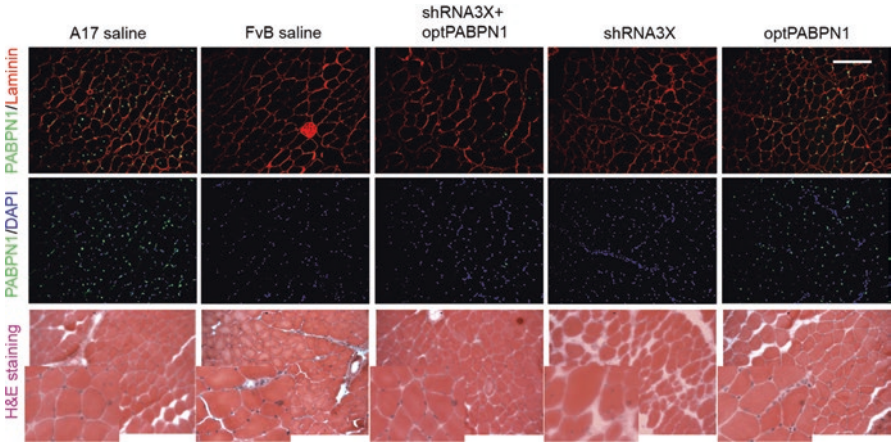

Fig. 31.1 Co-administration of AAVs expressing shRNA3X and optPABPN1 inhibits intranuclear aggregates in A17 muscles. Detection of PABPN1 inclusions (green) and laminin (red) by immunofluorescence in sections of treated muscles. Sections were pretreated with 1 M KCl to discard all soluble PABPN1 from the tissue. Nuclei are counterstained with DAPI (blue). The bottom panel shows representative images of histological staining for hematoxylin and eosin: several centrally nucleated fibres are shown in shRNA3X-treated muscles indicating muscle degeneration/regeneration processes ongoing in these muscles. Co-expression of optPABPN1 prevents muscle degeneration as indicated by the normal histology detected in muscles treated with both AAV-shRNA3X and AAV-optPABPN1. Bar, 200 μm

no signs of tissue degeneration were detectable (Fig. 31.1). Furthermore, crucial markers of muscle fibrosis such as collagens I, III and VI and fibronectin were decreased, while the average myofibre cross-sectional area was increased suggesting that the gene therapy treatment was able to partially counteract some pathological features of human OPMD, muscle fibrosis and atrophy. Muscle strength was assessed in TA muscles by in situ muscle physiology. While muscle weight was unchanged after treatments, the maximal tetanic force of muscles treated with the combination of AAV-shRNA3X and AAV-optPBAPN1 was increased, and the maximal specific force was normalized to the level of wild-type FvB muscles. Finally, a detailed analysis of the general muscle transcriptome was performed using microarray Affymetrix. 865 genes were differentially (452 up- and 413 down-, respectively) regulated (>1.5-fold change and $p < 0.05$) between wild-type and A17 tibialis anterior muscles. The single treatments only induced limited changes in gene expression, with some of them being actually detrimental as shown by downregulated muscle-specific genes in degenerating muscles after treatment with AA-shRNA3X

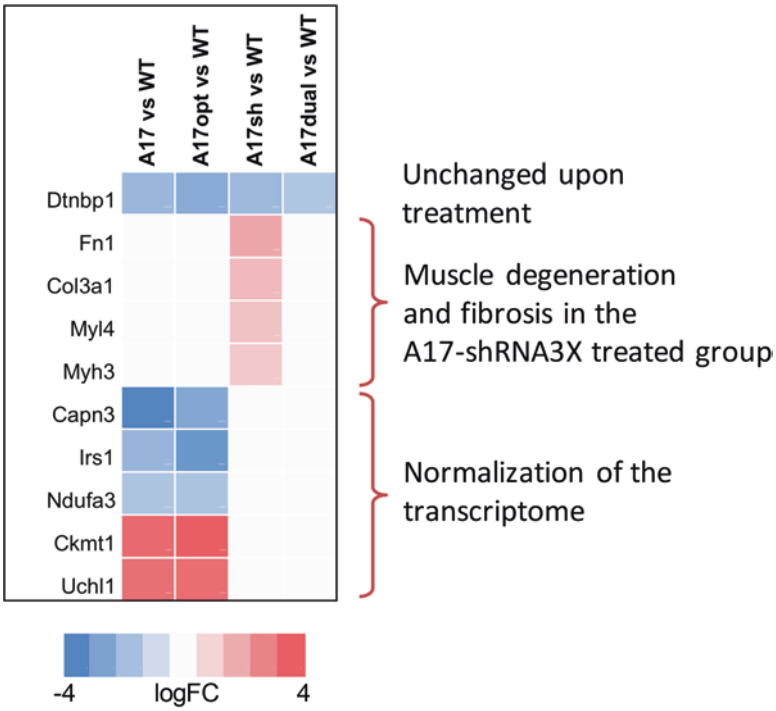

Fig. 31.2 Transcriptome of A17 muscles: examples of transcript expression for some selected genes after treatments. Example of genes detected in the transcriptomic analysis. Some transcripts were unchanged in A17 and FvB muscles (e.g. Dtnb1). Others were changed after treatment with AAV-shRNA3X where muscle degeneration was induced (e.g. Fn1, Col3a1, Myl4, Myh3). Most of the transcripts that were dysregulated (either up- or downregulated) in A17 mice or A17 mice treated with only optPABPN1 showed a complete return to normal expression after the gene therapy treatment (e.g. Capn3, Irs1, Ndufa3, Ckmt1, Uchl1)

(Fig. 31.2). On the other hand, the pathophysiological improvements observed with the combination of the two vectors were accompanied by the almost complete normalization of gene expression to wild-type muscles with 98% of deregulated genes that were correctly expressed after the treatment (Fig. 31.2). In order to demonstrate the applicability of such an approach in human subjects, myoblasts extracted from biopsies of OPMD patients were cultured in vitro. OPMD myoblasts do not show an obvious phenotype in vitro (i.e. no difference in proliferation/differentiation ability compared to myoblasts from normal muscles and absence of aggregates). However, when transduced with lentiviral vectors expressing the same shRNA3X cassette used in vivo, cell survival was strongly compromised, suggesting that human cells are sensitive to lower than normal levels of PABPN1. However, the transduction with lentiviral vectors delivering both shRNA3X and optPABPN1 cassettes rescued survival of human OPMD myoblasts showing that this combined suppression/replacement strategy is also functional in a human context.

31.6 Future Perspectives of Gene Therapy for OPMD

OPMD, with a limited number of muscles to treat, is particularly well-suited for a gene therapy approach as only a local administration directly into affected muscles would likely be required. Adeno-associated virus (AAV) is currently amongst the most promising of viral vectors for in vivo gene therapy applications due to its non-pathogenicity, natural efficient infection in primates for some serotypes and negligible risk of insertional mutagenesis and would be an ideal vector for gene therapy applications in OPMD. In this chapter, we described the most advanced gene therapy strategy for OPMD which is based on delivering two AAV vectors to down-regulate endogenous PABPN1 and newly express a cleavage-resistant human PABPN1 as targeting expPABPN1 invariably affects wild-type PABPN1 expression. Within the 4.7 kb vector packaging capacity, AAV vectors can accommodate both transgenes and shRNA/miRNA expression cassettes. The generation of a single construct, incorporating both suppression and replacement cassettes, is feasible and required for the translation of the approach into the clinic. A single construct would also increase the safety profile. Furthermore, the ddRNAi strategy can be modified to include the siRNA sequences modelled into a microRNA (miRNA)-based cassette mimicking the natural structure of miRNA [78]. This allows the enhanced control by a muscle-specific polymerase II promoter, a faster cleavage of the shRNA which prevents massive build-up of unprocessed, potentially toxic shRNA as well as a substantial reduction in the amount of unspecific siRNA produced. Benitec Biopharma is currently pursuing the advancement of BB-301, a next-generation, follow-up ddRNAi therapeutic for the treatment of OPMD that combines both the ‘silence and replace strategy’ of mutant PABPN1 into a miRNA-based single vector.

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Chapter 32
Gene Therapy for X-Linked Myotubular Myopathy

Jean-Baptiste Dupont, Michael W. Lawlor, and Martin K. Childers

Abstract X-linked myotubular myopathy (XLMTM) emerges from mutations in the *MTM1* gene and affects around 1 in 50,000 live-born male infants. This congenital myopathy has currently no treatment and leads to a severe impairment of motor skills and ventilation and premature death. In this chapter, we synthetize the results of gene therapy studies using recombinant adeno-associated vectors in preclinical models of X-linked myotubular myopathy. Over the past few years, the field has rapidly moved from myotubularin-deficient mice to dogs and has now begun the first clinical gene therapy trial for XLMTM. In both mice and dogs, a single intravenous injection of adeno-associated vector leads to a complete rescue of key pathological phenotypes, including motor and respiratory functions, and life expectancy. Despite the treatment being well tolerated in both animal models, we also interrogated some of the issues commonly encountered in gene therapy studies, notably immune responses against the vector capsid or the transgene product, genotoxicity, and off-target effects.

Keywords XLMTM · MTM1 · Myotubularin · Gene therapy · Adeno-associated virus · rAAV

32.1 Introduction

The use of adeno-associated virus (AAV)-derived vectors as molecular carriers to deliver transgenes in vivo has shown tremendous developments in the last 10 years for the treatment of monogenic disorders [1]. Patients suffering from some forms of

J.-B. Dupont (✉) · M. K. Childers
Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle, WA, USA
e-mail: jbd7@uw.edu; MChilders@askbio.com

M. W. Lawlor
Division of Pediatric Pathology, Department of Pathology and Laboratory Medicine, Medical College of Wisconsin, Milwaukee, WI, USA
e-mail: mlawlor@mcw.edu

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blindness or hemophilia B and treated by recombinant AAV (rAAV)-mediated gene therapy have experienced drastic improvements of their life conditions, and successful preclinical studies have raised hope for a cure to numerous additional diseases. To fulfill these early promises, researchers must manage the difficult translation to human patients by keeping similar therapeutic efficiency while devoting extra attention to the biosafety in this innovative area of molecular medicine. In the context of inherited muscular disorders, several interspecies differences—notably the overall morphology and the severity of the disease—often make it difficult to determine effective rAAV doses and therapeutic windows. The need to target a widespread territory that can encompass the entire axial, appendicular, and respiratory musculature is particularly challenging, and another layer of complexity arises sometimes from the continuous degeneration of target cells. This is particularly true for muscular dystrophies caused by mutations in genes coding for structural proteins involved in the generation or transmission of cell contraction, and in which muscle wasting is a prominent feature [2–4]. In contrast, rAAV vectors have shown spectacular efficiency in animal models of X-linked myotubular myopathy (XLMTM), a form of congenital centronuclear myopathy. In the past few years, the field has moved from the first proof of concept in mice to its translation in a large animal model [5, 6], and to initiation of the first clinical trial, which began in 2017. The goals of this chapter are to understand how this fast and efficient preclinical development was realized and to try to identify potential hurdles that might occur in clinical translation.

32.2 Characteristics of XLMTM Pathophysiology

XLMTM (OMIM #310400) results from mutations in the *MTM1* gene and occurs in around 1 in 50,000 live-born male infants [7]. Patients exhibit hypotonia at birth and rapidly develop severe impairment of motor and respiratory function. Many do not survive beyond their second birthday, and the majority of surviving patients experience disabling symptoms and a reduced lifespan despite intensive medical support [8, 9]. *MTM1* encodes the founding member of the myotubularin family of phosphoinositide phosphatases and transforms phosphatidylinositol 3-phosphate (PtdIns3P) and PtdIns(3,5)P2 into PtdIns and PtdIns5P, respectively. These lipids are important constituents of cellular membranes, playing various roles in vesicle trafficking and signaling cascades, and thus myotubularin is considered a crucial factor controlling membrane maintenance and integrity [10]. This feature can explain at least part of the pathological phenotypes observed in XLMTM patients and in the different animal models of the disease, such as *Mtm1*-deficient mice and XLMTM (p.N155K) dogs, or in *mtm* knockout and knockdown zebra fish models [11].

Even though *MTM1* is expressed ubiquitously, its absence results exclusively in neuromuscular phenotypes in most patients and all animal models. Myotubularin-deficient muscle fibers are small (likely due to a combination of hypotrophy and

atrophy) and display abnormal localization of nuclei and other organelles (mitochondria, sarcoplasmic reticulum, Golgi apparatus). This mislocalization of organelles results in central or internal placement of nuclei within myofibers (placing XLMTM in the "centronuclear myopathy" category of congenital myopathies), and oxidative histochemical stains reveal a variety of organelle aggregation patterns reflective of aggregations and abnormal placement of mitochondria and sarcotubular components (Fig. 32.1) [11, 12]. In contrast with muscular dystrophies, scant myofiber degeneration, fibrosis, or fat accumulation is seen in XLMTM muscles until terminal stages of disease. Yet, the number of satellite cells is significantly reduced in patients [12] and *Mtm1*-deficient mice [13], suggesting the inability to maintain or self-renew the muscle stem cell pool. While these abnormalities have the potential to affect muscle function, an overwhelming cause of weakness in XLMTM is a marked impairment of muscle contraction despite adequate innervation, and impairment of neuromuscular transmission and excitation-contraction

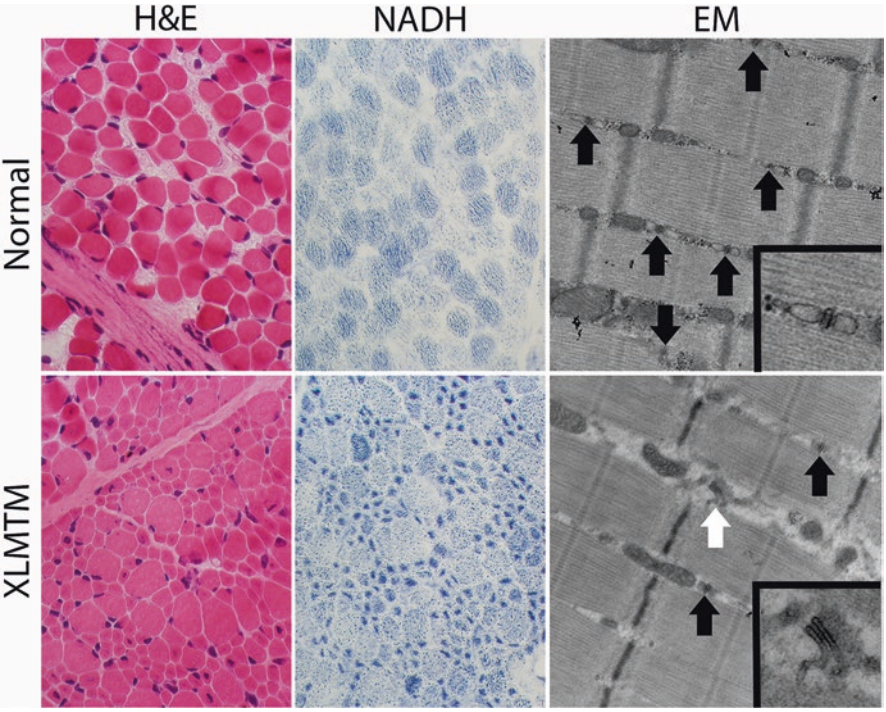

Fig. 32.1 Pathological findings in human XLMTM. Hematoxylin and eosin (H&E) staining reveals myofiber smallness and increased numbers of internally nucleated fibers in an XLMTM patient in comparison to a normal biopsy from an age-matched patient. NADH staining illustrates aggregation of organelles in some myofibers in XLMTM, in comparison to a regular and diffuse distribution of organelles in the normal biopsy. Electron microscopy (EM) reveals numerous triad structures with appropriate morphology (black arrows, inset) in the normal biopsy. While some normal triads are present in XLMTM, they are decreased in number, and abnormally formed and oriented triads (white arrow, inset) may be apparent

coupling have been described. *Mtm1*-deficient mice show enlarged and less complex neuromuscular junctions, leading to inefficient neurotransmission [14]. Of note, treatment with the acetylcholinesterase inhibitor, pyridostigmine, can improve several motor phenotypes such as grip fatigue and, to a greater extent, treadmill endurance up to 40% of the WT level [14]. In addition, the close associations between the transverse tubules and the SR terminal cisternae—called muscle triads—are less abundant and have an abnormal morphology (Fig. 32.1) [15, 16]. In normal skeletal muscle myofibers, these triads are an essential component that translates sarcolemmal depolarization into cytoplasmic calcium release. Abnormalities of triad formation and structure in XLMTM cells lead to marked defects in excitation-contraction coupling [15, 16], producing weakness far more profound than would be expected of the other pathology (such as myofiber smallness) that is observed in XLMTM [17]. The importance of excitation-contraction coupling is further underscored by several studies of myostatin inhibition in *Mtm1*-deficient mice [13], where marked treatment-associated myofiber growth was not capable of improving muscle strength [18, 19]. Finally, specific signaling pathways classically associated with denervation-induced muscle atrophy are found dysregulated, notably the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) hub and the ubiquitin-proteasome pathway [20–22]. It remains elusive how mutations in a lipid phosphatase lead to XLMTM pathophysiology, but a recent study brought strong evidence that it could be prevented by crossing *Mtm1*-deficient mice with mice knocked-out for phosphoinositide 3-kinase (PI3K), which reduces PtdIns3P accumulation [23]. Furthermore, the pharmacological inhibition of PI3K using wortmannin led to a partial improvement of the phenotype and the survival of *Mtm1*-deficient mice [23]. Overall, these findings indicate that XLMTM might be treated pharmacologically, at least to some extent. However, the most successful preclinical studies so far have attempted to increase the amount of intracellular myotubularin, either directly by enzyme replacement therapy [17] or by gene therapy using rAAV-derived vectors. The following sections of this chapter are specifically focused on the latter strategy.

32.3 Preclinical Studies Using rAAV Vectors in XLMTM Animal Models

Gene therapy in the context of XLMTM aims at delivering an expression cassette encoding myotubularin to skeletal myofibers. For this purpose, rAAV-derived vectors have proved particularly efficient [24]. The AAV was first characterized as a by-product in *Adenovirus*-infected cells and has subsequently been established as an ideal vector for in vivo gene transfer in postmitotic cells [25, 26]. To date, 173 clinical trials have been initiated with rAAV vectors for a wide range of diseases such as retinal disorders, neurological diseases, or hemophilia B (http://www.abedia.com/wiley/). In addition, the first medicine based on the use of this vector—Glybera®—was approved by the European Medicines Agency in 2012 for lipoprotein lipase

deficiency [27]. The existence of a dozen natural AAV serotypes and multiple artificial variants that all exhibit a specific tropism allows the targeting of numerous key organs and the transduction of specific cell populations [28, 29]. Concerning the neuromuscular and cardiac systems, rAAV vectors (composed mostly of serotypes 1, 6, 8, or 9) have been used in animal models of multiple genetic disorders including Duchenne muscular dystrophy (DMD) [30–32], limb girdle muscular dystrophies (LGMD) [33, 34], spinal muscular atrophy (SMA) [35, 36], or Pompe disease [37]. Most studies have reported gene transfer efficiency for weeks to months following rAAV injection, sometimes correlated with a drastic improvement of the pathological phenotypes.

The initial gene transfer attempt in the context of XLMTM was published in 2008 by the team of Jean-Louis Mandel [5]. Myotubularin-deficient mice created by knocking out the *Mtm1* gene normally exhibit a progressive myopathic phenotype that faithfully recapitulates the human pathology [38]. Their muscle fibers show typical XLMTM features at the histological level, such as a marked hypotrophy and mislocalized or aggregated organelles. These mice develop severe motor impairment associated with sharply reduced survival to no more than 14 weeks. Myotubularin-deficient mice that received a single intramuscular injection of a rAAV1 vector expressing a recombinant *Mtm1* cDNA resulted in an improved pathological phenotype. Both the structure (muscle weight and cellular architecture) and the function (contractile activity) of the injected muscle were markedly improved. In a later study, *Mtm1*-KO mice were injected intravenously with a rAAV8 vector expressing a similar transgene under the control of a muscle-specific promoter (desmin) [6]. Strikingly, the phenotype of treated mice was completely rescued, and their survival was normal, whether they received the vector at an early or late disease stage. Other physiological and functional parameters such as body weight and global strength or muscle architecture and specific force were also substantially improved at 6 months post-rAAV injection. Of note, mutant forms of myotubularin disrupting the phosphatase activity of the enzyme were shown to be as efficient as the WT protein at improving most of the muscle phenotypes in mutant mice [39]. The authors suggested that myotubularin enzyme activity is important at the age of XLMTM onset but that the maintenance of the pathological state in adult muscle cells rather depends upon other functions of this protein. A link between myotubularin and important signaling pathways, particularly those controlling muscle cell growth or atrophy, may explain this observation. Indeed, results published in 2013 demonstrated that rAAV-mediated transfer of a WT copy of *Mtm1* corrected the molecular signature associated with atrophy in XLMTM mice [22]. The same experiment with a phosphatase-dead enzyme was not carried out but could be of interest to decipher additional structure-function relationships in myotubularin.

While results of AAV gene transfer in small rodents were already very promising, similar results were subsequently observed in much larger animals with a muscle volume and morphology more representative of what would be found in XLMTM patients. In 2010, a naturally occurring p.N155K missense mutation in the *MTM1* gene was identified in Labrador Retrievers, and a colony of XLMTM dogs harboring this mutation was established by one of the authors (MKC) [40]. These dogs

display patellar hyporeflexia, dysphagia, dropped jaw, and a hoarse bark that develops over time. Unlike the golden retriever muscular dystrophy (GRMD) model, we have observed little variation in the phenotype of XLMTM dogs in the research setting, despite detailed physiological and clinical observations in ~ 30 affected dogs since 2009. Moreover, the disease onset in canine XLMTM is more rapid than in GRMD and universally results in markedly progressive weakness and muscle atrophy leading to loss of ambulation, necessitating euthanasia by 4–6 months of age [41]. Histological evaluation confirms some of the findings made in patients, notably a severe hypotrophy with internal nucleation, myofibril disarray, and triad abnormalities (Fig. 32.1) [11, 40]. Muscle function is also profoundly altered in this model, as assessed by isometric torque measurement or by scoring multiple neuromuscular parameters [42, 43]. Using an in vivo system to measure tibiotarsal joint torque, forces generated by XLMTM dogs at 10 weeks are ~40% lower than normal. By 18 weeks of age, torque generated by affected dogs is only ~15% of that of wild-type dogs [42].

Myotubularin-deficient dogs were first treated with a rAAV8 vector expressing the canine *MTM1* cDNA downstream the muscle-specific desmin promoter (rAAV8-cMTM1) injected into the *Cranial tibialis* muscle [6]. Similar to the results obtained in mice, the growth and the cellular architecture of the injected muscle were substantially improved compared with the saline-injected contralateral muscle, with particularly significant improvement by 4–6 weeks postinjection. This prompted the authors to assess the efficacy of gene therapy in an entire limb isolated from the general circulation by a tourniquet. In this condition, the injection of the vector under pressure allows its widespread dissemination in muscle groups of the injected limb and also leads to a slight leakage into the circulation and in distant tissues and organs [44, 45]. Three XLMTM dogs were injected by this "locoregional" route, which resulted in systemic beneficial effects, dramatic improvement of breathing functions, and survival, with treated dogs remaining ambulant and healthy beyond 1 year of age [6].

Subsequent to this surprising observation of phenotype rescue following "quasi-systemic" infusion, an AAV dose-finding experiment was conducted in a large cohort of XLMTM dogs. rAAV8-cMTM1 was administered by simple peripheral venous infusion in XLMTM dogs at 10 weeks of age, when signs of the disease were beginning to become evident. A comprehensive analysis of survival, limb strength, gait, respiratory function, neurological assessment, histology, vector biodistribution, transgene expression, and immune response was performed over a 9-month study period. Escalating doses of rAAV8-cMTM1 (0.3, 2, and 5×10^{14} vg/kg) were given into the cephalic vein of XLMTM dogs ($n = 3$ per dose), and saline was given in age-matched mutant and normal littermates ($n = 6$ per group) as controls. Results indicated that, in a dose-responsive manner, rAAV8-mediated gene therapy effectively corrected the entire skeletal musculature in a large animal model of an inherited fatal myopathy following a single intravenous vector administration [46].

In this large cohort study of XLMTM dogs, intravenous administration of rAAV8-cMTM1 at mid (2×10^{14} vg/kg) and high (5×10^{14} vg/kg) doses conferred long-term survival until the end of the 9-month observation period, whereas rAAV8-cMTM1 at low dose (0.3×10^{14} vg/kg) did not provide a major survival benefit. A

dose-dependent improvement in strength followed *MTM1* gene transfer. Because severe weakness is the key clinical characteristic of the disease, restoration to normal levels of muscle strength (measured by limb torque) following gene transfer represented the most important readout in this study. Similar to the response seen in torque, we observed a dose-response to AAV in walking gait. Gait assessment provides a functional, behavioral measure in awake animals, while muscle torque isolates function of a given muscle (or muscle group) and is assessed in anesthetized animals. Similarly, respiratory function, which involves the strength of various skeletal muscles including the diaphragm, was also comparable to normal controls following mid- and high-dose AAV.

XLMTM dogs that received either the mid or high dose of rAAV8-cMTM1 demonstrated near-complete reversal of XLMTM-related pathology, displaying essentially normal skeletal muscle histology in the majority of samples evaluated (also discussed and shown in Chap. 12). The normalized myofiber morphology and orientation of the triads suggest excitation-contraction coupling (ECC), organelle function, and myofiber size improvement to support greater muscle torque production. Finally, dogs were assessed for improvement in neurological function using a validated clinical scoring instrument developed for dogs, the neurological assessment score (NAS) [43]. Results clearly demonstrated a dose-dependent effect of rAAV8-cMTM1. Before infusion, XLMTM dogs scored slightly lower than normal controls. After infusion, at the 17-week time point, NAS declined markedly in saline- and low-dose-infused dogs, whereas normal controls and XLMTM dogs given mid- or high-dose rAAV8-cMTM1 achieved comparable neurological scores. By the end of the 9-month study, XLMTM dogs in the mid- and high-dose groups maintained neurological scores comparable to their age-matched normal controls, with dogs treated at high-dose performing the best of the three dosing groups. Together, these results identified an effective threshold dose of 2×10^{14} vg/kg of rAAV8-cMTM1 in dogs, consistently measured by separate and distinct study endpoints, a finding similar to the dose (2×10^{14} vg/kg) that we initially tested in our previous locoregional studies.

Table 32.1 recapitulates the main parameters of the gene therapy studies published so far and describes the use of rAAV vectors in animal models of XLMTM. The spectacular recovery observed in two of these models has highlighted the strong potential of rAAV-mediated gene therapy for this disease, which has now moved to clinical phase. For this purpose, longitudinal studies in XLMTM patients are ongoing and should help document the natural history of the disease before any intervention (https://clinicaltrials.gov/, NCT02704273, NCT02231697, and NCT02453152).

32.4 Remaining Challenges Toward a Successful Clinical Transfer

Despite intensive efforts, the safety and efficiency of gene therapy in human clinical trials are difficult to anticipate from preclinical studies in animal models. Even if mice or larger animal models faithfully reproduce the pathological state described in patients,

Table 32.1 Summary of the preclinical gene therapy studies in XLMTM animal models

Ref.	Model	AAV serotype	Promoter	Transgene	Dose	Administration	Age at injection	Follow-up
[5]	*Mtm1* muscle KO mice	rAAV2/1	CMV	Mtm1 cDNA	9.00×10^{10} vg	IM (TA)	4 weeks	4 weeks
					2.5×10^{10} vg	IM (EDL)		
					1.2×10^{11} vg	IM (TA)	4 weeks	6–8 weeks
[44]	*Mtm1* KO mice	rAAV2/1	CMV	Mtm1 cDNA WT or phosphatase-dead (C375S/S376N)	1.4×10^{10} vg	IM (TA)	2–3 weeks	4 weeks
[18]	*Mtm1* KO mice	rAAV2/1	CMV	Mtm1 cDNA	$1.0–1.4 \times 10^{10}$ vg	IM (TA and Quad)	2 weeks	2 weeks
[6]	*Mtm1* KO mice	rAAV2/8	Desmin	Mtm1 cDNA	3.0×10^{13} vg/kg	IV (tail vein)	3 weeks	6 months
							5 weeks	
					5.0×10^{12} vg/kg	IV (tail vein)	3 weeks	
	XLMTM dogs	rAAV2/8	Desmin	Mtm1 cDNA	4.0×10^{11} vg	IM (CT)	10 weeks	4–6 weeks
					2.5×10^{13} vg/kg	LR (isolated limb)		>4 years [58]
[51]	XLMTM dogs	rAAV2/8	Desmin	Mtm1 cDNA	0.3×10^{14} vg/kg	IV (cephalic vein)	10 weeks	9 months
					2.0×10^{14} vg/kg			
					5.0×10^{14} vg/kg			

IM intramuscular injection, *TA tibialis anterior* muscle, *EDL extensor digitorum longus* muscle, *Quad quadriceps femoris* muscle, *IV* intravenous injection, *CT cranial tibialis* muscle, *LR* locoregional injection

species-specific features related to host-virus (rAAV vector in this case) interactions may lead to dramatically different outcomes. Serious adverse events after gene therapy clinical trials using retroviral and adenoviral vectors have already been observed in the past and had not been anticipated in preclinical studies [47, 48]. In the context of AAV, natural infections with the wild-type virus prior to gene therapy can cause the formation of neutralizing antibodies and memory T cells directed against the capsid, which have the potential to limit gene transfer efficacy in patients [49, 50]. In addition, the immune reactions mounted against the vector capsid or the recombinant transgene protein after the injection of the vector are crucial issues to consider in terms of interspecies differences. This was notably the case during the initial clinical trials for hemophilia B or Duchenne muscular dystrophy [49, 51, 52]. In the latter, several patients showed preexisting immunity against dystrophin, most likely due to the spontaneous formation of revertant fibers. These patients developed a T-cell-mediated immune response which might have led to the destruction of transduced cells [52]. This immunity against the recombinant protein had not been described in animal models of DMD, highlighting the difficulties to translate preclinical studies in human patients. Thus, the last part of this chapter intends to anticipate potential pitfalls that gene therapy for XLMTM may encounter, in the light of previous data on rAAV vectors and muscle disorders.

In XLMTM dogs, anti-AAV8 capsid antibodies can also be detected after vector injection—independent of the dose and the delivery route—but are not associated with any T-cell-mediated immune response against either the rAAV capsid or the transgene protein [6, 46]. However, tackling this issue will be critical in view of a potential readministration of the vector in XLMTM patients.

One major challenge to address for gene transfer in the entire striated muscular system relates to the ability to deliver the vector and express the transgene in a widespread territory with high and long-lasting efficacy. Previous reports of rAAV gene therapy in animal models of dystrophic disorders showed a progressive loss of transduced cells when transgene expression is sub-therapeutic and not sufficient to counteract muscle wasting [2–4]. As previously mentioned, the absence of myotubularin has not been associated with muscle fiber degeneration, and thus rAAV genomes should be maintained in target cells for the long-term persistence. The initial study conducted in XLMTM dogs treated by locoregional injection suggested that functional benefits could last at least over 1 year, even though transgene expression was measured well under the WT level at necropsy in one of the treated dogs [6]. An additional study in our laboratory has confirmed and extended this observation: at 4 years postinjection, intravenously injected XLMTM dogs still show a robust phenotype correction and maintain a stable muscle transduction [53]. Nevertheless, this long-lasting effect depends on the initial dose of rAAV delivered in vivo, as previously suggested in *Mtm1*-deficient mice [6] and confirmed in another recent study in our laboratory [46]. In this latter publication, our team has demonstrated that 2×10^{14} vg/kg are required to achieve phenotype correction in XLMTM dogs, which should inform the dose to be injected in the first XLMTM patients.

Given this large vector dose, another concern is the potential for rAAV genome integration in the vicinity of oncogenes/tumor suppressors, which could lead to genotoxicity and tumorigenesis [54]. In muscle cells, rAAV is mostly maintained as

circularized and concatemerized episomes and does not actively integrate [55, 56]. However, muscle samples from nonhuman primates and human patients injected with rAAV have shown random integration profiles into the host genome at a low frequency estimated between 1×10^{-4} to 1×10^{-5} vg [57, 58]. If the vector loads delivered to patients led to an optimal transduction, this would give rise to at least 1×10^{9} independent integration events at random genomic locations. Despite this high frequency, no genotoxicity relative to vector genome insertion in muscle nuclei has been reported in preclinical studies.

Additional adverse events of rAAV overdosing can result from the toxic expression of the transgene in tissues which accumulate high amounts of rAAV vectors, whether they are therapeutic targets or not. In the first XLMTM gene therapy study, mice were injected intramuscularly with a high dose of rAAV vector expressing *Mtm1* downstream of a strong and constitutive promoter to force the overexpression of the transgene. This led to the accumulation of membrane stacks in transduced muscle fibers, but the functional significance of these structures is unclear [5]. In addition, mice treated intravenously with the highest dose of vector showed scar tissue and focal inflammation in the heart [6]. These two adverse events had no detectable functional impact, and none of them was ever reproduced in XLMTM dogs, even at a dose twofold higher than the therapeutic dose [46]. In addition to striated muscles, AAV8 is known to have a high tropism for the liver, as shown by the high vector genome copy numbers detected in injected XLMTM mice and dogs. The use of a muscle-oriented promoter (desmin) resulted in a very low hepatic expression of the transgene and did not alter its functions or the level of liver-specific enzymes in the blood [6, 46].

Finally, these concerns bring an interesting question about the identity of target organs. It is well accepted that XLMTM is primarily a muscle disorder, and so far, the prime objective has been to target the skeletal musculature. However, *MTM1* is ubiquitously expressed, and the effect of myotubularin deficiency in other tissues may be masked by the severity of the muscle pathology. What are these tissues and to what extent will they suffer from secondary phenotypes if transgene expression is maintained long enough only in skeletal muscles are currently unknown and will need to be carefully monitored as XLMTM gene therapy moves to clinical trials.

Previous studies have often shown the difficulty to translate preclinical gene therapy studies to human application, which can be explained by inherent interspecies differences in size and morphology, disease manifestation, and host-vector interactions. To ensure the success of this translation, it seems critical to broaden our understanding of basic XLMTM phenotypes in muscles and other organs, together with the biology of rAAV vectors specifically in this pathological context.

32.5 Conclusion

XLMTM gene therapy has known a fast development in preclinical studies following the systemic delivery of therapeutic rAAV vectors in affected mice and dogs. These spectacular preclinical results, with a complete and long-term rescue of the pathological phenotypes, have allowed the translation of rAAV gene therapy to a

clinical trial. As gene therapy for XLMTM has entered the clinic, the results look very promising. In May 2018, Audentes Therapeutics, the sponsor of the clinical trial, reported significant improvement in neuromuscular function, including respiratory function, in a treated subject at week 24 and three others as early as the 4-week time point. The clinical trial is still underway.

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Chapter 33
Preclinical Gene Therapy Studies for Metabolic Myopathy

Stephanie Salabarria, Barry J. Byrne, Cristina Liberati, and Manuela Corti

Abstract The application of adeno-associated virus (AAV) and adenovirus (AdV) gene therapy has become increasingly important as a therapeutic modality since its first application in humans in 1990. However, over the past 20 years, the field has matured, and tangible clinical outcomes have been achieved in a limited number of studies. Establishing proof of concept and overcoming some of the technical challenges and establishing safety are the focus of preclinical studies on gene therapy to date. Gene therapy has been extensively investigated for some metabolic myopathies such as Pompe, but it has just begun for many others. This chapter will evaluate animal models for future research in metabolic myopathies as well as preclinical gene therapy trials for glycogen storage diseases (GSD) I, III, and V, Barth syndrome, Friedreich's ataxia, and very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency . These animal models will provide a great opportunity to test novel forms of gene editing and gene replacement therapies.

Keywords Metabolic myopathies · Glycogen storage diseases · Mitochondrial disorders · Vector

33.1 Introduction

Metabolic myopathies refer to a group of hereditary muscle disorders due to genetic defects leading to a specific enzymatic deficiency. Metabolic myopathies are heterogeneous conditions that have common abnormalities of muscle energy metabolism

S. Salabarria · C. Liberati
Department of Pediatrics, College of Medicine, University of Florida, Gainesville, FL, USA
e-mail: ssalabarria@ufl.edu; c.liberati@peds.ufl.edu

B. J. Byrne · M. Corti (✉)
Department of Pediatrics, College of Medicine, University of Florida, Gainesville, FL, USA

Powell Gene Therapy Center, University of Florida, Gainesville, FL, USA
e-mail: bbyrne@ufl.edu; m.corti@peds.ufl.edu

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that result in skeletal muscle dysfunction [1]. As referenced by Berardo et al. [2], there are three main classes of metabolic myopathies including glycogen storage diseases (glycogenosis), mitochondrial disorders, and fatty oxidation defects. In this chapter, we review the development of gene therapy for all three classes of metabolic myopathies.

33.2 Glycogen Storage Diseases (Glycogenosis)

Glycogen storage diseases are caused by a genetic deficiency in the enzymes that either degrade or synthesize glycogen [3]. The glycogen storage diseases that will be discussed in this section are glycogenosis types I, II, III, and V.

33.2.1 Glycogenosis Type I

33.2.1.1 Clinical Aspects of Glycogenosis Type Ia

Glycogen storage disease type Ia (GSDIa), also known as von Gierke disease, is an inherited metabolic storage disorder with life-threatening complications due to hypoglycemia. Even though GSDIa is predominantly a liver glycogenosis, there are lessons learned from the gene therapy approach to GSDIa which are applicable in this category of disease. The condition is due to a deficiency of glucose 6-phosphatase (G6Pase), a key enzyme that controls the breakdown of glycogen to glucose for energy production [4]. The deficiency in G6Pase results in secondary accumulation of glycogen and lipids in tissues especially the liver, kidneys, and small intestine. Individuals affected by GSDIa suffer from severe hypoglycemia during fasting, excess production of lactic acid from increased glycolysis, and hyperlipidemia. Long-term complications include growth failure, pulmonary hypertension, formation of hepatic adenomas, and, occasionally, hepatocellular carcinoma (HCC) and renal failure within the first year of life [5]. The only treatment available for GSDIa is a strict dietary supplementation or intragastric infusion of carbohydrates [6].

33.2.1.2 Adeno-associated Virus (AAV) Vectors in a Murine Model of von Gierke Disease

Gene therapy is currently the only promising treatment option to correct the primary metabolic defect by delivering G6Pase to the cells of target tissues. Initial studies in mice and dogs used AAV serotype 2 (AAV2) vectors containing either

the cytomegalovirus (CMV) or mouse albumin promoter/enhancer and led to incomplete correction of G6Pase deficiency [7]. The efficacy of the gene therapy treatment was improved with the discovery of new AAV serotypes and promoters/enhancers. Improvements were also observed with the use of self-complementary (sc), double-stranded, AAV vectors. Since the liver is the main target tissue for gene therapy delivery in GSDIa, a highly liver-tropic vector such as AAV serotype 8 (AAV8) has been preferred. In fact, scAAV8 vector expressing human G6Pase-α from a minimal human G6Pase promoter led to restoration of hepatic G6Pase-α and normalization of fasting glucose and other biochemical parameters in G6pc$^{-/-}$ mice and GSDIa dogs [8]. The best results were obtained by another study in G6pc$^{-/-}$ mice using AAV8-GPE, expressing human G6Pase-α driven by an extended human region of the human G6Pase promoter (GPE), which showed improved G6Pase-α expression and complete normalization of G6Pase-α deficiency in the liver for 24 weeks [9]. The use of human G6Pase promoter regulates G6Pase-α expression preventing potential overexpression of the enzyme. Further, the use of G6Pase promoter has some advantages including expression in other target tissues besides the liver such as the kidney and with limited cytotoxic T-cell response. The extensive non-clinical studies published to date are expected to lead to human clinical studies [10, 11].

33.2.2 Pompe Disease

Pompe disease (also known as glycogenosis type II (GSDII)) is a glycogen storage disorder due to a deficiency or absence in acid α-glucosidase (GAA) [12, 13]. This autosomal recessive disorder has been traditionally thought to occur in every 1:40,000 live births and is characterized by glycogen accumulation causing muscle atrophy and severe cardiopulmonary dysfunction [14]. Recently, newborn screening efforts have discovered a significantly higher incidence of up to 1:9500. The severity of the disease is inversely correlated to GAA activity, and therefore, there are two broad categories of note—severe/early onset that results from a complete absence of the GAA enzyme and mild/late onset that results from 5 to 15% of normal GAA activity [15–17]. Common clinical pathology includes profound weakness and hypotonia, cardiac hypertrophy, and cardiorespiratory failure in patients with severe-/early-onset Pompe disease, as well as skeletal muscle and neuronal dysfunction leading to ventilatory insufficiency [16, 18–20].

Current treatment for Pompe disease involves replacement of the missing enzyme by repeated protein infusions, known as enzyme replacement therapy (ERT). However, ERT does not cross the blood-brain barrier only mitigating the cardiomyopathy and skeletal myopathy associated with Pompe disease [14, 17, 21].

For a more detailed description of the non-clinical and clinical studies for Pompe disease, please refer to Chap. 44.

33.2.3 Glycogenosis Type III

33.2.3.1 Clinical Aspects of Glycogenosis Type III

Glycogenosis type III (GSDIII), also known as debrancher enzyme deficiency and Cori-Forbes disease, is a glycogen storage disease due to a mutation in the Agl gene triggering a deficiency in the glycogen debranching debrancher enzyme (GDE). GDE, in tandem with glycogen phosphorylase, is responsible for degrading non-membranous glycogen [22–26]. This genetic deficiency causes an accretion of cytoplasmic glycogen disturbing glucose homeostasis and the accumulation and storage of phosphorylase-limit dextrin (PLD) in the tissues [3, 26–28]. There are two types of GSDIII—GSDIIIa and GSDIIIb. The difference between the two is GSDIIIa involves the liver and muscle, while GSDIIIb only involves the liver [3]. This rare autosomal recessive disorder occurs in 1:100,000 births [28, 29].

Clinical signs and symptoms vary from one patient to the next depending on disease phenotype; however, the most common symptoms for a patient with GSDIIIa are progressive skeletal myopathy, hypoglycemia, hepatomegaly, hyperlipidemia, cardiac hypertrophy, short stature, and elevated serum concentrations of liver transaminases (ALT, AST, and ALP) and muscle enzymes (creatine kinase, CK) [3, 23, 25, 30]. Of note, progressive skeletal myopathy is one of the major causes of morbidity in this population. Muscle weakness begins in late childhood and becomes more predominant with age [23, 25, 31, 32].

Currently, there is no effective treatment or adequate therapy for GSDIII, only dietary interventions. In efforts to promote normoglycemia, control hypoglycemia, and prevent hyperketonemia, patients with GSDIII are encouraged to either have frequent meals, high in carbohydrates with additional proteins, or nocturnal gastric feedings. Additionally, a high-protein diet is recommended for patients with myopathy, growth retardation, and cardiomyopathy [3, 23, 26, 33, 34].

33.2.3.2 Cori-Forbes Disease Animal Models for Future Research

To date there are only two GSDIIIa animal models—the first is a large animal model in curly-coated retriever (CCR) dogs and the second is a murine model in Agl knockout mice.

The study identifying the GDE frameshift mutation in CCR dogs was initially conducted by Gregory et al. [3, 27]. They followed a liter of six purebred CCR dogs for 42 months [27]. Of the six dogs, both females had elevated aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and CK ~~CPK~~ levels. Following liver biopsies, both affected dogs were found to have hepatomegaly and severe glycogen accumulation in their liver hepatocytes. Both CRC females also exhibited signs of skeletal myopathy. Serum enzymes and glucose concentration indicated leakage of liver enzymes and hypoglycemia. Biochemical studies showed an increase in glycogen content in the liver. Glycogen structure analysis exhibited glycogen with short outer chains and suggested debrancher enzyme defi-

ciency [27]. The protein coding sequence for the CCR Agl cDNA showed a deletion of an adenine in exon 32 leading to the curtail of the debranching enzyme by 126 amino acids [3, 27]. Absence of enzyme activity in the muscle and liver samples confirmed the GSDIIIa diagnosis [27].

A long-term follow-up study was later conducted by Brooks et al. [30] on the same six CCR dogs with GSDIIIa used by Gregory and colleagues aimed at collecting biochemical data and further information on organ and tissue involvement [27, 29].

Brooks et al. found glycogen deposition in the diaphragm, gastrocnemius, and quadriceps of all three dogs and in the cardiomyocytes of the eldest dog. Hepatic and muscle glycogen content decreased over time in conjunction with a decrease in CK levels in all affected canines. Biochemistry and urinary biomarker analysis showed liver enzymes AST and ALT decreasing over time, a sign of progressive hepatic disease [29, 35, 36]. The trend of decreased liver enzymes is like that found in GSDIIIa patients [32, 37, 38]. Hepatomegaly was also found in three of the affected dogs and was shown to decrease over time as in affected patients. Heart-associated abnormalities in this cohort were limited to the single case of cardiomyocytes in the eldest dog providing some supporting evidence that a high-protein diet can slow the progression of cardiac disease in GSDIIIa patients [29, 39, 40].

Likewise, Yi and colleagues performed a 16-month observational study to better understand the GSDIIIa phenotype and pathophysiology in eight curly-coated retrievers [25]. Biochemistry panels revealed gradually increasing ALT, AST, ALP, and CK with increasing age, and biopsies revealed high glycogen content in the liver and muscle of all dogs. Elevated AST, more so ALT, was found to be a strong indicator of liver impairment. Liver glycogen content was more than fourfold of that found in controls; however, its eventual decrease could be attributed to an increase in fibrous tissue. Liver biopsies revealed moderately smooth surfaces at baseline with an increase in the number of nodules on the surface with age. By 16 months, livers were engorged with evidence of cirrhosis and hepatocellular disorganization. As the CCR colony aged, glycogen accumulation increased in the skeletal muscle causing symptomatic myopathy much like in patients with GSDIIIa [23, 25].

In addition to these large animal models, two murine models have been generated to understand the pathology of GSDIII and recapitulate humanlike disease signs and symptoms—the first by Liu et al. [26] and the second by Pagliarani et al. [41].

In efforts to find a small animal model to study the pathology of GSDIIIa, Liu and colleagues generated the first GDE-deficient murine model that reiterated humanlike GSDIIIa by removing exons downstream of exon 5 deleting 1310 amino acids in the Agl gene, effectively deleting the GDE [26]. Muscle weakness was detected in both the forelimb and hind legs, and blood chemistry revealed elevated AST, ALT, ALP, and CK levels in affected mice. Hypoglycemia was present just as it is in human GSDIIIa patients; however, there was no evidence of hyperlipidemia [23, 26]. Western blot analysis illustrated the lack of GDE protein and a reduction of GDE enzyme activity in the knockout mice. Hepatomegaly, enlarged hepatocytes, and liver fibrosis were all observed, and heavy glycogen

accumulation was found in the liver, in the heart, and some in the skeletal muscle. In fact, glycogen accumulation by weight in the liver, heart, diaphragm, quadriceps, and gastrocnemius in homozygous mice was 2–20 times higher than in wild-type or heterozygous mice [26].

Pagliarani et al. also generated a novel Agl knockout (KO) mouse by introducing a Neo cassette that deleted the last 114 amino acids of the debranching enzyme containing the glucosidase domain and the glycogen-binding domain [41]. No GDE activity was found in the skeletal muscle, brain, liver, and heart of the Agl-KO mice. Livers of the Agl-KO mice were also found to be enlarged; however, there were no nodules, unlike in Yi et al.'s canines. Quantification revealed a large amount of glycogen in the skeletal muscle, liver, and heart tissue. Likewise, small periodic acid-Schiff (PAS)-positive glycogen deposits were found in the cerebellum of the Agl-KO mice. Of note glycogen accumulation was found in the diaphragm and tongue. Glycogen infiltration in both of these muscles has also been found in patients with GSDIII and in the canine model by Yi and colleagues' [25, 41, 42]. Additionally, AST, ALT, CK, and ALP levels were found to be significantly elevated. Muscle performance evaluations revealed Agl-KO to be slower and less coordinated. The decrease in coordination and defective equilibrium could be attributed to the PAS-positive glycogen deposits found in the cerebellum, a feature of the hindbrain that plays a critical role in motor coordination and balance [41]. Unlike in previously reported GSDIII animal models [25, 26], there were no signs of liver fibrosis present in the Agl-KO mice. Additionally, unlike in human GSDIII patients, hyperlipidemia was not observed. The Agl-KO murine models generated by Pagliarani et al. and Liu et al., as well as the canine models generated by Yi et al. and Gregory et al., all proved to be reliable models for GSDIIIa and suitable for the development of future gene therapies [23, 26, 41].

33.2.4 Glycogenosis Type V

33.2.4.1 Clinical Aspects of Glycogenosis Type V

Glycogenosis type V (GSDV), also known as myophosphorylase deficiency and McArdle disease, is a glycogen storage disease due to a mutation in Pygm gene, encoding the skeletal muscle isoform of glycogen phosphorylase GP-MM, also known as myophosphorylase [43, 44]. Myophosphorylase is responsible for initiating the breakdown of glycogen in muscles, without which patients are unable to obtain energy from their endogenous muscle glycogen stores impairing muscle function and basic cellular events [43, 45, 46]. This recessive disorder is the most common muscle glycogenolysis and one of the most frequently encountered genetic myopathies [43, 44].

Clinically, patients with GSDV present with exercise intolerance in the form of reversible, acute crises of premature muscle fatigue and contracture, which can be accompanied by a breakdown of muscle tissue releasing myoglobin into the blood inducing myoglobinuria [43, 44]. Although muscle glycogenolysis is blocked,

glucose utilization is not; therefore, patients with McArdle disease can still utilize blood glucose. Sufficient oral carbohydrates prior to exercise can improve exercise tolerance [43, 47].

33.2.4.2 McArdle Disease Animal Models for Future Research

Currently, there are only three GSDV animal models [3]. The first animal model was discovered in Charolais cattle which had a C-to-T substitution, a conservative mutation that caused arginine, and aliphatic amino acid, to change into tryptophan, an aromatic amino acid at codon 489 [3, 48].

The second model was discovered in a Merino sheep flock by Tan and colleagues [3, 49]. In sequencing the ovine cDNA, polymorphisms were detected in two of the six overlapping fragments using single-strand conformation polymorphism analysis. The second of the polymorphisms contained an eight-base deletion in exon 20 that lead to the removal of the last 31 amino acids from the protein. The creation of this splice site mutation was the most likely cause of the ovine McArdle's disease and is similar to a previously reported case in humans [49, 50].

Following the sequencing of the GSDV ovine myophosphorylase cDNAs, the cDNA was found to be 91.1% homologous to the human sequence and 95.7% homologous at the amino acid level [49, 51]. Additionally, the Merino sheep have similar anthropometric proportions to humans displaying similar GSDV disease phenotypes ideal for testing new therapeutic approaches [49].

The third is the murine model that has been described by both Nogales-Gadea et al. and Brull et al. [3, 44, 52]. Nogales-Gadea and colleagues developed a knock-in mouse model by replacing the wild-type Pygm with a modified allele carrying a p.R50X mutation, the most common Pygm mutation among Caucasian GSDV patients [44, 53]. Biochemical and histochemical analysis revealed glycogen phosphorylase activity was absent in the skeletal muscle of all knock-in mice studied. Periodic acid-Schiff staining of glycogen findings reported accumulation of glycogen beneath the cell membranes of muscle cells like those reported in patients with McArdle disease. Following a wired grip test and the treadmill test, the p.R50X/p.R50X mice showed clear exercise intolerance and exercise-induced myoglobinuria, a clinical phenotype that is present in all GSDV patients [43, 44].

To further analyze the effects of myophosphorylase dysfunction in different muscle types, Brull and colleagues used the knock-in mouse model developed by Nogales-Gadea et al. to analyze the consequences of the absence of myophosphorylase on slow-twitch, intermediate, and fast-twitch muscles [44, 52]. The study corroborated the finding that knock-in mice exhibited a McArdle-like phenotype in the same way as the line developed by Brull et al., confirming impaired exercise capacity and hyperCKemia, and proved that glycogen regulatory enzyme expression differed depending on the muscle fiber type. The fast-twitch muscles extensor digitorum longus (EDL) exhibited the highest catabolic enzyme levels and glycogen content, while the oxidative, or slow-twitch, muscles (soleus) displayed the highest glycogen anabolic enzyme levels [44].

All three models recapitulate the main features of human GSDV and can serve as valuable tool for evaluating which of the current delivery systems would be best for future gene therapy studies of McArdle disease [44, 49, 52, 53].

33.2.4.3 Adenovirus (AdV) and AAV Vectors in the Ovine Model of McArdle Disease

Presently, there is no definitive treatment for McArdle's disease; however, new therapeutic approaches are being evaluated using the animal models described in Sect. 33.2.4.2. One such study was conducted by Howell et al., who used the in vivo application of myophosphorylase gene transfer by using adenovirus 5 (AdV5) vector and AAV2 in the ovine model of McArdle's disease [54].

For the experiments using the AdV constructs, 16 affected neonatal lambs were injected with the AdV5 carrying LacZ reporter gene, and the other 19 were injected with AdV5 carrying human myophosphorylase cDNA. For the experiments involving the use of the AAV constructs, AAV2 was used as a vector to transport either the LacZ reporter gene or the human myophosphorylase cDNA into the semitendinosus muscle of 26 affected lambs. AdV5 constructs were regulated and transduced using either a CMV or a Rous sarcoma virus (RVS), and the AAV2 vector was regulated using a CMV promoter [3, 54].

Both AdV5 and AAV2 vectors produced expression of functional myophosphorylase in the area surrounding the injection site and were able to transduce ovine skeletal muscle; however, AdV5 was identified as the better vector for transduction of phosphorylase-positive fibers. AdV5 had both a higher percentage of sections with phosphorylase-positive fibers and a higher percentage of sections with 1000 or more positive fibers following injections. Expression of functional myophosphorylase lessened with time [3, 54].

33.3 Mitochondrial Disorders

Mitochondrial disorders are collectively one of the most common types of inherited metabolic disorders affecting around 1:5000 live births [55–59]. The pathogenic mechanisms of this multisystemic condition are heterogeneous, and the variability of disease onset makes generating novel therapies challenging [2, 60]. Clinically, organs that are highly dependent on aerobic metabolism are most likely to be affected. The mitochondrial dysfunction as a result of disruption in ATP synthesis in the skeletal muscle and the nervous system is most commonly expressed as exercise intolerance [55, 61].

Symptomatic therapy has become the standard of care for mitochondrial disorders since no effective therapies have been identified.

33.3.1 Barth Syndrome

33.3.1.1 Clinical Aspects of Barth Syndrome

Barth syndrome (BTHS, also called 3-methylglutaconic aciduria type II) is a rare (prevalence of 1:300,000–400,000 live births) X-linked disorder caused by defects in the *tafazzin* (*TAZ*) gene. The mutation results in the loss of function of tafazzin, an acyltransferase highly expressed in the cardiac and skeletal muscles and involved in the metabolism of the cardiolipin, one of the major phospholipids of the inner mitochondrial membrane. Tafazzin deficiency results in abnormal cardiolipin content and a reduction in mature tetralinoleoyl-cardiolipin [62, 63]. Clinical presentation of the disease is variable, but symptoms include cardiomyopathy, extreme fatigue, exercise intolerance, skeletal myopathy, neutropenia, early diminished growth, and associated biochemical abnormalities such as low plasma cholesterol and 3-methylglutaconic aciduria. Currently there are no effective therapies for BTHS other than supportive cardiac care [64–66].

The natural history of BTHS is variable; however, the onset of cardio-skeletal myopathy typically occurs by 2 years of age with fluctuating morbidity severity throughout childhood, adolescence, and young adulthood [64]. BTHS is a particularly devastating disease as it is often fatal in childhood, with the highest risk during infancy and adolescence [67]. Even after infancy, many patients experience unpredictable deterioration in cardiac function in adolescence or young adulthood, resulting in premature death.

33.3.1.2 Preclinical Studies of Barth Syndrome

Several models have been generated, including yeast, flies, zebrafish, and patients' cell lines in order to understand the role of TAZ and the mechanisms involved in TAZ deficiency. However, advances in development and testing of potential therapies came from a partial knockdown of TAZ function via RNA interference technology (TaconicArtemis GmbH, Cologne, Germany). The model shows alteration of cardiolipin profiles, abnormal mitochondrial morphology, skeletal muscle weakness, and cardiomyopathy [68].

Preliminary data on the effect of AAV-mediated gene therapy in the shRNA knockdown model was presented at the 20th American Society of Gene and Cell Therapy, Washington, DC, 2017 [69]. Suzuki-Hatano et al. compared intravenous injection of $1x10^{13}$ vg/kg of dsAAV9-desmin-TAZ, dsAAV9-cytomegalovirus-TAZ, or dsAAV9-tafazzin-TAZ. Mice from all treatment groups displayed improvements in multiple parameters including cardiac dimensions, body weight, muscle strength and fatigability, oxygen consumption, and mitochondrial quality. Results from this preliminary work suggest that gene therapy is a promising therapeutic treatment for Barth syndrome.

33.3.2 Friedreich's Ataxia (FRDA)

33.3.2.1 Clinical Aspects of FRDA

FRDA is an autosomal recessive and progressive condition caused by abnormal expansion of GAA repeat in intron 1 of the *frataxin* (*FXN*) gene. The mutation results in reduction of frataxin levels in mitochondria, a mitochondrial protein that is responsible for regulating iron-sulfur cluster enzymes within the cell. Reduced levels of frataxin lead to iron accumulation and oxidative stress [70, 71].

The clinical manifestation of the disease is variable, but symptoms include slowly progressive ataxia, dysarthria, muscle weakness, spasticity particularly in the lower limbs, scoliosis, bladder dysfunction, absent lower limb reflexes, and loss of position and vibration sense. In addition, approximately two thirds of individuals with FRDA have cardiomyopathy, as many as 30% have diabetes mellitus, and approximately 25% have an "atypical" presentation with later onset or retained tendon reflexes [72, 73]. Individuals with typical FRDA develop progressive ataxia with onset from early childhood through to early adulthood, starting with poor balance when walking, followed by slurred speech and upper-limb ataxia. The rate of progression of FRDA is variable with an average time from symptom onset to wheelchair dependence around 10 years and with a more rapid progression in those with earlier disease onset. The mean and the median age of death is 36.5 and 30, respectively. The most common cause of death is related to cardiac events [74].

33.3.2.2 Preclinical Studies of FRDA

Currently, there is no definitive therapy for FRDA. Over the past several years, multiple mouse models of frataxin deficiency have been generated [75] including a knock-in knockout model and repeat expansion knock-in model [76]; transgenic mice containing the entire Fxn gene within a human yeast artificial chromosome, YG8R and YG22R [77–79]; as well as a conditional Fxn knockout mouse, including the cardiac-specific [80, 81] and a neuron-specific model [82]. These transgenic and heterozygous knockout FRDA animal models have the limitations to be mildly symptomatic and/or restricted to specific tissues. Recently, a frataxin knockdown model via RNA interference technology, like the shRNA BTHS model, was developed. The shRNA model presents multiple features observed in human patients and enables to control onset and progression of the disease phenotype by modulation of frataxin levels [83].

Presently, the potential of gene therapy for the treatment of FRDA has been investigated using several approaches. Perdomini et al.[84] showed that intravenous (IV) delivery of an adeno-associated virus rh10 vector expressing human frataxin could prevent the onset of cardiac disease in a conditional model of FA with partial frataxin depletion in cardiac muscle. Although this approach may

pave the way for an isolated cardiac gene therapy-based clinical trial, it does not address the neurological degeneration, which is one of the most debilitating aspects of FA. Similar work was described after intraperitoneal injection of AAV9-hFXN [85].

The use of lentivirus-meditated frataxin gene delivery to reverse genome instability in Friedreich ataxia patient and mouse model fibroblasts has also been tested [86]. Lentivirus frataxin gene delivery to FRDA patient and YG8sR FRDA mouse model fibroblast cells induced long-term overexpression of FXN mRNA and frataxin protein levels with reduced double-strand break levels toward normal. Furthermore, γ-irradiation of FRDA patient and YG8sR cells revealed impaired double-strand break repair that was recovered on FXN gene transfer.

Ouellet et al. recently reported the use of the clustered regularly interspaced short palindromic repeat (CRISPR) system, using either SpCas9 or SaCas9 in combination with a pair of single-guide RNA (sgRNA), to delete the GAA trinucleotide repeats in vitro in YG8R26 and YG8sR29 mouse fibroblasts and in vivo in a YG8R-derived mouse line. The authors identified the YG8sR as a more suitable in vitro model to study CRISPR edition for FRDA as it has only one copy of the human FRDA FXN transgene. The authors suggest the use of YG8sR mouse model to study GAA correction using an AAV vector coding for the SaCas9 and two sgRNAs targeting the pre- and the post-GAA repeat [87].

Preliminary data on the effect of AAV-mediated gene therapy in the shRNA knockdown model was presented at the 20th American Society of Gene and Cell Therapy [88]. Nair et al. showed that IV injection of AAV9-CBA-FXN prevented weight loss and death in the conditional knockdown model. Additional work from the same group was presented at the International Ataxia Research Conference. Byrne and Corti showed the combination of IV and intrathecal (IT) injections of AAV9-CBA-FXN in the conditional knockdown model and in nonhuman primates to treat both the cardiac and neurological symptoms of the disease. Besides the dual route of administration, an additional novelty of this approach is the implementation of a large-scale manufacturing process using a recombinant herpes simplex virus (rHSV) for AAV production described in Chap. 15.

33.4 Fatty Oxidation Defects

Mitochondrial fatty acid oxidation defects account for a substantial amount of both acute and chronic liver, heart, and skeletal muscle diseases in patients of all ages [89]. Clinically, the defects mimic Reye's syndrome and are characterized by episodic nonketotic hypoglycemia and periods of decompensation when carbohydrate levels are low [90]. The fatty acid oxidation disorder reviewed in this section will be very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency.

33.4.1 Clinical Aspects of VLCAD

According to Leslie and colleagues, VLCAD deficiency, a deficiency affecting between 1:40,000 and 1:120,000 persons, is associated with three phenotypes including severe-/early-onset multisystem failure, hypoketotic hypoglycemia, and later-onset recurrent rhabdomyolysis [91, 92].

Clinically, all three phenotypes present differently. The first phenotype presents as a Reye-like syndrome characterized by severe cardiomyopathy, pericardial effusion, muscle weakness, enlarged livers, and periodic hypoglycemia. This phenotype is often considered lethal without early intervention. The second phenotype lacks cardiomyopathy but presents with hepatomegaly and episodic hypoglycemia due to hypoketosis, which refers to reduced levels of ketones produced during the breakdown of fats and used for energy. The final and most common phenotype is characterized by exercise intolerance resulting in rhabdomyolysis accompanied by an increase in serum and urine myoglobin [89, 91].

33.4.2 AAV Gene Therapy in Murine Models for VLCAD

Currently, there is no curative treatment for VLCAD, only dietary therapies [89, 91]. In an effort to find a human gene replacement strategy for VLCAD deficiency, Merritt and colleagues designed an AAV stereotype 2/8 vector (AAV2/8) containing human VLCAD cDNA (AAV8-hvLCAD). AAV8-hvLCAD, regulated by a CMV promoter expressed in murine hepatocytes, was administered into the tail vein of VLCAD knockout mice in vivo [93].

Postinjection Western blot analysis revealed hvLCAD in the liver of the early treatment group but absent in the late treatment group. However, the histological analysis of both treatment groups revealed no adipose degeneration. PCR analysis demonstrated more vector sequences and a greater proportion of hepatocytes expressing AAV8-hvLCAD in the VLCAD knockout mice than in the VLCAD-deficient mice. Furthermore, strong expression of hvLCAD was seen in the heart and liver with further increases in the heart as time passed. However, the vector seemed unable to cross the blood-muscle barrier seeing as there was no expression in the skeletal muscle. VLCAD knockout mice also exhibited a reduction in acylcarnitines and demonstrated fasting serum glucose levels with no hypoglycemia [93]. Merritt et al. were able to demonstrate limited biochemical correction in VLCAD-deficient mice.

Keeler et al. also demonstrated a transduction profile for AAV inclusive of a wide distribution of tissues using AAV9. The authors treated VLCAD$^{-/-}$ mice with an AAV9-CBA-VLCAD vector and compared them to both VLCAD$^{-/-}$ PBS controls and VLCAD$^{+/+}$ mice. The AAV9-treated VLCAD$^{-/-}$ mice displayed concentrations of rAAV9-VLCAD in the liver and cardiac and skeletal muscle and transduced a wider network of organs than seen with AAV8-hvLCAD. Additionally, AAV9-treated

VLCAD$^{-/-}$ mice also demonstrated both blood and tissue biochemical correction. Acyl carnitine accumulation in the blood was largely reduced in the rAAV9-treated group as well as in the liver, skeletal (EDL and soleus), and cardiac muscle [94].

Phenotypically, the AAV9-treated VLCAD$^{-/-}$ mice also saw disease correction when compared to both VLCAD$^{-/-}$ PBS controls and VLCAD$^{+/+}$ mice. The AAV9-treated VLCAD$^{-/-}$ mice challenged by cold exposure were able to maintain average core temperature, did not display lethargy or muscle weakness, and were able to maintain glucose levels without signs of hypoglycemia [94]. Taken together, the results do show promise using AAV9 gene therapy as a possible treatment for this Reye-like syndrome.

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Chapter 34
Elimination of Mutant Mitochondrial DNA in Mitochondrial Myopathies Using Gene-Editing Enzymes

Sandra R. Bacman and Carlos T. Moraes

Abstract Mitochondrial diseases form a genetically and clinically heterogeneous group of disorders that result in the dysfunction of the mitochondria oxidative phosphorylation (OXPHOS). This system is responsible for the generation of the cellular energy required for the function of cells, tissues, and organs. Skeletal and cardiac muscle dysfunction is also a common feature of mitochondrial diseases. Effective treatments have not been developed and are mostly related to supportive management and palliative therapies. Most pathogenic mitochondrial DNA (mtDNA) mutations are in a heteroplasmic state, and high levels of mutated mtDNA within a cell are required to exceed a critical threshold to cause a phenotype. Therefore, the goal of a therapeutic intervention would be to eliminate or decrease the amount of mutated mtDNA below a certain threshold to avoid clinical and biochemical manifestations of the disease. Our group and others have made several advances over the last 15 years inducing heteroplasmy shift as a potential strategy to treat mtDNA disorders. Although mitochondrial-targeted restriction endonucleases can efficiently change mtDNA heteroplasmy both ex vivo and in vivo, this approach can be used therapeutically only if a unique restriction site is created by a mtDNA mutation. To overcome this, non-specific endonucleases targeted to mitochondrial mutations have been developed using gene-editing nucleases such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). These are being used to reduce mutant mtDNA in the muscle and heart of mouse models. Although some limitations and concerns exist, future experiments should make this approach safe to treat patients.

Keywords Mitochondria · Gene therapy · Myopathies · Endonucleases · TALEN ZFN

S. R. Bacman · C. T. Moraes (✉)
Department of Neurology, University of Miami Miller School of Medicine, Miami, FL, USA
e-mail: SBacman@med.miami.edu; cmoraes@med.miami.edu

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34.1 Introduction

Mitochondrial diseases form a genetically and clinically heterogeneous group of disorders that result in the dysfunction of the mitochondria oxidative phosphorylation (OXPHOS), which is responsible for the generation of the cellular energy required for the function of cells, tissues, and organs [1]. As skeletal and cardiac tissues demand high energy, mitochondrial diseases present very frequently as myopathies and cardiomyopathies [2]. They also present as syndromes with variable clinical features [3] with onset in either adult or childhood [4].

Cardiomyopathies can present with arrhythmias and conduction defects, pulmonary hypertension, pericardial effusion, dilated aortic root, and coronary heart disease [5]. Myopathies and cardiomyopathies are frequent manifestations of mitochondrial diseases associated with defects in OXPHOS, involving complex subunits and their assembly factors, mitochondrial tRNAs, rRNAs, ribosomal proteins, translation factors, mtDNA maintenance, and CoQ_{10} synthesis [1]. For example, complex I deficiency can present with hypertrophic cardiomyopathy as the sole symptom or associated with a multi-organ disease. Cardiomyopathy can be caused by mutations in the mitochondrial DNA involving complex I subunits (MTND1 and MTND5) or nuclear gene mutations encoding subunits (NDUFS2, NDUFV2, NDUFA2, and NDUFA4) [6] or assembly factors (ACAD9 and NDUFAF1) [7, 8]. Mutations in complex II subunit genes (SDHA and SDHD) have been described in patients with hypertrophic and dilated cardiomyopathies [9]. Mutations in the MTCYB gene that encodes cytochrome b were also reported in patients with hypertrophic and dilated cardiomyopathies [10] as well as in patients with isolated myopathies and exercise intolerance [11, 12]. Mutations in COX6B1 manifest with encephalomyopathy, hydrocephalus, and cardiomyopathy [13]. Heart involvement was also observed in patients with mutations in complex IV assembly factor genes (COX10, SURF1, SCO2, and C2orf64) [13–15].

Mutations in mitochondrial tRNA genes, such as the m.8344A> G in MTTK (encoding mitochondrial tRNA lysine) responsible for myoclonic epilepsy with ragged-red fiber (MERRF) [16], or MTTL1 m.3243A> G mutation, causing MELAS syndrome [17–19], were found in patients with multi-organ mitochondrial diseases or isolated cardiomyopathies [20]. Mutations in the mt-$tRNA^{Ala}$ are also associated with isolated myopathy [21].

Mutations in mitochondrial-encoded MT-ATP6 (mitochondrial ATP synthase 6), associated with maternally inherited Leigh syndrome (MILS) [22], neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP) [23], and chronic progressive external ophthalmalgia (CPEO) [24], caused complex clinical presentations also including severe heart and skeletal muscle involvement [25].

Skeletal muscle dysfunction is also a common feature of mitochondrial diseases, resulting in progressive external ophthalmoplegia and exercise intolerance [2, 26]. Mutations in the mtDNA commonly cause myopathies and are diagnosed by the presence of ragged-red fibers and cytochrome oxidase-negative fibers in muscle biopsies [27, 28]. Large deletions of mtDNA as well as point mutations in some

tRNA genes are frequently observed in patients with PEO or a more severe and generalized Kearns-Sayre syndrome [29, 30]. Mutations in cytochrome b have been associated with exercise intolerance, proximal weakness, and occasional myoglobinuria [31, 32].

Although the speed of mitochondrial research has increased since the first description of pathogenic mtDNA mutations in the late 1980s, and great progress has been made in understanding the fundamental pathogenic processes causal of mitochondrial disease, effective treatments have not been developed and are mostly related to supportive management and palliative therapies [33, 34]. Therefore, gene therapy is an attractive therapeutic strategy for the treatment of mitochondrial diseases. In sections below, we review strategies that have been developed to replace the mutated gene and to manipulate mtDNA heteroplasmy by restriction enzymes and gene-editing enzymes and selective elimination of mtDNA mutations in the germline by genome editing. We also discuss limitations, concerns, and future direction of mitochondrial disease gene therapy.

34.2 Re-expression of Mutated Genes

Expressing the wild-type gene to correct mutation in critical organs or tissues and produce normal proteins has long been used to treat genetic diseases [34]. One example is its applicability to mitochondrial neurogastrointestinal encephalopathy (MNGIE) syndrome, which is caused by mutations in the thymidine phosphorylase (TYMP) gene. The TP deficiency results in systemic accumulation of thymidine and deoxyuridine, interfering with mitochondrial DNA replication and leading to mitochondrial dysfunction. Previous treatments available for MNGIE patients were allogeneic hematopoietic stem cell transplantation, which was associated with high morbidity and mortality [35, 36]. Other work done on a murine model of MNGIE demonstrated that gene therapy-expressing TYMP was a useful approach to normalize the biochemical abnormalities using either ex vivo transduction of a lentiviral vector targeted at hematopoietic stem cells [37] or an adeno-associated virus vector (AAV) with targeted expression at the liver [38]. TYMP was able to restore nucleoside homeostasis in the animal model of MNGIE during the entire life of the mice using AAV2/8 vector targeted to the liver with long-term restoration of dCTP and dTTP levels in plasma and tissue for up to 8 months [39]. The nucleoside reduction achieved by the treatment prevented deoxycytidine triphosphate (dCTP) depletion, which is the limiting factor affecting mtDNA replication in this disease [40].

In another example of re-expressing mutated genes, the heart-muscle isoform of the mitochondrial adenine nucleotide translocator (ANT1), associated with mitochondrial myopathy, was reintroduced in a mouse model. An AAV2 vector carrying the mouse Ant1 cDNA was used to transduce muscle cells and muscle from Ant1 mutant mice, which has a mitochondrial myopathy. AAV-ANT1 injection in the heart and muscle resulted in long-term, stable expression of the Ant1 transgene and functional ADP/ATP carrier increasing the mitochondrial export of

ATP and reversing the histopathological changes associated with the mitochondrial myopathy. This approach has the potential to provide symptomatic relief for the ophthalmoplegia and ptosis resulting from paralysis of the extraocular eye muscles caused by mutations in the Ant1 gene [41].

Ethylmalonic encephalopathy (EE) is an invariably fatal disease, characterized by the accumulation of hydrogen sulfide, a highly toxic compound. ETHE1 "ethylmalonic encephalopathy 1 protein and per sulfur dioxygenase (SDO)" encodes for a protein which takes part in the mitochondrial pathway that converts sulfide into harmless sulfate. A mouse model was described lacking the Ethe1 gene, Ethe1(−/−) that showed features of ethylmalonic encephalopathy with thiosulfate excreted in massive amounts in urine, resembling humans with ethylmalonic encephalopathy. Sulfur dioxygenase activity was absent in Ethe1(−/−) mice [42]. Using AAV2/8-mediated, ETHE1-gene transfer to the liver of Ethe1(−/−) mouse resulted in full restoration of sulfur dioxygenase (SDO) activity, correction of plasma thiosulfate, and clinical improvement with increased survival [43].

34.3 Manipulation of mtDNA Heteroplasmy

Most pathogenic mitochondrial DNA mutations are in a heteroplasmic state (mutated and WT mtDNA coexist in the same cell). The levels of mtDNA mutation within a cell or tissue required to exceed a critical threshold to cause a phenotype are relatively high (>70% mutant) [44, 45]. As most pathogenic mutations of mtDNA behave as "recessive-like" mutations, the principal goal of therapeutic intervention would be to eliminate or decrease the amount of mutated mitochondrial DNA below a certain threshold to avoid clinical and biochemical manifestations of the disease. This threshold level varies for each mutation and tissue and is dependent on several factors including OXPHOS requirements [46]. In general, mtDNA deletions cause disease at lower tissue mutation loads than point mutations, 60% versus 80% [33]. Even within the tissue of the same individual, higher mutation loads are associated with strong biochemical phenotypes [47].

34.3.1 Manipulating mtDNA Heteroplasmy with Specific Restriction Endonucleases

Heteroplasmy shift is a potentially useful strategy to treat mtDNA disorders, and our group has made several observations over the last 15 years. Table 34.1 summarizes the models published. The first study using restriction endonucleases (RE) to modify mtDNA heteroplasmy was published by Srivastava and Moraes [48]. In this case, *Pst*I was used to differentiate between mouse and rat mtDNA (rat mtDNA does not have *Pst*I sites). This interesting model allowed RE to be manipulated to reduce the levels of the mouse mtDNA in xenomitochondrial cybrids harboring both rat and mouse mtDNA.

Table 34.1 Comparison of different endonucleases targeted to mitochondria

Endonuclease	Target	Vector/delivery	In vitro	In vivo	Comments	Citation
PstI (2 sites in LM[mouse], none in NRK[rat])	LM/NRK hybrid	pCDNA3- CMV promoter/transfection	Yes	No	mtDNA heteroplasmy shift	Srivastava and Moraes [48]
SmaI/XmaI (1 site in m.8993 T>G cybrids)	m.8993T>G cybrids NARP/ MILs mutation MT-ATP6	rAd5-CMV promoter/ viral infection	Yes	Yes	mtDNA heteroplasmy shift OXPHOS improvement No nuclear damage	Tanaka et al. [49] Alexeyev et al. [52]
ApaLI (1 site in BALB/ none in NZB)	NZB/BALB derived hepatocytes	RU486 inducible system/ transfection	Yes	No	mtDNA heteroplasmy shift	Bayona-Bafaluy [53]#240
	NZB/BALB skeletal muscle and brain	rAd5/rAAV1,2-CMV promoter/focal injection	No	Yes	mtDNA heteroplasmy shift No mtDNA depletion	Bayona-Bafaluy et al. [101]
	NZB/BALB heart and liver	rAAV6/rAd5-CMV promoter/systemic intra-jugular	No	Yes	mtDNA heteroplasmy shift No mtDNA depletion	Bacman et al. [56]
	NZB/BALB neonates heart and skeletal muscle	rAAV9/rAd5-CMV promoter systemic IP and temporal	Yes	Yes	mtDNA heteroplasmy shift No mtDNA depletion	Bacman et al. [57]
	NZB/BALB oocytes and embryos	mRNA/injection	Yes	Yes	mtDNA heteroplasmy shift Normal offspring	Reddy et al. [100]
ScaI (3 sites in BALB/5 sites in NZB)	NZB/BALB liver and skeletal muscle	rAd5-CMV promoter/ systemic intra-jugular and focally	No	Yes	mtDNA heteroplasmy shift Transient COX deficiency and mtDNA depletion	Bacman et al. [55]
TALEN						
5 kb-common deletion (CD) (m.8483_13459 del4977)	Human osteosarcoma cybrids with CD	Two mitoTALENs monomers CMV promoter/transfection	Yes	No	mtDNA heteroplasmy shift	Bacman et al. [89]

(continued)

Table 34.1 (continued)

Endonuclease	Target	Vector/delivery	In vitro	In vivo	Comments	Citation
m.G14459 G>A	m.G14459A cybrids MT-ND6/ LHOND	Two mitoTALENs monomers CMV promoter/transfection	Yes	No	mtDNA heteroplasmy shift Complex I recovery Transient mtDNA depletion	Bacman et al. [89]
	Fused mouse oocytes with cybrids m.G14459A MT-ND6/ LHOND	Two mitoTALENs monomers CMV promoter/mRNA injection	Yes	Yes	mtDNA heteroplasmy shift Reduction in mtDNA copy number	Reddy et al. [100]
m.8344 A>G	m.8344 A>G cybrids tRNALys/MERRF	Two mitoTALENs monomers CMV promoter/transfection	Yes	No	mtDNA heteroplasmy shift OXPHOS improvement	Hashimoto et al. [94]
m.13531G>A	m.13531G>A cybrids ND5/ MELAS/Leigh syndrome	Two mitoTALENs monomers CMV promoter/transfection	Yes	No	mtDNA heteroplasmy shift Complex I recovery	Hashimoto et al. [94]
m.9176 T>C	m.9176 T>C NARP cybrids and fused mouse oocytes with m.9176 T>C cybrids	Two mitoTALENs monomers CMV promoter/mRNA Injection	Yes	Yes	mtDNA heteroplasmy shift	Reddy et al. [100]
NZB mtDNA	NZB/BALB Oocytes	Two mitoTALENs monomers CMV promoter/injection	Yes	Yes	mtDNA heteroplasmy shift Decrease mtDNA copy number	Reddy et al. [100]
ZFN						
m.8993 T>G	m.8993T>G cybrids NARP/ MILs mutation MT-ATP6	Two mitoZFNs monomers/transfection	Yes	No	mtDNA heteroplasmy shift/ OXPHOS and metabolic improvements/mild depletion	Gammage et al. [76] Gammage et al. [77]
5 kb-common deletion (CD) (m.8483_13459 del4977)	Human osteosarcoma cybrids CD	Two mitoZFNs monomers/transfection	Yes	No	mtDNA heteroplasmy shift/ OCR improvement/mild depletion	Gammage et al. [76]
CRISPR/Cas9						
Cox1 and Cox3	HEK-293T	Lentivirus and plasmid transfection	Yes	No	30% reduction in mtDNA copy number. Not effective in our lab	Jo et al. [99]

RE was also used to target the human mtDNA mutation T8993G (also known as the NARP mutation in the MT-ATP6 gene) [49]. This mtDNA mutation is associated with neuropathy ataxia and retinitis pigmentosa (NARP) or maternally inherited Leigh syndrome (MILS) [50, 51]. The authors were able to reduce the level of the mutation in heteroplasmic cybrids cells using a mitochondria-targeted *Sma*I restriction endonuclease. Further studies were done using the *Sma*I isoschizomer *Xma*I RE expressed from an adenovirus system. Mitochondria-targeted *Xma*I lead to selective destruction of the mutant mtDNA in a time and dose-dependent manner, restoring some of the phenotypes caused by the mutation. These included oxygen consumption and ATP levels that were decreased in the original cybrids carrying the mtDNA mutation. In addition, lactic acid production was decreased after the treatment showing that this approach can be useful for the treatment of NARP and MILS [52]. Another approach using restriction endonucleases to digest mtDNA was developed by Bayona-Bafaluy et al. in our laboratory [53]. The goal was to decrease specific mtDNA haplotypes by expressing a mitochondrially targeted restriction endonuclease, *Apa*LI, in cells of heteroplasmic mice that carry two mtDNA haplotypes (BALB/NZB) [54], only one of which contains an *ApaL*I site (BALB mtDNA). After transfection of cultured hepatocytes derived from the heteroplasmy mice with mitochondrially targeted *ApaL*I, the authors found a rapid, directional, and robust shift in mtDNA heteroplasmy in 2–6 h after transfection, with an increase in the NZB haplotype. The approach was also tested in vivo, by using recombinant viral vectors expressing the mitochondrially targeted *ApaL*I. A significant shift in mtDNA heteroplasmy was observed in the muscle and brain transduced with recombinant viruses (rAd and rAAV1 and 2, respectively). The use of restriction endonucleases to specifically digest the mtDNA in vivo was further explored in our laboratory. Bacman and colleagues published a system of a "differential multiple cleavage-site" in the same heteroplasmic mouse model described above, using a different restriction endonuclease. In this study the authors used *Sca*I that recognizes five sites in the BALB mtDNA and three sites in the NZB mtDNA [55]. The mitochondrial-targeted *Sca*l restriction endonuclease was delivered to different mouse tissues and showed that changes in mtDNA heteroplasmy were obtained after expression of mitochondria-targeted *Sca*I, in both livers after intravenous injection and in skeletal muscle after intramuscular injection using a recombinant adenovirus as a vector.

Continuing with this line of investigation, we took advantage of the specificity of AAV serotypes to target different tissues in vivo [56]. In this case, the mitochondrial-targeted *ApaL*I was able to increase the proportion of NZB mtDNA in targeted tissues. This was observed after systemic injections of a cardiotropic AAV6 or in the liver, using the hepatotropic adenovirus type-5 (Ad5). No loss of cytochrome c oxidase activity was observed in any of these tissues. The same principle was used with neonates [57]. In this case, a single injection in P2-P3 mice was delivered intraperitoneal (IP) or in the temporal vein (TV) using a recombinant AAV9 carrying the mito-*ApaL*I construct described above. The same heteroplasmic mice NZB/BALB were used, and the shift of heteroplasmy was found in all the skeletal muscle tissues and heart, confirming previous reports which showed a robust muscle transduction with AAV9 when injected systemically [58, 59]. These experiments showed that not

only the use of mitochondrial-targeted restriction endonuclease is a useful tool to change mtDNA heteroplasmy for specific mtDNA mutations but also revealed its applicability in vivo and the benefit that a unique injection, when using the adequate viral vector, could be enough to promote a long-lasting and probably definite change in heteroplasmy.

The approach requires a highly specific nuclease that recognizes one haplotype but not the other(s). Upon double-strand breaks, linearized mtDNA is quickly degraded [53]. The residual mtDNA will replicate to make up for the possible depletion.

34.3.2 Manipulating mtDNA Heteroplasmy with Gene-Editing Enzymes

Although mitochondrial-targeted restriction endonucleases can efficiently change mtDNA heteroplasmy both ex vivo and in vivo, this approach can be used therapeutically only if a unique restriction site is created by a mtDNA mutation, as in the case of the T>G NARP mutation. This has limited their further development. However, the recent development of gene-editing nucleases has broadened the spectrum of mtDNA mutation that can be targeted [60].

Our group and others have investigated the use of endonucleases with modular DNA recognition domains, which can be designed to bind almost any predetermined DNA sequence. Various gene-editing nucleases are actually available to modify nuclear genes, such as bacterial *Streptococcus pyogenes* clustered regularly interspaced short palindromic repeats, CRISPR-associated protein-9 nuclease (Cas9) [61]. Other available tools include zinc finger nucleases (ZFNs), artificial restriction enzymes generated by fusing a zinc finger DNA-binding domain to a DNA-cleavage domain [62], and transcription activator-like effector nucleases (TALENs) that are secreted by *Xanthomonas* bacteria via their type III secretion system [63] (Figs. 34.1 and 34.2; Table 34.1).

These gene-editing tools are commonly used to modify nuclear genes. Because the goal is usually to knock out the gene, nuclear DNA can be targeted at different positions of the genome to achieve optimal recognition sites, avoiding sites with similar sequences elsewhere in the genome. The use of these gene-editing tools to eliminate mutant mtDNA is more challenging as mtDNA point mutations require the specific recognition of one base to be discriminated from the non-targeted DNA. Furthermore, import of endonucleases to mitochondria is also not trivial, especially for ZFNs which have strong nuclear tropism [64, 65]. Even more challenging, CRISPR-Cas9 requires an RNA template, which cannot be easily imported into the mitochondria. In fact, we have attempted to use CRISPR-Cas9 in our lab, but were not able to promote mutant mtDNA elimination, even if Cas9 was imported into mitochondria (unpublished).

Both the TALEN and the zinc finger systems share a common basic structure utilizing sequencing-independent endonuclease domain from *Fok*I coupled to a

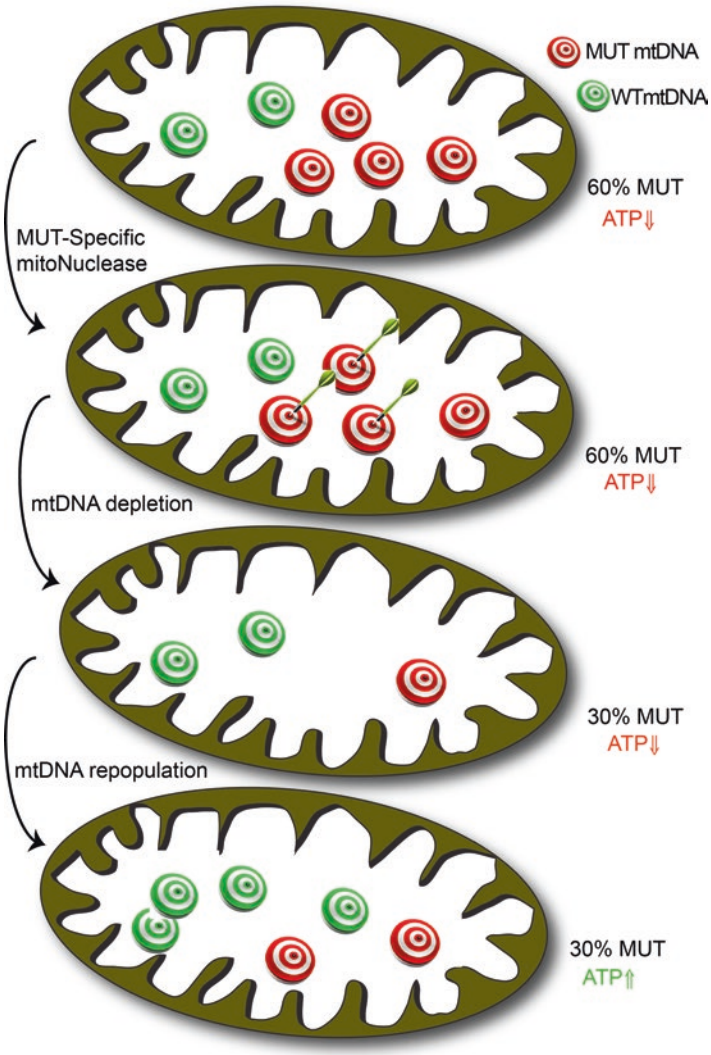

Fig. 34.1 Changing mtDNA heteroplasmy with site-specific mitochondrial-targeted nucleases. Cells with predominant levels of mutant mtDNA are subjected to mitochondrial-targeted nucleases that specifically promote the elimination of mutant genomes. Residual mtDNA replicates and restores the normal copy number, which will contain lower percentages of mutant mtDNA

sequence-specific modular DNA-binding domain [65, 66]. As *Fok*I creates double-strand breaks (DSBs) as a dimer, both enzyme systems require the design of pairs of monomers that bind specifically the region of interest in close proximity, enabling the dimerization of *Fok*I domains and double-strand cleavage between the monomer-binding sites. Once the DSB has been made, at least in nuclear genes, the lesion may be repaired by either nonhomologous end joining (NHEJ) or homologous recombi-

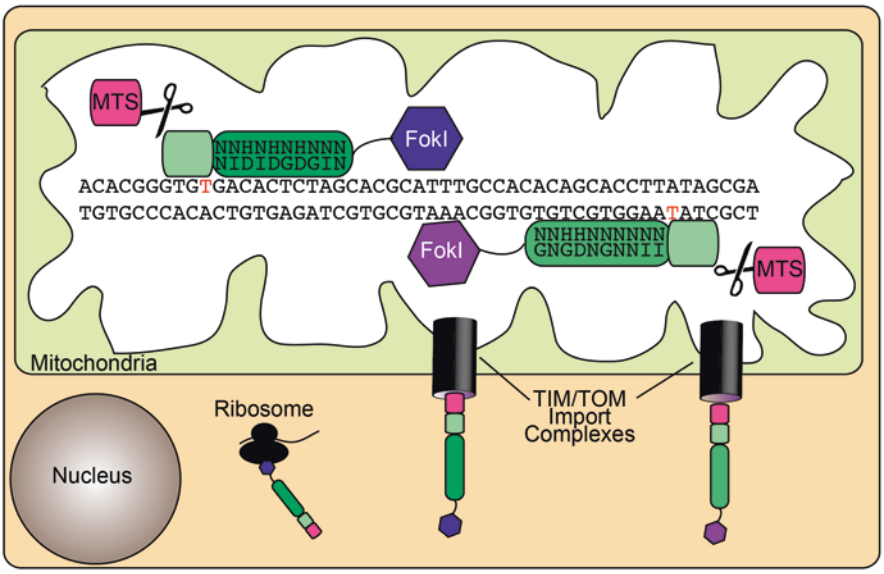

Fig. 34.2 MitoTALEN structure and function. Genes coding for mitoTALENs are introduced in the nuclear genome, where it is expressed according to its promoter. The protein is made in the cytosolic ribosomes and imported into the mitochondria through the translocases of the inner and outer membrane complexes (TIM/TOM). Once imported, the mitochondrial targeting sequence (MTS) is cleaved by endogenous peptidases and the TALENs find their target in the mtDNA. Double-strand break is performed by the *Fok*I domain that works as an obligatory heterodimer, increasing specificity. The DNA-binding domain is formed by 34 amino acid repeats (RVDs), where two of the variable residues define specificity (A = NI; T = NG; C=HD; G = NN)

nation [67]. Mutations can be permanently introduced into the genome upon repair. Since NHEJ is error-prone, repair can result in deletions or insertions at the break site, potentially resulting in frameshifts. During homologous recombination, if a repair template is introduced that contains a mutation, that mutation will be permanently introduced into the genome upon repair.

34.3.2.1 Zinc Finger Nucleases Targeted to Mitochondria

Zinc finger nucleases (ZFNs) are chimeric enzymes in which the modular Cys2-His2-zinc finger protein (ZFP) forms three to six individual zinc finger repeats that account for a total recognition of a unique 9–18 bp sequence [71–73]. Fusing a particular ZFP to a nuclease domain creates a zinc finger nuclease (ZFN) that can cleave DNA adjacent to the specific ZFP-binding site, providing sequence specificity to cleave the DNA [66]. This specific DNA sequence when conjugated with a type II restriction enzyme such as *Fok*I, after dimerization, can cleave the double-strand DNA [69]. To achieve dimerization, pairs of ZFNs are used to bind adjacent sequences of the double-strand DNA to cleave it [70]. Zinc finger nucleases (ZFNs)

improved the efficiency of gene targeting by generating DSBs at preselected sites on the chromosome [71, 72]. The custom-designed ZFN modules recognize 3–4 bp that account for a total recognition of a unique 9–18 bp DNA target sequences [68] with 4–7 bp spacers between each of the half sites. ZFN technology has been successfully applied to nuclear genome editing in many organisms including human cell lines [73] to correct specific genes via homologous recombination after achieving double-strand brakes of nuclear DNA [64]. ZF-based DNA modifications were successfully achieved in mtDNA by Minczuk et al. [74] to specifically modify methylation in mtDNA using a chimeric zinc finger methylase. Later on, the same group developed a strategy with a single-chain ZFNs conjugating two *Fok*I nuclease domains, connected by a flexible linker to a ZFP with an N-terminal mitochondrial targeting sequence (MTS) and nuclear export signal (NES) peptides to be targeted specifically to mitochondria [75]. ZFNs were shown to be efficiently transported and expressed into the mitochondria in heteroplasmic human cybrids carrying the m.8993T>C mutation and bind the DNA in a sequence-specific manner discriminating between two 12-bp-long sequences that differ in a unique base pair. With an improved design of mtZFNs, two single monomers of the engineered ZFNs were targeted to bind adjacent sites on complementary DNA strands spanning the targeted sequences and allowing the dimerization of the *Fok*I nuclease domains needed to cleave double-strand DNA [76]. This approach was successfully used to shift heteroplasmy in the human cybrids carrying the m.8993T>C mutation, accompanied by biochemical phenotype improvements such as restoring mitochondrial respiration, oxygen consumption, and ATP production and improving energy states [77]. They also applied this gene-editing technique in the large-scale mtDNA deletion of 4977 bp, called "common deletion," which is associated with adult-onset chronic progressive ophthalmoplegia (CPEO) and in Kearns-Sayre and Pearson's syndromes [76, 78]. The defect in mtDNA gene expression was corrected by mtZFNs and resulted in rescue of oxygen consumption rate and increased abundance of respiratory complex subunits.

34.3.2.2 TALE Nucleases Targeted to Mitochondria

Transcription activator-like (TAL) effectors recognize DNA in a modular fashion. Found in plant pathogenic bacteria as members of the genus *Xanthomonas*, TAL effectors are positive-acting transcription factors activating expression of downstream genes, which may contribute to bacterial colonization, symptom development, or pathogen dissemination [63]. TAL effectors recognize DNA through the amino acid repeats as the targeting domain of the protein, composed of 34 amino acids. Because the last repeat is truncated and contains only 20 amino acids (instead of 34), it is referred to as 0.5 repeat. Most TAL effectors have between 15.5 and 22.5 TALE repeats per monomer to avoid off-target cleavage [79, 80] with a recognition site of 14–20 bp per TALEN monomer, 28–40 bp per TALEN pair [68].

Each repeat is identical, and the polymorphism among the repeats is almost exclusively localized to a pair of residues at positions 12 and 13, called the repeat-

variable di-residue (RVD), with the four most common RVDs (HD, NG, NI, and NN) accounting for binding to each of the four nucleotides (C, T, A, and G, respectively) providing the basis to engineering novel specificities [81–83]. This simple DNA recognition code and its modular nature makes TALEs an ideal platform for constructing custom-designed artificial DNA nucleases [84, 85]. The traditional TAL N-terminus requires a "T" base at position 0 for binding (Fig. 34.2). We often take advantage of this requirement when designing mutant-specific mitoTALENs [86]. The high specificity of the TALENs is due to, in great part, the combination of both sequence specificity of TALE binding and the positional requirements of *Fok*I cleavage (Fig. 34.2). The two monomers that bind to the DNA are attached to *Fok*I endonucleases that work as dimers. Therefore, double-strand breaks can be generated similarly to the ones formed by ZFNs. In both ZFN and mitoTALENs, the *Fok*I moieties have been engineered to work as obligatory heterodimers, minimizing off-target DSB [87, 88]

This approach was used to specifically target mtDNA mutations by our group [89]. We designed mitochondrial-targeted transcription activator-like effector nucleases or mitoTALENs to cleave specific sequences in the mtDNA with the goal of eliminating mtDNA-carrying pathogenic point mutations. The mitoTALEN construct basically consist of the following:

1. A basic TAL-binding domain [86], which is relatively short (10–16 repeats)
2. A mitochondrial localization signal in the N-terminus
3. Each TALEN monomer has a unique tag (hemagglutinin (HA) or Flag) for immunological detection
4. Each construct also contains a GFP or mCherry for sorting of transfected cells
5. Inclusion of 3'UTR untranslated region from a nuclear gene (ATP5B or SOD2 mRNA) known to localize mRNA to ribosomes contacting mitochondria
6. Both mitoTALEN and the fluorescence marker genes are expressed from a unique promoter (CMV) using of a recoded picoviral 2A-like sequence (T2A) [90] between the mitoTALEN and the fluorescence marker that allows translation of different proteins from a unique transcript.

We tested a mitoTALEN in human osteosarcoma cells heteroplasmic for the mtDNA "common deletion" (m.8483_13459del4977) that is presented in approximately 30% of all patients with mtDNA deletions [91] and also in normal aging tissues [92]. The monomers bind specifically to a WT-sequence flanking the region to be removed. When bound to the deletion-mutant DNA, the mitoTALENs are close enough to allow the *Fok*I dimerization and subsequent cleavage. The "common deletion"-mitoTALEN (Δ5-mitoTALEN) co-localized to mitochondria and was tested in human heteroplasmic cybrids for the deletion by transfecting two monomer-containing plasmids. This analysis showed that the Δ5-mitoTALEN was effective in reducing the mtDNA deletion load and changing mtDNA heteroplasmy to a predominance of wild-type mtDNA. We then measured the levels of the different mtDNA species by quantitative PCR (qPCR) and found that the change in heteroplasmy was primarily caused by a reduction in the absolute levels of deletion-mutant mtDNA with the trend

toward a reduction in the total mtDNA levels after 2 days, compensated by an increase in wild-type mtDNA levels at 14 days.

The other mtDNA mutation that we tested was the point mutation m.14459G>A in MT-ND6 that causes the Leber's hereditary optic neuropathy plus dystonia [93]. In this case one monomer binds the wild-type sequence adjacent to the mutation (or wild-type strand), and the cleavage is dictated by the binding of the recognition sequence where the m.14459A is present (mutant strand). Transfection of heteroplasmic cells showed a significant increase in the wild-type mtDNA that persisted for 14 days, and complex I activity, which was defective in the cells carrying the point mutation in the MT-ND6 before the mitoTALENs transfection was improved after the mitoTALENs treatment [89].

The ability of TALENs to differentiate targets with only single nucleotide differences can be difficult, but we showed that this can be accomplished for different point mutations. Besides the m.14459A, we designed mitoTALENs [94] to target two relatively common pathogenic mtDNA point mutations associated with mitochondrial diseases: the m.8344A>G tRNALys gene mutation associated with myoclonic epilepsy with ragged-red fibers (MERRF) [95, 96] and the m.13513G>A ND5 mutation associated with MELAS/Leigh syndrome [97, 98]. MitoTALENs were co-localized to mitochondria and were able to promote a robust change in mtDNA heteroplasmy in both mtDNA point mutations while improving the OXPHOS function in the case of the m.8344A>G tRNALys gene mutation associated with MERRF syndrome.

We were able to reduce the size of the TALE-binding domain, downsizing the original m.8344A>G mitoTALEN sense and antisense monomers, which originally had 15.5 and 9.5 RVDs, to 10.5 and 7.5 RVDs, respectively. These shorter mitoTALENs were still very effective in recognizing a single base difference and shifting heteroplasmy. This is important because mitoTALENs are possible tools for gene therapy in patients, and size of constructs are limiting for viral vectors, particularly AAV.

34.3.2.3 CRISPR-Cas9 Targeted to Mitochondria

Jo et al. [99] express FLAG-Cas9 together with gRNA-targeting Cox1 and Cox3 in HEK-293 cells which lead to cleavage of the specific mtDNA loci. With a mitochondria-targeted Cas9 (mitoCas9) added together with gRNA-targeting Cox1 and Cox3, they reported specific cleavage of mtDNA, leading to decrease copy number, mitochondrial membrane potential disruption, and cell growth inhibition. We have not been able to observe mtDNA cleavage in our lab when using a similar approach. Cas9 was imported into mitochondria, but no change in heteroplasmy was observed for two different mtDNA mutations (Hashimoto, Bacman, and Moraes, unpublished). Even though we used different forms of gRNA, which included RNA structures previously described as import structures, we suspect that the gRNA cannot be efficiently imported into mammalian mitochondria.

34.4 Selective Elimination of mtDNA Mutations in the Germline by Genome Editing

Reddy, Ocampo, and colleagues using the NZB/BALB heteroplasmic mice, which contain two mtDNA haplotypes, selectively reduced one of the haplotypes and prevented their germline transmission using either mitochondria-targeted restriction endonucleases or mitoTALENs [100]. They were able to specifically reduce mitochondrial genomes in oocytes and embryos using the mito-*ApaL*I construct previously developed in our lab [56, 101]. Mitochondrial localization was observed in the NZB/BALB metaphase II in oocyte injected with mRNA encoding mito-*ApaL*I by immunocytochemistry and showed specific reduction of BALB mtDNA after 48 h. The studies in 1-cell NZB/BALB embryos also showed decrease in the BALB mtDNA. The embryos injected were grown in culture and transferred to pseudopregnant mice that gave birth to healthy pups with significant reduction of BALB mtDNA in different tissues. They also used mitoTALENs against the NZB mtDNA, which triggered a heteroplasmy shift in NZB/BALB toward a decrease of the NZB haplotype when tested in MII oocytes.

By generating artificial mammalian oocytes carrying mutated human mitochondrial genomes by cellular fusion of patient cells and mouse oocytes, they were able to test mitoTALENs specific for two mitochondrial diseases: Leber's hereditary optic neuropathy and dystonia (LHOND) m.14459G>A [89] and NARP [102, 103]. After injection of RNA coding for mitoTALENs, they were able to reduce of LHOND mtDNA in MII oocytes. The same approach was used against the mutation NARP m.9176T>C using mitoTALENs in immortalized NARP patient cells. Again, patient cells harboring the NARP m.9176T>C mutation were fused to MII oocytes, and 48 h after mRNA injection, a specific reduction of NARP mtDNA was achieved, but they failed to detect a significant increase in wild-type human mtDNA [100]. This report expanded the use of specific and non-specific nucleases to germline transmission.

34.5 Limitations and Concerns for Gene Therapy of Mitochondrial Diseases

Here we have described novel strategies to reduce or eliminate mitochondrial DNA mutations from patient cells or from germline transmission in models of mtDNA heteroplasmy. As a proof of principle, these engineering nucleases open the possibility to treat mtDNA diseases. However, many questions and limitations remain. Below we discuss some of these concerns.

1. There are some limitations in the design of the TALENs that may limit its applicability to all mtDNA mutations. It is generally accepted that the N-terminus proximal bases have a higher impact on binding [104], such as the presence of a T at position 0 (before the first repeat module-RVD binds) that is a required feature for the N-terminus of most TALEN architectures [86]. Different specificities

for N-terminus binding have also been developed [105] to expand the potential binding sites. Limitations in the construct of zinc finger nucleases reside on the lack of binding of guanine-poor sequences and also requiring some substantial protein engineering [68].

2. The delivery of therapeutic genes and proteins with high titer remains a barrier to the fast implementation of genetic therapies. However, the application of gene transfer in mitochondrial diseases has the advantage that transient expression of the mito-TALEN should be sufficient to produce lasting effects and changes in heteroplasmy [56, 89], what is not true for gene therapy of nuclear genes. For in vivo applications, the most promising delivery systems are viral vectors, particularly AAV, which have been approved for clinical use [106]. Several serotypes of AAV have been described to efficiently target a variety of tissues [107], including the eye, brain, liver, heart, and muscle [56, 108, 109]. The use of viral vectors as vehicles has been explored for both adults and neonates: adenovirus [110–112], AAV [108, 113, 114], lentivirus [115], retrovirus [116], etc. as well as the different delivery options to reach the affected organs and tissues involved in mitochondrial diseases [117–119].
3. The size of the monomer or monomers to be delivered should be suitable to package in a vector system, such as AAV that have relatively small packaging capacity. While ZFNs are relatively small and they can be packaged into a single AAV, a dimeric TALEN pair is much larger. This limitation can be resolved by packaging each monomer into two separate AAV vectors, decreasing the numbers of RVDs in the TALEN monomers [94], or using monomeric TALENs [120].
4. The absence of available animal models with heteroplasmic mtDNA mutations has been an obstacle to study gene therapy approaches in vivo. A mouse model carrying the common deletion has been published but was not made available to the scientific community at large [121]. Recently, a mouse model with a pathogenic mutation in the mitochondrial $tRNA^{Ala}$ gene, with defects in mtDNA translation and histochemical symptoms of a human mitochondrial disease [122], has been described [123].
5. Mitochondrial DNA depletion could be a problem when targeting mtDNA mutations, especially if the load of the mutant mtDNA is very high [89]. More controlled delivery systems may need to be developed to overcome this concern and minimize the in vivo risk [60, 124].
6. A major complication with engineered nucleases is the binding to unintended genomic sites that share sequence homology with the on-target site. Cleavage of these off-target sites followed by DNA repair can cause gene mutation or gross chromosome rearrangement. Bioinformatics analysis can be used to identify and avoid the most likely putative off-target sites and modifications of the structure of the nucleases to boost the nuclease-targeting specificity [125–127].
7. Immunological and toxicity reaction has always been a concern when viral vectors are used. These include humoral response of memory B cells when AAV vectors are used for gene therapies [108] and an immunological barrier composed of pre-existing neutralizing antibodies and CD8(+) T-cell response against AAV capsid in humans [128]. Furthermore, recent studies have demonstrated that Cas9 (part of the CRISPR-Cas9 system) evokes cellular immune responses [129], whereas TALENs showed less undesired immune responses [130].

34.6 Conclusions and Future Perspectives

The approaches described in this chapter show that it is possible to reduce the levels of mutated mtDNA in a targeted manner and give new hope for genetic therapies. However, these advances are still limited by the efficiency of delivery mechanisms, something that must be solved in order to deliver mitochondrial nucleases to all affected tissues, including skeletal and cardiac muscles. Furthermore, it is important to avoid off-target sequences (both in mitochondria and the nucleus) and demonstrate that this approach can be used safely in vivo.

During the production of this book, two important studies were published. These reports showed that either mitoTALEN or mitoZFN could change mtDNA heteroplasmy in a mouse model harboring a pathogenic heteroplasmic mtDNA mutation. Recombinant AAV9 delivered these gene editing enzymes to skeletal muscle and heart reducing the molecular phenotypes [131, 132].

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Chapter 35
Gene Therapy for CMT Inherited Neuropathy

Kleopas A. Kleopa, Alexia Kagiava, and Irene Sargiannidou

Abstract Non-syndromic inherited neuropathies of the peripheral nervous system, collectively known as Charcot-Marie-Tooth (CMT) disease, with prevalence as high as 1:2500 worldwide, are genetically extremely heterogeneous. Most CMT forms share the clinical features of gait dysfunction, progressive muscle weakness, and atrophy with sensory loss in distal limbs, leading to variable degrees of disability over the lifespan. So far, genetic studies in CMT have identified mutations in at least 80 different causative genes with all inheritance patterns and highly variable molecular genetic mechanisms including both loss-of-function and gain-of-function effects. Mutations in neuronal genes usually cause axonal neuropathies, while mutations in genes expressed in myelinating Schwann cells cause demyelinating neuropathies. Treatment for CMT has so far been supportive, and there are currently no effective therapies for any of the CMT forms. The discovery of causative genes and increasing insights into CMT molecular mechanisms facilitated also by the study of disease models provide new possibilities for the development of gene therapy approaches to treat CMT. Recent progress in optimizing gene delivery methods, including vectors and administration routes to target the peripheral nerves, offers promise for future therapies. This chapter summarizes the molecular genetic mechanisms of the disease and what has been developed in recent years toward a gene therapy for some of the CMT forms.

Keywords Charcot-Marie-Tooth disease · Schwann cells · Axons · Viral vectors Gene replacement · Gene silencing · Gene editing

K. A. Kleopa (✉)
Neuroscience Laboratory, The Cyprus Institute of Neurology and Genetics and Cyprus School of Molecular Medicine, Nicosia, Cyprus

Neurology Clinics, The Cyprus Institute of Neurology and Genetics and Cyprus School of Molecular Medicine, Nicosia, Cyprus
e-mail: kleopa@cing.ac.cy

A. Kagiava · I. Sargiannidou
Neuroscience Laboratory, The Cyprus Institute of Neurology and Genetics and Cyprus School of Molecular Medicine, Nicosia, Cyprus
e-mail: alexiak@cing.ac.cy; irenes@cing.ac.cy

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35.1 Introduction

Charcot-Marie-Tooth (CMT) disease encompasses numerous types of non-syndromic inherited neuropathies, which all together are considered to be one of the most common neurogenetic disorders, with a frequency of affected individuals reaching 1:2500 of the general population [1, 2]. CMT neuropathies are characterized by an ever increasing number of causative genes involved and overlapping phenotypes caused by different genes (Table 35.1). Moreover, several different genes may cause identical phenotypes making a consistent phenotype-genotype correlation as well as an overall classification of CMT forms challenging. Databases that offer updated list of CMT genes and associated phenotypes include, besides the Online Mendelian Inheritance in Man (OMIM; http://www.ncbi.nlm.nih.gov/omim), the Inherited Neuropathies Consortium (http://rarediseasesnetwork.epi.usf.edu/INC/) and GeneReviews (http://www.ncbi.nlm.nih.gov/books/NBK1358/),

Table 35.1 Classification and genetics of CMT disease

Inheritance	Pathology	Type and frequency	Common genes (subtype frequency)	Other associated genes (rare or frequency unknown)
Autosomal dominant	Myelinopathy	CMT1 (40–50%)	*PMP22* (CMT1A, 70–80%) *MPZ* (CMT1B, 10–12%)	*LITAF/SIMPLE*, *EGR2*, *NEFL*, *FBLN5*
	Axonopathy	CMT2 (10–15%)	*MFN2* (CMT2A2 20%)	*KIF1B*, *RAB7*, *TRPV4*, *GARS*, *NEFL*, *HSPB1*, *MPZ*, *GDAP1*, *HSPB8*, *DNM2*, *AARS*, *DYNC1H1*, *LRSAM1*, *DHTKD1*, *DNAJB2*, *HARS*, *MARS*, *MT-ATP6*, *TFG*
	Intermediate[a]	CMTDI (rare)		*DNM2*, *YARS*, *MPZ*, *IFN2*, *GNB4*
Autosomal recessive	Myelinopathy	CMT4 (rare)	*SH3TC2* (CMT4C 43%)	*GDAP1*, *MTMR2*, *MTMR13 (SBF2)*, *SBF1*, *NDRG1*, *EGR2*, *PRX*, *HK1*, *FGD4*, *FIG4*, *SURF1*
	Axonopathy	CMT2[b] (rare)		*LMNA*, *MED25*, *GDAP1*, *MFN2*, *NEFL*, *HINT1*, *TRIM2*, *IGHMBP2*, *GAN*
	Intermediate[a]	CMTRI (rare)		*GDAP1*, *KARS*, *PLEKHG5*, *COX6A1*
X-linked	Intermediate[a] or axonal	CMTX (10–15%)	*GJB1* (CMTX1, 90%)	*AIFM1*, *PRPS1*, *PDK3*

Subtype frequencies are based on reports by Saporta et al. [6] and Fridman et al. [74]. For complete classification of CMT neuropathies, see GeneReviews (http://www.ncbi.nlm.nih.gov/books/NBK1358/, last updated in 2018) and Scherer et al. [12]

[a]Combination of myelinopathy and axonopathy

[b]Also referred to as CMT3

while the inherited neuropathy variant browser (http://hihg.med.miami.edu/code/http/cmt/public_html/index.html#/) is a valuable tool to make sense out of the increasing number of mutations in CMT-related genes. Despite the increasing understanding of the complex genetic basis and diverse disease mechanisms underpinning CMT neuropathies, there is currently no effective treatment for any of the CMT forms, and only symptomatic and supportive therapy can be offered to patients. Thus, there is a great need for new treatment strategies for CMT. In the last two decades, there has been an effort to develop gene therapies for the treatment of CMT. While different gene therapy approaches hold promise for the future to treat diseases of the central and peripheral nervous system (PNS), multiple challenges remain to be overcome along this effort. In this review, we will first discuss the genetic and biological basis of CMT neuropathies followed by some recent advances in the development of gene therapy approaches.

35.2 Clinical Manifestations and Classification of CMT Neuropathies

Patients with CMT neuropathies typically present within the first or second decade of life with a history of abnormal gait, tripping, and falling. The foot and foreleg muscle atrophy and weakness cause characteristic foot deformities with high arches (pes cavus) and hammer toes that may appear even in childhood. Weakness progresses slowly to more proximal lower limb muscles and to the distal upper limbs with subsequent "claw hand" deformities resulting from weakness and atrophy of intrinsic hand muscles. Deep tendon reflexes are diminished or absent [3]. Sensory nerves are involved in most typical CMT forms, but sensory disturbances such as paresthesias and neuropathic pain are rarely reported by patients, except in certain variants with predominantly sensory neuropathy. These clinical manifestations reflect the underlying slowly progressive, length-dependent axonal degeneration, which occurs as primary pathology in axonal types and secondary to demyelination in the demyelinating types, correlating with the overall disability [4].

Since the first description of the disease in the late 1800s by Charcot, Marie, and Tooth, the heterogeneity of CMT neuropathies has expanded to a large number of genetic types, with partly overlapping clinical phenotypes and mode of inheritance [1, 5, 6] resulting in increasing challenges to classify them (Table 35.1). The term hereditary motor and sensory neuropathy (HMSN) was introduced based on clinical, electrophysiological, and histological features. Dominantly inherited HMSN/CMT1/2 forms are the most common. CMT1/HMSN-I is characterized by upper limb motor nerve conduction velocities (NCVs) below 38 m/s and segmental demyelination and remyelination with onion bulb formation in nerve biopsies [7]. CMT2/HMSN-II forms show NCVs above 38 m/s and loss of myelinated axons in nerve biopsies. Certain CMT types with NCVs in the intermediate range (25–45 m/s) did not fit these categories and were grouped under "dominant-intermediate" CMT

(CMTDI). CMT1X, some cases of CMT1B, and even some CMT2 and autosomal recessive types with mixed axonal and demyelinating findings also show intermediate NCVs (Table 35.1).

Recessive CMT forms are much rarer than dominant ones and extend across the whole spectrum of demyelinating, intermediate, and axonal phenotypes. Interestingly, some of the recessive forms share the same genes with dominant forms. The term CMT4/HMSN-IV described initially recessive demyelinating CMT forms but is also used for recessive axonal forms [8]. Déjérine-Sottas neuropathy (DSN)/CMT3/HMSN-III and congenital hypomyelinating neuropathy (CHN) are the most severe CMT phenotypes. DSN presents with delayed motor development before 3 years of age and progressive weakness leading to wheelchair dependency, kyphoscoliosis, short stature, foot deformities, profound sensory loss, and even respiratory failure. Motor NCVs are below 10 m/s, and nerve biopsies show a complete absence of normally myelinated axons. CHN presents with hypotonic weakness at birth, arthrogryposis caused by a prenatal onset, and swallowing or respiratory difficulties. NCVs are often below 5 m/s. DSN and CHN may overlap with severe CMT1 and CMT4 forms and are often caused by mutations in the same genes [9].

Some CMT forms affect predominantly sensory neurons and their axons and are called hereditary sensory and autonomic neuropathies (HSAN), or hereditary sensory neuropathy (HSN) since symptoms of autonomic dysfunction are mostly subtle [10]. Disorders that affect mainly motor axons in a length-dependent manner are called hereditary motor neuropathies (HMN) or distal spinal muscular atrophy (DSMA). Given the complexity of CMT classification that relies on phenotypes, a genetic and neurobiological classification according to causative genes and disease mechanisms (Fig. 35.1, Table 35.2) is more relevant for planning future gene therapy approaches.

35.3 Molecular Genetics and Neurobiology of CMT Neuropathies

CMT-associated genes show a great diversity of cellular functions spanning both neurons and Schwann cells leading to a multitude of disease-causing pathomechanisms [11, 12]. Most demyelinating CMT types result from mutations in genes expressed by Schwann cells, whereas axonal types result from mutations expressed by neurons and their axons. Demyelinating CMT forms are the most common and result from cell autonomous effects of the mutations in myelinating Schwann cells [12, 13]. Furthermore, dominantly inherited types are likely to be caused by gain-of-function mechanisms, while recessively inherited forms result from loss-of-function mutations. The importance of the specific cellular mechanisms of particular mutations is highlighted by the fact that both dominant and recessive mutations

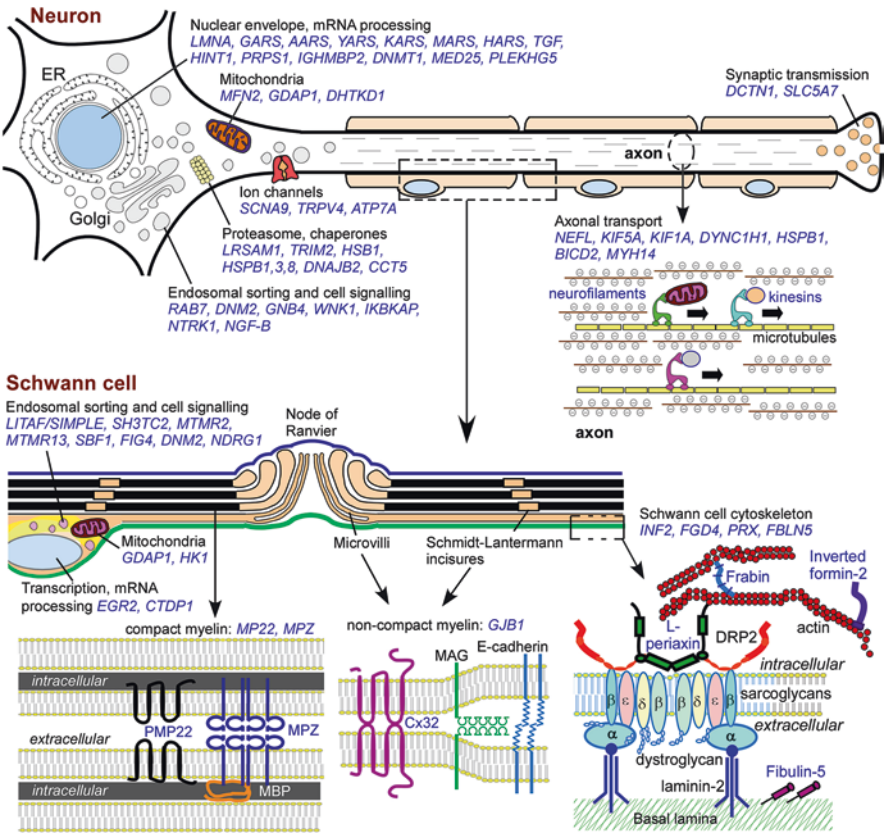

Fig. 35.1 CMT-associated genes and their function. Diagram showing schematically a neuron with its axon with myelinating Schwann cells. Neuropathy-associated genes and proteins are indicated in blue and categorized according to cellular function. Associated CMT types can be found in Tables 1 and 2 and in OMIM (https://www.omim.org/)

have been described in the same genes, while different phenotypes and nerve pathologies are sometimes caused by mutations in the same gene (Table 35.1).

In order to plan an effective gene therapy approach for CMT, the functional effects of the mutations in each type and often in each patient have to be clarified, since different approaches may be needed for loss-of-function mutations (e.g., gene replacement, gene addition) as opposed to gain-of-function dominant ones (e.g., gene editing, silencing approaches, etc.). Examples of common and representative forms of CMT and associated genes are discussed in more detail below, while an overview of known molecular mechanisms in different CMT forms is shown in Table 35.2 and Fig. 35.1.

Table 35.2 Basic neurobiological mechanisms in CMT and related neuropathies

Gene function category	Examples of genes[a] (and associated CMT types[b])	Cellular mechanisms of disease[b]
Genes expressed mainly in Schwann cells (mostly demyelinating or intermediate CMT)		
Myelin proteins	*PMP22* 601097 (CMT1A, CMT1E, HNPP, DSS)	Impaired regulation of myelin biosynthesis, alteration of mRNA processing, demyelination
	MPZ 159440 (CMT1B, CMT2I, CMT2J, DSS)	Myelin discompaction, unfolded protein response, demyelination
Gap junction channel protein	*GJB1/Cx32* (304040) (CMT1X)	Disturbed axonal and myelin homeostasis, impaired Schwann cell-axon signaling, axonal degeneration and demyelination
Transcription and mRNA processing	*EGR2* (129010) (CMT1D, DSS, CHN)	Impaired expression of myelin-related genes Phosphatase of RNA polymerase II, impaired
	CTDP1 (604927) (CCFDN, 604168)	Disturbed RNA processing
Schwann cell cytoskeleton and basal lamina adhesion	*INF2* (610982) (CMTDIE)	Disruption of actin dynamics in Schwann cells, disturbed myelin formation and maintenance
	FGD4 (611104) (CMT4H)	Actin binding, disturbed cytoskeleton
	PRX (605725) (CMT4F, DSS)	Interacts with dystroglycan complex: disrupted link between Schwann cell cytoskeleton and basal membrane
	FBLN5 (604580) (HNARMD 608895)	Impaired extracellular matrix
Endosomal sorting and cell signaling	*LITAF/SIMPLE* (603795) (CMT1C)	Impaired protein degradation in early endosomes
	SH3TC2 (608206) (CMT4C)	Impaired perinuclear endocytic recycling compartment, dysregulation of myelination
	*MTMR2 (*603557) (CMT4B1) *MTMR13* (607697) (CMT4B2) *SBF1 (603560)* (CMT4B3)	Dysregulated membrane homeostasis
	DMN2 (602378 (CMT2M, CMTDIB)	Impaired endocytosis and intracellular membrane trafficking
	NDRG1 (605262) (CMT4D)	Impaired Schwann cell signaling and demyelination
Genes expressed mainly in neurons and their axons (mostly axonal CMT, HMN/distal SMA, or HSAN)		
Ion channels	*TRPV4* (605427) (CMT2C, distal SMA)	Cation channel mediating calcium influx: mutations cause gain of function
	ATP7A (300011) (SMAX3, 300489)	Dysfunction of transmembrane copper-transporting P-type ATPase
	SCN9A (603415) (HSAN2D, congenital insensitivity to pain 243000) (paroxysmal extreme pain disorder 167400)	Voltage-gated sodium channel: gain of function causing axonal degeneration, loss of function causes insensitivity to pain, impaired inactivation causes paroxysmal extreme pain

(continued)

Table 35.2 (continued)

Gene function category	Examples of genes[a] (and associated CMT types[b])	Cellular mechanisms of disease[b]
Nuclear envelope and mRNA processing	*LMNA* (150330) (*CMT2B1*)	Nuclear envelope protein: impaired transcriptional regulation, axonal degeneration
	GARS (600287) (CMT2D, HMN5A) *AARS* (601065) (CMT2N), *YARS* (603623)(CMTDIC), *KARS (*601421) (CMTRIB), *MARS* (156560)(CMT2U), *HARS (*142810) (CMT2W)	Various tRNA synthetases: impaired mRNA processing and translation
	HINT1 (601314) (NMAN; 137200)	Histidine binding: gene expression dysregulation
	PRPS1 (311850) (CMTX5)	Phosphoribosylpyrophosphate synthetase: impaired purine and pyrimidine biosynthesis, reduced GTP and possibly ATP levels
	IGHMBP2 (600502) (CMT2S, HMN VI/ SMARD1, 604320)	Transcription factor and component of translational machinery
	DNMT1 (126375) (HSN1E 614116)	DNA (cytosine-5)-methyltransferase, role in transcriptional gene silencing
	MED25 (610197) (ARCMT2B2)	RNA polymerase II transcriptional regulator complex
	PLEKHG5 (611101) (CMTRIC, AR distal SMA 4)	Activates the nuclear factor kappa-B (NFKB1; 164011) signaling pathway
Endosomal sorting and cell signaling	*RAB7* (602298) (CMT2B)	GTPase regulating trafficking, maturation, and fusion of endocytic and autophagic vesicles: impaired transition of early endosomes into the late endosomal/lysosomal system
	DNM2 (602378) (CMT2M, CMTDIB)	GTPase involved in endocytosis and intracellular membrane trafficking
	GNB4 (610863) (*CMTDIF*)	G protein subunit, signals from cell surface receptors to internal effectors
	WNK1 (605232) (HSAN2)	Interacts with the endocytic scaffold protein
	IKBKAP (603722) (HSAN3/familial dysautonomia)	Transcription elongation factor: transcription impairment and cell migration defects
	NTRK1 (191315) (HSANIV/CIPA)	Neurotrophic tyrosine kinase receptor: impaired survival of sensory neurons
	NGF-B (162030) (HSAN5)	Nerve growth factor regulating growth and differentiation of sympathetic and certain sensory neurons

(continued)

Table 35.2 (continued)

Gene function category	Examples of genes[a] (and associated CMT types[b])	Cellular mechanisms of disease[b]
Axonal transport	*HSPB1* (*602195*) (*CMT2F*, *distal HMN*)	Disrupted neurofilament network, impaired mitochondrial axonal transport
	NEFL (*162280*) (*CMT2E*, *CMT1F*)	Aberrant neurofilament assembly and transport, protein aggregations
	KIF1A (601255) (*HSN2C*, (614213))	Impaired anterograde axonal transport of membranous organelles and synaptic vesicles
	DYNC1H1 (600112) (CMT2O, SMALED1, 158600)	Impaired retrograde axonal transport and protein sorting
	BICD2 (*609797*) (*SMALED2*, 615290)	Impaired dynein-mediated retrograde axonal and Golgi dynamics
Synaptic transmission	*DCTN1* (*601143*), *HMN VIIB* (607641)	Microtubule binding, loss of synapse stability at the neuromuscular junction
	SLC5A7 (608761), *HMB VIIA* (158580)	Disturbed choline transport at the neuromuscular junction
Genes expressed in both Schwann cells and neurons or ubiquitously		
Mitochondrial proteins	*MFN2* (608507) (CMT2A, ARCMT2)	Impaired fusion and distribution of mitochondria, reduced efficiency of oxidative phosphorylation
	GDAP1 (606598) (CMT4A, CMT2K, CMTRIA)	Protein of the outer mitochondrial membrane, regulates mitochondrial dynamics in both axons and Schwann cells, both axonal and demyelinating CMT
	HK1 (CMT4G) (142600)	Glucose metabolism at the outer mitochondrial membrane
	DHTKD1 (CMT2Q) (614984)	Axonal neuropathy, mitochondrial vacuolization

HNPP hereditary neuropathy with liability to pressure palsies, *CCFDN* congenital cataracts, facial dysmorphism, and neuropathy, *HMN* hereditary motor neuropathy, *HS(A)N* hereditary sensory (and autonomic) neuropathy, *SMA* spinal muscular atrophy, *SMALED* SMA lower extremity predominant, *NMAN* neuromyotonia and axonal neuropathy, *CIPA* congenital insensitivity to pain with anhidrosis

[a]Hyperlinked OMIM gene numbers for gene function

[b]Hyperlinked OMIM disease numbers for some included syndromic neuropathies sharing molecular mechanisms with non-syndromic CMT types

35.3.1 CMT1A

CMT1A (OMIM 118220) is the most common subtype accounting for the majority of CMT1 cases and results from an intrachromosomal duplication spanning 1.4 Mb on human chromosome 17p12 [14]. The genetically related hereditary neuropathy with pressure palsies (HNPP) resulting from deletion or point mutations in *PMP22* gene is equally common but milder and likely underdiagnosed. The responsible disease gene within this duplicated or deleted region encodes the peripheral myelin protein of 22 kDa (PMP22) [15–18]. Patients with CMT1A develop the typical

CMT1 phenotype, with slow progression and high variability in disease severity even within the same family [19, 20]. NCVs are typically around 20 m/s and do not change significantly over decades in contrast to motor amplitudes that decrease slowly reflecting axonal loss, correlating with clinical disability.

PMP22 is a 22-kDa tetraspan glycoprotein mainly expressed by myelinating Schwann cells and localized in compact myelin [21] but also in other cell types [22]. In normal myelinating and non-myelinating Schwann cells, approximately 20% of the newly synthesized PMP22 is glycosylated, while the remaining ~80% is targeted for proteasomal endoplasmic reticulum (ER)-associated degradation (ERAD) [23]. Overexpression of *PMP22* mRNA and protein in CMT1A nerve biopsies suggested that an increased dosage of PMP22 is the most likely disease mechanism in *PMP22* duplication-related CMT1A patients [24–26]. However, PMP22 was not overexpressed in nerve and skin biopsies from adult CMT1A patients, and expression levels did not correlate with disease severity [27]. The exact consequences of PMP22 overexpression remain unclear. Increased amount of protein is thought to exceed the capacity of the proteasome for degradation, resulting in PMP22 accumulation in perinuclear aggresomes [28, 29] and in reduced overall proteasome activity [30], likely destabilizing the myelin sheath and Schwann cells. However, studies in *PMP22*$^{0/0}$ mice [31, 32], as well as natural mouse mutants [33] and overexpressing mutants (below), indicate that PMP22 is also involved in early steps of myelinogenesis, in the determination myelin thickness and maintenance. CMT1A models show persistent differentiation defect in Schwann cells during early postnatal development with imbalanced activity of the PI3K-Akt and Mek-Erk signaling pathways, which could be overcome by soluble neuregulin-1 [34]. Altered mRNA processing with changes in the ratio of different PMP22 transcripts [35], as well as posttranslational modifications, may also play a role in disease pathogenesis.

Despite the complexity of CMT1A pathogenesis beyond the gene dosage effect, therapeutic efforts undertaken so far to treat CMT1A have focused on the gene dosage factor and aim to silence PMP22 overexpression. In this regard transgenic rodent CMT1A models have confirmed that PMP22 overexpression causes peripheral neuropathy [36–39] and have been used for developing treatments. Transgenic CMT rats harbor three copies of the mouse *Pmp22* gene, resulting in a 1.6-fold mRNA expression and closely reproduce the CMT1A pathology and phenotype [39, 40]. CMT1A transgenic mouse lines have been generated by the integration of extra copies of the *PMP22* gene [36, 37, 41]. High copy number PMP22 transgenic lines [42] display severe developmental histological and behavioral phenotypes [38] providing more relevant models for severe and early-onset CMT1A, DSS, or CHN, while low copy number PMP22 transgenic models show a milder phenotype and reproduce more faithfully classic CMT1A. An inducible overexpressing CMT1A model demonstrated that demyelinating pathology is reversible if overexpression is interrupted [43]. Thus, overexpressing CMT1A models are a valuable resource for developing gene and other therapies for this CMT type. Given the emerging developmental effects of *PMP22* overexpression, the earliest possible intervention to silence PMP22 expression may be needed to achieve a therapeutic benefit in CMT1A models and patients.

35.3.2 CMT1X

CMT1X (OMIM 302800) is the next most common CMT form [44, 45], after CMT1A and HNPP. More than 400 different mutations in the *GJB1* gene encoding the gap junction (GJ) protein Cx32 cause CMT1X. Cx32 is expressed by several cell types besides myelinating Schwann cells and oligodendrocytes [46–48]. However, peripheral neuropathy and in some cases mild or transient CNS phenotypes are usually the only clinical manifestations of CMT1X. *GJB1* mutations are mostly missense, but also nonsense and deletions, affecting all domains of the protein and sometimes noncoding regions including the promoter sequence. Cx32 is a gap junction (GJ) protein forming hexameric hemichannels which establish a full GJ channel by interacting with another hemichannel from an apposing membrane. Cx32 GJ channels are formed in the non-compact myelin areas of paranodal loops and Schmidt-Lantermann incisures and provide a communication pathway serving homeostatic and signaling functions that are essential for both the myelin and axon [49, 50].

CMT1X affects male patients earlier and more severely with a phenotype and clinical course characteristic of most dominant CMT forms [51–53], while heterozygous females are usually asymptomatic, subclinically or mildly affected at an older age [54, 55]. Acute transient CNS manifestations may occur in a subset of mostly younger CMT1X patients [56]. Motor NCVs usually show intermediate slowing (30–40 m/s) reflecting a mixed axonal and demyelinating pathology revealed by nerve biopsies [57–59]. Clinical studies of large CMT1X cohorts with different *GJB1* mutations showed that disability increases with age and that the degree of disability is comparable with that observed in patients with a documented *GJB1* deletion [53]. Thus, most *GJB1* mutations likely cause loss of Cx32 function. Likewise, pathological studies showed that the severity of changes in CMT1X nerve biopsies are not associated with particular *GJB1* mutations [58, 60].

Expression of CMT1X mutations in vitro revealed that many Cx32 mutants are often retained intracellularly [61–63] in the ER and/or Golgi [63–67] and fail to form functional GJ channels or form channels with altered biophysical characteristics [65]. Cx32 knockout (KO) mice with deletion of the *Gjb1/cx32* gene develop a progressive, predominantly motor demyelinating peripheral neuropathy beginning at about 3 months of age [68, 69], which can be prevented by transgenic expression of wild-type (WT) Cx32 protein in Schwann cells [70], confirming that Schwann cell autonomous loss of Cx32 function is sufficient to cause CMT1X pathology. CMT1X mutants expressed in transgenic mice showed similar intracellular localization as in vitro and no other toxic effects or trans-dominant effects on other connexins expressed by myelinating cells. However, certain Golgi-retained mutants had a dominant negative effect on co-expressed WT Cx32 [71, 72]. Although this is not clinically relevant in patients with CMT1X as only one *GJB1* allele is expressed in each cell, it should be taken into account when planning a gene therapy approach. Cx32 KO mice as well as mice expressing CMT1X mutants on Cx32 KO background provide useful models to test gene therapy approaches for CMT1X.

35.3.3 CMT2A

CMT2A (OMIM 609260) is the most prevalent axonal CMT form with a frequency of up to 30% among all CMT2 patients and 10–15% among all CMT forms [73, 74]. Patients present with progressive distal limb muscle weakness and atrophy, foot deformities, and gait difficulty which can lead to wheelchair dependency [75, 76]. Age at disease onset is variable, but most patients present within the first decade of life [77]. Early onset of disease is associated with a more severe phenotype and development of proximal weakness and optic neuropathy. Later onset after 10 years of age is associated with a more benign course and a higher frequency of unusual findings such as tremor, pain, and hearing loss [78].

More than 60 mutations in the *MFN2* gene encoding mitofusin-2 have been identified so far in CMT2A patients. Mitofusin-2 is a GTPase protein anchored in the outer mitochondrial membrane through two transmembrane domains situated close to the C-terminus. Mutations are preferentially located within the GTPase domain and in the downstream region before the hydrophobic heptad repeat domain 1 (HR1) localized at the base of the protein arm [73]. Recessive compound heterozygous *MFN2* mutations have been rarely reported, with early disease onset [79].

Mitofusin-2 plays a fundamental role in the mitochondrial fusion, fission, and trafficking [80]. Cellular mechanisms proposed to play a role in CMT2A pathogenesis include a deficiency in energy production and altered axonal transport of mitochondria [81], reduction of mitochondrial membrane potentials and coupling efficiency [82], and impaired ER-mitochondrial apposition [83]. Transgenic models of CMT2A include the T105M-expressing mouse under the control of HB9 motor-neuronal promoter [84] which showed hind limb gait defects, severe muscle atrophy, and motor axon degeneration. R94Q transgenic animals showed motor defects also mimicking CMT2A neuropathy [85]. However, these mice became symptomatic after 5 months of age, representing a model of late-onset CMT2A. A knock-in model of the R94W mutation displayed many of the pathological defects that characterize the human disease [86]. CMT2A models provide insights into the pathophysiology of the disease and highlight the dominant effects of the mutated MFN2 causing dysregulation of mitochondrial dynamics and ultimately axonal degeneration. Thus, gene editing and allele-specific silencing approaches are more likely to address the disease pathogenesis.

35.3.4 CMT4C

CMT4C (OMIM 601596) is the most prevalent among the rare recessive neuropathies accounting for almost half of all CMT4 cases [74]. In addition to typical early CMT manifestations, almost all CMT4C patients develop foot deformities and scoliosis, often requiring surgery [87–89]. Many have cranial nerve involvement with deafness, while phenotypic variations are common in patients with identical

mutations [90–92]. A combination of proprioceptive loss and vestibular neuropathy may cause profound and disabling imbalance early in disease evolution [93]. Several truncating but also missense mutations affecting the *SH3TC2* gene have been described in CMT4C patients [94], with higher frequency among certain ethnic groups with likely founder effects [90, 95]. *SH3TC2* encodes a protein of 1288 aa containing 2 Src homology 3 (SH3) and 10 tetratricopeptide repeat (TPR) domains sharing no overall significant similarity to any other human protein with known function. The SH3 and TPR domains suggest that SH3TC2 could act as a scaffold protein [94]. SH3TC2 is present in several components of the endocytic pathway including early and late endosomes, in clathrin-coated vesicles close to the trans-Golgi network, and in the plasma membrane. This localization is altered in CMT4C mutants examined in vitro [96].

Sh3tc2 KO mice developed an early-onset (by P5) but progressive hypomyelinating neuropathy [97]. In addition they showed abnormal organization of the node of Ranvier present already at P4, a phenotype that was confirmed in CMT4C patient nerve biopsies. These alterations were associated with changes in the Nrg1/ErbB pathway involved in control of myelination [98]. SH3TC2 was also found to be an effector of the small GTPase Rab11, a key regulator of recycling endosome functions. CMT4C mutations disrupt this interaction [99], a likely mechanism for the progressive nature of CMT4C neuropathy. Both the clinical phenotype and molecular basis of CMT4C suggest loss of function of SH3TC2, resulting in early-onset and slowly progressive hypomyelination. The Sh3tc2 KO mouse model recapitulates all major aspects of the disease and offers the opportunity to test potential treatments for CMT4C including a gene replacement approach as a paradigm for recessive CMT forms.

35.4 Gene Therapy for CMT Neuropathies

35.4.1 Vectors for Gene Delivery to Peripheral Nerves

One of the main challenges in gene therapy for CMT neuropathies is the need for cell-targeted gene expression since most CMT forms result from mutations in genes expressed by myelinating Schwann cells or by neurons with cell autonomous effects. Targeting of gene expression can be achieved either by using vectors with highly selective cell tropism, such as certain AAV serotypes, or by the use of cell-specific promoters largely restricting gene expression to the targeted cell type. A variety of delivery systems such as viral vectors and non-viral plasmids have been studied for the transfer of therapeutic genes to the PNS [100]. Viral vectors have proven to be the most efficient approach for gene delivery in different neuropathy models. Herpes simplex viral (HSV) vectors, adenoviral (Ad) vectors, adeno-associated viral (AAV) vectors, and lentiviral (LV) vectors are currently the most commonly used vectors for gene delivery.

Earlier studies of gene delivery to peripheral nerves for expression in Schwann cells used mostly Ad vectors and ubiquitous promoters [101–104]. Immunological reaction limited the duration of expression in most of these studies [102, 103, 105, 106]. Intraneural gene transfer of a replication-defective Ad vector expressing *lacZ* driven by a myelin protein zero/P0 (*Mpz*) promoter resulted in Schwann cell expression only for 2 weeks that could be extended to 8 weeks with immunosuppression. Although P0 protein was detected in P0 KO mice, no improvement of nerve pathology was reported [103]. In another study Ad-delivered reporter gene expression was only detectable in injured nerves or under immunosuppression [102]. HSV vectors have been used mainly for infecting DRG cells because of their tropism for sensory neurons to treat models of mostly acquired and predominantly sensory neuropathies [107]. Drawbacks include their limited tropism for other types of neurons and toxicity to target cells.

AAV vectors have the advantage of high tropism for PNS neurons and much lower immunogenicity compared to Ad vectors. The risk for insertional mutagenesis is low since most of the virally delivered DNA remains episomal. A number of different AAV serotypes have been studied showing different cell tropism profiles in the PNS and CNS [108–110]. After intrasciatic injection, AAV2/1 transduced both Schwann cells and neurons, AAV2/2 infected only sensory neurons, and AAV2/8 preferentially transduced Schwann cells. Although expression was detected up to 10 weeks after administration, neutralizing antibodies against all AAVs tested were detected [111]. For expression in sensory neurons, injecting AAV vectors directly into the dorsal root ganglia (DRG) was superior to intraneural injection. Expression lasted for 50 days and could be extended up to 100 days with immunosuppression [104]. Overall, AAVs and especially AAV2/8 may be promising tools for targeted gene delivery to PNS [111, 112].

LV vectors have been increasingly tried because of their larger transgene capacity and ability to integrate into the host genome providing stable long-term gene expression. They cause no significant immunogenicity, and there is no evidence so far of insertional mutagenesis in preclinical [113, 114] or clinical [115] studies. LV vector delivery to sciatic nerve has been used for gene silencing to study gene function [116] or for reporter gene expression [105]. Although initial reports suggested that VSV-G-pseudotyped LV vectors infect Schwann cells only at early stages of development, subsequent studies showed infection of up to 50% of Schwann cells in adult mouse sciatic nerve as well using a myelin-specific instead of an ubiquitous promoter [117].

Although not extensively studied for the PNS, gene therapy approaches using non-viral vectors may offer a promising alternative to overcome some of the problems associated with the use of viral vectors such as insertional mutagenesis and immunogenicity [118]. The primary concerns of the non-viral vectors are the poor transfection efficiency, the endosomal degradation, and low specificity of cell targeting. Nevertheless, recent advances in the development of more stable nanoparticles [119] or modifications on the lipopolyplexes have overcome many of the biological barriers in the use of the non-viral vectors treating neurons. Progress remains to be made in directing the nanoparticles to specific cell types or intracellular organelles in the PNS [120, 121].

35.4.2 Gene Delivery Routes Targeting the PNS

For gene therapy of CMT neuropathies, the gene of interest will have to be delivered either to Schwann cells or to neurons (motor and sensory) or to both cell types, depending on the biological basis of the specific form (Table 35.2). Peripheral nerves are not easily accessible to systemically delivered viral vectors due to the presence of the blood-nerve barrier. Therefore, various delivery approaches directly to the PNS have been tried for the successful targeting either of Schwann cells or neurons in a variety of preclinical studies. Intramuscular, intrathecal, intraneural, and even intraganglial injections have been used for the delivery of plasmids and viral vectors to the PNS.

Direct injection of AAV vector into the DRGs of the pig resulted in expression of the viral vector only in the injected DRG transducing about 30% of neurons [122], while injection of LV vector led to 20% expression of the viral vector in the rat DRG neurons [123] and resulted in suppression of neuropathic pain factors expressed in DRGs by delivering a silencing RNA [124]. Although this method proved to be successful, there are limitations for clinical use due to safety concerns for placing a needle into the human DRG for the infusion.

Intraneural injection directly into the sciatic nerve has been used successfully for gene delivery in Schwann cells using AAV [111] and LV [117] vectors as well as free plasmids with the aid of electroporation [125]. Although intraneural injections using atraumatic methods did not produce detectable demyelination or Wallerian degeneration [104, 105, 117], their utility for clinical translation remains limited due to the invasiveness of the technique and the fact that multiple nerves will need to be injected to achieve a therapeutic result. Intramuscular injections are clinically more feasible and have been used for plasmid and viral vector delivery [126]. The rationale for intramuscular delivery is that vectors can be taken up by the supplying peripheral nerve through the neuromuscular junction [127].

Intrathecal delivery has been tried based on the fact that the cerebrospinal fluid (CSF) and endoneurial fluid are in continuity. The epineurial connective tissue layers merge with the dura matter at the central ends of peripheral nerves [128] so that the subarachnoid and endoneurial space merge [129] allowing CSF to enter the endoneurial fluid. Diffusion of molecules injected into the mid-sciatic nerve has been shown up to 10 mm proximal and distal to the injection site, consistent with flow of endoneurial fluid [130, 131]. Particles may diffuse from subarachnoid space into the peripheral nerves due to pressure gradients. Intrathecal CSF pressure is higher at about 10 mmHg compared to 3–5 mmHg in DRGs and 1–2 mmHg in peripheral nerves [132]. Different studies demonstrated that AAV and LV can be easily delivered by lumbar intrathecal injection and can access both the CNS and PNS resulting in efficient gene expression [104, 133–139]. Overall, gene delivery methods used in preclinical trials have a variable potential to be applied in humans, with intrathecal injections being the most translatable. In contrast, direct intraneural or intraganglial injections are limited due to the invasiveness and because they are likely to achieve only localized gene expression. The latter is also a limitation for intramuscular injection. Further validation and optimization of gene delivery methods to PNS will be needed because of significant size and anatomical differences between humans and animal models.

35.4.3 Gene Therapy Studies in Neuropathy Models

Intraneural injection of AAV2/8, which preferentially transduces Schwann cells, delivering the ciliary neurotrophic factor (CNTF) gene led to an upregulation of P0 and PMP22 myelin proteins 4 weeks after transduction of injured sciatic nerves. CNTF-injected mice showed a significant increase in both GAP43 expression in sensory neurons, a marker of axonal regeneration, and functional improvement as indicated by the increased muscle compound action potential (CMAP) [111], suggesting a potential gene therapy approach for treating myelin disorders.

In an attempt to treat the most common form of CMT, CMT1A, an AAV vector, was injected intramuscularly in the *trembler J* (Tr^{J}) mouse model of CMT1A. AAV1 carrying the gene for neurotrophin-3 (NT-3) driven by the CMV promoter was administered in the gastrocnemius muscle, and NT-3 levels were monitored. Higher vector titers and the use of the CMV promoter proved to be more efficient for obtaining long-lasting (up to 10 months) expression. NT-3 expression ameliorated the hypomyelination and increased myelin fiber density. Furthermore, NT-3 improved the abnormality of increased neurofilament packing density that is characteristic of the Tr^{J} mice. Electrophysiological results were in accordance with the histological findings showing increased CMAPs and faster NCVs accompanied by improved grip strength of treated mice [127].

Based on the fact that most CMT1X mutations appear to cause loss of function, recent efforts have focused on a Schwann cell-targeted gene replacement therapy using the Cx32 KO model of CMT1X. To achieve stable and cell-specific expression, a third-generation LV vector carrying the human *GJB1* gene encoding Cx32 driven by the rat *Mpz* promoter was used to achieve expression in myelinating Schwann cells. This LV vector was delivered once directly into the mid-sciatic nerve. Expression analysis showed that the vector was transported and expressed throughout the length of the nerve. Up to 50% of myelinating Schwann cells showed expression in injected nerves lasting at least 16 weeks [117]. A treatment trial in the *Gjb1*-null/Cx32 KO mouse model of CMT1X using the intraneural LV vector injection resulted in expression of virally delivered Cx32 in the sciatic nerve and improvement of demyelination and inflammation that is characteristic of this model.

In order to develop a less invasive and more widespread gene therapy approach, the same LV vector was injected in the intrathecal space at L5–L6 spinal level resulting in Schwann cell-specific expression not only in lumbar spinal roots but also in sciatic, femoral, and trigeminal nerves and intramuscular nerves. Expression rates throughout the PNS were similar to those achieved by intraneural delivery and remained stable for at least 16 weeks [139]. A treatment trial using the CMT1X model resulted not only in improved pathology in peripheral nerves and spinal roots but also in improved performance in motor behavioral tests. Sciatic NCV and quadriceps muscle force generation were also significantly improved in treated compared to mock-treated animals [139]. Thus, intrathecal gene delivery using LV or AAV vectors may hold promise for a clinically translatable gene therapy for CMT1X and other CMT forms.

35.5 Conclusions and Future Perspectives

CMT neuropathies are a phenotypically and genetically very diverse group of disorders characterized by a variety of cellular and molecular mechanisms that ultimately lead to peripheral nerve degeneration. This plethora of underlying mechanisms means that different approaches will be needed for each CMT type that should be tailored to the function of specific gene or even to effects of different mutations occurring in the same gene. Thus, gene replacement will be needed for loss-of-function mutations, whereas gene silencing or gene editing will have to be developed for gene overexpression with dosage effect or for expression of toxic mutants. Recent advances in optimizing and characterizing vectors and administration methods for efficient gene delivery to PNS hold promise for developing effective treatments that may benefit several CMT types.

Despite this progress many challenges remain along the way to offer treatments to patients. Besides further optimization and validation of clinically applicable vectors, delivery methods, and stability of expression, as well as safety concerns with some vectors, one of the major challenges is the variability in the age of onset and severity, even between patients with the same mutation. Future clinical trials will need to include sensitive outcome measures because of the variable and slow progression of the disease in most cases making the demonstration of a therapeutic benefit more difficult. Furthermore, gene therapy will need to be offered as early in the disease course as possible because several CMT genes appear to have a developmentally crucial role, with early-onset pathological changes that cannot be reversed at later stages. Even in types with later onset of pathology, gene therapy will need to be offered before chronic axonal loss, and disability has been established, as this may not be reversible despite effective gene delivery methods. Nevertheless, gene therapy remains the major hope for treating CMT neuropathies in the near future, and the steps already taken have opened the way for further research and progress ahead.

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Chapter 36
Muscle as a Potent Target in Vaccination

Axel Rossi and Hildegard Büning

Abstract "Immunology" and "vaccines" are terms inevitably connected with the name of Edward Jenner, a country doctor from Berkley (UK), who submitted back in 1796 a report on 13 cases of protection against small pox infection through inoculation with cowpox. Since then vaccination has become the most efficient strategy to protect humans and livestock from infectious diseases. With exception of Japan, intramuscular injection serves as a standard vaccination route. Besides its easy accessibility, skeletal muscle presents several unique properties marking this tissue an ideal theater for vaccination. Muscle cells or more specifically myofibers act as unconventional antigen-presenting cells (APCs) supporting the activity of conventional APCs (e.g., dendritic cells (DCs)) rapidly recruited to the site of "damage." Depending on the vaccine, humoral and/or cytotoxic T cell responses are elicited. Moreover, unique control circuits involving hormone regulation and unconventional co-stimulatory molecules as well as muscle resident stem cells permit balanced immune responses and tissue integrity. The plasticity of this tissue regarding recruitment and regulation of immune cells as well as its regenerative capacity holds promise for the advent of new sophisticated vaccination strategies for targets that so far "escaped" from vaccine-mediated protection or treatment.

Keywords Skeletal muscle · Innate immune system · Adaptive immune system Routes of administration · Vaccination strategies

A. Rossi (✉)
Laboratory for Infection Biology & Gene Transfer, Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany
e-mail: Rossi.Axel@mh-hannover.de

H. Büning
Laboratory for Infection Biology & Gene Transfer, Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany

Laboratory for AAV Vector Development, Center for Molecular Medicine Cologne (CMMC), University of Cologne, Cologne, Germany

German Center for Infection Research (DZIF), Partner Site Hannover-Braunschweig, Braunschweig, Germany
e-mail: Buening.Hildegard@mh-hannover.de

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36.1 Introduction

Incited by the success of the vaccinia virus-based vaccine eradicating small pox [1], vaccination programs have been brought to life to protect humans and livestock from severe and/or life-threatening infections [2, 3]. The latter has dramatically reduced the level of poliovirus infection and demonstrated efficacy in preventing a broad range of diseases caused by viruses (e.g., yellow fever, measles, rubella, mumps) or bacteria (e.g., tuberculosis, plague, cholera) [3]. This success has fostered efforts to develop both preventive and therapeutic vaccines to enhance pathogen clearance and protect against/treat cancer, respectively [3–6]. In the early days of vaccination, attenuated pathogens were the vaccines of choice. They induce a mild, commonly asymptomatic, infection that owing to the presence of the full set of pathogen-specific antigens elicits the most appropriate kind of immune response conferring durable immunity against its pathogenic counterpart. Since they are attenuated, but biologically active, such vaccines may sometime cause severe side effects if mutations responsible for the attenuated pathogenicity are reverted to the wild type. In addition, attenuated pathogens cannot be applied to children, the elderly or immunocompromised patients as a fully competent immune system is required for their control. As an alternative, killed or inactivated pathogens have been explored as vaccines. They are safe, but less immunogenic, and are therefore applied together with an adjuvant that either directly enhances innate immune responses or indirectly supports pathogen uptake into antigen-presenting cells (APCs) [7]. In addition, booster immunization is required to induce a long-term protection [3]. With the advent of genetic engineering, a cornucopia of new opportunities became available resembling a “tool box” for the design of safe and efficacious vaccines [7, 8]. This offered moving from trial and error approaches to rational design in which a combination of features provided by the vaccine itself and the choice of the adjuvant/s as well as target tissue are used for shaping the adaptive immune response toward its humoral and/or cellular arm [9]. Co-administration of MF59 (squalene-in-water emulsions) or Alum (aluminum salt), for example, is reported to polarize the immune response toward antibody production, while antigen-specific cytotoxic T cell responses are enhanced by adjuvants like ISCOMs (saponins) or agonists of endosome-located sensors of the innate immune system (reviewed in [9]). Regarding target tissue accessibility, the ability to recover from vaccine treatment without detrimental effects for the host as well as the specific set of resident professional and nonprofessional APCs and thus the potency of a given target tissue to promote the desired immunological response are generally considered as decision-making parameters.

36.2 Skeletal Muscle: A Singular Immunological Environment

Skeletal muscle tissue has emerged as the most used target tissue for clinical vaccination in humans. It is easily accessible, possesses a remarkable regenerative potential, and orchestrates potent immune responses (Fig. 36.1) [10]. The muscle

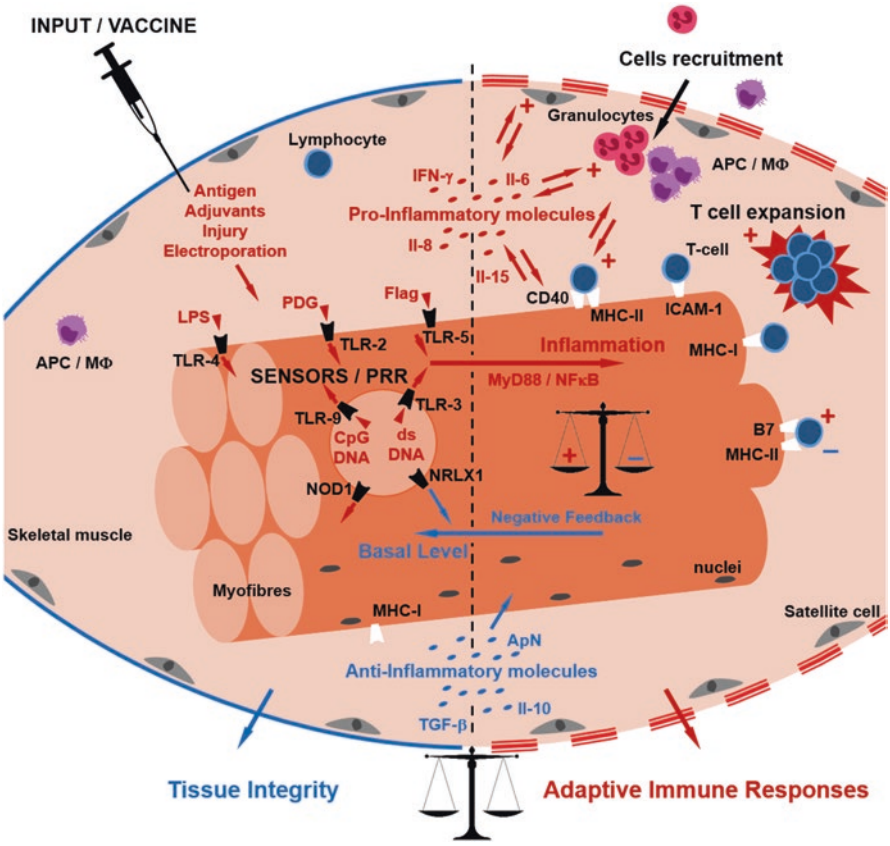

Fig. 36.1 Skeletal muscle as immune theater upon vaccination. Upon intramuscular vaccination, immune sensors (PRRs) recognize PAMPs or DAMPs of the vaccine and activate signal cascades that result in upregulation of pro-inflammatory cytokines and chemokines. As a consequence, resident immune cells and unconventional APCs (myofibers) are activated, and further immune cells are recruited, which support the local inflammation process. The reaction leads eventually to a cytotoxic or humoral adaptive immune response. Myofibers themselves and satellite cells allow control of tissue integrity by anti-inflammatory pathways or tissue repairing mechanism, respectively

parenchymal cells are myofibers, terminally differentiated from myoblast to multinuclear cells and responsible for the mechanical functions of the muscle [10]. Myofibers are interspersed with satellite cells, the muscle stem cells, and resident immune cells. Compared to the skin (epidermal, dermal, and subcutaneous layers) or mucosa, the proportion of resident immune cells in muscle tissue is low. Upon inflammation, however, the pool of professional APCs becomes enlarged by recruiting immune cells such as monocytes to the site of tissue damage [10]. In addition, myofibers, which are per definition nonprofessional APCs, are turned into important regulators of the immune responses during inflammation [10–12].

36.2.1 A Brief Introduction to the Innate and Adaptive Immune Response

The innate immune system is referred to as the body's first line of defense. Its function comprises recognition of pathogens or of danger signals and mounting responses to clear the injured site and to induce and shape adaptive immune responses against the culprit. Innate immune sensors are pattern recognition receptors (PRRs) [13]. They are germ line encoded, and the repertoire as well as level of expression varies between cell types. In addition, depending on specific environmental conditions, expression becomes up- or downregulated. PRRs recognize distinct patterns (pathogen-associated molecular patterns (PAMPs), danger-associated molecular pattern (DAMP)) that are hallmarks of classes of stress such as pathogens (PAMP) or dysregulation of cell homeostasis (DAMPs). The best-characterized PRRs are the Toll-like-receptors (TLRs) which induce inflammatory responses after ligand binding [14]. TLRs are type I transmembrane glycoproteins composed of extracellular leucine-rich repeat (LRR) motifs serving as recognition domains, a single transmembrane domain, and a cytoplasmic toll/interleukin (IL)-1 receptor homology (TIR) signaling domain [13, 15]. To date, ten different human TLRs (TLR-1–TLR-10) have been described. They form homo- or heterodimers and are either located at the cell membrane (e.g., TLR-1, TLR-2, TLR-4, TLR-5, TLR-6, and TLR-10) for recognizing distinct sets of PAMPs derived from the microbial outer surface or in the membranes of intracellular vesicles (TLR-3, TLR-7, TLR-8, and TLR-9) for detecting "nonself" nucleic acids. PAMP sensing activates a downstream signaling mediated by TIR domain containing adaptor molecules inclusive of a myeloid differentiation factor 88 (MyD88), the MyD88 adaptor-like protein (MAL or TIRAP), or the TIR domain-containing adaptor-inducing IFN-β (TRIF). TLRs differ in the choice of adaptor molecules and thereby in the respective innate immune response. TLR-2, for example, signals through MyD88 inducing upregulation of inflammatory cytokine and chemokine expression and secretion via nuclear factor kappa B (NFκB) and mitogen-activated protein kinases (MAPKs), while TLR-3 signals through TRIF leading to expression of type I interferon (IFN) as well as inflammatory cytokines. According to the released cytokine cocktail, various cell effectors including macrophages or granulocytes are recruited to participate in the clearance of the infection site [16]. In parallel, APCs activate naïve T lymphocytes to initiate adaptive immune responses directed against the presented antigen/s. Vaccination strategies use this immune response cascade from detection of the antigen down to the shaped adaptive immune response to induce the desired preventive or therapeutic effect.

36.2.2 Antigen Detection and Inflammation in Skeletal Muscle

Although it is difficult to trace the precise cellular origin of the different immune components (e.g., immune receptors, cytokines, chemokines, and other messengers) regulated in response to vaccine administration, gene expression arrays

revealed that in principle the whole subset of innate immune receptors is expressed in the muscle [17]. Examples of PRR in skeletal muscle, which responded toward stimuli confirming functionality, are TLR-2, TLR-3, TLR-5, and TLR-9. In addition, several intracellular sensors termed NLR (NOD-like receptors), including NOD1, sensitive to the bacterial component D-glutamyl-meso-diaminopimelic acid, and NRLX1, a negative regulator of the TLR/MyD88 pathway, are expressed. As already implied by NRLX1, innate immune activation is the subject of tight regulation to restrict tissue damage. Besides negative regulators such as NRLX1 or feedback loops (see below), sensitivity or reactivity is controlled by TLR expression, which differs depending on differentiation status (e.g., myofibers vs. myoblasts) and muscle type (e.g., soleus muscles vs. gastrocnemius muscle) [10, 17].

In addition to its function as a pathogen sensor, the muscle also plays an active role in the inflammation process itself by upregulating expression of cytokines, chemokines, and cell adhesion molecules. Of particular importance in this regard is interleukin (IL)-6 released by muscle cells in response to tumor necrosis factor (TNF)-α, IL-1α, IL1-β, lipopolysaccharide (LPS), or IFN-γ [10]. This pro-inflammatory cytokine triggers expression of monocyte chemoattractant protein (MCP)-1 and the intracellular adhesion molecule (ICAM)-1 by the muscle cells themselves (autocrine loop) using a mechanism termed trans-signaling mediated by IL-6R and gp130, both also expressed by the myofibers [10]. MCP-1 acts as a chemoattractant of monocytes and lymphocytes, leading to recruitment of further immune effector cells. Moreover, two different forms of ICAM-1 are overexpressed in response to IL-6; one is bound to the cell membrane and promotes the interaction between T lymphocytes and APCs or the cytotoxic effect of T lymphocytes on infected muscle cells, while the other one is secreted and functions as a competitor of the bound membrane form, triggering an inhibitory effect [18, 19]. The presence of pro-inflammatory cytokines such as IFN-γ in the skeletal muscle tissue also induces expression of the neutrophil chemoattractant IL-8 leading to the recruitment of neutrophils, which further replenishes the pool of secreted IL-6 [10].

Exaggeration of the immune response is avoided by secretion of anti-inflammatory cytokines, such as transforming growth factor (TGF)-β and IL-10, which counteract in particular the pro-inflammatory activities of IL-6. Moreover, evidence has been provided that besides anti-inflammatory cytokines, the hormone adiponectin (ApN) functions as a key regulator of immune responses against LPS in mice and in human primary myofibers. As a target for ApN, microRNA (miRNA) 711 was identified. This miRNA inhibits toll-interacting protein (TOLLIP), Fas-associated protein with death domain (FADD), TGF-β activated kinase 1-binding protein 1 (TAB1), phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit delta (PI3Kδ) and TNF-α, representing the TLR-4 downstream signaling cascade, thereby blocking NFκB and/or MAP kinase activation [20]. This and probably other to be identified pathways are maybe harnessed for providing a better control of the immunity for vaccination strategies [20, 21].

36.2.3 Promotion and Control of Adaptive Immune Responses in Skeletal Muscle

Vaccine strategies aim to induce antigen-specific cytotoxic and/or humoral immune responses. While $CD8^+$ T lymphocytes are the effector cells for the former, activated $CD4^+$ T helper cells and effector B cells are required for the latter. The quality of cellular as well as humoral immune responses depends, among others, on the magnitude of the local inflammation and the ability of the inflamed tissue to present antigens through major histocompatibility complex (MHC) classes I and II. Generally, MHC class I ligands are peptides newly produced within the cell, while peptides derived from exogenous sources are loaded on class II. However, antigens from the extracellular environment can also be presented on MHC class I, a phenomenon termed cross-presentation [22].

Recognition of antigens presented by MHC molecules lead to activation and clonal expansion of respective effector lymphocytes. Critical players in this process are the APCs. They possess a battery of PPRs, present antigens via MHC class I and II molecules, are particularly effective in cross-presentation, and link innate and adaptive immunity. The best characterized APCs are DCs. DCs maintain local inflammation by pro-inflammatory cytokine cocktail release and antigen uptake and presentation. As noted before, the skeletal muscle tissue contains a limited number of resident APCs compared with other vaccination targets such as the skin. This, however, is compensated by the muscle's ability to function as an "alternative" or nonprofessional APC in conditions of local inflammation (Fig. 36.1). Specifically, muscle cells gain the ability to express MHC class II molecules, and expression of class I molecules is upregulated. In addition, a nonconventional MHC-I molecule, the HLA-G, is expressed in muscle cells [23, 24]. This nonconventional MHC-I molecule is able to downregulate activity of immune cells including cytotoxic $CD8^+$ T cells and helper $CD4^+$ T cells providing thereby an effective regulation system involved in inducing antigen tolerance. Besides antigen presentation by MHC molecules, co-stimulatory molecules present on APCs and recognized by lymphocytes are required. Muscle cells are special in this regard as they lack the classical co-stimulatory molecules CD80 (B7-1) and CD86 (B7-2) but express instead BB1 and B7-H2—also B7 family members with a potential co-stimulatory role. Further, muscle expresses B7 homologs including B7-H1 and B7-H3 known for their capacity to reduce immune responses, a further example of a muscle-specific fine regulation mechanism to protect the integrity of the tissue. Skeletal muscle cells also differ from other non-APC cells in constitutively expressing CD40, a key molecule employed by APCs to communicate with $CD4^+$ and $CD8^+$ lymphocytes through CD40L. Local inflammation enhances expression of CD40 on myofibers resulting in a positive feedback loop through which further immune cells are recruited.

According to these unique immune properties, skeletal muscle tissue appears fully equipped as a potent target for human vaccination. Specifically, it recognizes a broad range of antigens through PRRs. Myofibers induce a local inflammation, are capable of antigen presentation, and fine regulate—together with several immune

effectors recruited to the site of injury/pathogen invasion—the local inflammatory response. As a consequence, an antigen-specific adaptive immune response is initiated which allows pathogen clearance and establishes a memory response, offering an effective protection against a potential second infection. Moreover, the skeletal muscle is a highly dynamic tissue. Thus, in case of cell loss, tissue repair mechanisms are initiated to maintain the tissue integrity.

36.3 Vaccination Strategies Using Muscles

Intramuscular (i.m.) injection is the standard method for vaccination in Europe, the United States, and other countries [25, 26]. Indeed, i.m. injection as delivery route appears safe (no serious side effects) and efficacious in inducing antigen-specific humoral and/or T cell-mediated immune responses. Examples of currently available vaccine treatments are shown in Table 36.1. In most of the cases, more than one vaccine is available to protect against a given pathogen, and combined vaccines have received marketing authorization (e.g., in Germany (http://www.pei.de/

Table 36.1 Available[a] viral and bacterial vaccines for use in human

	Live attenuated	Killed inactivated	Subunit
Viral	Vaccinia Polio (OPV) Yellow Fever Measles Mumps Rubella Influenza Rotavirus Dengue virus	Polio (IPV) Rabies Influenza Hepatitis A	Hepatitis B (HeB-surface antigen) Human papilloma virus (HPV)
Bacterial	BCG (tuberculosis) *Salmonella typhi* (oral)	*Bordetella pertussis* (whole cell) Cholera *Bacillus anthracis* *Leptospira interrogans*	Tetanus (toxoid) Diphteria (toxoid) *Neisseha meningitidis* (polysaccharide) *Bordetella pertussis* (acellular) *Streptococcis pneumoniae,* 23 valent (polysaccharide) *Hemophilus influenzae,* type b (Hib) (polysaccharide) *Neisseha meningitidis* (polysaccharide conjugate) *Streptococcis* pneumoniae, heptvalent (polysaccharide conjugate) *Salmonella typhi Vi* (capsular polysaccharide) *Borrelia burgdorferi* (outer-surface protein A (OspA)) *Leptospira interrogans*

[a]Principal available vaccines in West World. Adapted from Nascimento and Leite [8]

EN/medicinal-products/vaccines-human/vaccines-human-node.html)). Despite these achievements, infectious diseases remain one of the main causes of death around the world, and this is particularly true for low-income countries. In addition, in developed countries, the increase in life span calls for vaccines that can be applied to immunocompromised patients, patients with chronic infection or the elderly. In addition, cancer has been identified as a novel target in vaccine development [3]. This does not only include vaccination against pathogens related to cancer development such as hepatitis B virus (HBV) or human papillomavirus (HPV) but also employs vaccine strategies to induce adaptive immune responses against tumor cells itself. The latter is challenging as tolerance against "self" needs to be overcome. Finally, conventional vaccine strategies suffer from various drawbacks including failure to protect against major health burdens such as malaria, human immunodeficiency virus (HIV), hepatitis C virus (HCV), or influenza virus [27]. These challenges call for a new class of vaccines, designed on a rational basis and directed against pre-defined antigen(s) specifically inducing the type(s) of immune response required to confer protection or treatment. To develop this type of "modern vaccine," input from multiple disciplines is required. Specifically, there is a need for obtaining a deep understanding on the pathogenicity of microbes ("danger" to be addressed), on the type of immune responses that needs to be elicited, and on how these immune responses can be induced. Additionally, a "toolbox" must be available enabling designing a vaccine that can manipulate the host's immune system accordingly.

DNA-based vaccines represent one of the first examples of these modern vaccines. They are composed of plasmid DNA—produced in bacteria—that encode for the antigen along with a strong eukaryotic promoter to drive its continuous expression in the target cells [28]. Obvious advantages compared to attenuated pathogens or recombinant protein-based vaccines are high stability at room temperature (easy for storage and shipping) and the convenient way of developing and producing them. Specifically, antigen-coding sequences can be introduced by standard cloning techniques. Its sequence can de designed to function as innate immune activators along with plasmid backbone sequences. In addition, plasmids can be produced even in large amounts in bioreactors in an easy and cost-effective manner. Regarding gene transfer, muscle appears as an excellent target tissue. The large number of T-tubules and the caveolae structures of the myofibers promote uptake of DNA vaccines into the cell, while the unique organization of this tissue with peripheral location of the nuclei beneficially impacts on efficacy of transduction. The antigen—when designed as a secreted protein—is released from the transduced muscle cells and subsequently processed by APCs (e.g., DCs) or B cells triggering a humoral immune response. DCs may also cross-present secreted antigens inducing antigen-specific T cell responses. In addition, antigen is presented through MHC classes I and class II either by the muscle cells or following APC uptake to directly activate T cells. In animal models, DNA vaccines thereby trigger, both efficacious antigen-specific humoral and cellular immune responses. DNA vaccines received marketing authorization in veterinary medicine in Canada in 2005 [28, 29]. The first DNA vaccine in Europe was licensed in 2016 and is recommended for protecting

Atlantic salmon against salmon alphavirus subtype 3 (EMA/CVMP/281226/2016). The efficacy of DNA vaccines reported in veterinary medicine, however, is not translatable to humans [30]. A lower immunogenicity of DNA in humans as compared to other mammals is proposed as most likely explanation, although the underlining mechanism remains to be fully elucidated [28, 31]. Of interest in this regard is perhaps a recent report on species-specific differences in the cGAS/STING DNA sensing system [32, 33]. STING (stimulator of interferon gene) is the main PRR for DNA in muscle tissue [31]. STING is either directly activated by cyclic dinucleotides or upon binding of the second messenger cyclic GMP-AMP, produced by cyclic GMP-AMP synthase (cGAS) in response to cytosolic double-stranded DNA [34]. STING is located in the endoplasmic reticulum and mounts via TBK1 (TANK-binding kinase 1), a type I IFN response, the typical innate immune response toward nucleic acids. In addition, STING has been reported to activate NFκB, MAP kinase, and STAT6 immune signal pathways and may induce autophagy, an ancient cell response pathway that among others clears cells from invading pathogens [33, 35].

A totally different strategy for the design of modern vaccines is based on the use of antigens as recombinant proteins applied together with adjuvants or in form of so-called viruslike particles (VLP). An example for the latter is HPV vaccines. The purified L1 capsid protein of HPV self-assembles into icosahedral particles, which are recognized by the immune system after intramuscular injection. Following this line, L1 proteins of different HPV serotypes can be combined in a single vaccine such as in case of Gardasil9 [36, 37]. The latter contains L1 self-assembled VLP derived from nine different HPV serotypes and is reported to provide an efficient and reliable protection against HPV infection, which is cancer-associated. A very potent variation of this concept is the use of recombinant viral or bacterial vectors, for example, from adenovirus (AdV) or bacille Calmette-Guérin (BCG), as delivery tools. They encode for the target antigen/s and function simultaneously as multimodal adjuvants. In line, compared to DNA vaccines, these recombinant vectors/bacteria-based vaccines demonstrated significantly higher immunogenicity because multiple PRRs are addressed and antigen expression is improved as they possess a natural ability to efficiently cross host barriers. A more recent example targeting Nipah virus (NiV) uses vectors based on the adeno-associated virus (AAV), a non-pathogenic parvovirus otherwise used as delivery tool for in vivo gene therapy [38]. The natural hosts of NiV are fruit bats. NiV infection—classified by the WHO as emerging zoonosis—can cause fatal encephalitis in humans (http://www.who.int/csr/disease/nipah/en/). So far, no vaccine is available. An AAV serotype 8-derived vector was developed and tested in a proof-of-concept study [39]. The vector encoded for the G-glycoprotein gene of NiV and was applied by intramuscular injection. Of note, a single injection of this vaccine was sufficient to induce an effective protection in a hamster model. Interestingly, this vaccine also induced a cross-protective immune response in 50% of the hamsters against Hendra virus (HeV), another member of the *Henipavirus* family [39]. Recombinant viral vectors were also used in the battle against other emerging pathogens, the recent Ebola outbreak in West Africa being one example [40]. Ebola virus is a member of the *Filoviridae* that causes lethal hemorrhagic fever. In the most severe epidemic that

occurred between 2013 and 2016, 28,600 cases of Ebola virus disease (EVD) with more than 11,300 deaths were reported [40]. In a combined effort, already available vaccines—all belonging to the class of modern vaccines—were rapidly approved for clinical trials. They all focused on Ebola virus surface glycoprotein located on the envelope. Specifically, single-dose as well as prime-boost regimes were tested with vaccines based on AdV, vesicular stomatitis virus (VSV), and minute virus of mice (MVM) (for details see [40]). All vaccines proved to be safe with VSV-based vaccines likely being the most effective [40]. Although evaluations and developments are ongoing, results obtained from these trials allow concluding that in case of a new EVD emergency, outbreak control will be faster and more efficient. Similarly, the recent Zika virus outbreak fostered interdisciplinary collaborations to understand its pathogenicity, decipher its transmission route, and develop potent vaccine strategies [41].

36.4 Enhancing Vaccine Immunogenicity

Remarkable efforts are underway to address the WHO's list of emerging infections [42] in particular by using the abovementioned strategies of modern vaccine designs. However, a barrier toward reaching modern vaccine's full potential is frequently their comparison to live attenuated vaccines and lower immunogenicity. Besides further improvements in vector design (e.g., [43]) and adjuvants (for a recent review, see [16]), variations in application strategies are assayed to tackle this challenge. One strategy that resulted in considerable success regarding DNA vaccines is intramuscular electroporation (EP). Short electric pulses that destabilize the myofiber membranes, promoting DNA uptake and thus expression of the heterologous gene [44, 45]. Furthermore, EP enhances the strength of immune response due to local inflammation resulting from the electric stress and upregulation of TLR-9 and the inflammasome. The potency of such improved vaccine design may be well illustrated by a clinical vaccine trial for patients with chronic infection by HCV, a condition characterized by a dysregulated humoral and cellular immune responses [46]. Despite the abovementioned challenging condition, EP-mediated application of a DNA vaccine encoding for a conserved HCV antigen induced a $CD8^+$ T cell response and inhibited (albeit transient) HCV replication. Of note, no injury was reported allowing one to consider intramuscular EP as safe delivery strategy through which immunogenicity of a vaccine can be improved significantly.

Another approach to enhance the immunogenicity of vaccine consists of prime-boost strategies. For heterologous prime-boost strategies, antigen is inoculated into the host successively using two different formulations. DNA vaccines, for example, appear as excellent priming vehicles, while recombinant vectors showed promise as boosters [8, 47]. Alternatively, single-shot prime boost vaccines are developed in which capsid modified vectors are applied. The capsid of such vectors serve as scaffolds for antigen display (similarly to VLP) for priming an antigen-specific immune response upon vector administration. This maintains expression and secretion of

antigen/s following vector-mediated cell transduction and enhances the adaptive immune response [48]. To date, such prime-boost strategies are discussed as the most promising strategy to induce a strong and persistent adaptive immune response.

36.5 Skeletal Muscle vs. Alternative Vaccination Targets

The magnitude, the nature, and the kinetics of the immune responses are largely influenced by the delivery route [49]. Tissues localized at the interface between the host and its environment such as the skin and the mucosa appear more "immune competent" compared to the muscle. The inflammatory response is initiated faster and is stronger, mediated by a broader range of immune effectors (e.g., resident cells, immune sensors). This higher susceptibility, which is likely the cause of its function as natural barrier toward pathogens, is a clear advantage compared to the muscle as a vaccine target and is of importance in cases in which protective antigen-specific immune responses are difficult to achieve. However, this higher reactivity comes with the increased risk of unwanted local reactions. Skeletal muscle, on the contrary, while being in the "naïve" state and less "immune competent" than the skin or mucosa, fully compensates this disadvantage by efficient recruitment of APCs as well as by the potency of muscle cells in functioning as nonprofessional APCs. Thus, when receiving the correct "cocktail" of stimulation, skeletal muscle is equally competent to mount protective antigen-specific humoral and/or cellular immune responses. When comparing skeletal muscle with other vaccine targets, its regenerative capacity as well as the muscle-specific immune regulation circuits needs to be highlighted as it represents a unique "safety net" for maintaining tissue integrity despite mounting antigen-specific immune responses upon vaccination. The muscle and skin/mucosa also differ in the quality of the immune response induced following vaccine administration [50]. Targeting the gut mucosa, for example, results in a gut-associated cytotoxic T lymphocyte (CTL) response, while intramuscular administration allowed for CTL responses in blood and gut, revealing a distinct compartmentalization of the immune response [50]. Skeletal muscle also differs from the skin and mucosa in its turnover rate resulting in significant longer exposure of the immune system toward antigens expressed and released from DNA or vector-transduced cells. This seems to be a promising feature for vaccines designed for the elderly as exemplified by seasonal influenza vaccines [51].

36.6 Conclusion

The potency of vaccination programs in protecting humans and livestock from infectious diseases has been shown without doubt. Less efficient were attempts exploring therapeutic vaccines or vaccines that target tumors (not the cancer related pathogen). Further challenges comprise chronic infection, pathogens

with high antigenic variation, or use of vaccines in immune compromised subjects. In order to tackle these challenges and—in addition—to improve safety of vaccines, modern vaccines are developed in an interdisciplinary approach. These rational design-based vaccines increase in complexity with our growing knowledge on how our immune system works and with the advent of sophisticated techniques to mount (but also to control) antigen-specific immune responses required for long-term protection or efficient treatment. Here, we focused on the skeletal muscle, a tissue that represents itself with a long and successful record as a target for vaccine administration. This popularity is linked to its easy accessibility but also to its remarkable regenerative capacity. Owning to its natural function and location in the body, skeletal muscles are less well equipped with resident immune cells compared to other vaccine targets like the skin or mucosa and were therefore considered as being less competent in inducing protection against weak antigens. However, upon vaccination, myofibers, the main muscle cell type, gain the ability to function as nonprofessional APCs and thus to mount together with recruited and resident APCs an innate immune response that eventually results in a potent antigen-specific immune response. Based on this positive feedback loop and the availability of a "toolbox" to tailor modern vaccines, even a weak antigen ought to be turned into a potent i.m.-applicable vaccine. Moreover, in the muscle, tight regulation circuits are in place that—in conjunction with the regenerative capacity of skeletal muscles—protect the host, making skeletal muscle not only a promising but also safe target of vaccination.

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Part III
Clinical Muscle Gene Therapy

Chapter 37
Patient and Family Perspective on Muscle Gene Therapy

Pat Furlong

Abstract Gene therapy for Duchenne muscular dystrophy (DMD) has been a long time coming. Duchenne first identified this disease more than 100 years ago. In 1986, the genetic basis was identified, and the gene cloned was the largest in the human genome. In 1987, dystrophin, the protein product, was identified. Families watched as their sons lost the ability to walk and the ability to self-feed and died as teenagers. In the late 1980s and 1990s, the promise of gene therapy was thought to be "easy" through the use of a virus capable of delivering the 14.0 kilobases. Families were assured that restoring dystrophin would stop progression and delay the loss of motor milestones. Hope was high and "soon" seemed within reach. But many years have passed as the field moves forward, exploring both the safety and carrying capacity of viral vectors. And now, 30 years later and with intensive efforts to exploit gene therapy, we stand on the threshold. Gene therapy is on the horizon and the word "soon" is reality.

Keywords Duchenne · Rare disease · Genetic · Hope · Patients · Family Muscular dystrophy · Gene therapy

> Hope is a good thing, maybe the best of things, and no good thing ever dies.
> Shawshank Redemption

37.1 Rare Diseases: Legislation

Rare diseases affect more than 30 million Americans and 400 million people worldwide. In 1982, an informal coalition of patients and families with rare diseases formed the National Organization of Rare Disorders. The National Organization for Rare Disorders (NORD) and many other activists impacted by

P. Furlong (✉)
Parent Project Muscular Dystrophy, Hackensack, NJ, USA
e-mail: Pat@parentprojectmd.org

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rare diseases called for change, advocating for legislation intended to encourage, support, and incentivize drug development for treating these rare or orphan diseases. On January 4, 1983, President Ronald Reagan signed the Orphan Drug Act into law. Under the Orphan Drug Act, drugs, biologics, and vaccines qualify for orphan status if they are intended to treat a disease affecting less than 200,000 in the population. In an effort to encourage the development of drugs for rare diseases, the Act contained incentives, including 7-year market exclusivity for companies that developed orphan drug and tax credits that equal one half of the development costs. This was later changed to a 15-year carry-forward provision and a 3-year carry-back that can be applied in profitable years, grants for drug development, fast-track approvals of drugs indicated for rare diseases, and expanded access to the Investigational New Drug Program. The law was later amended to waive user fees charged under the Prescription Drug User Fee Act (or PDUFA). Until that time, only 38 orphan drugs had been approved by the US Food and Drug Administration (FDA). In 2014, 468 orphan designations covering 373 drugs had been approved. In part, and because of this legislation, Japan adopted an Orphan Drug Act in 1993 and the European Union in 2000. In 2017, the sales forecast for orphan drug sales is estimated to be $209 billion dollars and set to account for 21.4% of worldwide prescription sales by 2022.

Additional incentives have been put into place to ensure that more clinical studies are conducted on children. Congress enacted two laws that would serve to increase the study of drugs in children with rare diseases. First, the Best Pharmaceuticals for Children Act (BPCA) provides an incentive for drug companies to conduct FDA-requested pediatric studies by granting an additional 6 months of marketing exclusivity. Second, the Pediatric Research Equity Act (PREA) requires drug companies to study their products in children under certain circumstances. When pediatric studies are required, they must be conducted with the same drug and for the same use for which they were approved in adults. While these incentives were not specific to rare diseases, they enabled the rare disease community to engage with industry on products that may target pathways relevant to a rare disease.

In 1998, Parent Project Muscular Dystrophy (PPMD), an advocacy organization focused on Duchenne muscular dystrophy, made significant inroads within the National Institutes of Health (NIH) and Congress. In 2000, the NIH convened a workshop on Duchenne muscular dystrophy. It was the first time the NIH expressed any interest in this rare disease. The Muscular Dystrophy CARE Act (MDCA), legislation specifically focused on the muscular dystrophies, was introduced into the US Congress, both the House and Senate, on February 14, 2001. This legislation was signed into law in December 2001; this legislation would galvanize research, incentivize companies to invest in Duchenne, and restore hope in families worldwide.

37.2 Duchenne Muscular Dystrophy: Patients, Family, and Community

The dystrophin or DMD gene is the largest gene in the human genome consisting of 2.5 million base pairs and 79 exons. The size of the gene makes it susceptible to random and frequent mutation. The protein product, dystrophin, is present in all muscle: skeletal, cardiac, and smooth muscle. Dystrophin functions as a shock absorber for muscle and an anchor of sorts, stabilizing muscle fibers. Without dystrophin, the muscle cannot survive. It is estimated that 12,000 individuals are living with Duchenne in the United States and 250,000 worldwide. One in 4600 boys will be born with Duchenne. Approximately 30% of those diagnoses are spontaneous mutations. Similarly, 1 in 4600 women will be born carrying the genetic mutation on their X chromosome and, blissfully unaware, carrying a 50% risk of having a male child who will be diagnosed with Duchenne. A significant proportion of these carriers develops muscle disease, in particular cardiomyopathy.

In the confines of a physician's office, the diagnosis is made, translating concerns expressed by the parents into a single word: "Duchenne." The physician describes the loss of the structural protein, dystrophin, as well as the subsequent cascade of events that occur based on the absence of this critical protein in the muscle. The physician then outlines what the parents might expect over the trajectory of the illness.

Duchenne is characterized by skeletal muscle weakness, though dystrophin is present in every tissue. Typically, individuals lose ambulation in the early teens, become unable to lift their arms to their mouth, and will require ventilation in their late teens. Duchenne is 100% fatal with the mean age of death in the mid-20s. Life plans are changed, revised, and revised again and again as function is lost. Each day, families watch and wait. Hope often feels out of reach.

Parents search the Internet, seek expertise, and connect with researchers, clinicians, industry, and other families. They set up Google alerts, RSS feeds, list serves, PubMed criteria, industry alerts, and Facebook pages—living each day hoping to receive news. They review their dictionary of terms: dystrophin, genetic mutation, exon, and introns. They calculate what clinical trial or potential treatment may slow or halt progression and what opportunity may be "on the horizon" and "in time" for their son.

37.3 Gene Therapy for Duchenne: Hope

For families, dystrophin is the "holy grail," the belief that restoring dystrophin would have the potential to stop progression. Families become experts on their child's disease-causing mutation. They explore opportunities that might result in the expression of a truncated form of dystrophin, full-length dystrophin, or a replacement protein that may serve as an adequate substitute.

The cloning of the dystrophin gene in 1986, and the identification of the protein in 1987, was an exciting time—the potential to restore dystrophin seemed "soon" and "on the horizon." And in September 1990, doctors infused genetically altered white blood cells back into a young girl to correct her fatal illness, severe combined immune deficiency (SCID), the first successful gene therapy study. The rare disease community witnessed a new revolution in medicine. News traveled quickly throughout the Duchenne community; the hope and promise of restoring the missing dystrophin was within reach. But this was not to be the case. Setbacks and learnings occurred over the last 20 years. Today, though, promise and hope has reemerged.

During those intervening years, researchers have explored delivery vehicles, virus, plasmid, and other tools commonly used by molecular biologists (nanotherapy) to deliver genetic material into cells. They have explored the dystrophin gene to understand how to construct a smaller but effective version of dystrophin, one that could be packaged in a virus and safely delivered systemically, with widespread integration into both the heart and skeletal muscle. The single goal is to significantly slow or halt progression. Clinical trials first targeted single muscles and moved rapidly into limb delivery. Standing on the shoulders of success in hemophilia and, more recently, SMA Type 1, the Duchenne community believes treatments are now within reach. Clinicians, companies, and regulatory authorities around the world are aligned. The word "soon" means this generation of individuals diagnosed has hope. The word "promising" refers to incredibly promising and reproducible data. The words "every single one" mean application of this technology will apply to all diagnosed individuals.

And the word "hope" is bright with promise for this generation of individuals and all future generations.

Chapter 38
Design of Clinical Trials for Gene Therapy in Muscular Dystrophy

Jorge Quiroz and Kathryn Wagner

Abstract Gene therapy clinical trials in muscular dystrophy have commenced using intramuscular administration and have recently advanced to systemic administration of vector. This chapter will evaluate some of the elements of the design of clinical trials in gene therapy for muscular dystrophy that may optimize the safety and efficacy outcomes. Subject selection must consider preexisting immunity and the therapeutic window. While a placebo-controlled design is optimal, it may not be feasible in a pediatric trial. Efficacy outcome measures include muscle histology, muscle MRI, and functional tests. Safety outcome measures have special considerations for those with muscular dystrophy. Finally, ethical considerations important in design of clinical trials of gene therapy in muscular dystrophy include the principles of therapeutic misconception and justice.

Keywords Gene therapy · Clinical trials · Muscular dystrophy · Ethics

38.1 Introduction

Exogenous delivery of functional copies or knockdown of disease-causing genes is a leading therapeutic strategy for the muscular dystrophies. However, clinical gene therapy for muscle disorders is still in its infancy. While there have been thousands of gene therapy clinical trials, to date, only a handful have targeted skeletal muscle. Many of these trials were designed to use the muscle as a biofactory for a systemic disorder, while a smaller number were designed to test safety and efficacy in a

J. Quiroz
Solid Biosciences, Cambridge, MA, USA
e-mail: Jorge@solidbio.com

K. Wagner (✉)
Kennedy Krieger Institute, Johns Hopkins School of Medicine, Baltimore, MD, USA

Department of Neurology, Johns Hopkins School of Medicine, Baltimore, MD, USA

Department of Neuroscience, Johns Hopkins School of Medicine, Baltimore, MD, USA
e-mail: wagnerk@kennedykrieger.org

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primary muscle disorder. While the technology of vectors and transgenes has evolved in the decades since the original gene therapy trials in humans, lessons can be gleaned from these early studies.

Long-term follow-up of gene therapy studies in lipoprotein lipase deficiency (LPLD) and hemophilia B provides significant information on the persistence of the effects of adeno-associated virus (AAV)-based gene therapy in muscle tissue. The first gene therapy to be approved in the Western world, alipogene tiparvovec (Glybera), is an AAV1 vector carrying the human LPL^{S447X} gain-of-function variant for the treatment of a rare form of familial dyslipidemia (LPLD-induced pancreatitis). Individuals treated with a onetime intramuscular administration of alipogene tiparvovec had sustained transgene expression and were found to have an approximately 50% reduction in disease-related acute abdominal events in a 6-year follow-up [1, 2]. Despite pharmacological immune suppression, cellular infiltrates including CD8+ T cells were observed in the muscle injected with alipogene tiparvovec [2]. However, the lack of MHC molecules on muscle fiber membranes, elevation in serum creatine kinase (CK), or clinical symptoms suggests minimal immune-mediated damage to injected muscle [2]. The first hemophilia B gene therapy trial similarly used the muscle as a biofactory to produce human factor IX (hFIX) delivered by AAV2. Injections were well tolerated, and muscle biopsies showed no evidence of inflammation or muscle injury [3, 4]. However, efficacy was limited with only one of eight participants having detectable circulating hFIX [3, 4]. Importantly, for this individual from a low-dose cohort, transgene expression was sustained for more than 10 years [5].

Trials designed to test the safety and efficacy of gene therapy in primary muscle disorders have also all employed AAV. While trials of systemic delivery of AAV for primary muscle disorders have recently been initiated, to date, completed trials have all utilized local intramuscular delivery. In Duchenne muscular dystrophy (DMD), a modified AAV2 capsid with an insertion of five amino acids from AAV1 delivered a minidystrophin transgene, representing approximately 40% of the 11-kb coding sequence of the human dystrophin gene via intramuscular injection of the biceps [6]. Minidystrophin was detectable in two of the four subjects at 42 days but in neither of two subjects at 90 days postinjection. Although an immune response to AAV capsid was not observed, concern was raised for T-cell response to dystrophin epitopes resulting in low efficacy [6]. In limb-girdle muscular dystrophy (LGMD)2D, AAV1 was employed to deliver α-sarcoglycan to the extensor digitorum brevis [7]. Two of the three subjects displayed α-sarcoglycan expression at 6 months with the remaining subject exhibiting an early rise in neutralizing antibodies and early T-cell response to AAV1 capsid by interferon-γ enzyme-linked immunosorbent spot (ELISpot) [7]. Half the subjects exhibited local inflammatory cell infiltration at the site of injection, but these cells did not invade transduced myofibers [7]. In LGMD2C, AAV1 expressing γ-sarcoglycan was delivered locally to the extensor carpi radialis muscle [8]. At 30 days posttreatment, biopsies from low-dose cohorts did not express the transgene, while all of the biopsies from the high-dose cohort (4.5×10^{10} viral genomes) were positive for γ-sarcoglycan by immunohistochemistry but only one of the three by Western blot [8]. Although one subject receiving the

highest dose had a persistently detectable cytotoxic T lymphocyte response, there was no concomitant rise in creatine kinase, MHC class 1 overexpression, or inflammatory infiltration [8]. In Pompe disease, AAV1 expressing acid alpha-glucosidase (GAA) was delivered to the diaphragm muscle of five ventilator-dependent children. Transgene expression levels are unknown as the diaphragm cannot be safely biopsied, but some measures of respiratory function improved with increased unassisted tidal volumes and longer periods of unassisted breathing [9]. No T-cell-mediated immune responses to the vector were observed although one subject developed anti-GAA antibodies [9]. In a separate study, AAV1-CMV-GAA delivered to the diaphragms of Pompe children on partial/no mechanical ventilation and full-time mechanical ventilation were compared. Respiratory function as measured by flow and volume load compensation increased in those with a higher baseline respiratory function and was maintained for at least 6–12 months [10].

These early forays into gene therapy in skeletal muscle via local intramuscular injection of AAV demonstrated consistent safety but limited efficacy of persistent transgene expression. A combination of factors has been suggested to account for limited efficacy including preexisting immunity to AAV capsid proteins, preexisting immunity to transgene epitopes, development of immune response to AAV capsid proteins, dosing, or other factors. Here we discuss elements of the design of a clinical trial of gene therapy in muscular dystrophy to optimize the chances of demonstrating both safety and efficacy.

38.2 Subject Selection

Several parameters should be considered in patient selection in advance of the administration of novel gene transfer candidates: the level of preexisting immunity to the viral capsid, the potential immunogenic capacity of the expressed protein, the general condition of the patient, and the stage of disease. These are issues that may influence safety and efficacy of the gene transfer.

38.2.1 Preexisting Immunity

The response to the gene transfer candidate could be affected by mechanisms of innate immunity as well as adaptive immunity (humoral and cellular responses), both of which may affect the safety and efficacy of the drug candidate. While the former does not provide by itself long-lasting protection to the individual, the humoral and cellular responses do.

Innate immunity might play an important role in the early inflammatory responses mostly through direct action of macrophages and other resident cells in target organs and other infected tissues (e.g., Kupffer cells in the liver). Humoral response, instead, is mediated by the presence of anti-AAV antibodies

that will appear within days after a naturally occurring infection or after drug candidate administration. The newly created memory against AAV antigens is lifelong which may preclude any future readministration of the drug. For this reason, it is relevant to characterize the presence of circulating antibodies before drug administration in order to exclude patients that have prior exposure as they could have an exaggerated response to the AAV vector. Although the cutoff levels needed to induce life-threatening responses to AAV administration in previously immunized subjects have not been clearly established, experiments in nonhuman primates have shown that very high titers of circulating IgG antibodies posed a relevant threat to the animals re-exposed to AAV (Corti and Byrne, personal communication, [11]). Although the translatability of these effects to humans still needs to be fully understood, patients with high titers should be precluded from participating in these types of experimental studies, unless methods to eliminate the antibodies from circulation are developed and offered in advance of any drug infusion.

Neutralizing antibodies are a subset of circulating antibodies that not only contribute to the immune response but that can also effectively block the activity/function of new protein after drug candidate administration [12]. The presence of neutralizing antibodies would make the infusion of drug into patients a futile effort, dramatically modifying the risk/benefit ratio for a potential participant in clinical studies. It is important to note that the extent by which circulating antibodies and the subset of neutralizing antibodies are correlated may vary. Recent exploration of the strength of the correlation and prevalence of these two types of antibodies in nonhuman primates has shown great variation between colonies providing further evidence that neutralizing titer levels should be carefully evaluated and considered prior to patient selection [13]. The titer levels at which the neutralizing effects are observed (where the nature of the functional assays to evaluate the antibody neutralization may play an important role) have to be fully and specifically characterized for each drug candidate (vector and the gene of interest). Cutoff levels needed to significantly block translation after AAV administration have not been clearly established; however, in nonhuman primate experiments with an AAV8 vector, preexisting neutralizing antibodies at a titer of 1:20 were sufficient to reduce transduction considerably [14]. Importantly, in vivo neutralizing effects may depend greatly on the doses being administered, and therefore, it is theoretically possible that a higher dose of the drug candidate may initially surpass the neutralizing effect of antibodies before memory cells for these antigens are reactivated.

Efforts to decrease the levels of circulating and neutralizing antibodies through plasmapheresis have been explored for some time. Most recently, an extracorporeal immune-adsorption procedure was shown to reduce the levels of anti-AAV neutralizing antibodies in NHPs that previously received an AAV reporter construct (AAV5-hSEAP) by a mean factor of 12. Significantly, this reduction permitted the successful readministration of an AAV5 vector containing a different transgene (hFIX in this case), indicating the potential utility of this approach in previously immunized patients but naïve to treatment or those in potential need of

future gene transfer readministration [15]. Another approach to potentially enable the repeated administration of a vector of the same AAV serotype was explored through pharmacological modulation of the humoral immune response. In the study of AAV-GAA for Pompe disease, three subjects were treated daily with sirolimus, an inhibitor of the mammalian target of rapamycin (mTOR) which acts on B cells and T cells, and every 12 weeks with rituximab, a monoclonal antibody that induces B-cell depletion by binding CD20 found on the surface of B cells [16]. This strategy showed promising results in preventing immune responses to gene transfer [16].

38.2.2 Therapeutic Window

The muscular dystrophies are defined by their progressive nature and are characterized by fibrosis and fatty infiltration of affected muscles. It is reasonable to assume that gene transfer to adipose and fibrotic tissue will have no efficacy and that, therefore, the earlier the intervention, the better the probabilities of benefit in this population. In addition, there are safety concerns in enrolling individuals who have advanced respiratory and cardiac disease (who tend to be older) and very young individuals before safety and efficacy are fully understood. It seems reasonable that at these initial stages of clinical research, children and adolescent may present the best risk/benefit ratio for receiving gene transfer. An expansion to older and younger populations is expected to follow shortly after.

38.3 The Intervention Phase

38.3.1 Use of Glucocorticosteroids

Transaminase elevations (ALT and AST) have been reported in some patients after AAV administration for gene transfer by different groups at the clinical research stage of development [17–19]. These elevations have been reported to be transient and asymptomatic, without reported increases of total bilirubin beyond the upper limit of normal or variations of other markers of liver functionality. In many cases, the use of glucocorticoids was reported to ameliorate these transient elevations and has been used prophylactically before and during AAV administration or in response to ALT abnormalities. It is not known if patients with muscular dystrophies undergoing steroid treatment (which is standard of care in DMD) would prophylactically benefit from an increase in the dose of glucocorticoids to supra-therapeutic levels immediately before gene transfer. It is thought that in patients already being treated with glucocorticoids, the risk/benefit of increasing the dose may be favorable through provision of additional protection when utilized during the gene transfer administration (returning to basal levels thereafter).

38.3.2 Use of a Control Group

It has largely been established that a randomized double-blind placebo-controlled design in interventional clinical trials is the gold standard for generating the highest level of evidence when interpreting efficacy and safety signals. According to the FDA, "the placebo control design, by allowing blinding and randomization and including a group that receives an inert treatment, controls for all potential influences on the actual or apparent course of the disease other than those arising from the pharmacologic action of the test drug. These influences include spontaneous change (natural history of the disease and regression to the mean), subject or investigator expectations, the effect of being in a trial, use of other therapies, and subjective elements of diagnosis or assessment" [20]. However, the question of when it is possible to utilize such a design is a more controversial issue that should take several factors into consideration. These include but are not limited to the age group of the population to be investigated (pediatric vs. non-pediatric), the diagnoses (natural history, including level of disability and life-span), the nature of the intervention and associated risks, the burden of procedures during clinical investigation (e.g., biopsies, venous ports, radiation), and the availability of alternative treatments. Many muscular dystrophies are devastating pediatric diseases (where the majority of the patients affected with the disease are children and adolescents younger than 18 years old) with no efficacious and alternative therapeutic interventions that stop the advancement of the disease. Thus, any procedure associated with the placebo intervention needs to represent a minor increase over minimal risk for pediatric patients. Interestingly, the definition of this threshold has recently been deemed unclear, and a joint meeting of the FDA's Pediatric Advisory Committee and Pediatric Ethics Subcommittee was called to provide a recommendation on this respect [21]. It was asked if the placement of a central venous access port is considered to be beyond a minor increase over minimal risk for pediatric patients with DMD assigned to the placebo arm of a randomized, double-blind, placebo-controlled study. The committee recommended in this case that the use of central venous access ports in pediatric patients would be allowable in subjects randomized to receive investigational product or placebo (based on 21CFR50.54(b)(2) including that the clinical investigation presents a reasonable opportunity to further the understanding, prevention or alleviation of a serious problem affecting the health or welfare of children, among other regulations). Currently, studies including different active treatments as a concurrent control, different dose or regimen of the study treatment as a concurrent control, or historical controls are considered to be less optimal alternative trial designs.

38.4 Efficacy Outcome Measures

38.4.1 Muscle Histology

Determination of the efficacy of gene transfer requires the quantification of the transgene or cognate protein. In addition to quantification of the protein through mass spectrometry or semiquantitatively through immunoblot, the cellular

localization of the protein by immunohistochemistry is critical to confirm its functionality in muscle gene transfer. Unfortunately, the muscle community has not been consistently careful in the handling and analysis of muscle biopsies from research participants. Safeguards against degradation and artifact include detailed manual of procedures, surgeons' and technicians' experience with skeletal muscle, and a central laboratory with blinded readers.

Some skeletal muscles are affected disproportionately to others in the muscular dystrophies [22]. Many become replaced by fat and fibrosis early in the disease. In order to provide a true measure of efficacy, the specific muscle to be biopsied must be chosen with care as a fatty replaced muscle is unlikely to demonstrate transgene expression. Magnetic resonance imaging (MRI)-guided biopsy is helpful in this regard. However, even within an individual muscle, one should expect significant site-to-site variability which complicates analysis.

38.4.2 Muscle MRI

While muscle biopsy is invasive and provides information on a very limited region, MRI of the muscle is noninvasive and can provide information on several or all the muscles of the body. Transverse relaxation time (T2) MRI and magnetic resonance spectroscopy (MRS) are sensitive to extracellular water as occurs in inflammation and necrosis and to fatty replacement of the muscle. The MRI phenotype of most of the major muscular dystrophies has now been described [22]. Natural history studies have demonstrated disease progression by MRI in muscular dystrophies such as Duchenne, FSHD, and LGMD2I [23–27]. In ambulatory DMD, T2-MRI values and fat fraction (by H^1-MRS) in muscles of the lower extremities increased significantly over 12 months in all age groups [26]. Importantly, T2-MRI values decreased in several leg muscles of boys within 3 months of initiation of corticosteroids [28]. Although it has yet to be demonstrated if MRI is sensitive to transgene expression in muscle gene transfer, these studies suggest the potential to capture a reversal of inflammation/necrosis or a reduction in intramuscular fat accumulation.

38.4.3 Muscle Function

Multiple outcome measures have been found to be feasible and reliable in the testing of function in muscular dystrophy individuals. Most of these measures, such as the 10-m walk/run, timed up and go, rise from floor, four-stair climb, and the 6-min-walk test (6MWT), are focused on lower extremity function. Many lower extremity measures, often used routinely in clinical evaluations, have been extensively utilized in a number of clinical trials for various muscular dystrophies [29–32]. Measures of upper extremity function include the Performance of Upper Limb and more recently upper extremity reachable workspace outcome measures using a 3D vision-based sensor such as the Kinect system [33, 34]. Upper extremity function is

important to capture as an outcome measure that can be used across both ambulatory and nonambulatory populations. It also has special relevance to disorders such as FSHD with prominent arm and shoulder girdle involvement, in which the reachable workspace outcome measure has been found to be feasible and sensitive to differentiate a range of functional levels [35].

The 6MWT continues to be a frequent primary functional outcome measure in trials of muscular dystrophy. Originally developed as a global assessment of cardiopulmonary function, it was the basis for regulatory registration in Pompe and DMD [36, 37]. The 6MWT has been found to be reliable and correlate with the 10-m walk/run and the supine to stand time in BMD [30]. Similarly in FSHD, the 6MWT has been found to be reliable and correlates with other measures of disease severity [29]. In DMD it is considered by some to be an optimal test because of its low ratio of minimal clinically important difference to baseline relative to that of other endpoints [31]. However, like other timed function tests, it is not sensitive to disease progression in young DMD individuals under the age of 7 who are also an ideal age group for gene transfer studies [38].

Patient-reported outcome measures (PROMs) are instruments capable of obtaining a patient's insight into their own health state. If validated correctly, and used in the right setting, these instruments can be used to measure treatment benefits and risks. PROMs capture the status of the patient's health directly from the patient, often without requiring implementation or interpretation from an investigator or evaluator. Ideally, a PROM focuses on the symptoms that are most important to the study population. The FDA has established PROMs as part of its criteria for drug approval and labeling [39]. PROMs have been developed for DM1, FSHD, and DMD and may soon be seen as the primary outcome measure of clinical trials in muscular dystrophy [40–42].

38.5 Safety Outcome Measures

Monitoring of the safety of gene transfer includes both acute and chronic outcome measures. This includes evaluations of vital signs, physical examination, electrocardiography, and serology. There are some special considerations for monitoring muscular dystrophy patients.

38.5.1 Hepatotoxicity

It is important to note that patients with muscular dystrophies normally present with elevated levels of ALT and AST of muscular origin. These elevations may represent a potential confounder for signals of hepatic inflammation induced by candidate drug administrations. Therefore, efforts have been made to detect early abnormalities independent of transaminase level monitoring through more

specific biomarkers of hepatic integrity (in addition to monitoring levels of bilirubin, gamma-glutamyltransferase, or prothrombin time). It is postulated that exploratory biomarker measurements, including glutamate dehydrogenase (GLDH) levels, could be clinically relevant; in fact, GLDH serum levels elevated due to liver injury were shown to be unaffected by concurrent muscle disease in contrast to ALT levels, demonstrating the utility of this marker in this patient population [43].

38.5.2 Immune Response

In addition to a potential innate and humoral immune responses as described above, cellular responses to the capsid and transgene protein can also occur. These antigen-specific cytotoxic responses that are not mediated by antibodies may affect the efficacy and safety of the gene transfer drug candidate through destruction of the cells presenting antigenic segments of the vector or the antigenic segments of the new protein at the cell surfaces. These cytotoxic responses may be acute or chronic. One method to detect these responses is by measuring the frequency of cytokine-secreting cells, most commonly utilizing interferon-γ through ELISpot. This test is usually performed on easily accessible circulating cells (peripheral blood mononuclear cells) rather than on the tissue of interest (e.g., muscle). Intriguingly, in one account it was reported that circulating dystrophin-specific T cells were detected in patients with DMD not only after treatment (although the new protein was not detected in muscle) but also in the blood of patients before vector treatment [6]. It was hypothesized by the authors that these findings were explained by the presence of antigens derived from revertant dystrophin fiber containing epitopes targeted by the autoreactive T cells. These findings and interpretation have been challenged, and, additionally, it has been pointed out that a positive ELISpot signal for AAV vectors may not necessarily represent meaningful cytotoxic T lymphocyte activity in patients, questioning the clinical translatability of the test in blood to guide medical decisions. More research is needed to further understand the nature of these relationships, and efforts are underway to further explore potential cytotoxicity in preclinical models [44].

38.6 Ethical Considerations

Gene therapy trials and especially those in pediatric populations present unique ethical challenges for researchers designing clinical trials. Two important areas for consideration concern the principle of therapeutic misconception/misestimation and the principle of justice.

38.6.1 Therapeutic Misconception/Misestimation

Therapeutic misconception occurs when a research participant or their substitute decision-maker confuses the purpose of research participation with therapy [45]. Perhaps more common, therapeutic misestimation occurs when the participant or decision-maker overestimates the probability of benefit or underestimates the probability of risk in participating in research [46]. The name "gene therapy" itself, rather than "gene transfer," lends itself to therapeutic misconception. Researchers, patient advocates, and media may all contribute to the "hype" of a trial that inflates the probability and extent of benefit or minimizes potential risk. Decision-makers may agree to participation as a means to a disease cure. Compounding the difficulty in effectively communicating the true nature of research is that these facts must often be communicated to pediatric participants who potentially have a less-developed understanding of the medical world. In a telling example, Unguru et al. found that half the participants in a pediatric oncology trial did not understand they were participating in research rather than receiving a clinical treatment [47].

Therapeutic misconception and misestimation erode the process of meaningful informed consent and assent for research participation. True informed consent requires that not only does the participant have an understanding of the purpose of the research but also an understanding of how the research differs from clinical care. Moreover, researchers must ascertain that the potential research participant truly grasps the distinction, and this takes on particular importance in the pediatric population. Therapeutic misconception and misestimation alter the participants' decision-making process by misunderstanding the likelihood of benefit to risk, and avoidance of such requires deliberate care during the recruitment and enrollment of participants.

38.6.2 Justice

The principle of justice in clinical research requires fair processes and fair outcomes [48]. Applying the principle of justice ensures that all groups in the society are given the opportunity to benefit from being involved in research. The National Institutes of Health in fact requires all sponsored clinical trials to include women and minorities when applicable. A special challenge in gene therapy trials, particularly for phase 1 trials, is selecting a few participants of diverse backgrounds from the many interested and eligible. A "first-come, first-serve" approach as is employed in many trials benefits those who are best informed and often have higher socioeconomic status and are more highly educated. An alternative approach that fairly allocates a very limited number of participant slots is a lottery. Such an approach was taken in the endostatin phase 1 trial in which 1400 patients were enrolled in a lottery for three places in the first cohort [49]. However, for a lottery to be used fairly, those enrolled need to be representative of the population. Here engagement of patient foundations and other stakeholders is critical to the successful and just recruitment of study participants.

38.7 Conclusion

There have been only a handful of gene therapy clinical trials in muscular dystrophy to date, and the community is still on the steep slope of the learning curve. Lessons have been learned from these early trials as well as gene therapy trials in other disorders and pharmacological clinical trials in muscular dystrophy. As the field moves from intramuscular administration to systemic administration of vector, there will doubtlessly be new unforeseen challenges. However, with the careful design of clinical trials, the potential of fulfilling safety and efficacy outcome measures and eventually providing a meaningful therapeutic agent is near.

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Chapter 39
Path to Clinical Trials: Trial Design, Development of the Clinical Product, and Safety Concerns in the Implementation of Clinical Trials

Jerry R. Mendell, Louise R. Rodino-Klapac, and Christopher J. Shilling

Abstract The path to an IND from conception of a potentially promising mode of treatment to a clinical trial is often not fully understood. This chapter attempts to provide a template from early considerations, through safety concerns and product development. The regulatory agencies play a major role in this process and are often accessible to advise researchers about specific issues that may be relevant in the planning phase. The message from this chapter is that if researchers want to pursue clinical translation, it should be considered even before any preclinical studies are done. This will save both time and funds and avoid duplication and modifying previous experiments that showed promise but had not satisfied regulatory requirements. The preclinical studies must consider the vector used in gene delivery, dosing, and safety issues that are applicable to clinical trial. Taking advantage of opportunities to explore toxicity to the fullest for later presentation to regulatory agencies is an important consideration. The pre-IND meeting with the FDA is a pivotal step to get to a clinical trial and will provide a template to follow in preparation and planning for the IND. All of the agencies that are critical to the translation

J. R. Mendell (✉)
Departments of Pediatrics, Nationwide Children's Hospital and Research Institute, The Ohio State University, Columbus, OH, USA

Department of Neurology, Nationwide Children's Hospital and Research Institute, The Ohio State University, Columbus, OH, USA
e-mail: Jerry.Mendell@nationwidechildrens.org

L. R. Rodino-Klapac
Departments of Pediatrics, Nationwide Children's Hospital and Research Institute, The Ohio State University, Columbus, OH, USA

Vice President Gene Therapy, Sarepta Therapeutics, Columbus, OH, USA
e-mail: LRodinoKlapac@Sarepta.com

C. J. Shilling
Drug and Device Developmental Services, Nationwide Children's Hospital and Research Institute, The Ohio State University, Columbus, OH, USA
e-mail: Chris.Shilling@nationwidechildrens.org

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process are fully described in this chapter for fulfilling commitments to bring promising experiments from the laboratory to the clinic.

Keywords IND · FDA · Adeno-associated virus (AAV) · Toxicology Clinical trial

39.1 Designing and Implementing Clinical Gene Therapy Trials

Before the first experiment is done in the laboratory, the steps that are required to bring a project to the clinic (reviewed in the pages to follow) should be carefully considered. It is natural for the enthusiastic investigator initiating a novel approach that has the potential to advance to the clinic to overlook what will be required by the regulatory agencies for approval. Many of these considerations seem obvious but may be ignored because of lack of familiarity or simply ignored; however, careful consideration of the steps to achieve full translation saves both time and money.

39.2 Choice of Vector for Gene Transfer

Obviously the gene transfer vehicle is a critical issue. In the world of neuromuscular diseases, the decision to use adeno-associated virus (AAV) is an obvious outgrowth of experience. The Jesse Gelsinger case illustrated the potential vulnerability of subjects to an immune response related to adenovirus that was far more extensive than ever previously encountered resulting in multiple organ failure and brain death [1, 2]. Adenovirus was the viral vector used in gene transfer for treatment of ornithine transcarbamylase in Gelsinger, now steering investigators away from this vehicle.

Another major adverse event that framed future efforts for neuromuscular diseases occurred in the treatment of human severe combined immunodeficiency (SCID)-X1. The initial results using retroviral vectors for delivery appeared to be successful without problems [3]. However, a major complication occurred 2.5 years post treatment with the development of leukemia from insertional mutagenesis of the first intron of the LIM domain only-2 (LMO2) gene, a known transcription start site previously associated with T-cell acute lymphoblastic leukemia [4–6]. Twenty patients were treated in French and UK trials. Unfortunately, four patients in the French study and one patient in the UK trial developed T-cell leukemia 2–5.5 years after gene therapy. Four remained in long-term remission after chemotherapy and have fully recovered after treatment. The remaining patient died despite a range of therapeutic approaches. In all cases, further study of the adverse event, resulting from insertional oncogenesis, showed that the retroviral vector had integrated into malignant cells within or near tumor-promoting genes (mainly the LMO2 gene).

These studies directed the neuromuscular gene therapy community toward AAV as the delivery vehicle of choice for in vivo therapy targeted for treatment of inherited diseases of postmitotic tissues. A full discussion of AAV is provided in other chapters of the book (Chaps. 10 and 11), and comments here are only meant to guide the investigators planning preclinical research studies. AAV has proven to be a safe vehicle for gene transfer, but the limitations of packaging capacity to <5 kb remain an obstacle. AAV vectors are currently the safest and most efficient platform for gene transfer in mammalian cells, and by 2017 AAV vectors have been used in 204 clinical trials [7]. Very few safety issues have emerged, but like with other viruses, immune responses remain a concern. There was a patient who died in a clinical AAV trial, but the virus was exonerated by the Food and Drug Administration (FDA) as the responsible agent. In this Phase I clinical trial, a 36-year-old woman became extremely ill the day after a second injection in the knee joint using AAV.tgAAC94 to combat inflammatory arthritis. The death of this individual was due to disseminated *Histoplasma capsulatum*, a fungus endemic in the Midwest. The affected subject was simultaneously being treated for arthritis with adalimumab (Humira), a systemic anti-TNF-α antagonist. Fungal infections are known serious complications of this therapy. A Recombinant DNA Advisory Committee (RAC) investigation of the circumstances that surrounded the death concluded that the AAV vector carrying the transgene was not responsible and did not contribute to the untimely death. Thus, the FDA exonerated the AAV vector and permitted the clinical trial to resume [8].

39.2.1 Choice of AAV Serotype for Clinical Trial

There is a building literature and a growing experience demonstrating that the AAV serotype will target muscle and work well in clinic trial. Since gene therapy was introduced when AAV2 was the only choice, we are fortunate that the repertoire of serotypes has expanded significantly. Twelve human serotypes of AAV (AAV1 to AAV12) and more than 100 serotypes from nonhuman primates have been identified to date [9]. The lack of pathogenicity of this virus has increased AAV's potential as a delivery vehicle for gene therapy. Compared to AAV2, greater gene expression has been validated for the targeting muscle and heart with AAV serotypes 1, 6, 8, and 9. This increased expression extends to the central nervous system reflected in our own SMA type 1 trial demonstrating exceptional results in a cohort of 15 infants using AAV9 at Nationwide Children's Hospital [10]. In addition, a serotype designated rh74 was isolated at Nationwide Children's Hospital, sharing 93% homology to AAV8 and 98% homology to AAVrh10. AAVrh74 efficiently targets skeletal and cardiac muscle.

In planning preclinical studies with AAV, it is incumbent on the investigator to look ahead to the clinical application(s). Dose, safety, and efficacy must be considered, and this will include a consideration of the cassette and the promoter (see Chap. 8 for more discussion on designing the cassette). The outcome in the preclinical studies must be clearly defined in the animal model, and improvement following

delivery of recombinant AAV (rAAV) must be unequivocal. This can be challenging if there is no animal model or one with minimal manifestations. A muscle-specific promoter, including desmin, CK6, CK7, MCK, tMCK, and MHCK7, is not mandatory but may avoid off-target organ adversity.

Dosing issues cannot be overlooked and must be considered in relation to the target population. A major concern is whether the doses showing efficacy are applicable to the disease population (infants, children, adults). Given that the single most expensive item getting to clinical trial will be vector production, it is critical that the minimally and maximally effective dose be known before moving the project forward to the regulatory agencies.

One final issue to consider in vector planning is the potential benefit of new technologies developed to increase the genome capacity for AAV and enhance expression using a two-vector system. The trans-splicing approach takes advantage of AAV's ability to form head-to-head concatemers via recombination in the ITRs. In this approach, the transgene cassette is split between two rAAV vectors containing adequately placed splice donor and acceptor sites. Transcription from recombined AAV molecules, followed by the correct splicing of the mRNA transcript, results in a functional gene product. This application becomes useful for using AAV to deliver therapeutic genes up to 9 kb in size. Attempts in multiple organ gene delivery include the retina [11], lung [12], and muscle [13]. An alternative two-vector rAAV system approach that we find highly efficient is homologous recombination. We have used this extensively for delivery of the dysferlin cDNA using AAV serotype rh74 [14]. Through the use of two discrete vectors (rAAVrh74.DYSF) defined by a 1 kb region of homology, gene replacement via intramuscular and vascular delivery routes can be efficiently and safely delivered to dysferlin-deficient mice and nonhuman primates. This method is currently in clinical trial in LGMD2B patients.

39.3 Clinical Development for Biologics

At the time of Investigational New Drug (IND) application, documentation is required pertaining to product development (research, design, manufacturing) with assurance that safety has been thoroughly vetted in animal studies. The FDA has established a set of "good practices" that provide the framework for conduct of high-quality preclinical studies, product manufacture, and clinical trials, and these principles should be thoroughly understood by the sponsor of a gene therapy protocol. The Code of Federal Regulations (CFR) is posted annually at the US Government Publishing Office (http://www.gpo.gov/fdsys/). Title 21 of the CFR, Part 58, details the requirements for *good laboratory practice (GLP)*, a set of standards for the conduct of nonclinical laboratory studies used to support an IND application. In scenarios where the target indication meets certain criteria—such as a rare and designated orphan, a severe or life-threatening disorder, and the absence of an approved alternative treatment—the FDA has allowed greater consideration of preclinical studies that do not meet all the qualifications outlined in the GLPs. Title 21

of the CFR, Parts 210 and 211, describes the requirements for *good manufacturing practice (GMP)*. The principles of GMP apply to the manufacturing process and the manufacturing facility. The FDA is amendable to certain exceptions to GMP products manufactured to support Phase I clinical trials as outlined in the 2008 FDA issued "Guidance for Industry: cGMP for Phase 1 Investigational Drugs." Although there is some flexibility at early IND phases in the level of adherence to GLP and GMP regulations, application of these principles in the initial stages of product development establishes the guidelines necessary for the initiation of human clinical trials. *Good clinical practice (GCP)* guidelines apply to the conduct of the clinical trial and are described on the FDA Web site (http://www.fda.gov/oc/gcp/regulations.html). GCP guidelines implicitly require that clinical research be conducted for valid ethical and scientific reasons, performed by qualified investigators, and initiated only after Institutional Review Board (IRB) approval and valid informed consent have been obtained and documented. The objective of the International Conference on Harmonisation (ICH) on GCP is to provide a unified standard for the European Union (EU), Japan, and the United States to facilitate the mutual acceptance of clinical data by the regulatory authorities in these jurisdictions (http://www.ich.org/about/mission.html). There must also be periodic monitoring of the clinical trial to assess the quality of the research and integrity of the data. A second set of tools published by the FDA is available at the Center for Biologics Evaluation and Research (CBER) website descrbing specific policy, regulatory and expectations for gene therapy: (http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/default.htm).

The initial administration into humans is a *Phase I trial* (human pharmacology) with focus on safety. In 2015, the FDA issued "Guidance for Industry: Considerations for the Design of Early-Phase Clinical Trials of Cellular and Gene Therapy Products" which provides FDA's current recommendations regarding clinical trial design where the primary objectives are the initial assessments of safety, tolerability, or feasibility of administration of investigational products. Studies may be open, baseline controlled, or randomized with blinding. Randomizing subjects is usually not appropriate for early phase gene therapy trials, especially for the dose-escalation design, the usual paradigm for Phase I trials. Depending on study strategy, however, it is possible to randomize extremities for treatment (e.g., gene injection vs vehicle only into opposite limbs) [15, 16]. At the Phase I level of development, nontherapeutic, pharmacologic objectives are paramount. Data collection focuses on (1) safety and tolerability, (2) distribution and clearance (pharmacokinetics) from local and remote sites (e.g., urine, semen, and saliva), and (3) estimates of activity of the recombinant agent (usually a secondary outcome measure).

A *Phase II trial* (therapeutic exploratory) assesses initial efficacy as its primary objective. Goals include determination of dose, protocol for delivery, and establishing endpoints. Target populations can be clarified, such as mild vs severe disease. The Phase II concept of trial design may have safety as the primary outcome but includes a measurable functional outcome. It would be labeled a Phase I/IIa clinical trial if open label or a Phase I/IIb clinical trial with a control group. Substantiation

of efficacy is the goal of the *Phase III trial* (therapeutic confirmatory), providing a basis for licensure and marketing. Post marketing product modifications occur during a *Phase IV trial* (therapeutic use). So far, only one gene therapy product was approved by the FDA for treating retinal dystrophy using AAV2-mediated gene replacement with a normal RPE65 gene. This paucity of approvals includes challenges related to multiple issues. The resources needed to bring a product to market lead to excessive costs as do safety concerns. Limited vector production capacity and technical issues of scale-up also impose obstacles for some vector systems. Of note, Glybera, delivering the lipoprotein lipase gene in AAV directly to muscle, was approved by the European Medicines Agency (EMA), but the company that developed the product dropped plans for approval in the United States because of the cost necessary to achieve approval from the FDA.

39.4 Planning the Clinical Trial Is a Collaborative Process

Seeking regulatory approval for a first inhuman Phase I gene therapy protocol is the initial consideration for the investigator. The most efficient approach for a translational gene therapy is to give thought in defining what the clinical protocol will include. This will take into account the vector and transgene to be used, the dosing regimen, route of administration, safety monitoring system, and outcome measures. The strategy is benefitted by collaboration between the preclinical and clinical scientists. With the strategy assembled, the research team begins work to support product development that must follow regulatory guidelines considering that it will be closely scrutinized by the FDA, IRB, and Institutional Biosafety Committee (IBC).

Once the full development plan is agreed upon, laboratory-based preclinical studies should define (a) a relevant animal species/model, (b) the biologically effective dose range inclusive of the minimally effective dose (MED) and optimal biological dose (OBD) and/or a maximally tolerated dose (MTD), (c) optimization of the route of administration (ROA), (d) timing of administration relative to the onset of disease/injury, and (e) characterization of the purported mechanism of action (MOA). It is also worth the time and effort to look beyond gene expression in limb skeletal muscle with consideration of critical tissues of interest like gene expression and functional benefit to the diaphragm and heart. In addition, animal studies (mouse, canine, feline, or nonhuman primate) designed to show efficacy should gather as much information as possible to demonstrate a safety profile to defend the final product to be presented to the regulatory agencies. This includes immune studies for viral and transgene T-cell responses assessed by interferon gamma (IFN-γ) ELISpot assays and ELISA assays for antibody. In addition, complete autopsies with tissue studies on every major organ and chemistry batteries inclusive of assessment of the heart, liver, kidney, hematologic, and skeletal muscle should exclude adverse effects that can be presented with the pre-IND package or at the time of IND application to sup-

port a safety profile. Careful documentation, monitoring, and reporting of these preclinical studies in the spirit of GLP are necessary to allow the FDA to rely on the data. Study reports should include a detailed prospectively designed protocol, record of all amendments and deviations from protocol, and transparent and traceable results for all parameters for each animal on study. For muscular dystrophy, animal models should recognize cystatin C as a measure of kidney function because creatinine is often low when muscle mass is decreased [17]. Assessment should also include serum gamma-glutamyl transferase [18] to assess hepatocellular damage rather than transaminases that will usually be elevated when there is muscle damage related to the underlying disease. Having this information in well-defined data set will be necessary in moving things forward at every interaction with the FDA.

39.5 Regulatory Agencies Involved in Gene Therapy Protocols

It is important in translating basic or preclinical science to the clinic to understand the organizational structure of the Department of Health and Human Services (DHHS) (Fig. 39.1). The Secretary of DHHS is a Cabinet Post and is the chief policy officer and general manager who administers and oversees the organization, its programs, and its activities [19]. The Office of the Assistant Secretary of Health (OASH) is responsible for implementation of the goals of DHHS and protection of human subjects in biomedical and behavioral research. In collaboration with OASH, the Office for Human Research Protections (OHRP) and Secretary's Advisory Committee on Human Research Protections (SACHRP) report to OASH and are the most relevant promoting the objectives for gene therapy.

39.5.1 OHRP and SACHRP

This OHRP was created in June 2000 to lead the DHHS' efforts to protect human subjects in biomedical and behavioral research and to provide leadership for all federal agencies that conduct or support human subject's research under the Federal Policy for the Protection of Human Subjects. OHRP helps ensure this by providing clarification and guidance, developing educational programs and materials, maintaining regulatory oversight, and providing advice on ethical and regulatory issues in biomedical and social-behavioral research. The Director of OHRP reports to the Assistant Secretary of Health. OHRP has oversight over more than 13,000 institutions in the United States and worldwide that conduct HHS human subject research.

The SACHRP advises DHHS on issues related to protecting human subjects. The Committee advises, consults with, and makes recommendations on matters pertain-

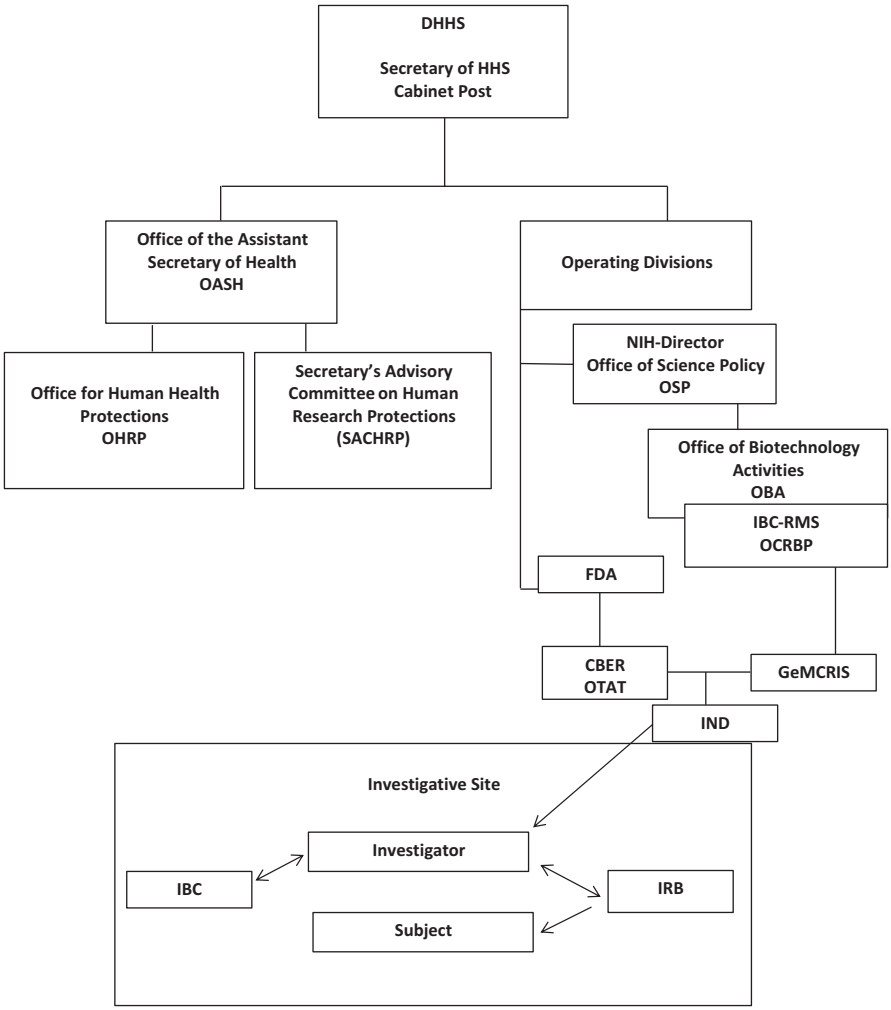

Fig. 39.1 Organizational chart for regulatory affairs. The Secretary of the Department of Health and Human Services (DHHS) is a Cabinet Post overseeing the programs and activities of this complex department. The Assistant Secretary of Health (ASH) is responsible for implementing the goals of HHS and protecting human subjects in biomedical and behavioral research. In relation to gene therapy, the Office for Human Research Protections (OHRP) and Secretary's Advisory Committee on Human Research Protections (SACHRP) report to OASH and provide leadership to protect the rights, welfare, and well-being of subjects involved in research. On the opposite side of the organizational chart, two human service agencies (of a total of 11) directly involved in gene therapy include the NIH and the FDA. The NIH has a highly integrated program that establishes guidelines for genetic research and executes policy through the Office of Science Policy (http://osp.od.nih.gov). OSP is the central site for planning, developing, and coordinating NIH-wide policy activities for clinical and healthcare research. The Office of Biotechnology Activities (OBA) reports to OSP regarding the conduct of clinical trials and oversight of gene therapy research. At the institutional level, the IBC updates OBA every year through the Institutional Biosafety Committee Registration Management System (IBC-RMS). The Center for Biologics Evaluation

ing to the continuance and improvement of functions within the authority of HHS directed toward protection for human subjects in research. Specifically, examples include advice relating to the responsible conduct of research involving human subjects with particular emphasis on:

- Special populations, such as neonates and children, prisoners, and the decisionally impaired

Fig. 39.1 (continued) and Research (CBER), an integral part of FDA, regulates cellular therapy products, human gene therapy products, and certain devices related to cell and gene therapy. CBER uses both the Public Health Service Act and the Federal Food Drug and Cosmetic Act as enabling statutes for oversight. Human gene therapy refers to products that introduce genetic material into a person's DNA to replace faulty or missing genetic material, thus treating a disease or abnormal medical condition. CBER has now approved one AAV-mediated product and sevaral cell-based products and the amount of cellular and gene therapy-related research and development occurring in the United States continues to grow at a rapid rate. All gene therapy clinical trial protocols must be conducted under Investigational New Drug (IND) application. The IND application is submitted to Office of Tissue and Advanced Therapies (OTAT) in CBER if the product has not previously been authorized for marketing in the United States and is intended to be used for the purposes of clinical investigation or, in certain cases, for the purposes of clinical treatment when no approved therapies are available. The sponsor of an IND application is the party who submits the application to FDA. This can be a pharmaceutical company, but in the early stages of translational gene therapy, it is the investigator conducting the proposed clinical trial who usually is the sponsor of the IND application. In collaboration with CBER, OBA has developed a registry of activities related to recombinant DNA research and human gene transfer: the Genetic Modification Clinical Research Information System (GeMCRIS), a Web-based information system for human gene transfer trials designed to facilitate safety reporting. Investigator and sponsors of a human gene transfer trials can utilize this system to report serious adverse events (SAEs) and annual reports. A hard copy of the electronic report can be printed and used as a template to fulfill FDA reporting requirements. GeMCRIS allows users to access an array of information about human gene transfer trials registered with the NIH, including medical conditions under study, institutions where trials are being conducted, investigators carrying out these trials, gene products being used, route of gene product delivery, and summaries of study protocols. This basic information is available to the public through GeMCRIS for gene transfer trials registered at the NIH. OBA provides guidelines for recombinant or synthetic nucleotides that have been published in the Federal Register on March 22, 2016 (effective April 27, 2016 http://osp.od.nih.gov) and updated August 16, 2018. The recent update eliminated the Recombinant DNA Advisory Committee. Other committees remain and are operative at the institutional site (may be public or private): the Institutional Review Board (IRB) and the Institutional Biosafety Committee (IBC). All human research requires IRB approval, and the functions, operations, and IRB committee membership follow policy defined in Title 45 of the CFR, Part 46 (http://www.hhs.gov/ohrp/regulations-and-policy/regulations/45-cfr-46/). This includes a requirement that IRBs register with OHRP. The IBC approves all experiments involving the transfer of recombinant DNA or DNA or RNA derived from recombinant DNA into human participants. The IBC is charged with the obligation to determine the risks and ensure public and environmental safety in the locale where the research takes place. Originally IBC oversight was restricted to recombinant DNA research, but over time responsibility has expanded to include a wide range of biohazardous materials (e.g., infectious agents and carcinogens). Members of the IBC committee must have expertise and training in recombinant DNA technology. Ad hoc consultants participate as required. The IBC files an annual report to NIH Office of Biotechnology Activities (OBA) (http://osp.od.nih.gov/office-biotechnology-activities)

- Pregnant women, embryos, and fetuses
- Individuals and populations in international studies
- Populations in which there are individually identifiable samples, data, or information
- Investigator conflicts of interest

39.5.2 National Institutes of Health (NIH) and Office of Science Policy (OSP)

On the opposite side of the organizational chart, two human service agencies (of a total of 11) directly involved in gene therapy include the NIH and the FDA (Fig. 39.1). The NIH has a highly integrated program that establishes guidelines for genetic research and executes policy through the OSP (http://osp.od.nih.gov). The OSP is the central site for planning, developing, and coordinating NIH-wide policy for clinical and healthcare research. *The Office of Biotechnology Activities (OBA)* reports to OSP regarding the conduct of clinical trials and oversight of gene therapy research. At the institutional level, the IBC updates OBA every year through the Institutional Biosafety Committee Registration Management System (IBC-RMS).

39.5.3 FDA

In 1993, the FDA issued a statement published in the Federal Register defining statutory authorities governing therapeutic products that apply to human cell therapy and gene therapy (http://www.fda.gov/cber/guidelines.htm) according to the Public Health Service Act and the Federal Food, Drug, and Cosmetic Act. The Center for Biologics Evaluation and Research (CBER), an integral part of the FDA, regulates cellular therapy products, human gene therapy products, and certain devices related to cell and gene therapy. CBER uses both the Public Health Service Act and the Federal Food Drug and Cosmetic Act as enabling statutes for oversight. Human gene therapy refers to products that introduce genetic material into a person's DNA to replace faulty or missing genetic material, thus treating a disease or abnormal medical condition. Although, CBER has only approved one human gene therapy product, the amount of cellular and gene therapy-related research and development occurring in the United States continues to grow at a fast rate.

All gene therapy clinical trial protocols must be conducted under *Investigational New Drug (IND)* application. The IND application is submitted to FDA's Office of Tissue and Advanced Therapies (OTAT) in CBER if the product has not previously been authorized for marketing in the United States and is intended to be

used for the purposes of clinical investigation or, in certain cases, for the purposes of clinical treatment when no approved therapies are available. The sponsor of an IND application is the party who submits the application to FDA and ultimately takes responsibility for the development of the investigational drug product. This can be a pharmaceutical company, but in the early stages of translational gene therapy, it is the academic-based investigator conducting the proposed clinical trial who usually is the sponsor of the IND application. The review time for initial submission of an IND application is 30 days from the date FDA receives the IND. An IND applicant may proceed with a clinical investigation once the applicant has been notified by FDA that the research study may be initiated or after 30 days from the FDA's receipt of the IND if there is no notification that the trial is on clinical hold. Regulations pertaining to this process appear in Title 21 of the CFR, Part 312.

In collaboration with CBER, OBA has developed a registry of activities related to recombinant DNA research and human gene transfer: *the Genetic Modification Clinical Research Information System (GeMCRIS)*, a Web-based information system for human gene transfer trials designed to facilitate safety reporting (https://www.gemcris.od.nih.gov). Investigator and sponsors of a human gene transfer trials can utilize this system to report serious adverse events (SAEs). A hard copy of the electronic report can be printed and used as a template to collect data that fulfills FDA reporting requirements. GeMCRIS allows users to access an array of information about human gene transfer trials registered with the NIH, including medical conditions under study, institutions where trials are being conducted, investigators carrying out these trials, gene products being used, route of gene product delivery, and summaries of study protocols. This basic information is available to the public through GeMCRIS for gene transfer trials registered at the NIH.

39.5.4 RAC

RAC had been a critical agency under OBA management for review and registration of humna gene transfer protocols. This has now changed as of August 16, 2018 with the announcement by NIH that reporting requirements were captured by existing agencies and in order to eliminate duplication, protocol submission, review and reporting would be eliminated. Thus, new proposals would no longer be acceptedand the NIH Office of Science Policy would no longer accept annual reports, safety reports, amendments or the documentation for any previously registered human gene transfer protocols.

39.5.5 Investigative or Institutional Site

Two committees are operative at the institutional site (may be public or private): the IRB and the IBC. All human research requires IRB approval, and the functions, operations, and IRB committee membership follow policy defined in Title 45 of the CFR, Part 46 (http://www.hhs.gov/ohrp/regulations-and-policy/regulations/45-cfr-46/). This includes a requirement that IRBs register with OHRP. The IBC approves all experiments involving the transfer of recombinant DNA or RNA, and committee participants must have expertise and training in recombinant DNA technology. The IBC is charged with the obligation to determine the risks and ensure public and environmental safety in the locale where the research takes place. Originally IBC oversight was restricted to recombinant DNA research, but over time responsibility has expanded to include a wide range of biohazardous materials (e.g., infectious agents and carcinogens). Ad hoc consultants participate as required to provide insight for this expanded responsibility. The IBC files an annual report with OBA (http://osp.od.nih.gov/office-biotechnology-activities/biosafety/institutional-biosafety-committees).

39.6 Summary of Sequential Steps in Implementation of a Clinical Gene Transfer Trial

Once there is understanding of the preclinical proof of concept data and there is intent to move to clinical trial with a well-defined protocol that matches the preliminary data that has been assembled, the investigators should seek guidance on the path forward for regulatory approval.

39.6.1 INitial Targeted Engagement for Regulatory Advice on CBER ProducTs (INTERACT)

In June 2018 the FDA implemented the INTERACT meeting program, designed to provide access to CBER regarding questions on the early development of products and clinical trials for gene therapy. This replaced the existing CBER pre-pre-Investigational New Drug (IND) meeting. The goal of these meetings was to allow sponsors to engage the FDA for advice on multiple phases of product development and in turn help facilitate progress and efficiency for bringing the product forward to the clinic. The INTERACT access to the FDA does not replace pre-IND meetings but allows potential sponsors to clarify expectations.

39.6.2 Pre-IND Meeting

This meeting is arranged at the request of the sponsor/investigator through a formal written letter sent to the FDA and is intended to be limited to 1 h. It is the first official step toward initiation of a Phase I gene therapy protocol and according to the FDA is considered a Type B meeting (21 CFR 312.82). Meetings should be scheduled to occur within 60 days of FDA receipt of the written request for a Type B meeting. The letter should request dates and times (morning and afternoon) and whether the meeting request is for a face-to-face or teleconference. A meeting request should include the product name, proposed indication, and a brief statement of the purpose and objectives of the meeting including background, brief summary of completed or planned studies and comments on the clinical trials, and a list of individuals who will attend the meeting. Questions of the specifics to clinical development of the product need to be included and should be grouped by discipline (e.g., pertaining to preclinical, toxicology, vector manufacturing, and clinical protocol). Once the date is established, the pre-IND package must be submitted by the sponsor 30 days before the meeting date. CBER can deny the meeting request based on what they consider a premature stage of development.

The meeting does not require a sponsor's presentation, but rather the focus of the meeting is on the comments supplied by the FDA reviewers. The FDA will provide written minutes documenting the discussion ~30 days after the meeting. Going through this critical review process defines a clear path for IND preparation that might include recommendations for additional preclinical studies to be done. In addition, the pre-IND meeting will strengthen the application for other regulatory submissions to IRB, and IBC.

39.6.3 Requesting IBC Approval

The timing for submission to IBC is somewhat arbitrary, but the committee must consider the issues raised in the pre-IND meeting and the response of the investigator and team. Information to IBC must include the source of the DNA, the nature of the inserted DNA sequences, the vectors to be used, the transgene and protein product, and its containment. The IBC then makes a decision as to whether the protocol needs review and sends its recommendation to the NIH in electronic form to the NIH OSP, preferably by e-mail (HGTprotocols@mail.nih.gov). The NIH will usually concur with IBC, but they reserve the right to override the decision if they feel there would be added benefit from a face-to-face meeting.

39.6.4 Seeking IRB Approval

The IRB approves all human gene therapy protocols and the informed consent documents before beginning a study. The functions, operations, and IRB committee membership follow policy defined in Title 45 of the CFR, Part 46. This includes a requirement that IRBs register with OHRP. The timing for this request is left to the investigator, keeping in mind that the initial IRB approval can only be tentative until input from the FDA (IND), and IBC is received. This highlights the highly integrated activities of these "independent" bodies in obtaining approval for gene therapy protocols.

39.6.5 IND Submission to the FDA (CBER)

Under current regulations, any product not previously authorized for marketing in the United States requires submission of an IND to the FDA. The specific requirements for content and format for an IND application are specified in Title 21 of the CFR, Part 312.23. In July 2003, the International Council for Harmonization introduced the Common Technical Document (http://www.ich.org/products/ctd.html) format adopted for drug applications in the EU and Japan and also acceptable but not mandatory for US FDA IND approval. A summary of IND requirements for investigational drug or biological products can be found at the following Web site: http://www.fda.gov/BiologicsBloodVaccines/DevelopmentApprovalProcess/InvestigationalNewDrugINDorDeviceExemptionIDEProcess/ucm094309.htm. Table 39.1 summarizes steps to IND submission and approval.

In May 2015, the FDA provided a draft guidance document titled "Investigational New Drug Applications Prepared and Submitted by Sponsor-Investigators" which summarizes the IND process. The basic elements of the IND include the following broad areas: (1) preclinical animal pharmacology establishing proof of concept and toxicology studies assessing product safety for human trials, (2) chemistry and manufacturing information of the vector, and (3) investigator information assessing risks based on qualifications of clinical investigators and the safety of proposed protocol. A commitment must also be given to obtain informed consent, obtain IRB approval, and adhere to the IND regulations. The IND application addresses all issues identified during the pre-IND meeting. The FDA is focused on the manufacturing summaries related to cell banks and viral banks, quality assurance and quality control programs for product manufacturing, the procedures ensuring compliance with GCP, a clinical monitoring plan, an organizational chart defining the role of individuals involved in the clinical trial, and the need for continued reporting of animal safety data that may raise awareness regarding clinical risk.

The IND is submitted to the CBER Document Control Center in triplicate. On receipt, the sponsor will be issued an acknowledgment letter containing the date of

Table 39.1 Steps involved for IND approval for human gene transfer clinical trials

Description	Implementation of process
1. Request type B meeting	Send cover letter requesting pre-IND meeting; letter should identify the product, the target disease, the indication, a proposed date, and the meeting attendees. Contact person for investigators should be identified; upon receiving a proposed date the meeting should be confirmed within 60 days
2. Preparation for pre-IND meeting	Briefing package sent to CBER at least 30 days in advance of meeting; package includes: Product name, chemical name and structure, proposed indication, dosage and route of administration, status of product development; chemistry, manufacturing, and controls information; pre-clinical data in support of product development summarized and description of prior and planned clinical trial; list of sponsors and attendees for meeting; list of questions for discussion grouped by discipline (pre-clinical, toxicology-biodistribution, CMC, and clinical). Keep in mind that pre-IND meetings are restricted to 1 h
3. Hold pre-IND meeting	Meeting focuses on submitted questions. Upon completion, CBER sends back minutes of the meeting and addresses plans acceptable for implementation or describes deficiencies that must be considered for IND approval. Post meeting, the FDA is receptive to the need for further clarification of any issue
4. Initiate IND-enabling preclinical and toxicology—biodistribution studies	Perform additional pre-clinical studies if necessary based on CBER recommendations; initiate toxicology-biodistribution studies according to recommendations from CBER
5. Initiate cGMP production to support clinical trials	Initiate cGMP vector production according to methods agreed upon with FDA. The FDA does not require manufacturing to be completed before approval of the IND
6. Submit IBC application	The IBC protocol is submitted at the site of the clinical trial; IBC will follow with recommendation to OSP for RAC meeting (determining if protocol requires a face to face RAC review)
7. Submit IND application	Submit full IND package inclusive of sections by discipline with careful consideration of all FDA pre-IND recommendations. Include all reports necessary to document safety and all data or certificates (if available) of the cGMP manufactured product
8. Submit IRB application	Following IND approval seek IRB approval for start of the clinical trial
9. Initiate trial enrollment	Approval by the FDA and IRB will dictate the timing of enrollment. If a DSMB is convened, timing may be altered at the committee's request

receipt and the assigned IND number. The sponsor's point of contact within CBER is typically through the Regulatory Project Manager, who is responsible for coordinating the review process. The sponsor will be contacted during the review if additional information is needed or to discuss deficiencies. INDs automatically become effective 30 days after receipt unless the FDA notifies the sponsor that the study is on clinical hold until concerns are resolved. This decision is communicated to the

sponsor by telephone and is followed by a letter that provides the hold comments and requests for additional information.

As detailed in Title 21 of the CFR, Part 312.42, Phase I INDs may be placed on clinical hold if human subjects are exposed to unreasonable and substantial risk of illness or injury; sufficient information is lacking to allow adequate assessment of risk; the information in the investigator's brochure is misleading, erroneous, or materially incomplete; or the clinical investigators are not qualified to conduct the study. In addition, Phase II and Phase III INDs may be put on hold if the protocol design is deficient to meet the objectives of the proposal. To proceed with the clinical study, the sponsor must correct the hold deficiencies and submit a response as an amendment to the IND (Title 21 of the CFR, Part 312.30). This amendment will be reviewed at CBER within 30 calendar days, and if satisfactory, the sponsor will be notified by telephone that the clinical trial may begin. A written letter will also be sent for validation.

All IND amendments are submitted to FDA and are used to report protocol changes, new protocols, or the addition of a new investigator or clinical site, as well as changes in the manufacturing process or new toxicology data. Annual reports (Title 21 of the CFR, Part 312.33) are due within 60 days of the anniversary of the IND. The sponsor must provide IND safety reports at 15 days for any serious and unexpected adverse event or within 7 days for fatal or life-threatening events (Title 21 of the CFR, Part 312.32).

39.7 Long-Term Follow-Up Evaluation

The investigator is required to prepare and maintain adequate and accurate case histories that record all observations and other data pertinent to the investigation on each subject given the investigational agent (see 21 CFR 312.62). In addition, an annual report for the IND must be submitted and include information obtained during the previous year's clinical and nonclinical investigations, a summary of all IND safety reports submitted during the past year, and a narrative or tabular summary showing the most frequent and most serious adverse experiences by body system (21 CFR 312.33(b)) [19].

Based on the 2006 FDA "Guidance for Industry: Gene Therapy Clinical Trials—Observing Subjects for Delayed Adverse Events," the length of the long-term follow-up will be influenced by the risk of integration of the viral vector and by the potential for adverse events. Considering the unlikely integration of AAV, CBER will not be overly demanding on length of follow-up. Investigators should clarify the demands at the time of presentation of the protocol in the pre-IND meeting. We have generally taken the position that a 2- to 3-year follow-up is a suitable time period to assess efficacy of gene transfer and is acceptable for assessing adverse events related to AAV gene transfer. It has been suitable for multiple gene therapy trials that have been done or are underway. CBER will provide written feedback following the pre-IND meeting and if they feel that longer follow-up should be

implemented. Their recommendation can be incorporated into the IND with a rationale for the need for longer follow-up.

In proposing a follow-up plan to CBER, investigators should keep in mind that monitoring patients post gene delivery should include toxicity to off-target organ systems (e.g., liver, renal, hematology, etc.) and the laboratory studies for monitoring these tissues should be included in the annual report. In addition, the risk of a delayed immune response is enhanced by persistent gene expression and triggers the need for IFN-γ ELISpot assays targeting AAV and transgene. If a T-cell response persists, there might be a need for corticosteroid suppression. Such findings might alter the long-term follow-up plan with a shift to yearly evaluations. This should be discussed with the clinical reviewer at CBER for final recommendations.

Subjects participating in clinical gene therapy projects should also be instructed to maintain a wallet-sized card with investigator contact information and to assist in reporting adverse events through the use of diaries of health-related events inclusive of both serious and unexpected (21 CFR 312.32). Investigators should provide contact numbers for themselves and office personnel and staff so that subjects participating in clinical trials have easy access to address questions and be available for adverse events.

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Chapter 40
Muscle MRI as an Endpoint in Clinical Trials

Dirk Fischer, Ulrike Bonati, and Mike P. Wattjes

Abstract In the last decade, there has been substantial progress in the genetic characterization and classification of inherited muscle disorders. In addition to clinical assessment, clinical neurophysiology, and the diagnostic gold standard of histopathology including immunohistochemistry, muscle imaging, and particularly magnetic resonance imaging (MRI), has increasingly been used in the diagnostic work-up of inherited muscle disease. Novel quantitative muscle MRI techniques have been developed in order to characterize and quantify the severity and pattern of muscle involvement in clinical routine as well as in therapeutic trials. This chapter provides a comprehensive overview of current MRI techniques in inherited muscle diseases with special emphasis on the use of quantitative muscle MRI in clinical therapeutic trials.

Keywords Muscle imaging · Quantitative muscle magnetic resonance imaging Muscular dystrophy · Clinical trials

40.1 Introduction

Neuromuscular imaging is used as a routine tool in the diagnosis of acquired and inherited muscle disorders [1–3]. The term "neuromuscular imaging" encompasses a broad variety of imaging modalities such as ultrasound (including quantitative

D. Fischer (✉) · U. Bonati
Division of Neuropaediatrics, University Children's Hospital Basel, University of Basel, Basel, Switzerland

Department of Neurology, University Hospital Basel, University of Basel, Basel, Switzerland
e-mail: Dirk.Fischer@ukbb.ch; Ulrike.Bonati@ukbb.ch

M. P. Wattjes
Department of Diagnostic and Interventional Neuroradiology, Hannover Medical School, Hannover, Germany
e-mail: Wattjes.Mike@mh-hannover.de

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ultrasound techniques), computed tomography (CT), conventional and quantitative magnetic resonance imaging (MRI), as well as nuclear medicine techniques. Various studies have shown that neuromuscular imaging, in particular MRI, can distinguish between different pathologic changes in the muscles such as fatty degeneration and signs suggestive of inflammation such as muscle edema. More advanced techniques allow the quantification and monitoring disease pathology in the individual muscle. Also, MRI helps to detect distinctive muscle involvement patterns that are sometimes specific for individual disease entities. This has coined the term "pattern recognition" aiding in the differential diagnostic work-up of inherited muscle diseases. Recently, advances in MRI techniques and the application of higher magnetic field strengths have enabled high-resolution whole-body examination, providing information on almost all skeletal muscles and possible comorbidities [4, 5]. The implementation and standardization of quantitative muscle MRI (qMRI) techniques allow monitoring disease progression and assessing treatment safety and efficacy in therapeutic trials [6, 7]. The following chapter will give an overview on some of the most widely used MRI techniques and their application in clinical routine as well as in research.

40.2 Neuromuscular Imaging in Inherited Muscle Diseases

Muscle imaging is increasingly used in patients with progressive weakness to help identify the underlying muscle disease. Muscle imaging is used to identify an affected area not yet completely replaced with fatty tissue suitable for biopsy. In muscular dystrophies, even if the clinical presentation in terms of the pattern of muscle weakness is characteristic, e.g., in limb-girdle muscular dystrophy or distal myopathy, there is still a wide genetic heterogeneity with several identified genetic mutations. While genetic analyses and whole-genome sequencing are used for routine work-up, the search for a causative genetic mutation can still be time consuming and expensive. At the present time, conventional muscle MRI (e.g., T2-weighted and T1-weighted (turbo/fast spin echo)) is used as an additional diagnostic tool to support the clinical diagnosis and to facilitate the identification of the underlying disease entity. Detecting a specific pattern of muscular involvement can help to narrow down the genes to be sequenced and evaluated. We and others have tried to identify pattern recognition algorithms as guidance for differential diagnosis. For more details the reader is referred to the following recently published reviews [1, 8]. An exhaustive description of known disorders, patterns, and algorithms was recently presented in a larger volume [3].

Conventional muscle MRI is capable of identifying characteristic patterns of muscle involvement in several neuromuscular disorders and has led to the development of new diagnosis algorithms. However, conventional muscle MRI and related algorithms have considerable limitations. First, most of the algorithms are based on results from retrospective studies. Identified patterns of different inherited muscle diseases may overlap and algorithms should therefore be verified and

validated in prospective studies. Second, most studies were limited to lower leg muscles only and measured muscle fatty degeneration, and less often muscle atrophy. Third, pattern analyses have relied on qualitative and not on quantitative assessment of muscle involvement. To overcome some of these drawbacks, promising new approaches such as whole-body MRI (WBMRI) assessing all muscles of the body and new techniques able to assess both fatty degeneration and muscle atrophy have recently been introduced [9, 10]. However, WBMRI evaluations can be difficult due to the large amount of data. Therefore, Hankiewicz et al. suggested the use of a consolidated graphical technique (heat map) allowing a visualization of all data at a glance [11]. For the individual disorders it is then easy to comprehend which muscles are commonly involved and which are commonly spared. Once a specific WBMRI pattern has been established for a known disorder, it can be applied to match patterns of patients with uncertain diagnosis and potentially confirm or discard the diagnosis [10].

40.3 MR Imaging Techniques

40.3.1 Conventional MRI

In the recent past, conventional muscle MRI has become a useful diagnostic tool for some degenerative neuromuscular disorders. One particular example is in facioscapulohumeral muscular dystrophy which by MRI defines a typical pattern of involvement that is unique, involving the trapezius, teres major, and serratus anterior [12, 13]. In congenital myasthenic syndromes, there are also imaging features such as a nonselective pattern of fat infiltration or a normal-appearing scan in the setting of significant weakness that favors this diagnosis [14]. Technological advances in image acquisition, such as whole-body MRI applying a high-spatial-resolution MRI acquisition, have contributed to the success of MRI in patient care and research. Since the introduction of WBMRI improvements in imaging hardware, such as improved coil technologies, more homogeneous magnetic fields, even at higher magnetic field strengths, have led to higher image quality and diagnostic accuracy (e.g., homogenous fat suppression on T2-weighted images with fat suppression) [5, 15]. However, the standardization of conventional MR image acquisition is still one of the major challenging factors, limiting its use especially for research purposes. One important aspect of standardization is (re)positioning of patients and the use of defined anatomical landmarks to improve the reproducibility [16]. This is of particular importance for repeated measurements and longitudinal assessments of disease progression and monitoring of treatment effects in therapeutic trials [17]. Therefore, standardized protocols or guidelines for image acquisition are needed to increase the validity of WBMRI for the scanning of the lower limbs but also other anatomic regions, such as the upper extremities. In terms of MRI acquisition protocols, T1 and fat-suppressed T2 (turbo/fast spin echo) sequences are the “work horses.” The degree of fatty degeneration should be

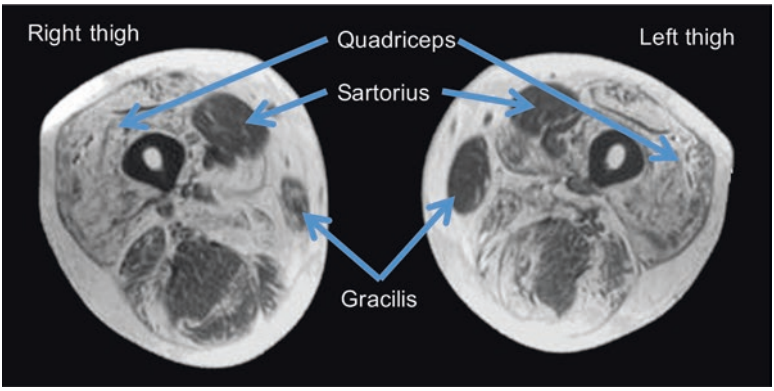

Fig. 40.1 T1-weighted thigh muscle MRI of a patient with Becker muscular dystrophy with prominent involvement of the quadriceps muscle, while gracilis and sartorius muscles are relatively spared

evaluated on the T1-weighted, while the possible presence and extent of muscle edema should be assessed on fat-suppressed T2-weighted images. Semiquantitative rating scales are helpful to further classify these changes. Figure 40.1 shows an example of a conventional thigh muscle T1-weighted image of a patient with Becker muscular dystrophy (BMD).

40.3.2 Quantitative MRI (qMRI) Techniques

In addition to conventional MRI, qMRI techniques are increasingly being used to assess muscle disease burden. It has been demonstrated that reliable fat quantification techniques are crucial for disease monitoring in neuromuscular disorders. A comprehensive discussion of all these techniques is beyond the scope of this chapter. Briefly, the Dixon fat quantification technique makes use of the fact that protons within water and lipids resonate at different frequencies [18]. Another widely used technique to reliably assess disease activity and fatty degeneration includes T2 mapping of skeletal muscle without fat suppression (global T2) and with fat suppression techniques (water T2) [6, 7, 19]. Other promising techniques such as diffusion tensor imaging, T2 relaxation, and ^{35}Cl and ^{23}Na MRI time measurements have been applied in skeletal muscle tissue recently but require further validation in larger studies [20–22]. These sensitive quantitative techniques could be of particular use in non-dystrophic muscle diseases with normal conventional MRI findings [23].

40.3.3 Nuclear Medicine Methods

Nuclear medicine methods such as scintigraphy, PET-computed tomography (CT), and single-photon emission CT have a certain value in the diagnosis of neoplastic, inflammatory, and degenerative muscle diseases. Interestingly, in addition to ^{18}fluorodeoxyglucose (FDG) PET, other PET tracers used for brain imaging such as Pittsburg compound B (^{11}C-PIB) were shown to be able to detect muscle diseases as well. The detection of amyloid in heart muscles or in skeletal muscle in patients with inclusion body myositis underpins the potential of these new techniques [24, 25].

40.4 Neuromuscular Imaging for Monitoring Disease Progression

In neuromuscular diseases, muscle MRI has become an important diagnostic tool able to identify target muscles for muscle biopsy and patterns of muscle involvement aiding diagnosis. With the development of new therapies for many forms of neuromuscular disorders, there is growing need for outcome measures highly sensitive to disease progression. Although validated functional scores exist for many neuromuscular disorders, they are all dependent on patient collaboration and fitness on the day of testing. In infants, ongoing fine and gross motor development can additionally mask disease progression. Also, neuromuscular disorders are rare diseases, progress slowly, and often have weak genotype-phenotype correlation.

In the recent past, semiquantitative and quantitative muscle MRI techniques were evaluated with regard to their sensitivity to detect disease progression, and these changes were correlated with and compared to changes in validated clinical assessments.

In most neuromuscular disorders, regardless of the underlying cause, accumulation of tissue water in the muscle characterizes the early stages of most diseases, while the chronic or end stages are characterized by fatty replacement of muscle tissue [21, 26].

One of the first studies comparing qMRI to a functional score regarding responsiveness was done in OPMD, a rare and slowly progressing muscular dystrophy [27]. It was shown that the motor function measure (MFM) and T1 images using Fischer's semiquantitative five-point (0–4) scale could not detect significant changes. In contrast, quantitative T2 values using a multi-contrast sequence and the MRI-measured fat fraction (MFF) using a two-point Dixon method increased significantly despite the relative short follow-up period. This demonstrated that quantitative muscle MRI is able to detect subclinical changes in patients with OPMD, while semiquantitative muscle MRI and functional scores remained stable.

40.4.1 Dystrophinopathies

40.4.1.1 Duchenne Muscular Dystrophy (DMD)

DMD is an orphan disease affecting 1 in 3500–6000 male births. In boys under the age of 7, ongoing fine and gross motor development may mask disease progression when only functional scores are used. QMRI was shown to detect pathological changes of muscle tissue even in children under the age of 7 [28]. In a large cross-sectional study in DMD boys, age-dependent changes in muscle tissue could be shown by quantitative MRI and spectroscopy. Older boys (11–14 years) had longer MRI-T2 times and greater lipid fraction compared to younger boys (5–6.9 years). In contrast $^{1}H^{2}O$ T2, representing inflammation and edema, was lower in the oldest age group compared to the young age group [29].

In a longitudinal study in 20 DMD patients, an annual increase in two-point Dixon acquired mean fat fraction of 5% was calculated. A cutoff for mean fat fraction of 50% predicted loss of ambulation with a sensitivity of 100% and a specificity of 91% [30].

MRI-measured fat fraction (MFF) in the skeletal muscles showed excellent correlation with most of the validated functional scores such as the 6-min walk test (6MWT), the motor function measure or the North Star Ambulatory Assessment, as well as myometric measures [28, 31].

In a cohort of 20 boys with DMD, the MFF using a two-point Dixon method negatively correlated with the MFM and its D1 subscores (measuring ambulation) cross-sectionally. In addition, the increase in fatty replacement and the decline in MFM from baseline to 1-year follow-up were also correlated. Power analysis showed that the two-point Dixon MFF had a much larger effect size compared to the MFM. For example, sample size estimations for qMRI data were up to 17-fold smaller compared to the MFM total score and up to sevenfold compared to the MFM D1 subscore, respectively [28].

40.4.1.2 Becker Muscular Dystrophy (BMD)

BMD is a less severe form of dystrophinopathy and with an incidence of 1 in 16,000 male births, even rarer than DMD. In a longitudinal study in BMD, it was shown that qMRI can detect disease progression in a small sample size and at relatively short imaging intervals [32]. In a natural history cross-sectional study in 20 BMD patients, qMRI-acquired muscle fat fraction and T2 relaxation times were highly negatively correlated with the MFM total, the MFM D1 subscore and 6MWT and positively correlated with the 10 m run/walk test. Age was not correlated with MFF, global T2 relaxation time, or clinical assessments showing that disease progression is difficult to predict in BMD [33].

40.4.2 Other Neuromuscular Disorders

In a recent 12-month natural history study in patients with Charcot-Marie-Tooth disease 1A and inclusion body myositis, the responsiveness of MRI outcome measures and their cross-sectional correlation with functionally relevant clinical measures, as well as the sensitivity of specific MRI indices to early muscle water changes before intramuscular fat accumulation, were studied [26]. Quantitative MRI measures of lower limb muscles changed significantly over the 12-month follow-up period. Again, MRI-MMF showed greater responsiveness compared to the validated functional scores and correlated with muscle strength and function.

To date, it remains a question of debate how treatment-related effects will affect skeletal muscles. One might speculate that early changes of muscle pathology such as water tissue accumulation due to edema and inflammation might respond more quickly to treatment than tissue fat accumulation or muscle mass. In Charcot-Marie-Tooth A1 and inclusion body myositis, T2 times (adjusted to fat fraction) were increased, and magnetization transfer ratio (MTR) was reduced in muscles without substantial intramuscular fat accumulation compared to controls. As shown above, measures of muscle edema and inflammation were also increased in the muscle of young boys with DMD compared with older boys and longer disease duration [29] Thus, fat quantification techniques were shown to be more sensitive to monitor disease progression in many neuromuscular disorders. T2 times, MTR, and MR spectroscopy (MRS) have the potential to serve as sensitive outcome measures to detect early and potentially reversible changes in muscle water distribution in therapeutic trials.

40.5 Muscle MRI as Endpoint in Interventional Clinical Trials

Arpan et al. evaluated cross-sectionally the effects of corticosteroids on the lower extremity muscles in DMD. They compared 15 DMD boys under corticosteroid treatment and 15 steroid naive boys using MRI and 1H-MRS at baseline and at 3, 6, and 12 months [34]. They demonstrated lower muscle global T2 tissue relaxation times and less intramuscular fat deposition in corticosteroid-treated patients at the baseline and a lower MFF increase at 1 year compared to steroid naive boys. In the group of boys starting steroid treatment during the trial, T2 tissue relaxation times acquired by MRI/MRS could detect effects of corticosteroids on muscle degeneration only 3 months after initiation. In conclusion, MRI and MRS were able to measure the effect of corticosteroids which are assumed to reduce short-term inflammatory processes and to lessen long-term degenerative changes in skeletal muscles. Their findings were later confirmed by Willcocks et al. in a large multicenter trial in 109 ambulatory DMD boys [35]. In this study, qMRI biomarkers were tested with regard to their responsiveness to disease progression. Measurements

were done at baseline and at 1-year follow-up. A subset of boys completed additional measurements after 3 or 6 months. Global T2 tissue relaxation times and MFF increased significantly over 12 months in all age groups, including in boys under the age of 7 as well as in boys who improved or remained stable in the 6MWT. Significant increases in vastus lateralis MFF were observed in 3 and 6 months, too. Of all the muscles examined, the vastus lateralis and biceps femoris long head were the most responsive to disease progression. Thus, both studies showed that qMRI can measure putative treatment effects even at very short follow-up. In a recent randomized, double-blind, placebo-controlled therapeutic trial testing the efficacy of L-citrulline and metformin versus placebo in DMD patients, qMRI was chosen as a surrogate endpoint alongside clinical assessments such as the MFM D1 subscore (primary endpoint) and other clinical and subclinical tests [36]. To the best of our knowledge, this is one of the first randomized, placebo-controlled trials in DMD using qMRI measured muscle fat fraction as an endpoint.

40.6 Summary

Today, the importance of muscle imaging in patients with inherited neuromuscular disorders is increasingly recognized. Conventional MRI has become the modality of choice in routine diagnostic work-up, often able to identify a distinctive pattern of muscle involvement. In the future, WBMRI protocols and hierarchical meta-analytical approaches with heat maps and fingerprints of individual diseases will probably further improve the validity of muscle MRI, especially for pattern recognition. Currently, MFF measurements using chemical shift differences and T2 relaxation times of separated fat and water components in skeletal muscle are the most reliable quantitative MRI techniques available. Recent research could prove for various disorders that qMRI is highly sensitive to changes and detects disease progression more sensitively than clinical scores. Therefore, in neuromuscular disorders monitoring natural history changes and therapeutic effects in therapy trials, the use of qMRI will likely soon be regarded as “state of the art.”

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Chapter 41
Gene Therapy Clinical Trials for Duchenne and Limb Girdle Muscular Dystrophies: Lessons Learned

Jerry R. Mendell, Louise R. Rodino-Klapac, and Christopher Walker

Abstract This chapter reviews the discovery of the Duchenne muscular dystrophy (DMD) gene, early attempts at plasmid-mediated *DMD* gene delivery and its limitations for clinical efficacy, and modifying the *DMD* cDNA to circumvent the obstacle of packaging in adeno-associated virus (AAV). The unfortunate events of the Jesse Gelsinger death are briefly discussed. The essence of the chapter is the review of the first clinical experience of DMD gene therapy using a mini-dystrophin and the challenges for immune responses. The potential way forward where possible to avoid these problems is fully described. In addition to DMD, our experience in limb girdle muscular dystrophy 2D (LGMD2D), alpha-sarcoglycan, gene therapy in clinical trial is also described. Success was achieved with long-term gene expression following intramuscular delivery, but again we encountered an obstacle for delivery related to pre-existing immunity to AAV. The lessons learned from these clinical trials provide a template and a path for additional clinical trials for muscular dystrophies.

Keywords DMD · Limb girdle muscular dystrophy · LGMD · Gene therapy clinical trials · Immune response

J. R. Mendell (✉)
Department of Pediatrics, Nationwide Children's Hospital and Research Institute, The Ohio State University, Columbus, OH, USA

Department of Neurology, Nationwide Children's Hospital and Research Institute, The Ohio State University, Columbus, OH, USA
e-mail: Jerry.Mendell@nationwidechildrens.org

L. R. Rodino-Klapac
Department of Pediatrics, Nationwide Children's Hospital and Research Institute, The Ohio State University, Columbus, OH, USA

Vice President Gene Therapy, Sarepta Therapeutics, Columbus, OH, USA
e-mail: LRodinoKlapac@Sarepta.com

C. Walker
Department of Pediatrics, Nationwide Children's Hospital and Research Institute, The Ohio State University, Columbus, OH, USA
e-mail: Christopher.Walker@nationwidechildrens.org

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41.1 Introduction to Gene Therapy for Duchenne Muscular Dystrophy (DMD)

DMD is caused by the absence of the muscle cytoskeleton protein, dystrophin encoded by the very large 2.4 Mb *DMD* gene [1]. Delivery of the cDNA encoding the full-length protein remains a major challenge and a potential obstacle to the success of gene replacement therapy. For the past two decades, scientists have employed viral and non-viral gene replacement/gene restoration strategies to replace the missing dystrophin protein. Most of this chapter will review the status of viral gene delivery, but comments on non-viral approaches are of interest. The use of antisense oligonucleotides to induce exon skipping has merits because it can slow the progress of the disease and preserve ambulation for 1–2 years beyond the use of corticosteroids alone [2]. Eteplirsen, a phosphorodiamidate morpholino oligomer, has recently been approved for DMD (commercial name Exondys 51, Sarepta Therapeutics) and has been discussed in Chap. 20. Gene editing using CRISPR (scientific name: clustered regularly interspaced short palindromic repeats) is one of the most talked about methods of gene editing [3]. Its ability to delete, add, activate, or suppress targeted genes makes this a powerful tool for gene editing. Understanding grew from appreciating that an adaptive immune system of bacteria enabling a defense against specific phages relies on a protein called Cas9, a specialized nuclease for cutting DNA. Since early recognition and its first applications for the development of mouse models of disease, the system has now matured enough to provide potential translation for gene editing in clinical disease. Further sophistication has been introduced permitting CRISPR/Cas9 to be delivered by AAV [4–6]. CRISPR provides a promising tool for the potential treatment of DMD, a devastating disease of childhood [7]. Chapter 22 provides a full discussion of CRISPR/Cas9 explaining its potential and its limitations.

41.2 Delivery of Naked DNA to Replace the Missing Gene

Plasmid-mediated gene therapy was introduced with great enthusiasm more than two decades ago [8]. In the quadriceps muscle of the mouse, following direct injection of the Lac-Z DNA, β-galactosidase was expressed in 60 of 4000 muscle fibers (1.5%). A dose-dependent effect was observed, and DNA was present in muscle for at least 30 days. A number of advantages over viral gene delivery were considered. The potential for safety of naked DNA delivered to muscle was considered to be a clear advantage [9, 10]. Plasmid DNA is far less expensive than production of virus for gene transfer. Another attractive feature is the lack of a humoral immune response using plasmid DNA, thus, enabling repeat gene delivery to enhance gene expression over time. Delivery to mice by intramuscular injection and electroporation also showed promise [11–13], but gene expression was limited to a few square

centimeters near the injection site. More ambitious methods of gene transfer using vascular delivery such as isolated limb infusion or delivery through the tail artery or vein of the mouse were also tried. Although more extensive gene expression was achieved, plasmid DNA-related gene expression peaked in 1–2 weeks and declined thereafter [14]. The duration of gene expression after intravascular infusion was disappointing, not much better than intramuscular injection. Limitations following plasmid DNA delivery were attributed to a vascular endothelial barrier limiting naked DNA access to muscle. Combating this obstacle requires infusion of relatively large volumes of solution with delivery under high pressure [15–17], a potentially unwanted introduction of safety concerns.

One point of interest for plasmid DNA is that, when introduced for the *DMD* gene, it was believed that in the absence of viral capsid, there would be little cause for concern for an immune response. Dystrophin expression from revertant fibers (muscle fibers with spontaneous somatic DMD mutations that restore the reading frame and DMD protein expression) was believed to be a built-in protection from newly expressed foreign protein introduced from naked DNA. However, gene transfer of the full-length dog dystrophin cDNA in the canine model induced a T-cell inflammatory response at the site of gene transfer [18] refuting notions of immune tolerance induced by revertants. Further experience seen in clinical trial again demonstrated an immune response to transgene supporting the notion that revertant fiber dystrophin expression did not induce tolerance [19].

The extensive preclinical work to establish plasmid-based DMD gene delivery as a viable option for clinical adaptation was tested in a phase I clinical trial in DMD and Becker muscular dystrophy (BMD) [20]. The aim of the study was to provide evidence demonstrating both safety and transgene expression following intramuscular delivery of pCMV-full-length dystrophin. Nine patients (three DMD, six BMD) were enrolled in the study and distributed into three cohorts. The first cohort received plasmid 200 μg, the second received 600 μg, and the third cohort was given two 600 μg injections separated by 2 weeks. The radialis muscle of the forearm was injected on one side, and biopsy was taken 3 weeks after plasmid injection. Control biopsies came from the opposite extremity or one previously available. Plasmid DNA was found in all patient biopsies indicative of the side of gene transfer. Overall, the efficiency of transfection for participating patients was modest. Plasmid DNA was found in six of nine patients, and a small percentage of muscle fibers expressed dystrophin (2–6% full sarcolemmal labeling and 3–26% weakly, partially labeled muscle fibers). This limited efficacy was attributed to both the dose and poor efficiency of local intramuscular administration of plasmid DNA. Clinically, no benefit was seen in wrist extension for these patients. Safety was the merit of this study, but without efficacy, moving forward proved to be a challenge. It was hoped at the time that plasmid DNA gene delivery pathway had been established for further DMD clinical trials, but additional trials have not yet been published.

41.3 Viral-Mediated Gene Delivery for DMD

41.3.1 Introducing DMD Gene Therapy to the Clinic

The first consideration for bringing viral-mediated gene delivery to the clinic for DMD was the concern over the size of the *DMD* gene and how to mediate the greatest potential for patients. The full-length cDNA of the *DMD* gene is approximately 14 kb, larger than the packaging capacity of AAV. Adenovirus appeared attractive to carry the burden of this large cDNA, and the introduction of a "gutted" adenoviral vector system lacking all viral genes looked very promising [21, 22]. A full-length, muscle-specific dystrophin expression cassette transduced muscle fibers efficiently in1-year-old *mdx* mice with restoration of contraction-induced injury 40% of normal [21]. A concern regarding adenovirus was that over 70% of the population carries antibodies impairing efficiency in transduction or associated with toxicity. Many of the concerns regarding gene therapy came to culmination when Jesse Gelsinger, an 18-year-old with deficiency of ornithine transcarbamylase (OTC) entered into a phase I OTC-adenoviral gene therapy clinical trial. Jesse was the first patient in whom death could be directly attributed to the vector-mediated gene therapy. An autopsy showed that although the vector had been infused directly into the liver through the hepatic artery, substantial amounts had disseminated into the circulation and accumulated in the spleen, lymph nodes, and bone marrow. The vector triggered a massive inflammatory response that led to disseminated intravascular coagulation, acute respiratory distress, and multiorgan failure [23–25]. Exactly why Gelsinger suffered such severe side effects, whereas a second patient tolerated a similar dose of the vector, remains unclear. However, it has been indicated that previous exposure to a wild-type virus infection might have sensitized his immune system to the vector [26]. The death of Jessie Gelsinger had a profound impact on the progress of gene therapy. For the most part, gene delivery for monogenic illness turned away from adenoviral vectors, and most of the entire field switched to the use of AAV. There was also more attention directed toward emphasizing the importance of pre-existing immunity especially to virus but also to transgene. Unfortunately, without the advantages of adenovirus, DMD gene therapy was handicapped, and the design of cassettes to circumvent cDNA size, while maintaining functional efficacy, became a primary focus.

41.3.2 Designing Cassettes for the DMD Gene

Attempts to develop gene therapy for DMD were dependent on developing small dystrophins that provided protection of sarcolemma. The Chamberlain laboratory showed that structural domains of the large dystrophin protein could be deleted creating mini- and micro-dystrophins that could be carried forward for clinical trials [27]. This was a major step in feasibility, potentially providing a path for gene

replacement for DMD, compatible with the limited packaging capacity of AAV. Varying the number of spectrin-like repeats and including or removing hinges enabled comparisons between constructs. Clones with four or fewer repeats were referred to as "micro-dystrophins" and the larger clones as "mini-dystrophins." The constructs were tested in both transgenic *mdx* mice and by direct muscle injection using AAV in immunocompetent mice that already showed pathology. These injections resulted in a reversal of myofiber central nucleation and fiber size variation. In contrast to the AAV method, transgenic mouse studies enabled functional analysis of whole animals and entire muscles, especially the severely affected diaphragm. The message from these studies had particular relevance with regard to rod domain function including the phasing of the repeats, the number and periodicity of repeats, and the presence or absence of internal hinge domains. Quantitative data were obtained by counting centrally nucleated myofibers and measuring fiber diameters before and after virus injection. An attractive cassette removed most of the 5′ and 3′ untranslated regions and portions of the C-terminal domain (Δexon71–78) [28–30]. This was coupled with the addition of the Kozak consensus sequence [31] and a muscle-specific promoter. The ΔR4–R23/Δ71–78 (hinge 1, rods 1, 2, and 3, hinge 2, rod 24, hinge 4) was most favorable reducing central nuclei to 14%, a reduction from 68% in *mdx* mice. More recent studies from the Chamberlain laboratory suggested replacing hinge 2 of micro-dystrophin with hinge 3-improved functional capacity preventing muscle degeneration, increasing muscle fiber area, and better preserving the neuromuscular junction [32].

Similar studies were carried out over a parallel time period by the Xiao laboratory, also making strides in gene delivery to the *mdx* mouse [33, 34]. They used a gene construct, Δ3990 (N-terminus, hinge1, rods 1 and 2, hinge 3, rods 22, 23, and 24, hinge 4 (Fig. 41.1), that would fall under the strict definition of a mini-dystrophin representing approximately 40% of the coding sequence of the human dystrophin gene. They demonstrated success in mdx mouse gene transfer by intramuscular

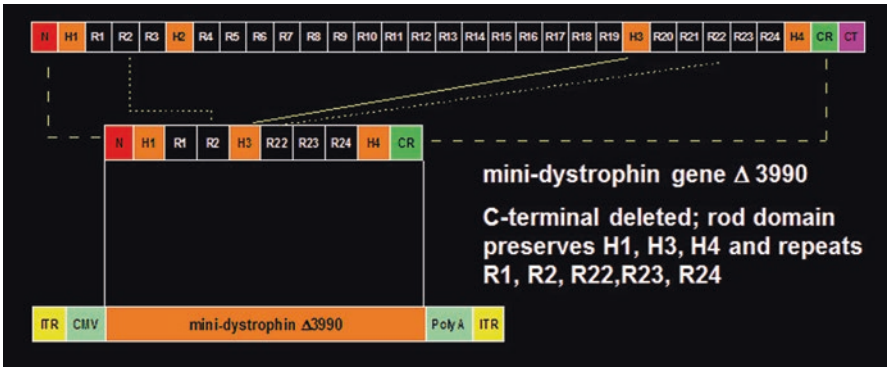

Fig. 41.1 Mini-dystrophin gene Δ3990 shows the C-terminal deleted and elements preserved in rod domain including hinge (H)1, H3, and H4 and spectrin repeats R1, R2, R22, R23, and R24. The genomic DMD gene copy is illustrated above Δ3990. Below is seen the cassette with ITRs, CMV promoter, and mini-dystrophin

injection to tibialis anterior muscle showing virtually no central nucleation when gene was delivered in the neonatal period and a 35–50% reduction when delivered to 45-day-old adult *mdx* mice. They also showed significant improvement in force generation and protection against eccentric contraction. These results were seen with either MCK or CMV promoters. The Xiao study laid the path for the first clinical gene therapy trial in DMD.

41.3.3 First In-Human Gene Therapy for DMD

41.3.3.1 Methods

The first in-human gene therapy trial in DMD was initiated on March 15, 2006 [19]. Six boys with frame-shifting deletions in the dystrophin gene were enrolled (Table 41.1). At enrollment, four patients were receiving daily glucocorticoid therapy, one deflazacort, two prednisone, and one taking weekend prednisone. Two others were not on corticosteroids. The clinical trial was approved by the Institutional Review Board (IRB) at Nationwide Children's Hospital. Written informed consent was obtained from the parents of all the patients, and participants 9 years of age and older provided assent in writing. The study was conducted under IND-BB #12936. Δ3990 under control of a CMV promoter (Fig. 41.1) was delivered in a modified rAAV2, referred to as AAV2.5. The objective was to diminish the potential for an immune response to neutralizing antibodies (Nab) through insertion of five amino acids of AAV type 1 into the type 2 capsid backbone. The biceps was the target of delivery by direct muscle injection. The sides for injection were randomized by the pharmacy, and one side received vector, while the opposite side was given placebo. In patients 1–4 the placebo consisted of saline, and in patients 5 and 6, empty capsids were delivered. This strategy pertained to an effort to distinguish an immune response to vector versus mini-dystrophin transgene.

Table 41.1 Clinical trial participants for intramuscular Δ3990 mini-dystrophin gene therapy

Subject	Age (years)	Deletion mutation	Corticosteroids	Pre-Nab	Viral dose
1	8	45	Deflazacort 18.0 mg/day	≤1:2	2.0×10^{10} vg/kg
2	9	50	Pred 18.0 mg/day	1:800	2.0×10^{10} vg/kg
3	9	46–50	None	≤1:2	2.0×10^{10} vg/kg
4	5	49–54	None	≤1:2	1.0×10^{11} vg/kg
5	11	3–17	Pred 150 mg Sat/Sun	1:100	1.0×10^{11} vg/kg
6	9	46–52	Pred 22.5 mg/day	1:2	1.0×10^{11} vg/kg

Participants in Δ3990 mini-dystrophin gene therapy trial showing subject number, ages at gene transfer, deletion mutations, corticosteroid doses and doses of rAAV.mini-dystrophin. Subject 2 showed distinctly higher titer to AAV pre-gene transfer compared to other subjects and had little or no humoral response following gene delivery suggesting blunted (blocked) muscle fiber transduction by neutralizing antibodies (Nab)

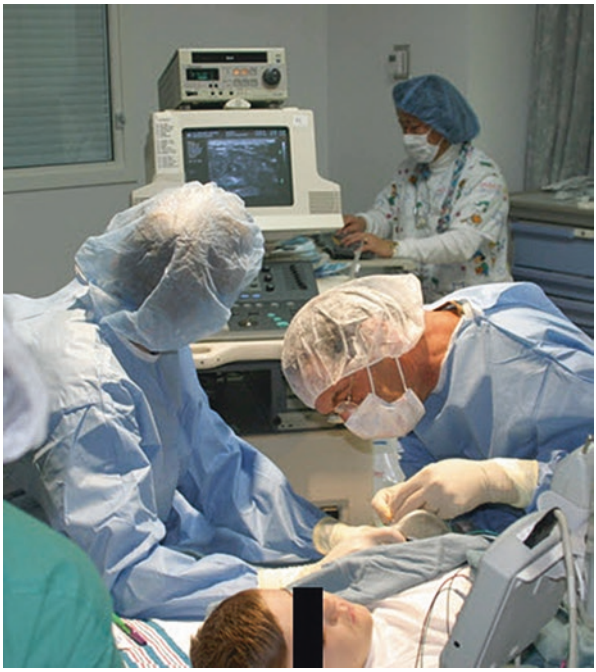

Fig. 41.2 Picture shows gene transfer to biceps muscle guided by ultrasonographer viewing liquid-crystal display (LCD) screen to make sure that needle is in skeletal muscle. In the foreground is an electromyography (EMG) monitor that is also recording muscle action potentials confirming injection to muscle

Treatment was done under conscious sedation in the interventional radiology suite (Fig. 41.2). Needle injection was guided by ultrasound and EMG monitoring to ensure skeletal muscle (not fibrotic tissue) vector delivery. A MyoJect hypodermic needle (Oxford Instruments, Hawthorne, NY) was used to daeliver 1.2 ml of vector in three equivalent boluses spaced 0.5 cm apart along an injection tract that was placed in a longitudinal trajectory relative to the biceps muscle orientation. This was a dose escalation trial. Cohort 1 (patients 1, 2, and 3) received 2.0×10^{10} vector genomes per kilogram of body weight, and patients 4, 5, and 6 received a dose that was higher by a factor of five (1.0×10^{11} vector genomes per kilogram) (Table 41.1). Because gene transfer for this study was a direct muscle injection, and previous experience showed that needle insertion into muscle aroused an inflammatory response, patients received intravenous methylprednisolone (2 mg/kg, limited to <1 g total) 4 h prior to vector administration, with repeat doses the following two mornings.

Immune responses were monitored by serum-binding antibody titers and interferon-γ enzyme-linked immunosorbent spot (ELISPOT) assay for reactivity to mini-dystrophin. Three pools of 40 overlapping synthetic peptides (designated MDP1, MDP2, and MDP3) that spanned the entire mini-dystrophin sequence were used to stimulate peripheral-blood mononuclear cells (PBMCs) in the ELISPOT assay. The biceps muscles were biopsied on day 42 (patients 1, 3, 4, 6) and day 90 (patients 2 and 5) and processed for gene expression, histology, and inflammatory reaction. Biopsies were read blindly without knowing the side receiving vector versus placebo.

Table 41.2 Clinical trial participants for intramuscular SGCA gene therapy

Subjects	Age gene transfer (years)	Age disease onset (years)	Post gene therapy muscle biopsy	AAV dose rAAV1.tMCK.hSGCA
1	13	9	6 weeks	3.25×10^{11} vg
2	12	3	3 months	3.25×10^{11} vg
3	14	8	6 weeks	3.25×10^{11} vg
4	43	10	6 months	3.25×10^{11} vg
5	34	10	6 months	3.25×10^{11} vg
6	23	10	6 months	3.25×10^{11} vg

Participants in SGCA gene therapy study are shown including age at gene transfer, age at onset of disease, timing of post gene therapy muscle biopsies and dose of viral mediated gene transfer

41.3.3.2 Results

Given that this was the first gene therapy for DMD, physical and laboratory studies following gene delivery were important. There were no significant adverse events encountered and specifically no fever, lymphadenopathy, and organomegaly and no signs of inflammation at the injection site. During the 2-year active phase of the trial, only a few minor adverse events commonly seen in this age group were observed unrelated to gene delivery including sore throats, rashes, and nausea (Table 41.2). Hematology and chemistry panels that included an assessment of liver function also indicated that the vector was well tolerated in all subjects. All blood samples showed normal laboratory values for CK, GGT, alkaline phosphatase, and lymphocyte counts.

Transduction efficiency of gene transfer was assessed by vector genome copies and by dystrophin muscle expression. In all the patients, vector DNA was detected in amounts ranging from 0.01 to 2.56 genome copies per diploid genome in the treated muscles, and none were detected in the untreated contralateral biceps muscles. Mini-dystrophin expression was not detected in myofibers in the two biopsy specimens examined on day 90 (patients 2 and 5). On day 42 muscle biopsies from only patients 3 and 6 showed a few mini-dystrophin-positive myofibers detected by N-terminus antibody (Dys3) while staining negative by C-terminus antibody (Dys2). No mini-dystrophin-positive myofibers (patients 1,2,4,5) suggested that transgene expression was either very poor or lost before the time of biopsy.

41.3.3.3 Humoral Immune Responses to Gene Transfer

In preparation for the mini-dystrophin gene therapy clinical trial, patients were screened for pre-existing antibody to AAV. Neutralizing antibodies (Nabs) to both AAV2 and the synthetic AAV2.5 capsids (Table 41.1) were found in Subject 2 with a baseline titer of 1:800. Titers for other subjects were very low (patients 1, 3, 4, and 6), although patient 5 exhibited a borderline titer of 1:100 targeting AAV2 and AAV2.5. Not unexpectedly humoral responses peaked from week 2 to week 6 and

ranged from 50- to 1000-times baseline titers with the exception of patient 2 with titers maintained within the pre-treatment range, 1:800 to 1:2000 (no more than a 2.5× increase). This pre-treatment evidence of a pre-existing immunity may have significance given the rapid rise in the interferon-γ ELISPOT assay to mini-dystrophin described below.

41.3.3.4 Cellular Immune Responses to Transgene Expression: Pathogenic Mechanisms

From this mini-dystrophin gene therapy trial, two paths to cellular immunity were identified using the interferon-γ ELISPOT assay. Patient 5 had a large DMD mutation inclusive of exons 3–17. On day 60 post-gene delivery, three discrete HLA restricted epitopes were targeted to MDP1 derived from the mini-dystrophin. A CD8+-specific T-cell response targeting an epitope that spanned amino acids 181–200, encoded by exon 7 (Fig. 41.3) was identified. We also found specificity for CD4+ T-cell immunogenesis with recognition of two epitopes that localized to amino acids 221–240 expressed from exon 8 and another that localized to amino acids 161–180 expressed from exon 6. These findings support the hypothesis that CD8+ and CD4+ T cells were primed by intramuscular gene transfer of the therapeutic mini-dystrophin cassette with expression into the domain of the patient's

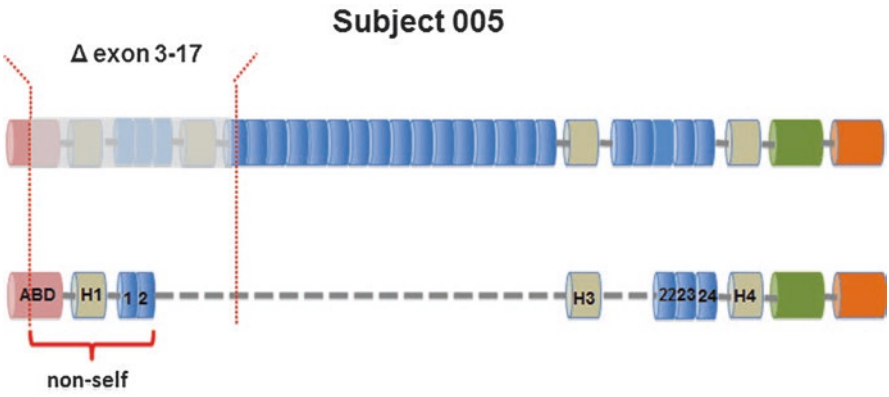

Fig. 41.3 In subject 5 with exon 3–17 deletion of the *DMD* gene, the actin-binding domain (ABD), H1, and repeats 1 and 2 express directly into the patient's gene deletion. We were able to identify a highly specific T-cell response using an IFN-γ ELISPOT assay. CD8+ T cells targeted an epitope in exon 8 [amino acids (aa) 181–200], and there were CD4+ cells targeting epitopes in exon 6 (aa 161–180) and exon 8 (aa 221–240)

deletion. The inflammatory response would likely have invaded and cleared any muscle cells expressing the transgene product.

We have incorporated the lesson from this observation into the planning for forthcoming clinical trials. In upcoming DMD trials, we will exclude patients with DMD gene deletions directly in the path of an expressed domain of the miniature dystrophin cDNA. Studies like this will be necessary to further our chances for success as we advance our efforts in gene delivery for DMD. Success using this strategy can be followed by more inclusive protocols to establish the full range of patients amenable to mini- or micro-dystrophin gene delivery for DMD.

The second path resulting in cellular immunity that we observed in this initial DMD gene therapy trial is also of interest. At day 15 post-gene delivery, patient 2 (exon 50 deletion) exhibited a rapid rise in cellular immune response to MDP2 (Fig. 41.4). The pre-gene delivery interferon-γ ELISPOT assay showed no pre-existing immunity to mini-dystrophin (<50 spot-forming cells (SPCs). Muscle biopsy post-gene transfer showed a small cluster of revertant myofibers [35] best explained by either spontaneous exon skipping or a second-site mutation that restores the reading frame in a patient with a frameshift mutation in exon 50 (Fig. 41.4b). As a general principle, the dystrophin expressed by revertant fibers is assumed to provide tolerance for DMD patients undergoing gene restoration or replacement. Observations in this trial do not support that hypothesis. In the post-gene transfer muscle biopsy from this patient, dystrophin expression initiates in exon 57 (Fig. 41.4) as evidenced by monoclonal antibody staining (antibodies provided by

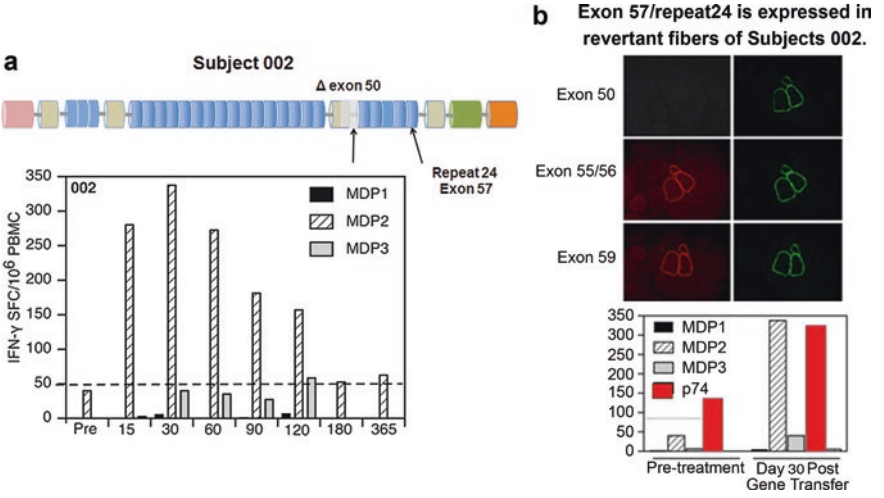

Fig. 41.4 (**a**) Patient 2 with exon an 50 deletion (bottom graph) exhibited a rapid rise in cellular immune response to an epitope specific to MDP2 not apparent in pretesting. This immune response targeted an epitope in exon 57 in repeat 24. (**b**) Revertant fiber cluster is shown in upper panel that expresses exon 57 (repeat 24) using monoclonal exon-specific antibodies. The ELISPOT graph (below) shows that T cells from a highly specific epitope in MPD2 target a 20 aa sequence (labeled p74) that accelerated the cellular immune response following delivery of mini-dystrophin (From Mendell et al. N Engl J Med 2010;363:1429–37)

Glen E Morris) [36]. The immunohistochemical profile favored that exon 57 encoded a highly specific 20 amino acid peptide sequence exhibiting pre-existing immunity that accelerated the cellular immune response following delivery of the therapeutic mini-dystrophin.

In summary, there are several lessons to be learned from this mini-dystrophin gene therapy trial. From patient 5 in the mini-dystrophin trial, we learned that a very large deletion of the *DMD* gene spanning exons 3–17 establishes a potential path for an immune response related to transgene expression. For future trials, investigators need to consider a protocol design that will prevent expression of the transgene into the deleted region of the host mutation. In the case of patient 5 in this trial, it may have been the large size of the mutation that predisposed to a problem, but as we move forward caution would be the best approach even for small deletions in relation to transgene expression. The situation is somewhat more complicated for patient 2 given that the immune response targeted a 20 amino acid epitope in an exon that is expressed in a small cluster of revertant fibers that was not initially identified. This highlights multiple factors that must be considered for patient enrollment into gene therapy trials.

41.4 Lessons from the First In-Human Limb Girdle Muscular Dystrophy 2D (LGMD2D) Gene Therapy Clinical Trial

41.4.1 Study Design

The trial design is similar to what has been presented in the DMD trial [19] and for the most part will not be reiterated. Six LGMD2D patients with proven alpha-sarcoglycan (SGCA, α-SG) mutations were enrolled in this clinical trial [37, 38] (Table 41.2). This was a double blind, randomized controlled trial of rAAV1 containing the full-length human SGCA under control of the tMCK promoter (rAAV1.tMCK.hSGCA) (Fig. 41.5) injected into the extensor digitorum brevis (EDB) muscle (3.25×10^{11} vector genomes). Gene transfer was performed in the intensive care unit at Nationwide Children's Hospital. Approximately 4 h before gene transfer, subjects received a dose of intravenous methylprednisolone, 2 mg/kg (not to exceed 1 g total), and dosing was repeated at 24 and 48 h. Injection sides (vector vs placebo)

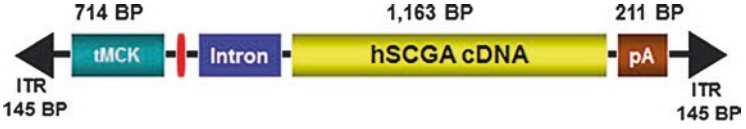

Fig. 41.5 The cassette used for gene transfer of the human alpha-sarcoglycan (hSGCA) cDNA shows a tMCK promoter with the addition of a Kozak consensus sequence (red) that plays a role in the initiation of translation and an intron to enhance gene expression

were determined by a computer-generated random number sequence. Gene delivery was guided by ultrasound and electromyography to ensure that muscle was the destination of the delivered product. EDB muscles were removed bilaterally from patients at varying times (Table 41.2).

41.4.2 Efficacy Evaluation

Efficacy was evaluated by blinded analyses assessing α-SG gene expression by immune stains to quantify the percentage of transduced muscle fibers and quantitative western blot analysis. In this first study of LGMD2D gene therapy, we deliberately studied gene expression by muscle biopsies post-gene delivery at intervals of 6 weeks (43 and 50 days), 12 weeks (92 days), and 6 months (176 and 183 days). There had been reports that overexpression of α-SG via AAV-mediated gene transfer resulted in poorly sustained gene expression related to transgene toxicity [39]. In our studies, we had not seen any signs of toxicity in any of the preclinical experiments or in the toxicology studies in preparation for the Investigational New Drug (IND) [40]. The objective of these multiple time-point muscle biopsy studies for patients enrolled in this trial was to determine if early gene expression would persist throughout the trial. Figure 41.6 shows unequivocal differentiation between the side of gene transfer and placebo-injected EDB with sustained expression at 6 months (also positive at ~6 and 12 weeks, not shown). In the biopsies from 6 to 26 weeks, there was no loss of α-SG staining. There was robust staining on only one side in each case that was easily distinguishable from low level or background α-SG gene expression observed on the control side. A further sign of gene restoration was illustrated by the documentation of full sarcoglycan complex staining including β-, γ-, and δ-sarcoglycan on the side of gene transfer. In addition, a vector-specific primer probe set permitted amplification of a unique 5' untranslated leader sequence of the α-SG cassette that differentiated transgene from endogenous alpha-sarcoglycan gene expression.

The single subject that departed from the findings of the first five receiving gene transfer was patient 6, the final patient enrolled in this study. In this case, the 6-month EDB muscle biopsies showed low-level gene expression on both sides compatible with biallelic missense mutations, and the side of gene transfer (left) could not be differentiated by western blots (Fig. 41.6). There was also a very striking paucity of transgene copy numbers per nucleus that was 3–30-fold lower compared to other patients in the trial. A one-time muscle biopsy makes it difficult to differentiate between loss of gene expression versus poor muscle transduction at the time of gene transfer. However, a distinctive feature in this muscle biopsy of patient 6 is the lack of expression of MHC I antigen on any muscle fiber in the biopsy. This is in direct contrast to the findings in all other patients in the trial. This patient also showed a well-defined and distinctive pattern of both early humoral and T-cell responses to AAV1 capsid. The IFN-γ ELISPOT assay demonstrated T-cell activation to AAV1 capsid as early as day 2 after gene transfer (Fig. 41.7). This is

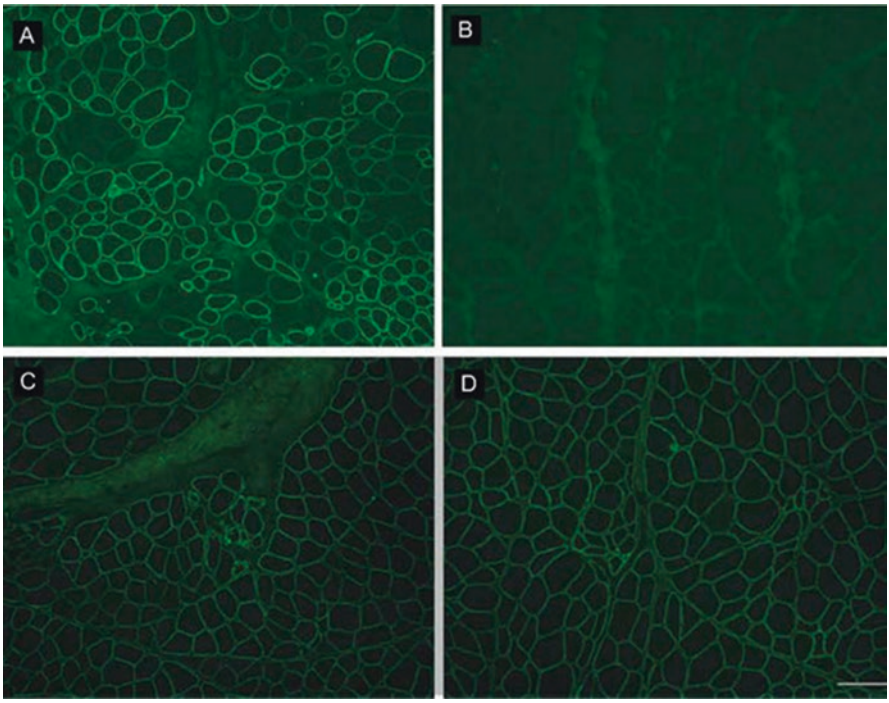

Fig. 41.6 Pre- and post-gene transfer muscle biopsies are shown on two patients in the LGMD2D clinical trial. (**a**) 6 months post-gene transfer with significant SGCA expression on individual muscle fibers compared to (**b**) pre-treatment from same patient showing all fibers negative for SGCA expression. (**c**) Post-gene transfer and (**d**) pre-gene transfer from same patient show no difference in SGCA expression. Partial expression of alpha-sarcoglycan related to patient's missense mutation (figures previously published: Mendell et al. Ann Neurol 2010;68:629–638)

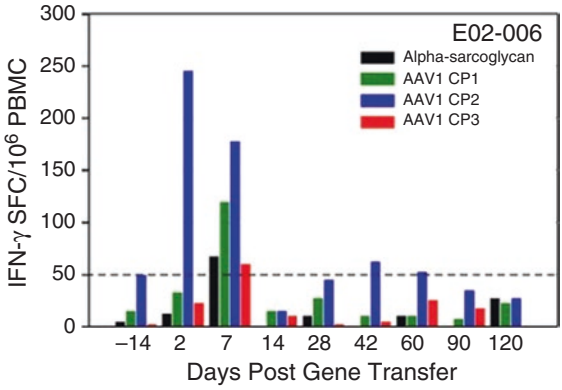

Fig. 41.7 Subject 6 (E02-006): AAV1 capsid stimulated IFN-γ ELISpot assays was negative prior to gene transfer but showed a prolific response as early as day 2 (AAV CP2) that was also present on day 7 with the addition of immune response to AAV CP1. This was distinctly earlier than other patients in this clinical trial (From Mendell et al. Ann Neurol 2010;68:629–638)

in clear contradistinction to every other patient undergoing gene transfer in this trial, where responses if present were not seen until day 14. Accompanying the AAV capsid-induced T-cell response in patient 6, we found a very rapid rise in AAV-neutralizing antibody titers reaching >30-fold higher compared to other cases. Collectively, these findings favor an amnestic response related to pre-existing immunity to AAV.

We attribute the lack of α-SG transgene expression and the early and exaggerated IFN-γ ELISPOT PBMC responses to AAV capsid to pre-existing immunity to AAV. The finding was twofold greater Nab titer compared to any other patient seen in the trial. The most revealing evidence of pre-existing immunity was the rise in Nabs at day 7 after gene transfer (baseline 1:1600 to >1:102,400 post-gene therapy). The Nab titer at this time point rose over 30-fold compared to other subjects in the trial.

The take home message from this LGMD2D clinical trial is that pre-existing antibodies to AAV capsid can preclude significant muscle fiber transduction. The exact titer resulting in poor outcome may vary between patients, but our results working with the Center for Biologics Evaluation and Research (CBER) provided a designated level for enrollment at no ≥1:50 to AAV, now typically used for enrollment in gene therapy trials employing AAV gene transfer. In the case under discussion, validation for the high antibody titer was demonstrated by binding antibody ratios against AAV1 that were >1000-fold higher compared to any other subject in the trial. The pre-existing AAV immunity predicted an amnestic response with early-onset humoral and T-cell immunity. It is also worth noting that the overall experience from this LGMD2D trial is different from our immunological findings related to the scenario observed in the DMD gene therapy trial [19]. In the DMD trial, we detected a T-cell response in the ELISPOT assay directed against an amino acid sequence present in the mini-dystrophin gene. This corresponded with a deleted region of the patient's endogenous dystrophin gene. This situation is unlikely to be encountered in LGMD2D, an autosomal recessive disease requiring mutations on both alleles to produce a clinical phenotype. Because missense mutations predominate in LGMD, even a heterozygous deletion mutation at one allele is not likely to predispose to an immune response from transgene expression. In addition, novel immunogenic epitopes on revertant muscle fibers might be encountered in DMD but a parallel scenario would be unlikely in LGMD2D. One final point based on this clinical experience is that we did not see any clinical manifestations of the immune responses in either DMD or LGMD2D gene therapy clinical trials. There was no evidence of a systemic reaction manifesting in fever, organ system involvement, or lymphadenopathy that we could be detected clinically or in clinical laboratory findings. This provides a degree of assurance for the safety of AAV gene delivery for future trials.

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Chapter 42
Duchenne Muscular Dystrophy Exon-Skipping Trials

Jerry R. Mendell, Zarife Sahenk, and Louise R. Rodino-Klapac

Abstract This chapter demonstrates an alternative mode of molecular therapy with the goal of restoring the *DMD* gene to achieve clinical improvement using antisense oligonucleotides (AONs). Preclinical studies were successful in mdx mice showing persistent production of dystrophin at significant levels in large numbers of muscle fibers. These experimental studies led to the clinical introduction of a 2′-*O*-methyl-phosphorothioate oligonucleotide (2'OMePS) and a phosphorodiamidate morpholino oligonucleotide (PMO) to induce skipping of one or more exons, restoring the reading frame and allowing for the production of a BMD-like dystrophin. This chapter reviews the clinical trial experience, the side effect profiles, and the basis for FDA approval of the PMO product, introduced as eteplirsen. Following approval the product is identified as Exondys 51®. The results of the long-term, 36-month clinical trial are described. The findings included a change in the rate of decline and prolonged ambulation in DMD boys compared to natural history controls. Exondys 51™ represents the first drug other than deflazacort (Emflaza®) ever approved for DMD.

Keywords Exon skipping · Duchenne muscular dystrophy · Antisense oligonucleotides · Phosphorothioate · Phosphorodiamidate · Morpholino

J. R. Mendell (✉) · Z. Sahenk
Department of Pediatrics, Nationwide Children's Hospital and Research Institute, The Ohio State University, Columbus, OH, USA

Department of Neurology, Nationwide Children's Hospital and Research Institute, The Ohio State University, Columbus, OH, USA
e-mail: Jerry.Mendell@nationwidechildrens.org; Zarife.Sahenk@nationwidechildrens.org

L. R. Rodino-Klapac
Department of Pediatrics, Nationwide Children's Hospital and Research Institute, The Ohio State University, Columbus, OH, USA

Vice President Gene Therapy, Sarepta Therapeutics, Columbus, OH, USA
e-mail: LRodinoKlapac@Sarepta.com

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42.1 Background

Kunkel and colleagues cloned the gene for Duchenne muscular dystrophy (DMD) in 1986 [1]. Its DNA sequence is encoded by 79 exons scattered along two and a half million base pairs of DNA. In DMD patients, a mutation shifts the reading frame so that the message cannot be read, and this abolishes the production of dystrophin. An allelic milder form of the disease is caused by in-frame mutations permitting production of a truncated dystrophin leading to Becker muscular dystrophy (BMD). In DMD, gene restoration can be achieved using antisense oligonucleotides (AONs) that can induce skipping of one or more exons, restoring the reading frame and allowing for the production of a BMD-like dystrophin. In a proof-of-principle study in 1999, Wilton demonstrated that the nonsense mutation in exon 23 of the mdx mouse could be removed from myoblast cultures by antisense 2′-*O*-methyl-oligonucleotides (2′OMeAO) targeting pre-mRNA [2]. Within 24 h the majority of transcripts showed skipping of the mutated exon allowing for the production of truncated protein. Subsequently, in vivo proof-of-concept studies were performed in mdx mice again using the 2′OMeAO. The treated mice showed persistent production of dystrophin at significant levels in large numbers of muscle fibers. Repeated administration enhanced dystrophin expression without eliciting an immune response [3]. The feasibility for translational studies was apparent demonstrating that skipping a single exon can induce a modified, smaller dystrophin without inducing an immune response. The foundation established in these proof-of-concept studies has provided the rationale for moving to clinical trials using exon skipping to upregulate dystrophin as a tool with intention to change the natural history of DMD.

42.2 Introducing Exon Skipping to the Clinic

The first exon-skipping studies in boys with DMD have targeted exon 51 (Fig. 42.1). The particular target was intended to skip an exon with potential for impacting the largest subgroup of DMD patients (approximately 13%), including those with deletions of exons 45–50, 48–50, 50 and 52 [4]. *Drisapersen* (introduced as PRO051 by Prosensa, later licensed to BioMarin under the commercial name Kyndrisa®) is a 2′-*O*-methyl-phosphorothioate oligonucleotide (2′OMePS) (Fig. 42.2). Common problems that have emerged in clinical trials are attributed to the negative charges on the inter-subunit linker permitting binding to renal tubular epithelium leading to proteinuria [5, 6]. In addition, binding immune cell receptors potentially activating Toll-like receptors (TLRs) may account for the localized inflammatory injection-site reactions seen frequently with drisapersen [7]. *Eteplirsen*, developed by AVI BioPharma (company name changed to Sarepta Therapeutics in July 2012), has a morpholino backbone (PMO) and a charge-neutral phosphorodiamidate linker (Fig. 42.2). In clinical trials this has proven safer and associated with a minimal side effect profile. Drisapersen and eteplirsen were introduced into the clinic at about the same time. The products had a clear distinction in their path to commercialization. Each will be discussed separately below.

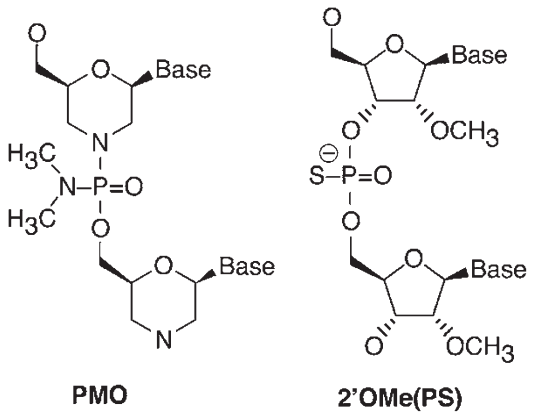

Fig. 42.1 Example of exon skipping using eteplirsen (Exondys 51) for treatment of exons 49–50 DMD gene deletion (X). Upper panel shows frameshift mutation preventing dystrophin translation. In lower panel skipping exon 51 puts the *DMD* gene in frame and permits translation of a smaller dystrophin without expression of exon 51 and exons 49–50. Other exons are highlighted that permit skipping exon 51 with in-frame restoration including exons 47–50 and 48–50. Other combinations also permit skipping exon 51 with restoration of frame (45–50, 50, 52)

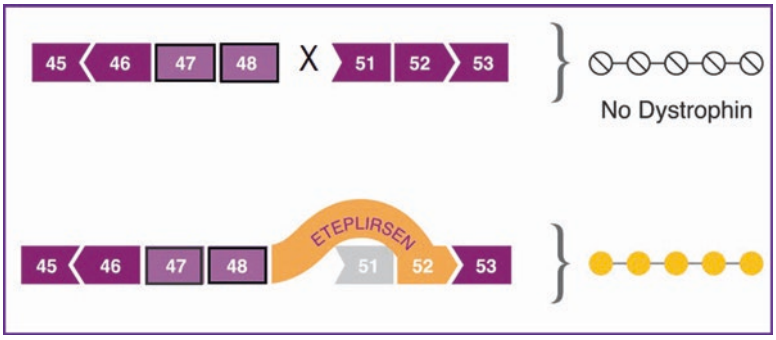

Fig. 42.2 Phosphorodiamidate morpholino oligomer (PMO) compared to 2'OMe(PS), a phosphorothioate oligonucleotide. The PMO linker is charge-neutral, while the 2'OMe(PS) has negative charges on the inter-subunit linker permitting binding to the renal epithelium leading to proteinuria

42.3 Drisapersen Meets Resistance for FDA Approval

PRO051 with its phosphorothioate linkage entered the clinic as a product for intramuscular injection. It was injected into the tibialis anterior muscle in four DMD patients [7]. The initial studies looked promising on muscle biopsy performed 28 days after direct muscle injection. Each patient showed precise skipping of exon 51 confirmed by sequencing. Sarcolemmal dystrophin was seen in more than 50% of muscle fibers. The amount of dystrophin in total protein extracts ranged from 3 to 12% of controls, and a quantitative ratio of dystrophin to laminin α2 showed

17–35% of that of control specimens. The injections were safe with few adverse events. No functional improvement in muscle strength was observed in the tibialis anterior muscle.

In follow-up to the promising results of intramuscular injection, PRO051 was advanced to a systemically administered Phase I/IIa (uncontrolled, safety and efficacy trial) dose-ranging study [8]. Weekly abdominal subcutaneous injections of PRO051 were administered, and after 12 weeks, at doses of 2.0–6.0 mg/kg, detectable exon 51 skipping-induced expression of dystrophin was seen in approximately 60–100% of muscle fibers in 10 of the 12 patients. Expression increased in a dose-dependent manner up to 15.6% of normal expression. Functionally there was a mean improvement of 35.2 ± 28.7 m (from the baseline of 384 ± 121 m) on the 6-min walk test (6MWT). Again the suggestion of a favorable exon-skipping product encouraged investigators to move to a 48-week double-blind, placebo-controlled, multicenter trial study done between Sept 1, 2010, and Sept 12, 2012 [9]. Drisapersen was given subcutaneously at 6 mg/kg. Recruitment included 53 patients: 18 were given once-weekly injections, 17 received intermittent drisapersen (9 doses over 10 weeks), and 18 were given placebo (participants received either continuous or intermittent). At week 25, mean distance on the 6MWT had increased by 31.5 ± 9.8 m from baseline for continuous drisapersen, with a mean difference in change from baseline of 35.09 m (95% confidence interval (CI) 7.59–62.60; $p = 0{\cdot}014$) versus placebo. As the studies extended beyond the 25th week, the difference between treated and placebo cohorts was less apparent and no longer statistically significant ($p = 0.051$) at week 49.

Adverse events in this trial (and in others administering subcutaneous drisapersen injections for systemic distribution) included injection-site reactions with varying time for skin recovery and in some cases sclerotic/fibrotic changes in the skin or skin fragility. Mild proteinuria was common, and raised urinary α1-microglobulin levels were seen. Occasional patients had elevation of cystatin C with abnormalities reversed during drug-free periods, which may indicate mild reversible interference with protein reabsorption in the proximal tubule. Rarely reduced platelet counts were encountered.

In addition to the 48-week Phase II [9], GlaxoSmithKline/Prosensa sponsored a Phase III, pivotal, placebo-controlled trial of 186 patients [10]. In this study, DMD boys were randomized to either drisapersen at a dose of 6 mg/kg/week ($n = 125$) or to placebo ($n = 61$) for 48 weeks. On September 20, 2013, it was announced that the study failed to achieve statistical significance in its primary endpoint, the 6MWT [10]. Following the disappointing results, BioMarin purchased and licensed the product with the intent to move forward with Phase III testing of drisapersen (referred to as Kyndrisa™). On January 7, 2016, the FDA, composed of a panel of outside advisors, voted that there was no conclusive benefit from Kyndrisa, and a few months later, BioMarin announced on May 31, 2016, that it was withdrawing Kyndrisa™ from clinical testing (https://www.insidertracking.com/biomarin-announces-withdrawal-of-market-authorization-application-for-kyndrisa-drisapersen-in-europe).

Any question about higher dosing of drisapersen to achieve greater benefit was addressed in a study assessing safety, tolerability, and pharmacokinetics after a single subcutaneous dose-ranging study (3–9 mg/kg) was completed in non-ambulatory DMD boys [11]. It was already known that antisense oligonucleotides with phosphorothioate linkage had potential adverse effects that fall into four main categories: inflammation, thrombocytopenia, accumulation in the kidneys and liver, and increases in activated partial thromboplastin time (aPTT). In this single administration trial, drisapersen at 3 and 6 mg/kg did not result in significant safety or tolerability concerns; however, at the 9 mg/kg dose, pyrexia and transient elevations in inflammatory parameters were seen. All subjects in this high-dose group experienced adverse effects (AEs) consisting of pyrexia and concomitant signs of inflammation with increases in C-reactive protein (CRP), monocyte chemoattractant protein-1 (MCP), and IL-6. These effects were time-limited and self-resolving, without any further clinical sequelae. Based on these studies, it was concluded that the maximum tolerated dose of drisapersen, 6 mg/kg, had been reached without efficacy in the DMD population.

The news that drisapersen (Kyndrisa™) was not approved by the FDA was not well received in the DMD community nor the foundations that supported exon skipping as a therapeutic strategy for this disease. CureDuchenne, the California-based nonprofit organization dedicated to finding cures for Duchenne muscular dystrophy, released a statement following the decision by the Food and Drug Administration. "We are disappointed that the FDA did not approve drisapersen, given the significant benefit that many Duchenne boys experienced when they were on an early and consistent treatment protocol of the drug." Valerie Cwik, President and Chief Medical and Scientific Officer at MDA at that time, also expressed frustration and disappointment on behalf of MDA families and study participants hoping for an effective treatment. "With more treatments staged to begin……, we remain optimistic that many of those living with DMD today will have safe and effective therapy options in the very near future."

Shortly after the disappointing experience of BioMarin in January 2016, Sarepta Therapeutics, the sponsor of the PMO eteplirsen (now known as Exondys 51®), was granted approval for use of this drug for DMD boys with confirmed mutations of the dystrophin gene amenable to skipping exon 51. This was a major milestone given that the only drug approved for DMD by the FDA was deflazacort.

42.4 Clinical Trial with Eteplirsen

42.4.1 Intramuscular Injection Trial of Eteplirsen

Eteplirsen was initially introduced for clinical trial by AVI BioPharma under agreement with the University of Western Australia. The PMO used for the trial was referred to as AVI-4658 (later eteplirsen) and injected directly into the extensor digitorum brevis in five DMD subjects [Phase I/II trial (NCT00159250)] [12].

Immunostaining revealed that the mean intensity of dystrophin expression in randomly chosen sections of eteplirsen-treated muscles was 22–32% of that seen in healthy muscles and 17% ($p = 0.002$) greater than in the saline-treated contralateral muscles. When only dystrophin-positive fibers were assessed, the intensity in the eteplirsen-treated muscles reached 42% of that in healthy muscle. The findings were confirmed in western blot and validated with co-localization of components of the dystrophin glycoprotein complex.

42.4.2 Systemic Delivery Clinical Trials with Eteplirsen

42.4.2.1 Great Ormond Street Hospital for Children

Following the direct intramuscular trial, an open-label, Phase II dose-escalation study of systemically delivered, intravenous (IV) eteplirsen was conducted in ambulatory DMD boys ages 5–15 ($n = 19$) [13]. This was a dose-ascending trial (0.5, 1.0, 2.0, 4.0, 10.0, and 20.0 mg/kg) that was done at the Dubowitz Neuromuscular Centre at Great Ormond Street Hospital for Children, London, UK. Participants had a muscle biopsy before starting treatment. DMD patients were ambulatory, age 5–15 years, and had mutations amenable to skipping exon 51. Nineteen patients received 12 weekly intravenous infusions of AVI-4658. The PMO was well tolerated with no drug-related serious adverse events. AVI-4658 induced exon 51 skipping in all cohorts, and new dystrophin protein expression was seen in a dose-dependent ($p = 0.0203$), but variable, manner in boys from dosing 2 mg/kg onward. Seven patients responded to treatment, demonstrating increased dystrophin fluorescence intensity from 8.9% (95% CI 7.1–10.6) to 16.4% (95% CI 10.8–22.0) of normal ($p = 0.0287$). The three patients with the greatest responses to treatment had 21%, 15%, and 55% dystrophin-positive fibers, and these findings were confirmed with western blot, which showed an increase after treatment of protein levels from 2% to 18%, from 0.9% to 17%, and from 0% to 7.7% of normal muscle, respectively. The functional properties of restored dystrophin were confirmed by quantification of α-sarcoglycan and neuronal nitric oxide synthase (nNOS) expression. Dystrophin-positive fibers had roughly a 30% average increase in α-sarcoglycan expression compared with dystrophin-negative fibers in the patient with the best response (deletions 49–50). Dystrophin upregulation was followed by restoration of nNOS at the sarcolemma, more so in patients with exon 49–50 deletions than in those with 45–50 deletions, which is consistent with the observation that the nNOS-binding domain is located in dystrophin exons 42–45 [14, 15]. Muscle biopsies from patients receiving the 10 or 20 mg/kg dosages showed a reduction in inflammatory infiltrate. DMD boys remained stable in this study, but considering the short period of observation (12 weeks), assessment of clinical outcome measures was limited.

42.4.2.2 Nationwide Children's Hospital (NCH) Study in Year 1

In the next 48-week phase of testing, eteplirsen clinical trials moved to NCH, Columbus, OH [16]. Sarepta Therapeutics acquired the exclusive license from AVI BioPharma to complete clinical studies with eteplirsen establishing a path for potential commercialization. Concomitant with Sarepta's leadership, the name eteplirsen became firmly associated with this PMO. A double-blind placebo-controlled protocol was introduced for further evaluation of treatment-induced dystrophin expression and possible effects on distance walked on the 6MWD. The next several years were devoted to clinical trials testing the efficacy of this exon-skipping agent (Fig. 42.3).

The trial at NCH (Eteplirsen 201 sponsored by Sarepta Therapeutics) began with enrollment of the first patient on July 18, 2011 [16]. A total of 12 DMD boys aged 7–13 years with confirmed out-of-frame DMD deletions potentially correctable by skipping exon 51 (representing 5 different out-of-frame deletions) were included (Table 42.1). All were on stable glucocorticoids (prednisone or deflazacort) for ≥24 weeks. Eteplirsen was introduced at NCH as a randomized, double-blind, placebo-controlled study consisting of three cohorts (placebo, 30 mg/kg/weeks, 50 mg/kg/weeks) (Study 201). After 24 weeks of double-blind dosing, the placebo-treated patients were randomized 1:1 to weekly 30 mg ($n = 2$) or 50 mg/kg ($n = 2$) eteplirsen

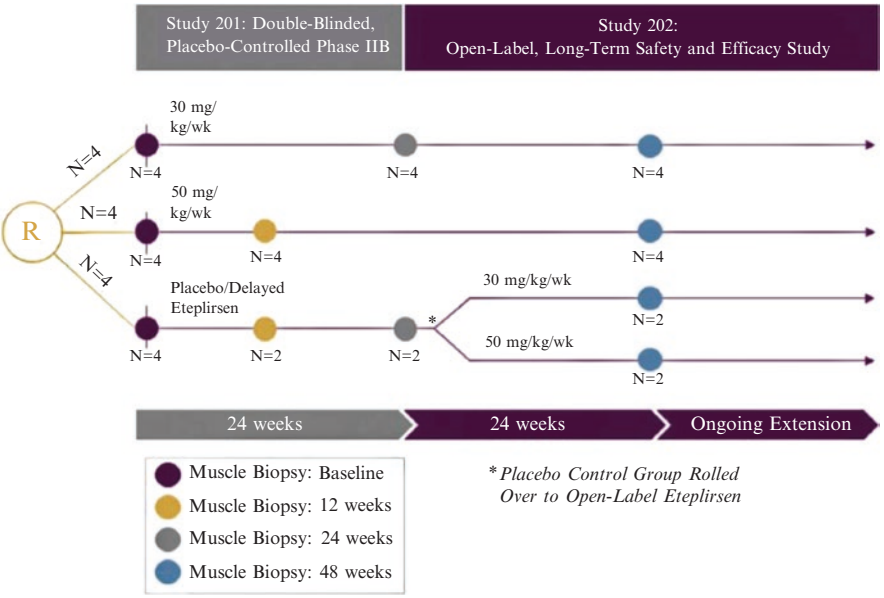

Fig. 42.3 Design of exon-skipping study inclusive of 168 weeks (3 years). Twelve subjects with mutations amenable to skipping exon 51 were enrolled into 3 cohorts receiving placebo vs eteplirsen 50 mg/kg and 30 mg/kg (blinded study). All patients had biceps muscle biopsies at baseline. Second muscle biopsies (opposite biceps) were done at week 12 (50 mg/kg cohort and two placebo-treated) and week 24 (30 mg/kg cohort and two placebo-treated patients). At week 48 a fourth muscle biopsy was on the left deltoid in all 12 subjects. Functional clinical assessments including the 6-min walk test and pulmonary function tests were performed at weeks shown on the time axis (figure reproduced from Mendell et al. Ann Neurol 2016; 79:257–271)

Table 42.1 Comparative groups eteplirsen-treated and historical controls

Group	Exon mutations each group	Age baseline Mean SD	6MWT baseline Mean SD
Historical controls amenable to exon 51 skipping, ≥7 years old	$n = 13$ Ex 45–50 $n = 3$ Ex 48–50 $n = 2$ Ex 49–50 $n = 3$ Ex 50 $n = 2$ Ex 52 $n = 3$	9.45 (1.454)	357.6 (66.75)
Eteplirsen-treated (ITT)	$n = 12$ Ex 45–50 $n = 3$ Ex 48–50 $n = 1$ Ex 49–50 $n = 5$ Ex 50 $n = 1$ Ex 52 $n = 2$	9.41 (1.183)	363.2 (42.19)

narrowing the study to two groups taking 30 mg/kg/weeks or 50 mg/kg/weeks (Study 202). The patients originally on placebo for the first 24 weeks were now labeled "Placebo-delayed." Because eteplirsen required weekly intravenous dosing and patients were traveling far distances to receive the IV dosing at NCH for the initial 24-week study, 10 additional research sites were asked to participate in an open-label extension of eteplirsen at 30 mg/kg/weeks or 50 mg/kg/weeks The evaluation of dystrophin expression by muscle biopsy and the functional motor assessments including the 6MWT were performed for all study participants by the staff at NCH.

For the initial 48-week study, dystrophin expression was the primary outcome measure. The evaluation of dystrophin included three muscle biopsies. At baseline a biceps muscle (the side randomly chosen) provided initial counts of dystrophin-positive fibers. At week 12, a second biopsy from the opposite biceps was collected from patients taking 50 mg/kg along with two placebo-treated patients and at week 24 patients taking 30 mg/kg/weeks, and the other two placebo-treated patients were sampled. To evaluate the effect of continued exposure to eteplirsen on dystrophin production, a third biopsy was done on the left deltoid in all 12 patients at week 48. The biopsies were read blindly by an experienced muscle pathologist without knowledge of trial assignment as to the cohorts: eteplirsen 50 mg/kg/weeks, 30 mg/kg/weeks, and placebo-controlled.

The first muscle biopsies were done after treatment with 50 mg/kg for 12 weeks. There was no increase in percentage of dystrophin-positive fibers compared to pre-treatment, and the change from baseline (mean = 0.8%, range = −9.3 to 7.4%) was not statistically different compared to the placebo cohorts. At week 24 (12 additional weeks of treatment with eteplirsen), the 30 mg/kg dose resulted in a 22.9% (range = 15.9–29.0%) increase in dystrophin-positive fibers from baseline ($p \leq 0.002$) compared to the placebo-treated patients. These data suggest that at least 24 weeks of treatment with eteplirsen is needed to produce definite increases in dystrophin production in muscle biopsies. The baseline vs week 24 comparison of the percentage of dystrophin-positive fibers for the 30 mg/kg cohort was significantly different ($p \leq 0.004$). At week 48 the 30 and 50 mg/kg cohorts showed significant increases ($p \leq 0.001$) in percentage of dystrophin-positive fibers (mean = 47.3%, range = 29.8–

60.3%). The four patients in the placebo-delayed cohort taking 30 mg/kg ($n = 2$) and 50 mg/kg ($n = 2$) of eteplirsen also showed significant increases ($p \leq 0.008$, mean = 37.7%, range = 28.4–55.1%). In agreement with these findings, eteplirsen significantly increased mean fluorescence signal intensity of muscle fibers (Bioquant analysis) expressing MANDYS106 (a monoclonal antibody that recognizes exon 43-encoded region in dystrophin) at week 48 compared to baseline in patients receiving 30 mg/kg ($p \leq 0.023$) and 50 mg/kg ($p \leq 0.005$) and in the placebo-delayed ($p \leq 0.001$). Confirmatory to these findings was the restoration of sarcolemma nNOS and β- and γ-sarcoglycan. Dystrophin expression and exon skipping were confirmed by reverse transcription polymerase chain reaction RT-PCR and western blot.

42.4.2.3 Motor Function in Year 1 NCH Study

The initial 24 weeks (6 months) of the trial assessed the 6MWT comparing treated to placebo (Fig. 42.4). Two patients lost ambulation prior to the 6-month time point. The data is of interest providing insight for understanding the time for induction of dystrophin expression extrapolating from 6MWD for the ambulatory patients in the trial. This required a modified intent-to-treat analysis excluding the two boys who stopped walking by 6 months. Figure 42.4 shows that both the placebo controls and eteplirsen-treated were indistinguishable for the first 12 weeks before diverging. The placebo-treated then lost 20 m from baseline distance on the 6MWT. At 6 months this cohort rolled over to

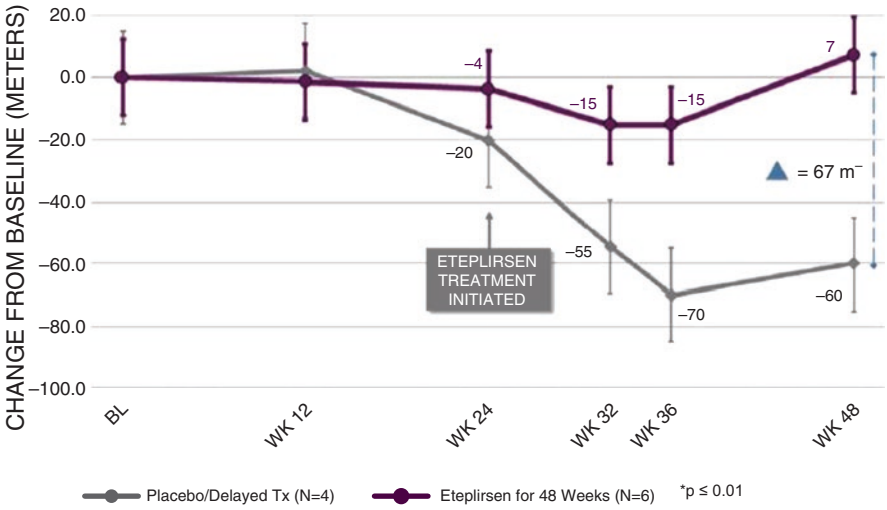

Fig. 42.4 Functional efficacy of eteplirsen at year 1 for ambulatory subjects. The dark purple line shows minimal change from baseline in distance walked on the 6-min walk test (6MWT) throughout year 1 for 6 patients receiving eteplirsen from the start of study. The dark gray line shows change from baseline in distance walked on the 6MWT for the 4 patients who received placebo for the first 24 weeks and then started eteplirsen at week 25 and continued through 48. Dystrophin expression studies and multiple biopsies support that dystrophin was produced after 12 weeks. The placebo-delayed cohort stabilized in function after week 36 (figure reproduced from Mendell et al. Ann Neurol 2013; 74:637–647)

open-label treatment and continued to decline for another 12 weeks. At that point they showed an improvement of 10 m. In contrast the eteplirsen-treated essentially maintained their plateau for the entire first year (48 weeks) of the trial. From the eteplirsen-treated, we learn that the effect of treatment takes approximately 12 weeks before a minimal efficacy is observed implying that dystrophin production will reach expression levels sufficient to maintain function. The course of the placebo-treated suggests confirmation of this conclusion. After week 12, they continue to decline, and then after starting on eteplirsen at week 24, it then takes another 12 weeks before their course is reversed at week 36 after which the 6MWD maintains a plateau for the remainder of the first year study (out to week 48). The first year of the eteplirsen trial predicted the outcome for the next 4 years showing efficacy compared to natural history controls [17].

42.4.2.4 NCH 36-Month Longitudinal Study

Following the first year of therapy, the eteplirsen study continued as an open-label clinical trial ending at week 168 (Fig. 42.3). The candidates initially enrolled ($n = 12$) continued for this entire period without dropouts and included the 2 boys who lost ambulation during the first 6 months of the trial (Table 42.1). The ability of eteplirsen to confer clinical benefit was primarily evaluated by the 6MWD with the addition of pulmonary functions including maximum inspiratory pressure (MIP), maximum expiratory pressure (MEP), and forced vital capacity (FVC) [18, 19]. For all analyses, the data for the placebo patients ($n = 4$) was incorporated into the overall data set based on duration of time that drug was given to these patients from week 48 to week 168.

Historical control data ($n = 116$) were provided by Eugenio Mercuri on behalf of the Italian DMD Registry database with contributions from 11 neuromuscular centers in Italy [20] and from the Belgium Registry (Leuven's Neuromuscular Reference Centre) provided by Nathalie Goemans [20, 21]. Historical control data complied with the eligibility criteria inclusive of the key parameters enabling a statistical comparison to eteplirsen-treated patients: (1) age-matched, (2) corticosteroid use, and (3) genotype amenable to skipping exon 51. Of the 116 patients identified in Italy and Belgium, 91 were ≥7 years old, 50 patients were amenable to exon skipping, and the final group fulfilling criteria included 13 subjects amenable to exon 51 skipping (Table 42.1).

The importance of the controls representing a cohort ≥7 and the same spectrum of mutations cannot be over emphasized. Consistent with previous reports, patients <7 years of age showed improvement over the first 2 years of observation, followed by a decline between months 24 and 36 [20, 21] (Fig. 42.5a). In contrast, the older

Fig. 42.5 (continued) amenable to exon skipping compared to those not amenable to exon skipping. Disease progression trajectory is compared in steroid-treated patients ≥7 years of age with genotypes amenable or not amenable to exon-skipping therapy. (**c**) 6MWT performance declines more rapidly in patients amenable to exon 51 skipping versus patients amenable to skipping other exons. Disease progression trajectory is shown in steroid-treated patients ≥7 years of age with genotypes amenable to exon 51 skipping or amenable to skipping other exons (figure reproduced from Mendell et al. Ann Neurol 2016; 79:257–271)

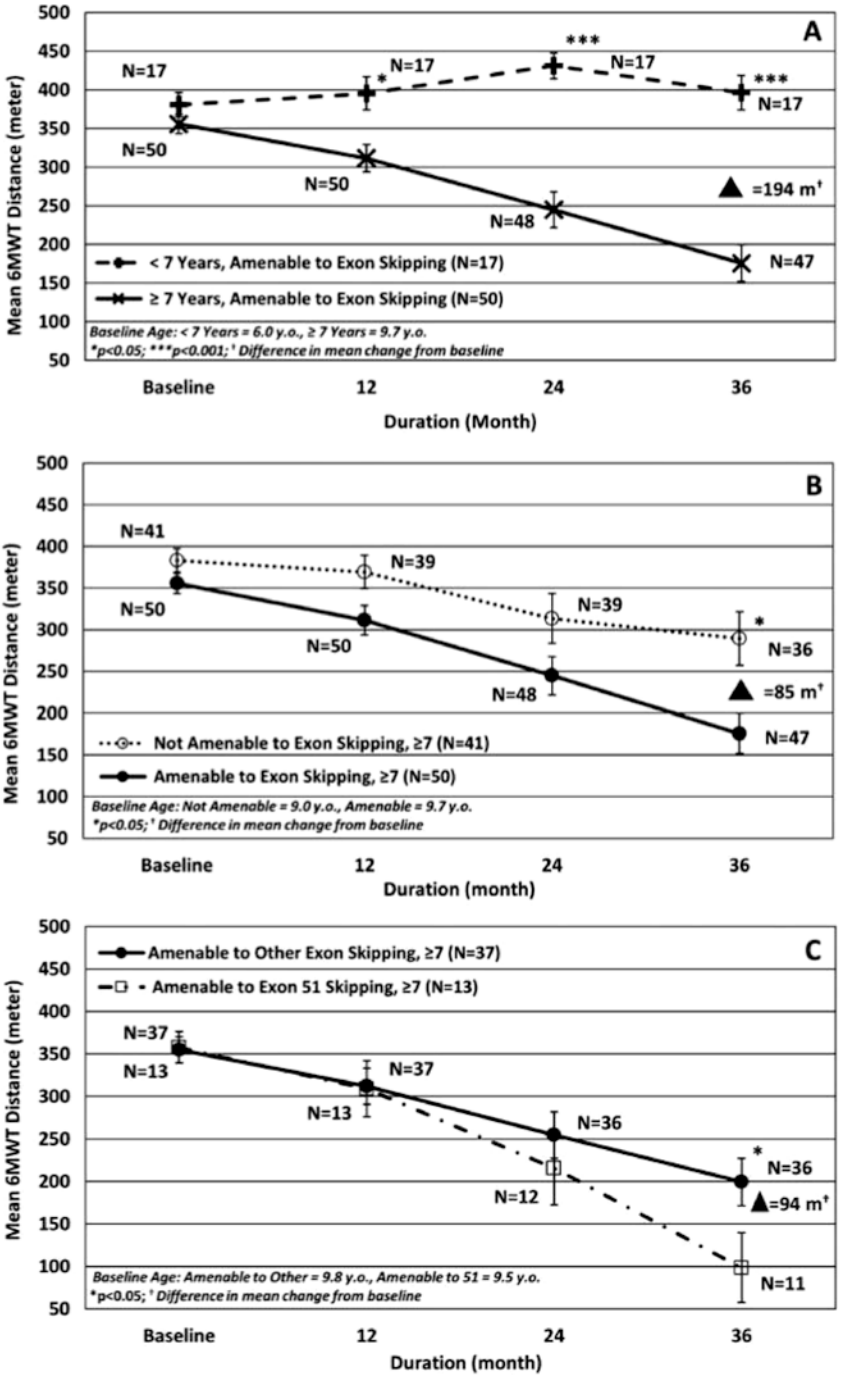

Fig. 42.5 (**a**) Disease progression trajectory is shown for steroid-treated Duchenne muscular dystrophy historical controls who were older and younger than 7 years and amenable to skipping any exon. (**b**) 6-min walk test (6MWT) performance declines more rapidly in patients with mutations

age group showed a disease progression trajectory characterized by greater declines at 24 and 36 months. Also of interest the comparison of patients ≥7 years old by genotype demonstrates that patients amenable to exon skipping experience more severe disease progression than those who are not (Fig. 42.5b). Further comparison of patients ≥7 years old by genotype demonstrates that patients amenable to exon 51 skipping experience even more rapid disease progression than patients amenable to skipping other exons. This difference is statistically significant by 36 months (Fig. 42.5c), with patients amenable to exon 51 skipping showing a 94 m greater decrease in 6MWT distance ($p < 0.05$).

42.4.2.5 Comparison of 6MWT in Eteplirsen-Treated Patients to Historical Controls

Evaluation of 6MWT results of eteplirsen-treated patients and historical control showed comparable baseline 6MWT distances that diverged through the second and third year, culminating in a 75 m difference in 6MWT decline between the groups by 24 months and a statistically significant ($p < 0.01$) and clinically meaningful [22] difference in 6MWT decline of 151 m between the groups by 36 months (Fig. 42.6). The proportion of patients who lost ambulation was also evaluated. Individual patient data provide detailed comparison of eteplirsen patients and historical controls (Fig. 42.7). Over a 3-year period, eteplirsen treatment markedly reduced loss of ambulation compared to matched historical controls. After 3 years, 2 of 12 (16.7%) eteplirsen-treated patients lost ambulation, compared with 6 of 13 (46.2%) historical control patients ≥7 years of age amenable to exon 51 skipping (Fig. 42.8).

Over 36 months of treatment, mean percentage of predicted MIP declined by 2.2% (from 91.7% at baseline to 89.5%), mean percentage of predicted MEP declined by 5.0% (79.3–74.3%), and mean percentage of predicted FVC declined by 9.4% (101.3–91.9%). Pulmonary function data from recent natural history studies in patients with DMD suggest that MEP and MIP decline at a rate of 4% per year for patients in the age range of 6–19 years, and FVC declines at a rate of 5% per year for patients in the age range of 5–24 years [18, 23].

42.4.2.6 Safety of Eteplirsen Treatment over 36 Months

Safety assessments included adverse event monitoring and clinical laboratory tests assessing possible organ system toxicity commonly observed with other oligonucleotide therapeutics such as inflammatory events, coagulopathies, and hepatic and renal toxicity. In the eteplirsen study, no adverse event led to treatment interruption

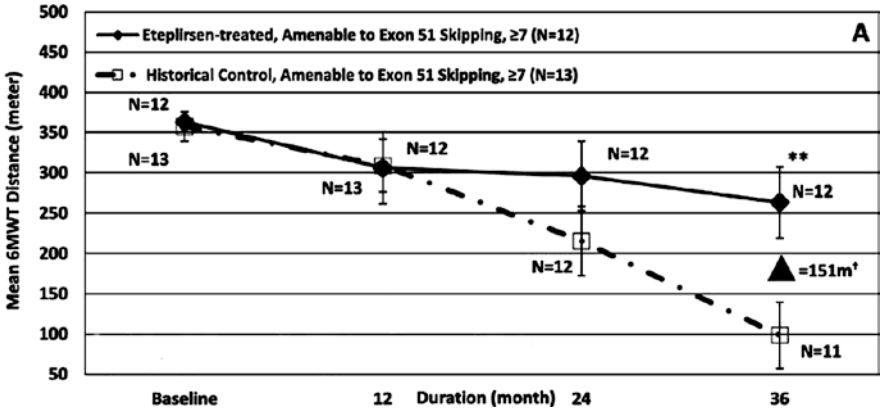

Fig. 42.6 Longitudinal 6-min walk distance (mean ± SE of the mean) and loss of ambulation over 3 years. (**a**) Eteplirsen-treated patients experience slower disease progression than matched historical controls. Disease progression trajectory is shown for steroid-treated historical controls, ≥7 years old amenable to exon 51 skipping ($n = 13$) and eteplirsen-treated patients ($n = 12$); Exon 51 amenable = 9.5 years old; eteplirsen-treated = 9.4 years old; †difference in mean change from baseline, $^{**}p < 0.01$. Table insert: the distance lost on the 6MWT was 151 m less over 3 years compared to historical controls age-matched and amenable to skipping exon 51 (figure reproduced from Mendell, et al. Ann Neurol 2016; 79:257–271)

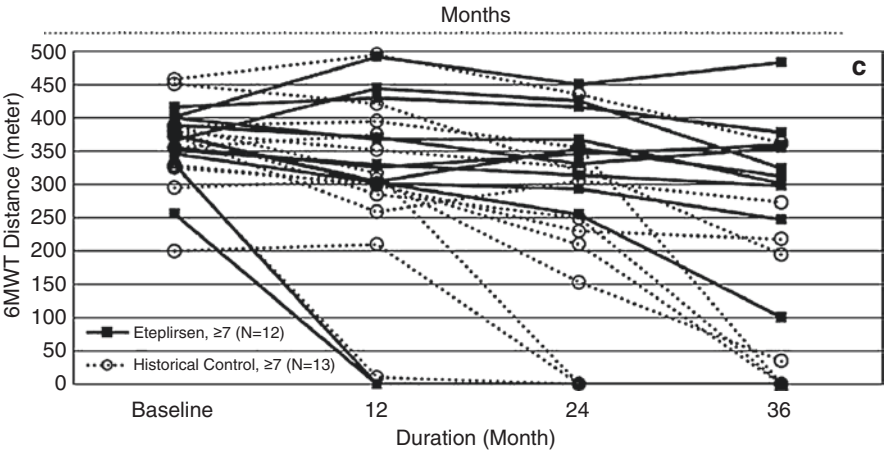

Fig. 42.7 Individual patient data is plotted and shows relative stabilization over time in eteplirsen-treated patients (solid lines) compared to matched historical controls (dotted lines) (figure reproduced from Mendell et al. Ann Neurol 2016; 79:257–271)

or dose change. Weekly eteplirsen infusions were well tolerated, with no reports of systemic reactions and no serious adverse events related to treatment. The most frequently reported adverse events on eteplirsen were headaches ($n = 8$), procedural pain related to biopsy and catheter placement ($n = 7$), and proteinuria ($n = 6$). A total of eight adverse events (seven while receiving eteplirsen, one on placebo) occurring

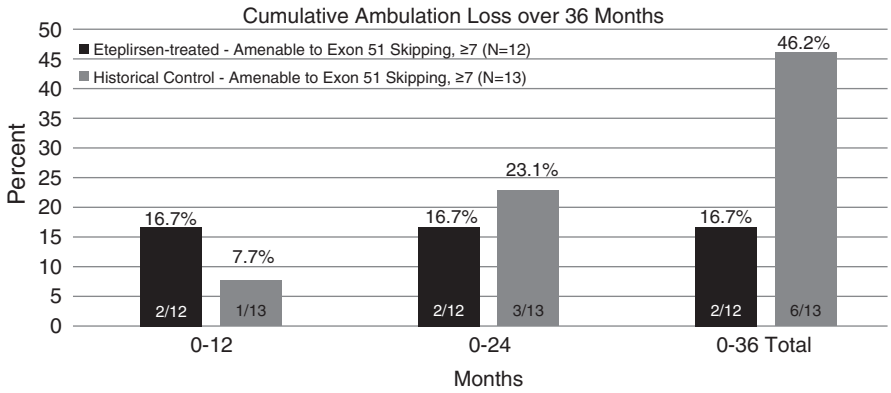

Fig. 42.8 Historical control populations experience greater loss of ambulation over a 36-month period (6 of 13, 46.2%) than eteplirsen-treated patients (2 of 12, 16.7%) (figure reproduced from Mendell et al. Ann Neurol 2016; 79:257–271)

in seven subjects were considered to be possibly or probably related to study drug. Treatment continued uninterrupted through these events, which involved two patients whose tunneled port catheters were observed to be thrombosed prior to study drug infusion; one placebo patient with mild nausea; one patient with two events of mild erythema (reddened cheeks); one patient with a previous history of low white blood cells whose counts fell to 3.70×10^9/l (lower limit of normal range = 4.00×10^9/l); and two patients with mild, transient proteinuria that resolved spontaneously. There were no signs of renal toxicity or elevation of cystatin C. Hematology and coagulation parameters were generally within normal range. Blood chemistry reflected the expected disease-related abnormalities, with markedly elevated creatine kinase, aspartate aminotransferase, and alanine aminotransferase, all of which decreased over the course of treatment with eteplirsen. There were no signs or symptoms of hepatic toxicity.

42.5 Conclusions

The major findings from this study were that eteplirsen-induced exon skipping prolonged ambulation and changed the rate of decline in the 6MWT compared to historical controls matched for age, mutations amenable to skipping exon 51, and baseline motor function (distance walked on 6MWT). The relative stability of respiratory muscle function over >3 years is supporting evidence of clinical efficacy for eteplirsen and may have contributed to the greater distance covered on the 6MWT. Two of 12 patients taking eteplirsen lost ambulation in the first 24 weeks of treatment (16.6%) vs 6 of 13 (46%) of historical controls. The final calculations of improved distance walked at 151 m for eteplirsen-treated vs historical controls included the data from these two unfortunate DMD boys losing ambulation early in the trial. The

early loss of ambulation of these two boys may have obscured differences in treatment effect until year 2 because of the small sample size. The small sample size of the 12 DMD boys participating in this study from the outset could also reasonably be considered the most significant limitation of the observations presented here. Eteplirsen was also found to increase the number of dystrophin-positive muscle fibers at both 30 mg/kg and 50 mg/kg. FDA representatives challenged this finding and suggested a recount of the percent increase of dystrophin-positive fibers. However, newly imposed criteria for positivity were used for the recount [24]. A closer look though revealed that the fold increase in percent of dystrophin-positive fibers was actually higher following the rescoring by the FDA despite a more conservative protocol reporting an overall fewer number of dystrophin-positive fibers than the values originally obtained [25].

It is also important to emphasize that eteplirsen treatment is not a cure for the disease but provides a path toward a milder phenotype. The low side effect profile of eteplirsen must be considered an advantage for this product compared to other RNA analogs that cause skin reactions at the site of injection, flu-like symptoms, coagulopathies, inflammatory response, and renal or hepatic toxicity [8, 9]. The lack of toxicity of eteplirsen is attributed to PMO chemistry, which is charge-neutral, largely unmetabolized, and not linked to immune activation, platelet activation, or hepatotoxicity [12, 26].

As a result of this study, Sarepta Therapeutics, the sponsor of eteplirsen (now known as Exondys 51), was granted approval for the use of this drug for DMD boys with confirmed mutations of the dystrophin gene amenable to exon 51 skipping. Coincident with the FDA approval, Janet Woodcock, M.D., Director of FDA's Center for Drug Evaluation and Research, described the event as follows: "Patients with a particular type of Duchenne muscular dystrophy will now have access to an approved treatment for this rare and devastating disease. In rare diseases, new drug development is especially challenging due to the small numbers of people affected by each disease and the lack of medical understanding of many disorders. Accelerated approval makes this drug available to patients based on initial data, but we eagerly await learning more about the efficacy of this drug through a confirmatory clinical trial that the company is conducting." In addition, similar exon-skipping protocols are in trial for exons 45 and 53.

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Chapter 43
What We Have Learned from 10 Years of DMD Exon-Skipping Trials

Svitlana Pasteuning-Vuhman and Annemieke Aartsma-Rus

Abstract Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder caused by truncating mutations in the *DMD* gene. These result in the absence of the muscle fibre stabilizing dystrophin protein and progressive loss of muscle tissue and function. In-frame mutations with partially functional dystrophin generally lead to Becker muscular dystrophy (BMD) with a milder disease phenotype. This was the inspiration for the antisense-mediated exon-skipping approach that restores the dystrophin reading frame to allow production of a Becker-type dystrophin. This approach is mutation specific. Since exon 51 skipping is applicable to the largest group of DMD patients, two antisense compounds targeting exon 51 were developed first, i.e. drisapersen and eteplirsen. Ten years have passed since the first exon-skipping antisense compound was tested clinically in DMD patients. If objectively evaluated, initial trials were suboptimal with modest clinical success. Major hurdles were that, at the time of trial planning, natural history data and reliable outcome measures to detect clinical benefit were not available. Moreover, the levels of dystrophin that are restored in DMD patients are lower than those observed in BMD patients. This chapter looks back at the lessons that were learned during the development of DMD exon skipping so far, to allow for more optimal exon-skipping trials in the future.

Keywords Duchenne muscular dystrophy · Exon skipping · Clinical trial
Dystrophin level · Natural history · Outcome measure · Disease heterogeneity

43.1 Exon Skipping for Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder affecting around 1 in 5000 newborn males worldwide [1, 2]. Patients progressively lose muscle and generally become wheelchair-dependent by the age of 12, require assisted

S. Pasteuning-Vuhman · A. Aartsma-Rus (✉)
Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands
e-mail: s.k.pasteuning-vuhman@umcutrecht.nl; A.M.Aartsma-Rus@lumc.nl

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ventilation by the age of 20 and usually die in the third or fourth decade due to pulmonary or cardiac failure [3, 4].

DMD is caused by out-of-frame mutations in the *DMD* gene that prevent the production of the muscle fibre stabilizing dystrophin protein [5, 6]. Lack of functional dystrophin makes muscle fibres more susceptible to damage resulting in chronic injury accompanied by inflammation and replacement of muscle fibres by adipose and fibrotic tissue [7]. Interestingly, the crucial functional parts of dystrophin are located at the beginning and at the end of the protein. Internal deletions or duplications in the *DMD* gene that maintain the reading frame give rise to partially functional dystrophins and generally lead to Becker muscular dystrophy (BMD) with a later onset and slower disease progression [4].

The finding that out-of-frame mutations generally lead to DMD while in-frame mutations generally lead to BMD was the inspiration for the antisense-mediated exon-skipping approach. Here, antisense oligonucleotides (AONs) are used as steric blockers that hide a targeted exon from the splicing machinery, causing it to be skipped so that the dystrophin reading frame is restored, allowing the production of a Becker-type dystrophin [8, 9]. The exon-skipping approach is mutation specific. DMD patients carry different types of mutations that vary in position and size within the *DMD* gene [5], and, as such, different exons need to be skipped to restore the reading frame for different mutations. In theory, the approach would be applicable to the majority of *DMD* gene mutations (55% of all patients and 80% of patients with deletions) [5, 10]. Moreover, the majority of the mutations are found at the 'hot spot' between exons 45 and 53; thus skipping of certain exons would apply to larger groups of patients, with exon 51 skipping being applicable to the largest group (13–14% of patients).

AONs are chemically modified DNA or RNA analogues. Early modifications involved phosphorothioate linkages to improve stability and pharmacokinetic properties and 2′-O-methyl RNA to render AONs RNase H resistant and making AONs suitable for splicing modulation [11]. These 2′-O-methyl RNA with a phosphorothioate backbone (2OMePS) AONs were the initial tool to modify splicing and skip one or more exons, thereby restoring dystrophin production in patient-derived cell models and animal models [9]. Phosphorodiamidate morpholino oligomers (PMOs), containing a six-membered morpholine moiety instead of ribose and phosphorodiamidate linkages [12], have been explored as another chemistry for *DMD* exon skipping [13–17]. The *mdx* mouse model was helpful to explore exon-skipping efficiency for both chemistries. Interestingly, the AON uptake after systemic delivery in dystrophic muscles was found to be tenfold higher than in healthy muscles. This suggests that the dystrophic phenotype of the muscles lacking dystrophin facilitates AON uptake [9].

After encouraging preclinical results, both chemistries were tested in DMD patients who were amenable to exon 51 skipping. It has now been more than 10 years after the first exon-skipping AON was tested clinically in DMD patients. This chapter will give an overview of the decade-long clinical journey for AONs and will outline the lessons learned along the way.

43.2 Clinical Trials

Since exon 51 skipping is applicable to the largest number of DMD patients, two antisense compounds targeting exon 51 were developed first, i.e. drisapersen and eteplirsen. These components differ in their oligonucleotide backbone chemistries (i.e. 2OMePS and PMO, respectively). Drisapersen was developed by Prosensa/GSK/BioMarin, while eteplirsen was produced by AVI Biopharma/Sarepta. Since the exon-skipping approach is a mutation-specific genetic approach, it was not tested in healthy volunteers. First, safety data was available for both chemistries from trials for other indications. Secondly, exon 51 skipping would disrupt the reading frame in healthy volunteers and thus have the opposite effect compared to DMD patients.

43.2.1 Drisapersen

43.2.1.1 Local Injections

Drisapersen was administrated intramuscularly into tibialis anterior muscles of four DMD patients. The injection was tolerated well, and no side effects were observed beyond some redness and swelling at the injection site. A biopsy taken from the injection site 28 days later showed that in all four patients, drisapersen induced specific skipping of exon 51 during pre-messenger RNA splicing of the dystrophin transcript and restored dystrophin locally [18]. Patients did not show any functional improvement, nor was this expected due to the localized nature of the treatment. Interestingly, the oldest patient, who had the most advanced stage of the disease as assessed by magnetic resonance imaging, showed dystrophin restoration in almost all muscle fibres. However, since he had only a limited number of fibres left, the absolute amount of dystrophin restored was much lower than those observed for the three younger patients. This result underlines that the therapeutic effect of exon-skipping treatment relies on the muscle quality at the time of treatment.

43.2.1.2 Systemic Phase 1–2a Trials

DMD is a disease that affects all skeletal muscles, and lifelong repeated AON treatment is required due to dystrophin mRNA transcript and protein turnover. This makes intramuscular injection of each muscle unfeasible. Therefore, subsequent trials involved systemic treatment, using subcutaneous injections as studies in the *mdx* mouse model had revealed that this resulted in lower kidney and liver exposure than intravenous delivery and speculating that this would be more patient-friendly than intravenous infusions.

First, drisapersen was tested for safety and efficacy in an open-label, dose-escalation phase 1–2a study, where 12 DMD patients were treated by weekly subcutaneous injections of drisapersen for 5 weeks, with groups of 3 patients receiving each of 4 possible doses (0.5, 2.0, 4.0 and 6.0 mg/kg) (PRO051-CLIN02, ClinicalTrials.gov Identifier NCT01910649) [19]. Treatment was tolerated well and resulted in detectable dystrophin production in patients treated with a dose of 2.0 mg/kg or higher. No functional effects were anticipated or observed after 5 weeks. After the dose-finding study, all 12 patients were enrolled in an open-label extension phase, during which they were treated subcutaneously weekly with a dose of 6.0 mg/kg (ClinicalTrials.gov identifier NCT01910649). Twelve weeks into this extension trial, drisapersen treatment was still well-tolerated without serious adverse events. Furthermore, the 10 ambulant patients showed a modest improvement in the distance walked in the 6-min walk test compared to the baseline at the initiation of the extension trial. Patients received weekly treatment for 72 weeks, followed by an 8-week treatment break and then cycles of 8 weekly treatments and 4-week treatment breaks of 6 mg/kg drisapersen for 188 weeks [20]. After 3.4 years, the most common observed adverse events were injection-site reactions and mild proteinuria and raised urinary α1-microglobulin levels. During the off-treatment periods, the proteinuria levels normalized. However, the injection-site reactions sometimes persisted.

Functionally, on average there was an improvement in 6-min walk test performances compared to the expected decline found in natural history studies of age-matched patients [21]. The distance walked in 6 min was stable for 8 of the 10 ambulant patients for the duration of the study, whereas 2 patients lost ambulation. While this finding was encouraging, it should be interpreted with caution, since it involved an open-label study and only a small number of patients.

43.2.1.3 Phase 2 Placebo-Controlled Trials

Prosensa had coordinated the local injection and the phase 2a dose-escalation trials. Following this, they in-licensed drisapersen to GlaxoSmithKline (GSK). GSK then planned and coordinated three placebo-controlled trials. In the first phase 2 double-blind, three-arm, placebo-controlled study, different dosing regimens were compared in patients (DMD 114117, ClinicalTrials.gov Identifier NCT01153932). The study involved 53 DMD patients aged 5 years and older from 13 specialized centres in 9 countries. Patients were all in the early stage of the disease, since they had to be able to rise from the floor in less than 7 s [22]. All patients first received twice weekly doses of 6 mg/kg drisapersen or placebo during a 3-week period. After this period, patients were treated either continuously (once weekly) or intermittently (twice weekly at weeks 1, 3 and 5; once weekly at weeks 2, 4 and 6; and no active drug in weeks 7–10 of each 10-week cycle) for a total duration of 48 weeks. Patients from the drisapersen continuous group showed a significant increase in 6-min walk distance at 25 weeks (34 m; $p = 0.01$), while no significant differences were found for patients from the intermittent group. At week 49, the 6-min walk distance

differed between drisapersen and placebo in 36 m and 27 m for the continuous and intermittent group, respectively (not statistically significant). Some decline towards baseline was observed in the continuous group between 25 and 49 weeks, whereas the intermittent group was relatively stable.

The second phase 2 placebo-controlled study compared different doses of drisapersen and involved 51 DMD patients in an early stage of the disease (6–8 years of age; time to rise from floor <15 s) (DMD114876, ClinicalTrials.gov Identifier NCT01462292). Patients were treated with placebo, 3 or 6 mg/kg drisapersen for 24 weeks. Patients treated with 6 mg/kg walked 27 m more than patients treated with placebo or 3 mg/kg; however this difference was not statistically significant [23].

Although ambulation improvements in this young population with early stage of the disease appear very encouraging, both phase 2 studies were exploratory and contained small numbers of patients in each treatment group. Moreover, both studies were not sufficiently powered to be able to detect significant differences and clinical benefits.

In all phase 2 trials and the following open-label studies using subcutaneous injections of drisapersen, injection-site reactions and proteinuria were more frequently reported in drisapersen-treated patients. Similar injection-site reactions have also been reported for mipomersen, an AON of comparable chemistry that was approved by the Food and Drug Administration (FDA, USA) for the treatment of familial hypercholesterolaemia [24]. These injection-site reactions do not occur after intravenous delivery, which has been explored in clinical trials for AONs targeting exons 44, 45 and 53.

43.2.1.4 Phase 3 Placebo-Controlled Trial

In parallel with the two phase 2 trials, the safety and effectiveness of treatment with drisapersen were tested in a large phase 3 trial involving 186 ambulant patients between 5 and 16 years (DMD114044, ClinicalTrials.gov Identifier NCT01254019). Patients were treated with placebo (n = 61) or 6 mg/kg drisapersen (n = 125) for 48 weeks, and the primary outcome measure was the 6-min walk test. At the end of the trial, drisapersen-treated patients walked 10.3 m more than the placebo group, which was not clinically relevant or statistically significant [25]. Consequently, GSK stopped the clinical development of drisapersen, and all rights returned to Prosensa. In early 2015, BioMarin acquired Prosensa and reanalysed the clinical data. Post hoc analysis of the data from the phase 2 and 3 trials revealed that patients in the phase 3 trial were on average older and had a more advanced disease stage than patients in the phase 2 trials. Therefore, analysis was performed on the subset of patients who would have met the selection criteria for phase 2 trials, revealing that for this group the treatment difference in 6-min walk test was 21.5 m (p = 0.131) [25]. Given that all studies had open-label extension arms, for a substantial number of patients, 96-week treatment data were available. Analysis of this data revealed that when compared to natural history data, longer-term drisapersen treatment appears to slow down disease progression in younger patients but also in older patients [26].

Based on these findings, drug registration applications were filed with the FDA and the European Medicines Agency (EMA). FDA declined approval for drisapersen, saying the 'standard of substantial evidence of effectiveness has not been met'. In May 2016, BioMarin announced they had withdrawn the application with EMA [27] and that they would stop the clinical developments of their current exon-skipping components, to focus on investing in research of next-generation oligonucleotides [28].

43.2.2 Eteplirsen

43.2.2.1 Local Injection Study

Like drisapersen, eteplirsen was also first tested in a local injection study. Here, the extensor digitorum brevis (EDB) muscles of seven DMD patients were injected with eteplirsen at doses of 0.09 mg (n = 2) and 0.9 mg (n = 5) (ClinicalTrials.gov Identifier NCT00159250). The contralateral EDB served as a control and received only saline injection [15]. EDB muscles were selected based on their preservation observed with magnetic resonance and the responsiveness to exon 51 skipping in cultured fibroblasts obtained from skin biopsies. Muscle biopsies taken between 3 and 4 weeks after injections showed dystrophin restoration in all 5 patients treated with the higher dose. Intramuscular administration of eteplirsen appeared to be safe and on average intensity of dystrophin staining was 17% higher in treated muscles than the intensity in the contralateral control muscles. This proof-of-concept study led to systemic clinical trials in DMD patients.

43.2.2.2 Dose-Funding and Efficacy Phase 2 Trials

Following proof-of-concept after the local injection study, systemic trials were performed for eteplirsen. The studies used intravenous infusion as a delivery route. Due to poorer solubility of the PMO compound, subcutaneous injections were not feasible.

The safety and biochemical efficacy of eteplirsen was first examined in an open-label, dose-escalation phase 2 study involving 19 ambulant patients with DMD aged 5–15 years (ClinicalTrials.gov Identifier NCT00844597). Several doses of eteplirsen were tested (0.5, 1.0, 2.0, 4.0, 10.0 and 20.0 mg/kg body weight), and muscle biopsies were taken from the biceps at the start and from the contralateral biceps after 12 weeks of weekly intravenous treatment [29]. Overall, eteplirsen was well-tolerated with no serious drug-related adverse effects. Seven patients responded to treatment showing exon 51 skipping and dystrophin restoration. Three patients showed a clear response to treatment with 21%, 15% and 55% of dystrophin-positive fibres, while the other four patients demonstrated only increases between 6 and 8%. Notably, newly produced dystrophin was functional, as the dystrophin-

associated glycoprotein complex (DGC) was restored at the sarcolemma. However, since even in the 20 mg/kg dose group, there were patients in whom no increase in dystrophin expression was observed; the conclusion was that probably a higher dose was needed.

A subsequent trial involved 12 patients with DMD aged 7–13 years. The trial started as a placebo-controlled, double-blind trial. Patients were randomized to weekly intravenous infusions of 30 or 50 mg/kg/weeks eteplirsen or placebo ($n = 4$/group) for 24 weeks [30]. At week 25, the study became an open-label trial, and placebo patients switched to 30 or 50 mg/kg eteplirsen ($n = 2$/group), and all patients have been receiving weekly intravenous infusions now for over 4 years (ClinicalTrials.gov Identifier NCT01396239). An increase in dystrophin production was the primary endpoint, but function was also assessed by the 6-min walk test. No increase in dystrophin was observed after 12 weeks of treatment with 50 mg/kg eteplirsen. In biopsies taken at week 24, however, the percentage of dystrophin-positive fibres was increased to 23% in patients treated with 30 mg/kg of eteplirsen, while no increase was found in placebo-treated patients. After longer treatment (48 weeks), even greater increases of dystrophin-positive fibres (52% and 43% in the 30 and 50 mg/kg cohorts, respectively) were observed. Furthermore, restored dystrophin appeared to be functional, since sarcoglycans and neuronal nitric oxide synthase were localized at the sarcolemma [31].

Two of the patients in the 30 mg/kg group lost ambulation within the first 3 months of the study. During the 3 years of follow-up, the 10 remaining ambulant patients showed a lower degree of decline in their 6-min walk distance than would be expected from the natural history. Namely, the eteplirsen-treated patients declined 100 m, while the cohort of 13 untreated Belgium and Italian DMD patients declined 250 m in a 3-year time frame [31]. As mentioned before, comparisons of small groups of patients should be interpreted with caution. Nevertheless, Sarepta filed for accelerated approval with the FDA. However, the FDA was hesitant to approve eteplirsen based on such a small number of patients and also questioned the robustness of the dystrophin quantification method, which involved manual counting of dystrophin-positive fibres by a pathologist, while information on the quantity of dystrophin was lacking. A fourth biopsy was taken from patients after 188 weeks of treatment. Western blot analysis quantification revealed an increase of dystrophin of 0.9% [32, 33].

43.2.2.3 Open-Labelled Confirmatory Phase 3 Trial

In September 2014, Sarepta initiated an open-labelled phase 3 trial to provide confirmatory evidence of eteplirsen efficacy (ClinicalTrials.gov Identifier: NCT02255552). The trial involved 80 ambulant DMD patients amenable to exon 51 skipping, who received weekly intravenous dosing of 30 mg/kg eteplirsen for up to 96 weeks, while 80 matched DMD patients with mutations not amenable to exon 51 skipping served as controls for safety and functional outcome measures.

FDA requested Sarepta to confirm increased dystrophin expression by western blot analysis from biopsies taken from these patients before and after 48 weeks of

eteplirsen treatment [34]. Western blot analysis of 13 patients showed an increase in dystrophin in some patients, ranging from 0.22% to 0.32% of normal [35]. Notably about half of the patients had no or minimal apparent increases in dystrophin expression. Although the levels of dystrophin restoration were lower than anticipated, eteplirsen was granted accelerated approval under provisions that Sarepta will confirm the drug's clinical benefit before 2021 [36].

43.3 Lesson Learned

There have been several lessons learnt from the exon-skipping studies. Sometimes things could not have been foreseen, e.g. the injection-site reactions after subcutaneous injections of drisapersen were never observed in mice. In retrospect, intravenous delivery would have been preferred and probably should be considered for future trials using high doses of PS-modified AONs.

However, some of the lessons learned relate to the field being unprepared for clinical trials.

At the onset of the clinical trials, neither natural history data of the disease were available nor did functional outcome measures exist. This realization inspired multiple stakeholder collaboration meetings involving academics, regulators and representatives from industry and patient advocacy groups to identify gaps, collect additional data and develop new outcome measures [3, 37, 38]. However, this is an effort that is still ongoing, while the first systemic trials were initiated in 2008.

The 6-min walk test was used in these trials, but this test was not developed for DMD but borrowed from the cardiovascular field to measure muscle function in ambulant patients. Since the test had not been performed by DMD patients and no natural history data for this test existed, the heterogeneity of the disease had not been fully appreciated. With the onset of therapy trials, the field started collecting natural history data for the 6-min walk test [39–41]. This revealed that generally the 6-min walk distance declines nonlinearly and younger patients ($\leq$7 years of age) are stable or can even increase in their walk distance within 1 year [39]. Later the 6-min walk distance stabilizes, followed by a slow decline and finally a rapid decline just before losing ambulation [42]. Given that the exon-skipping approach aims to slow down disease progression and prolong the ambulation period, ideally patients in the decline phase are selected for future clinical trials (it is not possible to measure a slower decline in stable patients) [32, 42]. However, once the rapid decline has started, it may be too late to achieve a therapeutic effect on walking function. Thus, currently a specific subset of patients is selected in clinical trials using the 6-min walk test, i.e. the patients where one expects to be able to detect a slower disease progression in a 1-year trial. This is generally assumed to be patients with a baseline 6-min walk distance near 350 m [3].

Looking back on past trials with the current knowledge, it is clear to see how initial trials may have been suboptimal. For instance, the phase 2 drisapersen trials involved only very young patients in a relatively stable phase of the disease [20],

while the phase 3 trial involved patients between 5 and 16 years of age [25], resulting in high variability. The unexpected heterogeneity can also give rise to uncertainty within trials, e.g. in the phase 2 drisapersen trial testing different dosing regimens, the continuous treatment regime appeared to contain higher number of younger and more functional patients compared to the intermitted regime explaining improved 6-min walk distance in the continuous but not in the intermitted group at 25 weeks [25].

As mentioned, DMD progresses slowly when measured with the 6-min walk test, and the exon-skipping compounds aim to slow down disease progression. This has an impact on trial duration. The EMA guidance recommends DMD trials to be placebo-controlled and lasting at least 1 year [43], while the draft FDA guidance suggests 18–24 months [44]. The phase 2 trial design for eteplirsen was originally not set up for drug registration, as underlined by the small number of patients and the fact that there was no placebo group beyond the first 24 weeks. Therefore, results of the 6-min walk test had to be compared between eteplirsen-treated patients and historical controls selected from natural history data of baseline-matched patients from Belgium and Italy [31, 32]. This is a challenging exercise, because variation in care in different countries will influence disease progression. As such it is not surprising that FDA was not convinced by this data and requested Sarepta to provide compelling functional data in future study as a condition of the accelerated approval [32].

Currently, the 6-min walk is often selected as the primary functional endpoint in phase 2 and 3 trials for DMD. However, it has several disadvantages. First, it was not developed for DMD. As such, a lot of effort was needed to define the clinically meaningful difference for patients as 30 m [41]. Being able to walk 30 m more in 6 min may not appear clinically relevant. However, it has become clear that the distance walked in 6 min is predictive for when patients will lose ambulation, which clearly is clinically relevant. Alternative outcome measures are now developed as well, such as the North Star Ambulatory Assessment, which captures multiple items that are relevant to patients, such as the ability to climb stairs (and therefore traverse thresholds) and get up from the floor. The performance upper limb (PUL) functional outcome measure was established in collaboration with patients and can also be used in non-ambulant patients. However, these outcome measures have been newly developed, and natural history data is only now being collected. If there is one lesson from this all, it is that ideally outcome measures should be available at the time first trials are initiated.

Another thing that has become clear is that it is unlikely that exon skipping will convert a DMD patient into a BMD patient. First, the levels of dystrophin that are restored in patients after exon skipping are a lot lower than those expressed in BMD patients. However, preclinical studies in mouse models revealed that very low levels (less than 4%) of dystrophin are beneficial for survival [45]. Furthermore, patients amenable to exon 44 skipping show higher baseline levels of dystrophin due to spontaneous exon 44 skipping, which result in clinical benefits such as prolonged ambulation and slower disease progression [46, 47]. However, the higher dystrophin levels are present from birth, while dystrophin expression will only be induced at

the time of intervention in DMD patients. At that time muscle damage will already have accumulated. It is currently not known how much dystrophin is required to slow down disease progression in DMD patients, but it is likely that the levels may vary for young patients with relatively good muscle quality and for older patients with progressive muscle tissue loss.

43.4 Future Perspectives

New oligonucleotide chemistries are currently in development aiming to achieve more widespread restoration of dystrophin throughout the whole body's muscle including the heart.

Heart failure is one of the main causes of death in DMD patients, and targeting heart remains one of the most significant challenges [14]. Among oligonucleotide chemistries tested in preclinical trials, cell-penetrating peptide-conjugated PMO (PPMO) efficiently induced dystrophin expression in whole body muscles and the heart and improved heart function [48, 49]. Although preclinical testing of PPMOs in *mdx* mice appeared to be safe, monkeys are more sensitive to dose-dependent PPMO-related toxicity, which can lead to kidney degeneration [50]. If it is possible to lower the toxicity, e.g. through structural modifications, PPMOs could be a promising therapeutic compound for DMD.

Another next-generation exon 51 DMD compound was recently developed by Wave Life Sciences Ltd. Wave has presented that their stereopure component induces higher exon-skipping levels and results in better uptake in skeletal muscle and the heart. Wave is planning to initiate their first clinical trial involving ambulatory and non-ambulatory DMD patients in 2017 [51].

A major hurdle of the exon-skipping approach is that the *DMD* mutations are very heterogeneous, while the exon-skipping approach is highly mutation specific. Each AON is considered as a new drug by the regulators. To address this, multi-exon skipping has been proposed as a method that is applicable for larger groups of patients. For example, skipping exons 45–55 would apply to 40% of all patients [10], and Becker patients with a deletion of exons 45–55 show a mild disease phenotype [52]. However, this approach needs 11 AONs targeting 11 exons, which is challenging [53]. Multi-exon skipping is currently at a preclinical stage, and several hurdles need to be addressed including low efficacy and potentially high toxicity. A better understanding of the *DMD* intron splicing order and usage of new-generation antisense oligonucleotides may reduce the number of AONs required to skip exons 45–55 and reduce the therapeutic of individual AONs [54].

Exon-skipping therapy development for DMD is very dynamic. New AON chemistries and modifications are tested in cell and animal models, and outcome measures have been developed and natural history collected. While initial trials perhaps were not optimal, it is hoped that future AON trials will benefit from the work that has been done so far.

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Chapter 44
Clinical Gene Therapy Trials for Pompe Disease

Cristina Liberati, Stephanie Salabarria, Manuela Corti, and Barry J. Byrne

Abstract Pompe disease is a metabolic myopathy which leads to severe and progressive weakness due to glycogen storage in striated muscle and neurons. Generalized weakness leads to cardiopulmonary insufficiency and early mortality. The spectrum of disease ranges from a fatal early-onset form to a more slowly progressive intermediate and adult-onset type. The severity of disease depends on the amount of residual acid α-glucosidase (GAA) activity which is determined by the nature of the two mutant alleles. The lack of GAA leads to accumulation of glycogen in lysosomes of neurons and striated muscle (especially skeletal and cardiac muscle). Lysosomal dysfunction and cellular autophagy result in neuronal cell loss over time. Enzyme replacement therapy (ERT) is the only currently approved treatment for Pompe disease; however, ERT does not effectively address the neural deficits; therefore, alternative approaches using gene therapy must be considered. The principal objective of gene therapy in Pompe disease is to increase the intrinsic ability of the cells to produce GAA. A variety of recombinant adeno-associated viral vectors (rAAV) are being studied to complete this task. The efficacy of gene therapy not only depends on the efficiency of the gene therapy agent but also on the host's immune response. The most critical immunological challenges are anti-AAV capsid antibodies and anti-GAA antibodies. In this chapter, we review the current status of AAV-mediated gene therapy for Pompe disease.

Keywords Pompe disease · Glycogen storage disorder · Gene therapy · Clinical trials · AAV · Immune response

C. Liberati · S. Salabarria
Department of Pediatrics, College of Medicine, University of Florida, Gainesville, FL, USA
e-mail: c.liberati@peds.ufl.edu; ssalabarria@ufl.edu

M. Corti · B. J. Byrne (✉)
Department of Pediatrics, College of Medicine, University of Florida, Gainesville, FL, USA

Powell Gene Therapy Center, University of Florida, Gainesville, FL, USA
e-mail: m.corti@peds.ufl.edu; bbyrne@ufl.edu

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44.1 Introduction

Pompe disease is a rare, life-threatening, autosomal recessive, glycogen storage disorder caused by mutations in the acid α-glucosidase (GAA) gene located on the long arm of chromosome 17 [1]. Deficiency of the GAA enzyme leads to accumulation of lysosomal glycogen in neurons and muscle cells, causing cellular dysfunction and apoptosis [2–4].

It is estimated that the incidence in the United States of individuals born with GAA mutations is approximately 1:40,000, but recently, new data has shown this estimate may not reflect the true incidence based on newborn screening [5]. Newborn screening efforts in several US states suggest there is a higher than anticipated disease frequency of 1:10,000, implying that there may be many subclinical cases of weakness attributed to other causes, especially among adult patients.

44.2 Clinical Findings

In unaffected individuals, normal GAA enzyme leads to degradation of lysosomal glycogen through the hydrolysis of α-1,4 and α-1,6 links [6], releasing glucose from the acidic lysosome. The cytosolic pathway, which operates at neutral pH, is unimpaired in Pompe disease, and glucose homeostasis following fasting is normal. The relationship between fasting, autophagy, and lysosomal dysfunction may be an important factor in disease severity as well as a link to other neurodegenerative diseases where this mechanism is part of the pathobiology. Therefore, lysosomal accumulation of glycogen is the primary pathogenic mechanism, which leads to the clinical manifestations observed in Pompe. All striated muscles, especially the body-wide musculature and the heart, are affected. Additionally, neurons, principally lower motor neurons and the phrenic motoneuron pool, are impacted, leading to significant respiratory symptoms [7–14]. The severity of the disease and the onset of symptoms, ranging from the neonatal period (infantile) to adulthood (juvenile/adult onset) [15], are usually related to amount of residual GAA. The range of enzyme's activity can vary between less than 1% and up to 20% of wild-type activity level [16–18], and those with 50% of wild-type activity level (GAA mutation carriers) are unaffected. Pompe disease patients face progressive muscle weakness, hypotonia, cardiomyopathy, and respiratory insufficiency. These symptoms eventually lead to respiratory distress and cardiorespiratory failure, which often results in early mortality [10, 19–22]. Even though infantile and late-onset patients have a different rate of progression, progressive respiratory insufficiency is one of the primary clinical findings. Ventilatory support is critical in management. However, prolonged use of mechanical ventilation has itself been associated with diaphragm dysfunction. It is important to consider that pediatric subjects are more susceptible to ventilator-induced diaphragm dysfunction [23–37].

44.3 Diagnosis

To pursue the diagnosis of Pompe disease, an adequate level of clinical suspicion of the disease is necessary. In infants, feeding difficulties and hypotonia, otherwise termed "floppy baby syndrome," and cardiomyopathy are most common initial findings of Pompe disease [38]. In adults, a limb-girdle pattern of weakness and nocturnal hypoventilation are common findings. Often, these symptoms contribute to fatigue and morning headaches as the respiratory insufficiency slowly becomes more severe. All patients have elevated blood levels of creatine kinase and the associated elevation of serum transaminase, which are often mistaken for liver disease. The mainstay of diagnosis is based on detecting deficient GAA activity in the peripheral blood or tissue samples, which is then confirmed by molecular diagnostics [16, 39].

Between 2013 and 2015, the Discretionary Advisory Committee on Heritable Disorders in Newborns and Children and the Secretary of Health and Human Services worked to add Pompe disease to the Recommended Uniform Screening Panel (RUSP), the statewide newborn screening program (more information is available on: www.hrsa.gov). Currently, seven states are using newborn screening to establish an early diagnosis of Pompe disease [39–42]. One important aspect of newborn screening is that some patients with late-onset disease will be identified during the newborn period.

44.4 Therapies

44.4.1 Enzyme Replacement Therapy

Enzyme replacement therapy (ERT) is the only currently approved treatment for Pompe disease. The strategy relies on IV infusion of the recombinant protein, on a weekly or biweekly basis. Alglucosidase alfa, marketed as Myozyme® and Lumizyme® by Genzyme Corporation (Cambridge, MA, USA), was approved in 2006 by the EMA and FDA. Initial clinical studies in early-onset Pompe disease were successful in showing a strong survival advantage compared to historical cohorts where early mortality was observed in the untreated population. The initial approval was granted for early-onset patients and later expanded to late-onset patients. Over the 10-year history of ERT use, there is evidence for improved survival in some infantile patients, yet there continues to be significant deficits in speech, motor function, and ventilatory function in the treated population [43–47]. In adult patients, the co-primary endpoints of forced vital capacity and 6-minute walk test were studied in a pivotal studies. In general, patients on long-term therapy have shown slight improvement or reduction in decline rate, although no studies have demonstrated significant long-term gain in function.

Central nervous system (CNS) manifestations of Pompe disease have come to light, and peripheral ERT has been demonstrated to be not effective in treating the CNS since the product is unable to cross the blood-brain barrier [48, 49]. Ongoing neural degeneration and motor unit dysfunction are major contributing factors to the symptom complex in infantile and late-onset Pompe disease [11–14, 50, 51].

Additionally, ERT can lead to an immune response as would be expected in a recessive condition with limited endogenous GAA activity. Patients with severe or null mutations, otherwise known as CRIM (cross-reactive immunologic material)-negative patients, have the least functional improvement following ERT [52–56]. A number of studies have been conducted to evaluate the impact of high-sustained antibodies against alglucosidase alfa and to design strategies to block antibody response. These studies are critical to the success of ERT in infants. Adult patients can also develop infusion reactions related to anti-GAA antibodies, but the full impact on efficacy versus safety is unknown. Another strategy to lower the immune response to ERT is to promote regulatory T-cells by gene transfer of GAA to the liver [57–61]. While this approach is very valuable for liver-derived proteins that function in the blood, hepatic-derived GAA will not substantially cross the blood-brain barrier, where a cell autonomous effect is required to rescue GAA deficiency in neurons. Finally, as the phenotype of long-term treated patients continues to be characterized, there is a distinct observation of the limitations to alglucosidase alfa effectiveness as a therapeutic strategy. Interest in developing alternative or second-generation therapies has led to additional clinical studies of alternatives or second-generation formulations.

44.4.2 Gene Therapy

Gene therapy is a rational solution for Pompe disease as well as for many other neuropathogenic lysosomal storage diseases. In the most common strategy of in vivo transduction, a viral vector is used to provide a supplemental copy of the cDNA for the dysfunctional allele in target cells. This approach should be distinguished from ex vivo gene therapy, where an integrating viral vector is used to modify a stem cell population for later delivery back to the patient. The most common viral vector currently used for in vivo gene transfer is the adeno-associated viral vector or recombinant AAV (rAAV). The primary benefit derived from this type of therapy in Pompe disease is an increase in the cell's intrinsic ability to produce alpha glucosidase. The processing of all lysosomal proteins involves a complex delivery process in which the new synthesized protein is moved from the rough endoplasmic reticulum to the trans-Golgi and then trafficked to the mature lysosome. The elegant process which results in delivery of a functional protein to the acidic compartment of the lysosome is most efficient when these proteins are delivered from within the cell in an autonomous manner. Exogenous supply of the protein, such as with ERT,

relies on a less efficient process of endocytosis after the binding of the mature protein to the mannose 6-phosphate receptor. Importantly, lysosomal proteins are inherently unstable at neutral pH during transit in the blood. These same fundamental principles apply to liver-directed gene therapy of a lysosomal protein. The amount of expression from the liver is several orders of magnitude greater than is needed in a target cell, mostly to overcome the inefficiency of the alternative uptake pathway. A second aspect which must be considered is that most lysosomal proteins, GAA included, do not cross the blood-brain barrier. To combat this, some alternative transport mechanisms do exist, such as loading of the protein in leukocytes, but currently the efficiency is very low.

44.4.2.1 Immune Response

One of the most critical questions regarding the efficacy and durability of gene therapy in any recessive disease is the likelihood of an anti-transgene immune response. The antibody response to ERT, especially in infantile Pompe disease, is entirely responsible for infusion reactions and diminished efficacy. Therefore, in infantile Pompe disease, it is common clinical practice to initiate immune management at the time of starting ERT. The amount of residual protein expression, or CRIM status, can then be investigate by further testing, and the duration of immune therapy can be established. Several immune management strategies have been evaluated in Pompe disease ERT, but the duration and precise regimen are still under investigation [59, 62, 63].

In addition to anti-GAA immune responses, it is also important to consider the anti-capsid response to the AAV vector. Viral shedding during natural AAV infection and the high rate of cross-reacting antibodies suggest that prior exposure to AAV often occurs in the early school-age years [64–66], resulting in acquired immunity to the vector capsid [67–70]. Anti-capsid antibodies are believed to have an impact on efficacy due to B-cell-mediated activation of cytotoxic T-lymphocytes and binding antibodies, which redirect vector capsids from the circulation. In addition to natural exposure to the virus, there may be a need for readministration of a therapeutic gene therapy vector when somatic growth or initial dose leads to inadequate expression at a later date [67–72].

To fully consider the immune challenges in Pompe disease gene therapy, we must address (a) how to control the humoral immune response to GAA in CRIM-negative subjects; (b) how to successfully deliver AAV in a patient population that may have pre-existing binding or neutralizing antibodies against AAV; (c) how to readminister the therapeutic agent to achieve lifelong correction of GAA deficiency. Our group has shown that attenuating or ablating the humoral immune response can (1) enhance initial safety of high-dose AAV by limiting immunotoxicity, (2) increase transgene expression by eliminating anti-GAA antibodies, and (3) allow subsequent exposure to AAV vectors [63, 71, 73–76].

44.4.2.2 Preclinical Studies

The original mouse model of Pompe disease was generated in collaboration with Dr. Nina Raben in 1998 [77], and several novel derivations have been created since that time. The benefit of a mouse model is that the behavioral and biochemical phenotype can be readily established and used to analyze the change from baseline after a therapy. Using a variety of AAV vector serotypes, many vector-promoter-capsid combinations have been evaluated [78–81]. The initial experiment was to show that an E1-deleted adenoviral vector expressing human GAA (rAd-hGAA) could be used to demonstrate cross correction in vitro (in deficient fibroblasts and myoblasts) and in vivo (in deficient mice) [82–87]. Subsequently, a myriad of nonclinical studies with AAV vectors were initiated to demonstrate the biochemical, physiological, and clinical responses to gene therapy with AAV-GAA [11, 71, 77, 88–90]. The initial observations with AAV serotype 2 have been amplified through the availability of second-generation AAV vectors, which have higher tropism for striated muscle or neurons.

One of the key outcome measures of nonclinical studies in the GAA knockout (KO) models assessed the effect of vector delivered systemically in the heart. Successful reversal of glycogen storage results in reduction in LV mass, restoration of the normal PR interval, and improvement in LV function [90–94].

Furthermore, important observations on the performance of skeletal muscle function showed that lysosomal glycogen levels can be reduced, facilitating the recovery of muscle function by several assays. An important area to assess is the ability to restore diaphragmatic and phrenic motor pool function [90, 95]. A novel delivery strategy for the correction of diaphragm muscle was described and helped establish the basis for a clinical study focused on the same outcomes (see below) [96–99].

Several nonclinical studies have focused on respiratory dysfunction, especially the impact of GAA deficiency on neuron motor units, neuromuscular junction, and the myofiber [12, 100]. Initial studies demonstrated the ability of AAV1 to transduce and impact all components of the motor unit [11, 98]. Lastly with both systemic and direct IM delivery, it has been possible to show that rAAV1-GAA leads to normalization of the enzyme amount, reduction of glycogen accumulation, and gain of function of the muscular system [94, 101–103]. A human clinical trial based on these supportive findings has been completed with evidence of a positive effect (see next section).

Comparative efficacy of ERT versus rAAV-GAA treatment in GAA KO mice has also been investigated, demonstrating promising AAV-based gene therapy outcomes [104]. Additional studies addressed the question of how the humoral immunological response against hGAA could be reduced using a recombinant AAV8-GAA vector controlled by a liver-specific promoter [81, 105–107]. Plans to test these approaches are being considered by regulatory agencies.

44.4.2.3 Clinical Trials

In Pompe disease gene therapy, only two gene therapy studies have achieved approval from the FDA, and no studies have been conducted thus far in the EU. The first gene therapy study in Pompe disease was for children with early-onset disease and is based on nonclinical studies described earlier in the chapter. The study, titled "Phase I/II Trial of Diaphragm Delivery of Recombinant Adeno-Associated Virus Acid Alpha-Glucosidase (rAAV1-CMV-GAA) Gene Vector in Patients with Pompe Disease" [NCT00976352], was initiated in 2010 [74, 76, 108]. This trial involved three cohorts of young patients (two dose cohorts) in a phase I/II open-label study. The purpose of the study was to test the safety and exploratory effects on ventilator function of an AAV1 vector expressing human GAA (rAAV1-CMV-hGAA) after intramuscular (IM) delivery [74, 76, 96, 97, 109]. Study subjects were initially required to have progressed to invasive ventilatory support and be on stable ERT therapy. The proof-of-concept data has been discussed previously, and it should be further emphasized that the study population was considered to have failed ERT. Study participants were between 2 and 15 years of age, and all subjects were fully dependent on mechanical ventilatory support in cohort 1 and varying degrees of dependence in cohorts 2 and 3. Two dosing levels were studied, and the vector was administered bilaterally at the anterior, middle, and posterior parts of the diaphragm muscle. The primary outcome of initial early-onset ERT studies was ventilator-free survival. Therefore, in assessing the ventilatory outcome of these patients, any observed changes could be attributed to the study agent [75, 96, 97, 110, 111]. The direct IM strategy was chosen because it has shown to be more successful than IV delivery when working with AAV serotype 1 at the time these studies began. Early work with AAV serotype 9 was underway, but sufficient nonclinical data of AAV9 dosing and longer-term efficacy studies had not yet been completed. Subsequent studies with AAV9 have confirmed an advantage for certain cellular targets and a systemic route of administration (see below). The primary outcome measure of the study was safety [75], and the exploratory efficacy endpoints related to ventilatory function were also reported [74, 108].

A unique aspect of the study was to assess the importance of inspiratory muscle strength training as part of respiratory rehabilitation. In order to establish uniform baseline functional status, each subject was asked to follow a standardized inspiratory muscle strength training (IMST) effort up to 12 weeks before study agent dosing. Several novel findings have been observed through direct and indirect observation of the study subjects. First, preconditioning with IMST was sufficient to improve any aspect of ventilatory function. In relation to safety, there were no study agent-related adverse events, although a number of adverse events and serious adverse events were described as related to disease progression [74, 75, 108]. An important distinction was made in the baseline status of subjects who had full-time mechanical ventilation versus part-time mechanical ventilatory support. Changes from baseline were only observed in the subjects with minimal or part-time ventilatory support, suggesting a

certain degree of disease burden may not be reversible [108]. Also, it is notable that subjects in the part-time mechanical ventilatory support group had significant positive gains from baseline, rather than a reduction in the rate of decline, as it had been observed in ERT studies.

Since those subjects with infantile disease often have null mutations in the GAA gene, it may be necessary to consider immunotherapy in order for ERT to be tolerated. Of the subjects in the study, three had received chronic immune management to maximize the benefit of ongoing ERT, through treatment with rituximab and sirolimus [71, 73, 74, 76, 108]. Most importantly, these patients did not have immune responses to AAV1 following dosing, indicating the ability to undergo additional AAV treatment at a later time [75].

Given the findings from this first gene therapy study in Pompe disease, a new series of nonclinical studies were completed to directly demonstrate the ability to deliver AAV in two sequential doses. The corresponding clinical study is being conducted in late-onset Pompe disease and is titled "Re-administration of Intramuscular AAV9 in Patients with Late-Onset Pompe Disease" [NCT02240407]. The study is a within-participant, double-blind, randomized, phase I controlled study where each participant will receive AAV dosing twice in a span of 4 months. The objectives are to evaluate the toxicology, biodistribution, and potential activity of rAAV9-DES-hGAA (with the Desmin promoter used to drive transgene expression) injected intramuscularly (IM) into the anterior muscle of the lower leg. Nine potential participants will be selected and screened until six subjects (aged 18–50 years old) will receive the study agent. Inclusion requires that the patient has no prior exposure to AAV so that blocking B-cells prior to initial AAV exposure will prevent the formation of anti-AAV antibodies. To date, two subjects have been enrolled and safety dosed, and preliminary data are being analyzed. The primary outcome is safety; however, important clinical assessments are being performed to confirm the active dose and other evidence of efficacy including biomarker and imaging findings. A unique aspect of the study is that the subjects received the active agent in one leg at the first dosing visit along with excipient in the contralateral leg. At the second dosing visit, the placebo-treated leg received the active agent. Such a study design allows for eventual redosing, which could ultimately lead to the possibility of initiating new studies consisting of dose-escalating within the same subject.

This study in late-onset Pompe disease is an important step toward the ultimate goal of systemic delivery of AAV9 to achieve body-wide correction of GAA deficiency. As noted earlier in the context of a recessive disease, immune response against the transgene product is a critical barrier to successful gene therapy, especially in Pompe disease. By applying the same principles described above and in the supporting nonclinical studies, we have established a strategy to allow for correction of GAA deficiency in infantile Pompe disease. An innovative study supported by the NHLBI Gene Therapy Resource Program and a new program at the NIH Clinical Center will study the systemic delivery of AAV9-GAA in infants with infantile Pompe disease and will use the same immune management strategy first observed to be effective in the AAV1 study and then evaluated with a series of nonclinical studies using AAV9. Confirmation of the study principles will be completed

with the late-onset Pompe disease IM study, and in mid-2019, the study in early-onset Pompe disease will be initiated. Six to eight subjects will be enrolled at the active dose, determined from the related study in adults.

To develop an effective treatment for Pompe disease, it is necessary to evaluate the findings in patients who have been managed by ERT alone and then establish strategies that confirm how vector-derived GAA expression will provide further benefit to this patient population. After 10 years of ERT experience, there are some clear benefits to patients; however, the effect is not sufficient to observe ongoing improvement and full restoration of neuromuscular function. Given the findings of neuronal and motor unit dysfunction, the approach to direct cellular transduction with AAV delivered systemically is a rational strategy to improve the long-term outcomes in Pompe disease. These important new strategies enabling early correction and maintenance of the effective level of gene expression have been developed and are being deployed in two studies. Future findings from these studies will impact the era of newborn screening for Pompe disease and early treatment of many other early-onset pediatric conditions.

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Chapter 45
Muscle-Directed Gene Therapy for Alpha-1 Antitrypsin Deficiency

Alisha M. Gruntman and Terence R. Flotte

Abstract Alpha-1 antitrypsin (AAT) deficiency is a common monogenic disorder resulting in emphysema, which is currently treated with weekly infusions of protein replacement. We have reported achieving plasma wild-type (M) AAT concentrations at greater than 2% of the therapeutic level at 1 and 5 years after intramuscular (IM) administration of 6×10^{12} vg/kg of a recombinant adeno-associated virus serotype 1 (rAAV1)-AAT vector in AAT-deficient patients. This persistent expression was associated with a regulatory T cell (Treg) response to AAV1 capsid epitopes in the absence of any exogenous immune suppression. The patients also showed partial correction of functional biomarkers of AAT expression, including an increase in antineutrophil elastase capacity and a decrease in markers of neutrophil degranulation. Muscle-based gene therapy has allowed us to avoid targeting the liver, therefore preventing potential toxicity in patients where the hepatocytes are burdened with mutant AAT protein. Future muscle gene therapy will likely require dose escalation using a limb perfusion delivery method in order to obtain therapeutic serum levels of AAT while still avoiding delivery to the liver.

Keywords Adeno-associated virus · AAV · Intramuscular · Limb perfusion

A. M. Gruntman (✉)
Department of Pediatrics and Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA, USA

Department of Clinical Sciences, Cummings School of Veterinary Medicine, Tufts University, North Grafton, MA, USA
e-mail: Alisha.Gruntman@umassmed.edu

T. R. Flotte
Department of Pediatrics and Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA, USA
e-mail: Terry.Flotte@umassmed.edu

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45.1 Background of Alpha-1 Antitrypsin Deficiency (AATD)

Alpha-1 antitrypsin (AAT) deficiency is a common single gene disorder first discovered by Laurell and Eriksson in 1963 as a cause of familial clusters of emphysema correlated with the absence of the alpha-1 globulin band on serum protein electrophoresis [1]. The alpha-1 fraction was determined to have antiprotease activity in the laboratory setting against trypsin and so was named alpha-1 antitrypsin (AAT). Subsequently, it has been determined that AAT is a 52 kDa glycoprotein comprising the most abundant serum antiprotease, with physiologic serum levels ranging from 20–50 μM (1.0–2.6 mg/ml; 100–280 mg/dl). AAT is a member of the serpin family of antiproteases, with homology to antithrombin 3 (AT3) and serpins found in humans and other mammals.

The primary substrate for AAT antiprotease activity is neutrophil elastase (NE). AAT also has activity against a number of other neutrophil-derived proteases, such as cathepsin G and proteinase-3, and may play a direct role in the inhibition of apoptosis in alveolar endothelial cells [2, 3]. AAT is primarily produced in hepatocytes and to a lesser extent in monocytes and macrophages. AAT is an IL-6 inducible gene and functions as an acute phase reactant [4–6]. Like other mammalian serpins, this acute increase in serum AAT facilitates its function in limiting the activity of NE to local tissue foci of infection where neutrophils accumulate and release granule contents as part of the innate immune response. AAT is inactivated by oxidation of methionine residues in its active site. Thus, reactive oxygen species released during the oxidative burst by clusters of neutrophils at foci of infection serve to counteract AAT within infected sites and allow NE to remain fully active against microbial invaders [7, 8].

Certain AAT deficiency alleles are very common in the populations of Scandinavia, Ireland, other countries of northern and western Europe, and North America [9–13]. The most common “severe” allele, glu342lys (E342K), is designated as proteinase inhibitor (Pi)-type Z, based on its migration on isoelectric focusing gel. PiZ AAT protein (Z-AAT) polymerizes by “loop-sheet” polymerization, where the reactive loop of one molecule of AAT inserts itself into the beta-sheet region of a neighboring molecule near the site where the E342K mutation interrupts a salt bridge [14, 15]. This polymerization process results in accumulation of Z-AAT in the endoplasmic reticulum (ER) of hepatocytes and ultimately leads to the formation of very large aggregates, detectable by periodic acid-Schiff (PAS) staining and resistance to diastase (glycogenase) digestion.

The most common manifestation of AATD is lung disease in Pi-ZZ homozygous patients due to the relative paucity of AAT in serum, secondary to the impairment of release of AAT from hepatocytes. Normally, AAT is present in molar excess of NE in serum and healthy tissue. Low levels of AAT in serum result in unopposed action of NE on elastin fibers in the pulmonary interstitium. This results in progressive loss of lung elasticity and the clinical picture of emphysema. In addition, the presence of elastin degradation products in the lung leads to release of chemokines and further infiltration of inflammatory cells. The classic histopathologic picture of AATD is panacinar emphysema, with a predilection for the lower lobes [16]. In reality, the

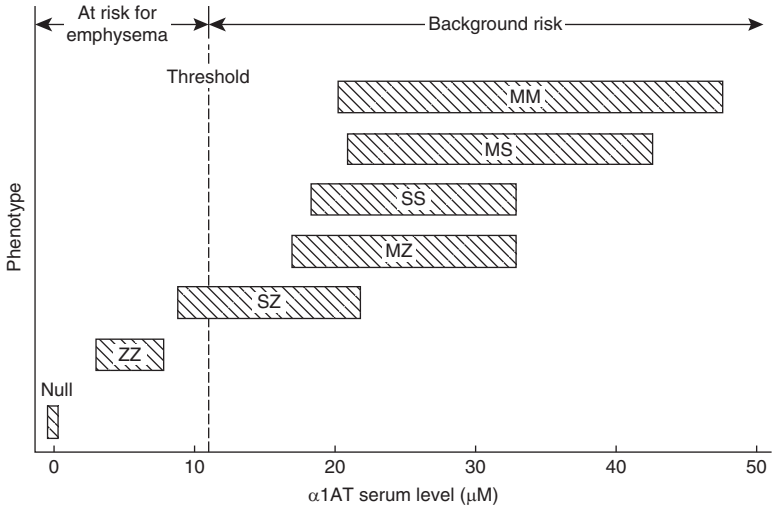

Fig. 45.1 Establishment of threshold level of AAT therapy. Reproduced with permission from Crystal, RG. *Trends in Genetics* 1989; 5:411–417

pulmonary presentation is quite varied. Most patients experience an asthma-like picture for years preceding the correct diagnosis and may respond partially to anti-inflammatory therapy.

Genotype-phenotype studies have shown that individuals heterozygous for PiZ with one wild-type (PiM) or one less severe missense mutation (e.g., PiS, glu264val) who display levels of AAT in serum that are lower than normal but higher than 11 μM (572 mcg/ml, 57 mg/dl) are at low risk of developing spontaneous lung disease (Fig. 45.1) [17]. Interestingly, such individuals are at increased risk of lung disease if they are exposed to tobacco smoking, either directly by smoking or as secondhand exposure. This may be due to oxidative inactivation of residual AAT within the lung by ROS released in response to smoke inhalation. Importantly, the 11 μM levels established from such studies have set the benchmark for FDA approval of IV protein replacement products, most of which are derived from pooled human plasma [18]. Other than protein replacement therapy, options for AATD lung disease patients are limited, consisting of tobacco avoidance, prompt treatment of infectious and inflammatory processes in the lung, oxygen supplementation as needed, and lung transplant, the latter reserved for patients with end-stage lung disease (Table 45.1).

In a subset of patients with AATD, the accumulation of mutant Z-AAT within hepatocytes triggers an ER stress response further triggering hepatocyte injury and inflammation [19]. In a smaller subset of such patients (approximately 10% of total AATD population), liver inflammation progresses to more serious liver disease [20]. Liver disease in AATD can present at any age but generally can be considered as bimodal, with a more rapidly progressing form presenting in infancy and early

Table 45.1 Currently available therapeutic options for alpha-1 antitrypsin deficiency lung and liver disease

Therapeutic target organ	Primary therapies	Adjunct therapies
Lung	Protein augmentation therapy—intravenous infusion of AAT protein	Inhaled bronchodilators and steroids
	Lung transplant	Pneumococcal and influenza vaccinations
	Surgical lung volume reduction	Supplemental oxygen
		Smoking cessation
		Pulmonary rehabilitation therapy
Liver	Liver transplant	

Table 45.2 Alpha-1 antitrypsin deficiency as a target for gene therapy

Advantages for gene therapy
Single gene target
AAT coding sequence is within AAV's packaging size
Multiple choices for target cells because AAT is a secreted protein
Easily assayed serum endpoint applicable for clinical trials
Wide therapeutic window based on protein replacement data

childhood with cholestasis and more rapid progression to cirrhosis and liver failure and an adult form, which is generally more slowly progressing and more often associated with gradual accumulation of fibrosis and an increased risk of hepatocellular carcinoma (HCC). Treatment options for AATD liver disease are even more limited than for AATD lung disease. Protein replacement therapy would not be expected to help with AATD liver disease, and is not indicated for this purpose. Liver transplant is an option for patients with end-stage liver disease (Table 45.1).

45.2 Gene Therapy for AATD

AAT has been viewed as a particularly favorable target for gene therapy, because of its secreted nature, the ease with which the therapeutic endpoint may be measured (i.e., the serum or plasma level) and the very wide therapeutic window (Table 45.2). AAT is a secreted protein, with a signal peptide suitable for secretion for any of a variety of cells, and capable of functioning properly when present in serum, regardless of the source. Thus, once the AAT gene was identified, attempts were made to develop gene therapies directed toward multiple different target tissues, including hepatocytes, pulmonary airways, pleura, monocytes, salivary glands, and muscle [21–34]. A number of different viral and non-viral vectors were developed in these efforts, including cationic liposomes, gammaretroviruses, recombinant adenoviruses, and recombinant adeno-associated virus (rAAV). Of these, the greatest promise to date has been with rAAV-based gene therapy vectors.

45.3 Concept of "Liver-Sparing" AAT Gene Therapy

At first consideration, one may presume that rAAV-based gene therapy might best be achieved by systemic (IV) injection of a rAAV vector capable of expressing high levels of wild-type PiM-AAT within hepatocytes. This can readily be achieved in mice with a normal AAT genotype, using gammaretroviruses, adenovirus, or rAAV vectors. Our group has articulated concerns regarding this approach, however. First, the variable levels of subclinical liver disease that have been described in some studies suggest that the hepatocytes of PiZ homozygotes, even those without AATD liver disease, carry a large burden of accumulated mutant Z-AAT and thus may be more susceptible to liver injury from systemic rAAV administration. Second, studies with IV rAAV8 gene therapy for hemophilia have demonstrated transaminase elevations in many patients. This appears to be primarily due to an effector T cell response to AAV capsid epitopes and may be blunted by prednisolone therapy. However, it is not clear whether such toxicity would be as well tolerated in AATD patients with Z-AAT aggregates as it is in hemophilia patients with normal livers. Finally, a simple rAAV-based M-AAT augmentation vector would have no potential for treating patients that have already manifested with AATD liver disease.

In response to this, our group has developed a number of "liver-sparing" gene therapy approaches for AATD lung disease. One such approach is the so-called "dual-function" vector approach in which a rAAV8 or rAAV9 vector is used to continuously deliver both a synthetic miRNA designed to knock down the endogenous PiZ-AAT allele and a PiM-AAT encoding augmentation allele which has been rendered resistant to degradation by the miRNA by the introduction of silent nucleotide changes. The rAAV9 dual-function construct was shown to be capable of 80% knockdown of PiZ mutant AAT in a PiZ-transgenic mouse with simultaneous, allele-specific augmentation [31].

45.4 Proof-of-Concept, Preclinical, and Clinical Studies of Muscle-Directed rAAV2-AAT Gene Therapy

The concept of using muscle-directed rAAV gene transfer for production of a secreted transfer was first developed in the mid-1990s with erythropoietin as the transgene [35]. The first proof of concept for intramuscular (IM) rAAV gene therapy for ectopic AAT secretion was performed with rAAV2 vectors expressing wild-type PiM-AAT from the cytomegalovirus immediate early promoter (CMV or CMV-IE), as compared with the elongation factor 1-alpha promoter, the U1a promoter, and the U1b promoter. The CMV promoter in these studies mediated the highest level of gene transfer [26], but subsequent studies were completed with the CBA cassette, consisting of the CMV-IE enhancer, the chicken beta actin promoter, and a hybrid intron consisting of portions of the beta actin first intron and the rabbit beta globin first intron [36]. In addition to providing evidence that a single IM injection of rAAV-AAT could mediate high-level expression of AAT for over a year, these studies demonstrated the episomal nature of rAAV and the fact that the DNA-PK pathway, important in nonhomologous end joining (NHEJ), is also involved in the formation

of stable episomal rAAV concatemers [37, 38]. Subsequently, rAAV2-AAT underwent formal preclinical studies and a phase 1 clinical trial in AAT-deficient adults [39, 40]. The phase I study showed an excellent safety profile but only short-term, low-level transgene expression [39].

45.5 Proof-of-Concept and Preclinical Studies of rAAV1-AAT Gene Therapy

The relatively low efficiency of rAAV2-AAT gene transfer in human muscle led to a broader comparison of various AAV serotype capsids [41]. These studies showed that rAAV1 capsid was the most efficient. These studies, and all subsequent ones, utilized a vector cassette with AAV2-ITRs and packaged with complementing constructs that include AAV2-Rep and AAV1-Cap genes, producing cross-packaged vectors called "AAV2/1" by some authors but abbreviated as rAAV1 here. Such constructs were also used to demonstrate that muscle-produced AAT was biochemically active in complexing and neutralizing human NE [41] and in prevention of emphysema in a mouse model [3]. Formal preclinical toxicology and distribution studies showed that rAAV1 were positive and the vector was then moved forward into clinical trials [42].

45.6 Clinical Studies of rAAV1-AAT Gene Therapy by Direct Intramuscular Injection

Clinical testing of IM rAAV1-AAT began with a phase 1 study in AAT-deficient adults at a dose range up to approximately 8×10^{11} vg/kg [39]. All patients tested demonstrated effector T cell responses to the capsid. However, all patients also demonstrated gene transfer, stable for up to 1 year after a single IM injection. Based on this, the dose escalation was continued through a phase 2a study in which the vector manufacturing process was modified to an HSV1 helper system, as compared with co-transfection-based packaging used for the phase 1 trial [43]. The phase 2a trial included dose up to 6×10^{12} vg/kg by IM injection in the high-dose cohort. Because the vector could not be concentrated any further, this amounted to an injection volume of 135 ml, accomplished by 100 IM injections, completed in a single session of injections.

Both the phase 1 and phase 2a trials demonstrated an absence of vector-related serious adverse events [39, 43–46]. Furthermore, the highest-dose IM injection group in the phase 2a trial showed a peak level of approximately 0.5 μM, 5% of the therapeutic target, and then leveling off at 2.5–3.0% of the therapeutic target level. The peak of gene expression was seen at 45 days after vector injection, and patients all demonstrated positive gamma interferon ELISPOT responses at this time point. Patients in the high-dose cohort demonstrated a rise in the creatine kinase level at this time point. Transgene expression decreased modestly but then stabilized, despite the fact that no corticosteroids or immune suppressive drugs were used.

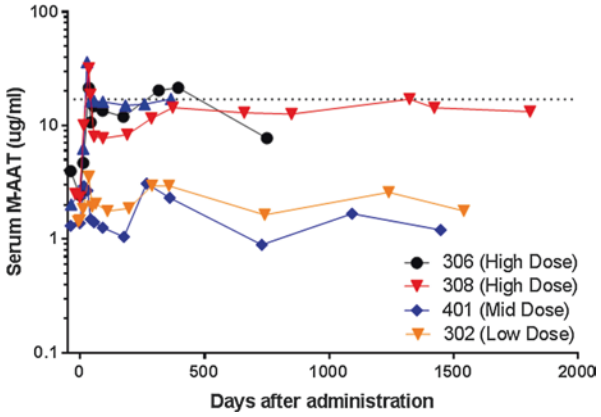

Fig. 45.2 Sustained expression of AAT for 5 years after a single IM dose in AAT-deficient patients. Low dose = 6×10^{11} vg/kg; mid-dose = 2×10^{12} vg/kg; high dose = 6×10^{12} vg/kg. Reproduced with permission from Mueller et al., *Molecular Therapy* 2017

A series of muscle biopsies were performed at 3 months, 12 months, and 5 years. At 3 and 12 months, there was very substantial immunohistochemical staining within the muscle that indicated robust gene expression was persisting at those time points, despite the fact that there were also substantial cellular infiltrates in those biopsy samples [45]. The explanation for this was provided by further studies, which indicated that infiltrating cells showed evidence of regulatory T cell markers. This was confirmed by bisulfite sequencing that showed that 10% of all T cells in the muscle were also FoxP3+, indicative of T_{reg} function. Peripheral blood T cell studies showed AAV1 capsid epitope-specific T_{reg} cells were present in these patients, and next-generation sequencing demonstrated identity between certain peripheral blood T cell clones and those persisting within the muscle. This strongly suggested that AAV1 capsid-specific T_{reg} cells were induced by IM rAAV1-AAT, resulting in functional tolerance to rAAV1-hAAT-transduced cells.

Patients in the phase 2a study were further characterized in a 5-year follow-up study, including assays for PiM-specific serum AAT levels, which showed persistence of levels from 2.5–3% of the therapeutic target (Fig. 45.2) [46]. In addition, muscle biopsy samples showed persistence of AAT-expressing myofibers and T_{reg} cell infiltrates. Interestingly, these patients showed partial correction of functional biomarkers of AAT expression as well, including an increase in antineutrophil elastase capacity and a decrease in markers of neutrophil degranulation [46].

45.7 Future Directions of Muscle-Directed AAT Gene Therapy

In order to continue the dose escalation of muscle-directed AAT gene therapy, the injection methodology has been transitioned to isolated limb perfusion. Previous investigations have demonstrated that either venous or arterial infusion of rAAV vectors can mediate widespread gene transfer to muscle tissue (Table 45.3) [57].

Table 45.3 A summary of methods used in limb infusion studies delivering rAAV to large animal models

	Femoral artery delivery				Peripheral venous delivery			
Study	Arruda et al. [47]	Rodino-Klapac et al. [48]	Rodino-Klapac et al. [49]	Chicoine et al. [50, 51]	Su et al. [52]	Toromanoff et al. [53, 54]	Arruda et al. [55]	Le Guiner et al. [56]
Species (weight)	Canine (12–22.5 kg)	Cynomolgus macaque (4–5 kg)	Rhesus macaque (4–8 kg)	Rhesus macaque	Canine (5–11 kg)	Cynomolgus macaque (3–5 kg)	Canine (8.7–24 kg)	Canine (8.7–24 kg)
Route	Infuse, dwell, flush—ipsilateral	Infuse and dwell—ipsilateral—gastric only	Infuse and dwell—ipsilateral	Infuse and dwell—ipsilateral—gastric only	Hydrodynamic (ATVRX)	Hydrodynamic	Hydrodynamic (ATVRX)	Hydrodynamic
Vector gene	LacZ and FIX	CMV eGFP	Microdystrophin	CMV eGFP or MCK.GALGT2	CMV lacZ	Human LEA29Y, cmEpo	cFIX	U7snRNA-E6/E8
Dose vector	1.7 × 10e12 to 3 × 10e12 vg/kg	2 × 10e12 vg/kg	2 × 10e12 vg/kg	2 × 10e12 vg/kg	1 × 12e14 gc	5 × 10e12 vg/kg	3 × 10e12 vg/kg	1 × 10e13 to 5 × 10e13 vg/kg
Serotype	AAV2	AAV8	AAV8	AAVrh.74	rAAV1	rAAV 1 ≥ rAAV8	AAV2 and AAV6	AAV8
Volume vector	2.5 ml/kg PBS followed by 10 ml/kg PBS	2 ml PBS over 60 s	2.5 ml/kg	2.5 ml/kg	500 ml PBS	50 ml/kg	20 ml/kg	12 ml/kg at 300 mmHg or 6–7 ml/kg at 10 or 35 ml/min
Vector dwell time	15–20 min	10 min	10 min	10 min	20 min	15 min	15 min	15 min

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In many of these studies, some method was used to enhance extravasation of the vector, either by co-administration of papaverine or histamine to endothelial tight junctions or by a high-pressure (regional hydrodynamic) infusion. In several cases these methods have been developed for use in patients with muscular dystrophies. It is hoped that in the future muscle-directed gene therapy with AAV can advance in parallel between primary muscle diseases and serum protein deficiencies, like AATD, which capitalize on the large mass of skeletal muscle as a site for production of large quantities of a secreted protein. Ideally, each approach can benefit from the lessons learned with the other, so that the use of muscle-directed therapy can be maximized in the creation of new therapies for both classes of diseases in future years.

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