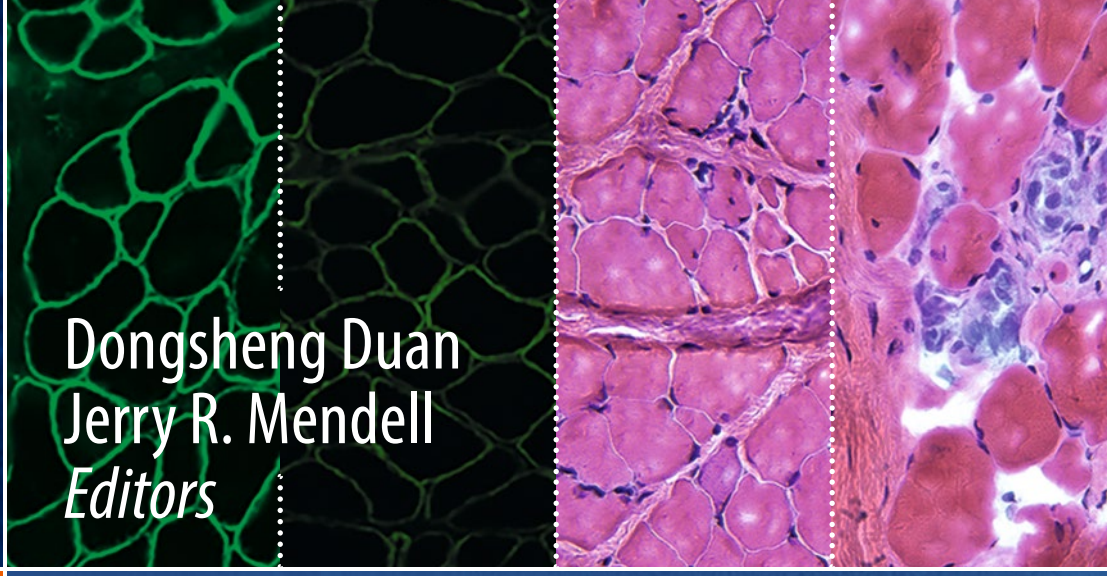

Dongsheng Duan
Jerry R. Mendell
Editors

Muscle Gene Therapy

Second Edition

Springer

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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

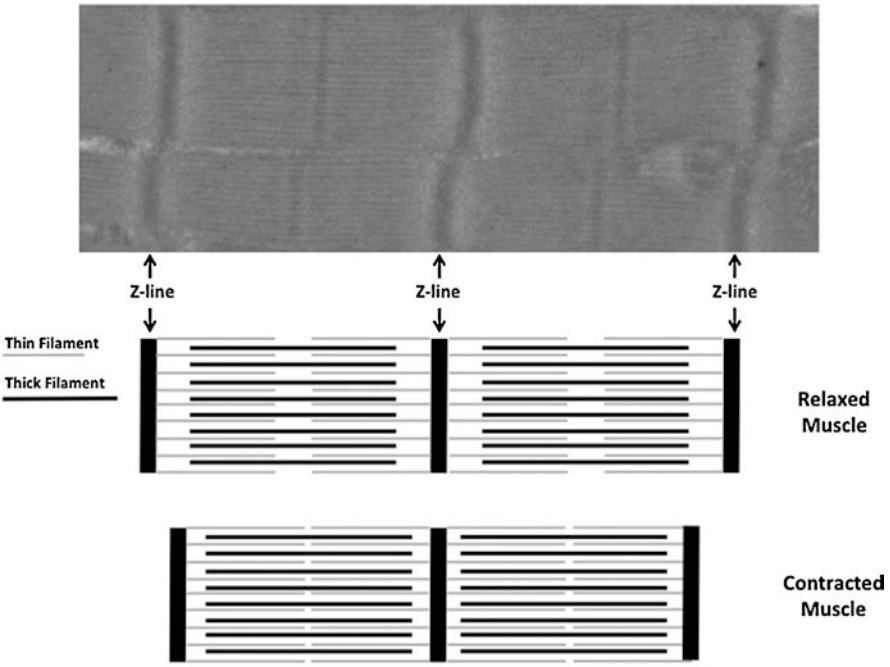

Fig. 1.1 Top: representative electron microscopic photograph of sarcomeres. Middle: arrangement of the thick and thin filaments in a sarcomere of a muscle in the relaxed state. Bottom: arrangement of the myofilaments in a contracting muscle. Photograph courtesy of Dr. Maegen Ackermann

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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