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Systems Biology of Skeletal Muscle: Fiber Type as an Organizing Principle

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Abstract

Skeletal muscle force generation and contraction are fundamental to countless aspects of human life. The complexity of skeletal muscle physiology is simplified by fiber type classification where differences are observed from neuromuscular transmission to release of intracellular Ca^{2+} from the sarcoplasmic reticulum and the resulting recruitment and cycling of cross-bridges. This review uses fiber type classification as an organizing and simplifying principle to explore the complex interactions between the major proteins involved in muscle force generation and contraction.

Skeletal muscle comprises a variety of interacting proteins, and understanding their functional roles is well-suited for a systems biology approach. As an organizing principle for this review, we will group sarcomeric proteins based on their functional roles. The usefulness of functional categorization is best illustrated by fiber type classification, which was initially based on differences in mechanical and fatigue properties of muscle fibers, but was later correlated with the expression of muscle contractile proteins, e.g., myosin heavy chain (MyHC) isoforms. Although overt fiber type distinctions are not as apparent for proteins related to membrane excitability, excitation-contraction coupling, and structural support and force transmission, differences do emerge based on fiber type comparisons. Thus, fiber type classification is a very useful organizing principle for a systems biology approach to simplify the complexity of muscle protein expression.

FIBER TYPE CLASSIFICATION

Muscle fiber type classification schemes have progressed over the years. The original distinction of fiber type was based on color, red (high myoglobin) and white (less myoglobin), and correlated with speed of contraction (slow vs. fast, respectively) and fatigability (fatigue resistance vs. fatigable, respectively). This advanced to different classification schemes: one based on contractile properties and histologic staining for metabolic enzyme activities - slow oxidative (SO), fast oxidative, glycolytic (FOG) and fast, glycolytic (FG) ¹; and a second based on the pH lability of actomyosin ATPase staining, with fibers classified as type I, IIa, IIb, or IIx. Corresponding to this latter classification scheme, fibers were found to be primarily composed of a single MyHC isoform, and specific

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antibodies were developed to identify the expression of different MyHC isoforms. Today, fiber type classification is primarily based on immunoreactivity to antibodies specific for different MyHC isoforms, and these antibodies can also be used in Western blot analysis to determine the MyHC isoform composition and content of individually dissected muscle fibers. In general, muscle fibers classified histochemically as type I, IIa, IIb, and IIx are composed of MyHC_{slow}, MyHC_{2A}, MyHC_{2B}, and MyHC_{2X} isoforms, respectively². Type IIb fibers in some muscles are an exception as these fibers often co-express MyHC_{2X} together with MyHC_{2B}^{3,4}.

In addition to the four adult MyHC isoforms, there are also embryonic (MyHC_{emb}) and neonatal (MyHC_{neo}) MyHC isoforms that are usually found in skeletal muscle fibers during embryonic and early postnatal development. During early myogenesis, primary myotubes express MyHC_{slow} in combination with the MyHC_{emb} isoform⁵. With the progression from primary to secondary myotubes, expression of MyHC_{neo} isoform appears and continues through the early stage of postnatal development as adult fiber types mature. Although muscle fibers singularly expressing MyHC_{slow} (type I fibers) are present during early postnatal development, singular expression of other MyHC isoforms appears only later in postnatal development⁶. Thus, distinction fiber type classification is limited to more mature muscle fibers. Pathophysiological conditions are also associated with MyHC co-expression. For example, MyHC isoform co-expression in single muscle fibers has been reported in conditions of hypothyroidism⁷, aging⁸, and neurodegenerative diseases such as spinal muscular atrophy⁹ and amyotrophic lateral sclerosis¹⁰. The presence of MyHC isoform co-expression may reflect fiber type conversion, although clear evidence for such conversion is lacking. It is clear however, that whenever MyHC isoform co-expression occurs, it blurs the unambiguous distinction of muscle fiber type.

Motor unit types – influence of innervation

There is substantial evidence that the central nervous system exerts a predominant influence over fiber type (MyHC isoform expression) in skeletal muscle. This is evident by the homogenous fiber type composition of motor units (Figure 1). Within a motor unit, a motor neuron action potential activates the group of muscle fibers it innervates in an all-or-none fashion¹¹⁻¹³. Motor units are classified into slow- (type S) or fast-twitch units, based on the mechanical properties of the motor unit muscle fibers¹². Fast-twitch motor units are further classified based on their fatigability as fast-twitch fatigue resistant (type FR), fast-twitch fatigue intermediate (type FInt), and fast-twitch fatigable (type FF). This classification of motor unit types parallels the classification of type IIa, IIx and IIb muscle fibers, respectively (Figure 1)^{14,15}. It remains controversial whether activation history or neurotrophic factors are responsible for the predominant influence of innervation on fiber type and MyHC isoform expression. In studies where the diaphragm muscle was inactivated for a prolonged period of time by cervical spinal cord hemisection, fiber type composition and singular MyHC isoform expression in fibers was unaffected^{16,17}. In contrast, when neural influence was removed by denervation, MyHC isoform co-expression occurs in diaphragm muscle fibers obviating unambiguous fiber type distinctions¹⁶⁻¹⁸. Neuregulin is a potential nerve-derived neurotropic factor that may influence contractile protein expression in muscle fibers^{19,20}.

SARCOMERIC PROTEINS

The organization of skeletal muscle from the whole muscle level down to individual sarcomeric proteins is crucial to the understanding of how muscle force is generated (Figure 2). Myofibrils are grouped into fiber bundles that align all the sarcomeres in series to facilitate force generation. Each sarcomere forms a highly organized unit of thick (myosin) and thin (actin) filaments representing the contractile apparatus of the skeletal muscle and

giving skeletal (and cardiac) muscle a striated appearance²¹. Sarcomeres are anchored and connected to neighboring sarcomeres through the Z-disc, which runs perpendicular to the filaments creating a structural and mechanical functional unit that allows for lateral and longitudinal transmission of force and contraction. Within each sarcomere, the elemental unit of force generation is the cross-bridge, formed by the binding of myosin to actin.

Sarcomere structure

Each sarcomere is composed of interdigitating thick and thin filaments, with dimensions of ~1 μm in diameter and ~2.5 μm in length (Figure 2). The overlap between thick and thin filaments is important in determining the number of cross-bridges that can form during muscle activation. The thick filament has a fixed length of ~1.6 μm , while the length of thin filaments ranges between 1.0 – 1.3 μm , with the length dependent upon both the species and muscle type²². During muscle fiber contraction both the thick and thin filaments maintain their intrinsic lengths. However, with cross-bridge formation actin filaments are pulled toward the midline of the sarcomere, thus causing the overlap between thick and thin filaments to increase (Figure 3). The force-length relationship of a muscle fiber reflects the overlap between thick and thin filaments within sarcomeres and the number of bound cross-bridges that contribute to force generation.

Myofilament lattice spacing—There is a fixed structural organization to the sarcomere based on the known stoichiometry between thick and thin filaments (myosin and actin proteins). A crystalline structure of thick and thin filaments is evident through electron microscopy and X-ray diffraction²³ of cross sections of muscle fibers (Figure 2). Six thin (actin) filaments surround each thick (myosin) filament, which is further surrounded by six myosin filaments with associated actin filaments. Thus, each actin filament is surrounded by three myosin filaments, and two actin filaments are shared by any given myosin filament. This arrangement creates the double hexagonal array of the myofilament lattice. The spacing between thick and thin filaments in this lattice largely determines the relative myosin and actin protein content within a muscle fiber (myosin 43-50% and actin 18-22% of total protein)^{24,25}. Since the sarcolemma (muscle fiber membrane) encompasses myofibrils in a relatively constant volume, osmolarity shifts within the muscle due to changes in ion concentrations across the membrane can cause osmotic compression or expansion of the lattice, and thus change the distance between thick and thin filaments. Accordingly, lattice spacing is decreased (compressed) under hypertonic conditions, and increased (expanded) during hypotonic conditions. Changes in myofilament lattice spacing can affect cross-bridge attachment and thus, force generation²³.

Thick filament proteins

The thick filament is primarily composed of the myosin protein (specifically myosin II), which is a hexameric protein comprising two MyHCs (~220 kDa), two essential myosin light chains (MyLC₁₇ - 17 kDa) and two regulatory myosin light chains (MyLC₂₀ - 20 kDa). The arrangement of the myosin molecule is such that the tail portion is positioned toward the midline of the sarcomere and the heads are directed outward to allow binding to actin. The myosin heads are positioned in a spiral around the thick filament to align with the position of the actin filaments.

The N-terminal domain of MyHC includes regions related to both the enzymatic (ATPase) and contractile (cross-bridge attachment) functions of myosin. The two MyHCs form a double helix by wrapping around each other, creating the tail region of myosin. A globular head region is located at the end of the N-terminal domain that forms the actin-binding site for cross-bridge formation.

The MyLC₁₇ primarily plays a structural role stabilizing the myosin head; thereby enhancing cross-bridge formation and cycling. Several MyLC₁₇ isoforms exist but their specific associations with different muscle fiber types are not clear at the present time. For example, two MyLC₁₇ isoforms are expressed in fast-twitch (type II) fibers, but how the expression of these two isoforms relates to the mechanical properties of type II fibers is unknown. In skeletal muscle fibers, Ca²⁺-calmodulin dependent myosin light chain kinase (MLCK) mediates phosphorylation of the serine-19 residue of MyLC₂₀. The effect of MyLC₂₀ phosphorylation in skeletal muscle fibers remains controversial, but it appears to increase cross-bridge cycling rate along with ATP consumption rate and may also affect Ca²⁺ sensitivity of force generation²⁶. These effects are most likely the result of an effect of MyLC₂₀ phosphorylation on the position of the myosin head (moving it away from the backbone of the myosin filament) in relation to the actin filament. Several MyLC₂₀ isoforms also exist but there appears to be no distinct relationship between the expression of a given MyLC₂₀ isoform with muscle fiber type nor the mechanical performance of muscle fibers.

Additional proteins associated with the thick filament help to maintain the alignment of sarcomeres in parallel within and across myofibrils. The M-line is a key landmark in the midline of the thick filament, and is composed of two proteins, myomesin and M-protein, that serve to anchor titin to myosin, and thus help stabilize and align adjacent sarcomeres. Myomesin is present in both slow- and fast-twitch muscle fibers, but M-protein is only found in fast-twitch fibers. Three isoforms of myomesin exist: one isoform (myomesin-1) exists in all fiber types, myomesin-2 is expressed only in type IIb fibers, and myomesin-3 is expressed in type IIa and IIx fibers. Titin is the largest known protein (3.2 to 3.8 MDa) and spans the sarcomere from the Z-disk to the M-line and is important in stabilizing the thick filament. Titin may also function to sense the strain on the thick filament and trigger downstream signaling pathways involved in maintaining the sarcomere^{27, 28}. Several isoforms of titin exist due to alternative splicing²⁹. Generally longer titin isoforms are expressed in slow fibers, and it has been suggested that this might impart differences in passive stiffness across muscle fiber types that could affect contractile performance. Myosin binding protein C is also located at regular intervals along the length of the thick filament protein and has binding sites for the myosin rod and titin. It is thought to be involved in assembling the backbone of the thick filament. Two isoforms of myosin binding protein C exist in skeletal muscle, associated with slow and fast fibers.

Thin filament proteins

The major contractile protein of the thin filament is α -actin, a globular protein (40 kDa) that contains the binding sites for myosin to form cross-bridges. Although different isoforms of α -actin exist, their relationship to fiber type or mechanical performance remains unclear. Globular actin monomers (G-actin) assemble into helical double stranded chain of filamentous actin (F-actin), which forms two strands of an alpha helix. Between six and seven globular actin monomers are attached to tropomyosin (TM), which in turn is associated with a troponin complex that together regulate exposure of the myosin binding site on actin. The thin filament also includes the nebulin protein, which is a large protein (0.6 to 0.9 MDa) that spans the length of the thin filament and is thought to regulate actin filament length³⁰. It has been suggested that variants in both nebulin and titin may affect passive and active mechanical properties of muscle fibers.

EXCITATION-CONTRACTION COUPLING

Excitation of a muscle fiber is initiated by a nerve action potential and is transmitted through the neuromuscular junction. Once an action potential is initiated in a muscle fiber, it is propagated along the sarcolemma and eventually depolarization is passively transmitted down deep transverse invaginations of the plasma membrane known as transverse tubules (t-

tubule). This depolarization is coupled to Ca^{2+} release from the sarcoplasmic reticulum via activation of dihydropyridine receptor (DHPR) channels at the triad junction. The release of Ca^{2+} from the sarcoplasmic reticulum triggers cross-bridge formation through an interaction with the troponin/TM complex on the thin filament.

Neuromuscular junction

The neuromuscular junction (NMJ) is the synapse between a motor neuron and the group of muscle fibers it innervates (Figure 4), which together constitute a motor unit. The NMJ comprises pre- and postsynaptic elements. At the presynaptic terminal of a motor neuron, synaptic vesicles contain acetylcholine (ACh) as a neurotransmitter to effect neuromuscular transmission. Synaptic vesicles dock with the presynaptic terminal membrane at specialized regions called active zones. The number of docked vesicles at each active zone is comparable across fiber types³¹. However, the total surface area of the presynaptic terminal is smaller at type I and IIa fibers compared to type IIx and IIb fibers. Thus, the total number of active zones at presynaptic terminals is greater at type IIx and IIb fibers, and accordingly, the total number of synaptic vesicles released in response to a nerve action potential (quantal content) is greater at these fibers compared to type I and IIa fibers³¹.

The motor end-plate is a specialized region of postsynaptic muscle fiber membrane that contains nicotinic cholinergic receptors that are activated by presynaptic release of ACh. To increase the surface area, there is both branching and postsynaptic folding at the motor end-plates. This postsynaptic branching and folding is far more complex at type IIx and IIb fibers compared to type I and IIa fibers³². The motor end-plates at type I and IIa fibers are further distinguished by the imposition of mitochondria, rough endoplasmic reticulum, and free polysomes between the postsynaptic specializations and myofibrils³². There are no apparent differences in the density of postsynaptic cholinergic receptors across fiber types; however, given the larger surface area of the postsynaptic membrane at type IIx and IIb fibers, the total number of cholinergic receptors is greater at these fibers. Accordingly, end-plate potential responses to a nerve action potential-induced release of ACh is greater at type IIx and IIb fibers. In addition, the density of voltage-gated Na^+ channels adjacent to the motor end-plate is higher at type IIx and IIb fibers than at type I and IIa fibers³³. Thus, the membrane depolarization required to initiate a muscle fiber action potential (threshold) is lower at type IIx and IIb fibers³⁴. Together, the greater end-plate potential response and lower threshold account for a higher safety factor for neuromuscular transmission at type IIx and IIb fibers. However, with repetitive stimulation, the amplitude of the evoked end-plate potential response decreases markedly in type IIx and IIb fibers, and with it the safety factor for neuromuscular transmission³⁴. Accordingly, these fibers are much more susceptible to neuromuscular transmission failure during repetitive stimulations compared to type I and IIa fibers³⁵.

T-tubule and triad junction

An action potential generated at the NMJ is propagated down the muscle fiber membrane (sarcolemma) by the activation of voltage-gated Na^+ channels. There does not appear to be any fiber type differences in the propagation of muscle fiber action potentials. The t-tubule membrane contains a lower density of voltage-gated Na^+ channels; thus, depolarization is passively conducted down the t-tubules where it activates DHPR channels, which are specialized voltage-sensing Ca^{2+} channels. In skeletal muscle fibers, it is unlikely that significant Ca^{2+} influx occurs when DHPR channels are activated. Instead, the DHPR channels allosterically interact with ryanodine receptor (RyR) channels located in the terminal cisternae of the sarcoplasmic reticulum that directly abut the t-tubule at the triad junction. During depolarization of the t-tubule, DHPR channels undergo a conformational change that is mechanically linked to the opening of RyR channels at the triad junction

allowing Ca^{2+} release from the sarcoplasmic reticulum. There is no evidence that there are fiber type differences in excitation-contraction coupling at the level of the t-tubule and triad junction.

Calcium release from the sarcoplasmic reticulum

The sarcoplasmic reticulum is the main storage site for Ca^{2+} within muscle fibers, and Ca^{2+} release is mediated through RyR channels. The RyR channel is a very large (2.3 MDa) tetrameric protein (565 kDa per subunit) that has three subtypes. The subtype predominantly found in skeletal muscle fibers is the RyR1 channel. The open probability of RyR1 channels is sensitive to the myoplasmic Ca^{2+} concentration. Accordingly, after the initial release of Ca^{2+} through RyR1 channels at the triad junction the open probability of nearby RyR1 channels increases, thus triggering additional Ca^{2+} release from the sarcoplasmic reticulum. The positive feedback that results from the Ca^{2+} sensitivity of RyR1 channel opening is termed Ca^{2+} -induced Ca^{2+} release and is important for rapidly flooding the myoplasmic space with Ca^{2+} (Figure 5). The Ca^{2+} flux through the RyR1 channel is modulated by a 12 kDa immunophilin FK506-binding protein (FKBP12). The FKBP12 protein is an integral component of the RyR1 channel and appears to mediate coupled gating of adjacent RyR1 channels, thus coordinating simultaneous opening and closing of adjacent RyR1 channels. Specifically, FKBP12 appears to be particularly important in stabilizing the closed state of the RYR1 channel. It has been shown that when permeabilized skeletal muscle fibers are exposed to the drug FK506, FKBP12 is dissociated from the RyR1 channel, and Ca^{2+} release is blocked. Calmodulin (CaM), a Ca^{2+} -sensing protein, also binds to the RyR1 channel with high affinity and modulates channel open probability in response to myoplasmic Ca^{2+} concentration.

The increase in myoplasmic Ca^{2+} is only transient as basal levels are restored by a rapid re-uptake of Ca^{2+} into the sarcoplasmic reticulum via a sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) pump. There are three specific SERCA genes with up to 10 distinct isoforms related to alternative splicing of each gene product. These SERCA isoforms display differences in the rate of Ca^{2+} pumping and ATPase activity that would affect myoplasmic Ca^{2+} transients (Figure 5) and mechanical responses³⁶. The sarcoplasmic reticulum has a much higher concentration of Ca^{2+} compared to the myoplasm; therefore, SERCA must pump Ca^{2+} against a high concentration gradient, thereby requiring energy in the form of ATP hydrolysis. Calsequestrin, another protein within the sarcoplasmic reticulum, binds Ca^{2+} thereby reducing the concentration of free Ca^{2+} . Calsequestrin has multiple binding sites for Ca^{2+} but binds with a very low affinity so Ca^{2+} is easily unbound. The binding of Ca^{2+} to calsequestrin within the sarcoplasmic reticulum serves to reduce the Ca^{2+} concentration gradient against which SERCA must pump Ca^{2+} while assuring a higher free Ca^{2+} concentration during release through RyR channels. The rate of SERCA pumping Ca^{2+} into the sarcoplasmic reticulum is also controlled by calmodulin kinase II (CaM K II) dependent phosphorylation of the protein phospholamban (PLB). It appears that little if any PLB is associated with SERCA in type I fibers, which may also underlie fiber type dependent differences in the Ca^{2+} transient induced by stimulation and the rate of Ca^{2+} clearance from the myoplasm³⁷.

Thin filament regulation of cross-bridge formation

The binding of myosin heads to actin to form cross-bridges is regulated by the interaction between Ca^{2+} released from the sarcoplasmic reticulum and the troponin/TM complex on the thin filament (Figure 6). There are three components of the troponin complex: troponin C (TnC – “C” for Ca^{2+}); troponin I (TnI – “I” for inhibition); and troponin T (TnT – “T” for tropomyosin). At low Ca^{2+} concentrations (resting muscle), TM, which is a rod shaped protein that aligns head to tail along the actin filament, is positioned such that it sterically

blocks the actin-binding site for myosin. With an increase in myoplasmic Ca^{2+} concentration, Ca^{2+} binds to TnC causing movement of TM, through an interaction with TnT, which is thought to be the connector for the troponin-tropomyosin-actin complex, since it binds to TnI, TnC, TM, and actin. As a result of the movement of TM, the actin-binding site for myosin is exposed allowing cross-bridge formation. In addition, Ca^{2+} binding to TnC displaces TnI, which is also in a position that inhibits the myosin binding site on actin.

There are two isoforms of TnC: the cTnC ("c" for cardiac) isoform is expressed in cardiac and slow skeletal muscle fibers, whereas sTnC ("s" for skeletal) is expressed only in fast muscle fibers. The TnC molecule has two active binding sites for Ca^{2+} on its carboxy-terminal region and the amino-terminal region contains either one (cTnC isoform) or two (sTnC isoform) active binding sites for Ca^{2+} ³⁸. This difference in the number of active Ca^{2+} binding on TnC sites affects the binding affinity for Ca^{2+} and thus, the sensitivity of the force- Ca^{2+} relationship (Figure 5). The amount of force that is generated depends on myoplasmic Ca^{2+} concentration and the Ca^{2+} sensitivity of thin filament regulation of cross-bridge formation. The force- Ca^{2+} relationship is the representation of force and the rate of force development by free Ca^{2+} concentration. The sensitivity of this relationship is commonly represented as the myoplasmic Ca^{2+} concentration at which 50% of maximal force is generated (pCa_{50}) which is an index of the myofibrillar affinity to Ca^{2+} . Several studies have shown that pCa_{50} is lower (greater Ca^{2+} sensitivity) in type I muscle fibers compared to all type II fibers³⁹. This fiber type difference in pCa_{50} relates to differences in Ca^{2+} binding affinity between cTnC and sTnC isoforms.

It has been reported that more than eight "fast" types and two "slow" types of TnT isoforms are expressed in skeletal muscle. However, it is unclear how these different TnT isoforms are expressed relative to specific fiber types; however the slow TnT isoforms are expressed in type I fibers, while fast TnT isoforms are expressed in type II fibers. Three different TM isoforms are also expressed in skeletal muscle fibers: the TM- α -slow isoform is expressed in type I fibers the TM- α -fast isoform is expressed in type IIb fibers; and a third TM- β isoform is expressed in type IIa and IIx fibers².

FORCE GENERATION AND CONTRACTION

The main functions of muscle fibers are force generation and contraction (change in muscle fiber length). The cross-bridge is the essential unit of contractile function (force generation and shortening), and is formed by the binding of the myosin head to actin (Figure 7), after which the actin filament is pulled toward the midline of the sarcomere (Figure 2)⁴⁰. The force generated by a muscle fiber depends on the number of cross-bridges formed in parallel per half sarcomere; thus the relationship of muscle force to fiber cross-sectional area. For this reason, muscle force is often normalized by cross-sectional area (specific force). It is also important to understand how the force generated by individual sarcomeres is transmitted to move entire limbs or objects.

Cross-bridge formation and cycling

The observation of an increase in the overlap between thick and thin filaments lead to the sliding filament theory of muscle contraction as proposed by Andrew Huxley⁴¹. In his simple two-state model of muscle contraction, cross-bridges cycle between two functional states: 1) an attached force-generating state in which myosin is strongly bound to actin; or 2) a detached non-force-generating state. More complex cross-bridge cycling models have been introduced that include a number of intermediate states including a power-stroke phase after the myosin head binds to actin, during which there is bending at the junction between the

head and neck regions of myosin, leading to force generation and pulling of the thin filament to the midline of the sarcomere (Figures 6 & 7).

In the simplified two-state model of cross-bridge cycling, force generation depends on: 1) the total number of myosin heads (n) in parallel per half sarcomere volume of a muscle fiber; 2) the average force per cross-bridge (F); and 3) the fraction of these myosin heads that are strongly bound to actin forming cross-bridges (α_{fs}). This mathematical model is illustrated in Figure 7.

Myosin heads per half sarcomere (n)—In single muscle fibers, the number of myosin heads per half sarcomere (n) can be estimated. Based on the length of the thick filament and measurements of the distance between myosin heads, it can be determined that each myosin filament has approximately 300 myosin heads. For a muscle fiber that has a cross-sectional area of $1,500 \mu\text{m}^2$, there would be approximately 1 million myosin filaments and 300 million myosin heads. MyHC content per half sarcomere can also be determined based on protein extraction from single muscle fiber segments of known length, and quantification of electrophoretically separated MyHC bands to determine protein concentration (Figure 8)⁴². In this technique, the cross-sectional area and the number of sarcomeres in series of the muscle fiber segment are determined before protein extraction to determine half sarcomere volume. Thus, the MyHC content (number of myosin heads) per half sarcomere volume (n in Figure 7) was estimated to be ~ 400 myosin heads per μm^3 half sarcomere volume. According to this model, when muscle fiber cross-sectional area increases (hypertrophy), there is an increase in the number of sarcomeres and myosin heads in parallel. Thus, if α_{fs} remains the same, the total number of cross-bridges that form and contribute to force generation increases. Based on results from single muscle fiber studies, type IIX and IIb fibers (which are larger) have higher MyHC content per half sarcomere compared to type IIA fibers with type I fibers (which are smaller) having the lowest MyHC content⁴².

Force per cross-bridge (F)—In single permeabilized muscle fibers during maximum Ca^{2+} activation at 37°C , the specific force (force per cross-sectional area) of a muscle fiber is $\sim 30 \text{ N per cm}^2$. Based on the estimated number of myosin heads per half sarcomere (n), the calculated force per myosin head (cross-bridge; F in Figure 7) is $\sim 0.5 \text{ pN}$. However, the estimated force per cross-bridge is $\sim 45\%$ lower in type I fibers compared to all fast fiber types^{3, 39, 42}. The molecular basis for this fiber type difference in force per cross-bridge is unclear.

Fraction of myosin heads forming cross-bridges (α_{fs})—Regulation of α_{fs} is important in the regulation of force generation, and α_{fs} can be estimated by measuring muscle fiber stiffness⁴². As the number of cross-bridges formed increases, longitudinal stiffness of the muscle fiber also increases. This can be measured in individual muscle fibers by imposing sinusoidal, high frequency (2 kHz), small amplitude length oscillation of 0.01% of optimal muscle length (L_o) and then measuring the resulting recoil force (stiffness as defined by the slope of the force-length relationship). The small amplitude length change does not disrupt cross-bridge binding, and the frequency of length oscillation exceeds the cross-bridge cycling rate, minimizing hysteresis. Thus, muscle fiber stiffness reflects the number of strongly bound cross-bridges under any condition of muscle fiber activation. Typically muscle fiber stiffness is normalized to a maximum stiffness induced by exposing permeabilized fibers to a solution that contains a Ca^{2+} concentration but does not contain ATP, thus creating a rigor condition in which cross-bridges are strongly bound. Using this technique, it has been estimated that $\sim 75\text{--}80\%$ of myosin heads are strongly bound to actin to form cross-bridges during maximum Ca^{2+} activation. There are no fiber type differences in α_{fs} during maximum or submaximal Ca^{2+} activation^{3, 39, 42}.

Muscle fiber weakness—Under a variety of conditions, specific force (force per muscle fiber cross-sectional area) is reduced. For example, specific force is lower at both ends of the age spectrum – during early postnatal development³ and during old age⁸. Similarly, specific force is reduced following muscle denervation⁴³ and as a result of an altered hormonal environment such as hypothyroidism⁷. In these conditions, the simplified two-state model of force generation provides a conceptual framework to assess possible underlying mechanisms for muscle weakness. For example, muscle fiber weakness induced by denervation is associated with a decrease in MyHC content per half sarcomere (decreased n in the equation) and a decrease in the estimated force per cross-bridge⁴³. However, α_{fs} is not affected by denervation. Similarly, hypothyroidism induces a decrease in MyHC content per half sarcomere but no change in the average force per cross-bridge or α_{fs} . These functional results point to the importance of assessing the balance between MyHC protein synthesis and degradation under these conditions.

Cross-bridge cycling and ATP consumption—Cross-bridge cycling is dependent on ATP hydrolysis and cross-bridge detachment (Figure 6). The myosin molecule is an ATPase that subserves a molecular motor function in skeletal muscle fibers converting the chemical energy of ATP into the mechanical energy of force and movement. Based on the simple two-state model of force generation introduced by Huxley, an analytical framework to explain the chemomechanical transduction of ATP to force was introduced by Brenner and Eisenberg^{44, 45}. In this framework, the cycling of cross-bridges is described by two apparent rate constants: one for cross-bridge attachment (f_{app}), and one for cross-bridge detachment (g_{app}) during which ATP is hydrolysed. If it is assumed that one ATP molecule is hydrolysed per cross-bridge cycle, total ATP consumption during muscle fiber force generation is dependent on: 1) the total number of myosin heads (n) in parallel per half sarcomere volume of a muscle fiber; 2) the number of half sarcomeres in series within the muscle fiber (b); 3) the fraction (α_{fs}) of these myosin heads that are strongly bound to actin forming cross-bridges; and 4) the apparent detachment rate constant (g_{app}) (Figure 7).

Cross-bridge cycling rate can be estimated by estimating the overall rate constant for force redevelopment (k_{tr}), which is the summation of f_{app} and g_{app} (Figure 7). In one experimental approach, single muscle fibers are activated at L_0 . Muscle length is then rapidly decreased by ~20% of L_0 during which all cross-bridges are broken and force decreases to zero. The muscle fiber is then rapidly restretched to L_0 , and force redevelops as cross-bridges reattach to actin. The rate constant for force redevelopment (k_{tr}) can then be calculated. In another experimental approach, a permeabilized muscle fiber can be rapidly exposed to either a high concentration of Ca^{2+} or ATP by photolytic release of a “caged” Ca^{2+} or ATP. In the case of photolytic release of caged Ca^{2+} cross-bridges will form and force will develop. Using photolytic release of caged ATP in the presence of high Ca^{2+} concentration, muscle fibers will transition from a state of rigor to cross-bridge cycling and ATP consumption, and thus the g_{app} estimated.

The rate of ATP consumption of single permeabilized muscle fibers can be measured based on extinction of NADH fluorescence using a stop flow technique (Figure 8)⁴⁶. In this technique, ATP hydrolysis during cross-bridge cycling is coupled to ATP regeneration via an enzymatic reaction that leads to the reduction of NADH (fluorescent) to NAD (non-fluorescent). The rate of NADH fluorescence extinction is linearly related to ATP consumption rate in the permeabilized muscle fiber. Experimentally, it was shown that the rate of ATP consumption in muscle fibers varies with the total number of myosin heads (n) in parallel per half sarcomere, the fraction of myosin heads forming cross-bridges (α_{fs}), and the apparent rate constant for cross-bridge detachment (g_{app}). For example, isometric ATP consumption rate decreases following denervation as the number of myosin heads (n) in parallel per half sarcomere decreases⁴⁷. As predicted by the Fenn effect^{48, 49}, the maximum

ATP consumption rate of a muscle fiber was found to correspond to peak power output of a muscle fiber.

Muscle fiber shortening velocity is dependent on the load against which the muscle fiber is contracting and is associated with the rate of cross-bridge cycling (g_{app}). The force (load)-velocity curve defines this relationship (Figure 8). The slack test is used to determine the maximum unloaded shortening velocity (V_o) of a muscle fiber. In this test, permeabilized muscle fibers are maximally activated with Ca^{2+} at optimal length (L_0), and fiber length is shortened systematically in steps ranging from 4-12% of L_0 . The shortening of fiber length results in an unloading (or “slack”) of the fiber during which force drops to zero. The time required to redevelop force is measured and related to slack length to determine V_o . Using this test, V_o is fastest in type IIx and IIb fibers and slowest in type I fibers⁵⁰. At maximum load, muscle fibers do not contract, and at L_0 this defines the maximum isometric force (F_{max}) of a muscle fiber, which also varies across fiber types (see above).

The velocity of shortening of a muscle fiber increases against submaximal loads (reflecting an increasing g_{app}) while force decreases (due to a decrease in the fraction of myosin heads forming cross-bridges - α_{fs}) (Figure 8). Power output of a muscle fiber is determined by the product of force and velocity, and peak power output occurs at ~33% of both V_o and F_{max} (Figure 8). Maximum ATP consumption rate within a muscle fiber occurs at this peak power output reflecting a balance between the effects of an increase in g_{app} and a decrease in α_{fs} . There are fiber type differences in maximum ATP consumption rate at peak power output, and these differences reflect not only differences in the number of cross-bridges consuming ATP but also cross-bridge cycling rate (g_{app}) reflecting the myosin ATPase activity of the specific MyHC isoforms – $MyHC_{2B} > MyHC_{2X} > MyHC_{2A} > MyHC_{slow}$ ^{50, 51}.

Force transmission

Force is generated by cross-bridge formation and transmitted longitudinally through the attachment of thin filaments to the Z-disk. Longitudinal force transmission then proceeds in series through the sarcomeres eventually ending at the myotendinous junction. Although we commonly think of force transmission as occurring only in the longitudinal direction, lateral force transmission also occurs and stabilization of this force can be quite important for maintaining the integrity of the sarcomere. Lateral force transmission also originates with cross-bridge formation and sarcomere shortening but is transmitted across myofibrils to the sarcolemma and eventually to the extracellular matrix.

Longitudinal force transmission—Thin filaments originate from the Z-disk, which acts as a major site for longitudinal transmission of force developed by cross-bridge formation. The Z-disk is composed primarily of α -actinin, which is necessary for the attachment of F-actin. A number of additional proteins comprise the Z-disk (e.g., telethonin, talin, desmin, myotilin, obscurin, and filamin C). Of note, desmin comprises intermediate filaments that are important in aligning adjacent Z-disks and thus sarcomeres. Desmin-containing intermediate filaments also connect the Z-disk to the sarcolemma and extracellular matrix via the dystrophin-glycoprotein complex and integrins. Disorders of proteins comprising the Z-disk have been implicated in limb-girdle muscular dystrophies, resulting in progressive myopathies²⁸.

Force generated by cross-bridges depends on the extent of overlap between thick and thin filaments and thus on sarcomere length (Figure 3). The dependency of force generation on sarcomere length is reflected by the length-tension relationship of a muscle and is a representation of the fraction of strongly bound cross-bridges during contraction. Force generated by cross-bridge formation is transmitted longitudinally to the Z-disk and from sarcomere to sarcomere in series.

The connecting unit between a muscle and its bony attachments is a tendon, and attachments occur through the extracellular matrix. Tendons are composed of various types of collagen, primarily collagen I and III, and are subjected to tensile loading. The function of tendons is to passively transmit contractile force and thus cause movement⁵². Thus, the efficacy of contraction will depend on the specific characteristics of the tendinous attachments (at the muscle and bone levels) as well as the composition and mechanical properties of the tendon itself. The myotendinous junction connects the myofibril to the tendon at a specialized area of the muscle membrane that has deep invaginations created by varying myofibrils. Force generated within the sarcomeres is transmitted from the sarcomere through the tendon connected to the origin and insertion of muscle, usually bony attachments. The myotendinous junction is known to contain numerous proteins such as vinculin, talin, integrin, desmin, and fibronectin which aid in the transmission of force⁵³. Of importance at the myotendinous junction is structural folding that functions to increase the surface area, and thus these folds may function to reduce membrane stress associated with load⁵⁴.

Lateral force transmission—Many aspects of the cytoskeleton and its function in lateral force transmission are crucial to controlling sarcomere length and ultimately optimizing force generation. A significant amount of force is also transmitted laterally through the myofibrils⁵⁵. Proteins involved in lateral force transmission include the dystrophin-glycoprotein complex and integrins. Of importance for the dystrophin-glycoprotein complex are structures that comprise the cytoskeleton of muscle fibers, organized as the costamere, a complex composed of dystroglycan proteins, dystrophin, vinculin, spectrin, and laminin (for review see⁵⁶). Importantly, the costamere aligns with the Z-disk of peripherally located myofibrils. The costamere functions to link the sarcomere contractile apparatus with the sarcolemma, and thereby transmit force laterally through the sarcolemma to the basal lamina.

REGULATION OF PROTEIN EXPRESSION

Specific control mechanisms are in place to keep muscle structure and function optimal, as well as to balance synthesis and degradation. These control mechanisms likely exert their effect via specific molecular cues that influence the complement of muscle fiber and motor unit properties. Gene transcription is a highly complex nuclear process that results from the interaction of multiple proteins (usually transcription factors and co-activators), RNA moieties (e.g., microRNAs or miRNA), and various enzymatic processes.

Myonuclear domain

Skeletal muscle fibers are multinucleated cells with each myonucleus influencing gene expression within a volume of muscle fiber – the average volume per myonucleus is termed the myonuclear domain (Figure 9). It has been suggested that myonuclear domain size is a controlled variable such that the number of myonuclei in a muscle fiber changes in proportion to the change in fiber volume (cross-sectional area). However, myonuclear domain size varies across muscle fiber types in relation to differences in fiber cross-sectional area^{57, 58}. The myonuclear domain of type I and IIa fibers is smaller than that of type IIx and IIb fibers^{57, 58}. Moreover, there is little evidence to support the concept that myonuclear domain size is maintained in conditions of atrophy or hypertrophy even within a fiber type. For example, when fiber size is reduced (atrophy) following denervation⁵⁷ or corticosteroid treatment⁵⁸, the total number of myonuclei does not change, but myonuclear domain size decreases in proportion to the decrease in cross-sectional area. Thus, it does not appear that during these conditions of atrophy, myonuclear domain size is being controlled by myonuclear loss (apoptosis). Conversely, during early postnatal development, when there is dramatic growth of muscle fibers (hypertrophy), there is also an increase in the total

number of myonuclei. However, the growth in fiber cross-sectional area is proportionately greater and as a result myonuclear domain size increases during postnatal development⁵⁹.

Regulation of gene expression

Even within the context of a multinucleated single muscle fiber, unravelling the complexity of coordinated gene expression is daunting. At the level of the single type-identified fibers, newer techniques are available to examine mRNA expression for numerous proteins within the fiber.

Transcriptional control—Networks of transcription factors and microRNAs function to orchestrate gene expression within skeletal muscle fibers. For instance, a number of skeletal muscle specific microRNAs have been identified including mi-R1, mi-R206, and mi-R133. Although the role of these microRNAs in differentiation and development via interaction with myogenic transcription factors such as serum response factor (SRF), MyoD, and myocyte enhancer factor-2 (MEF2) have been described^{60, 61}, whether they play a role in coordinating gene expression across myonuclei within a single muscle fiber has not been explored. It is also unclear whether microRNAs/myogenic transcription factor interactions play a role in fiber type specializations. The coordinated expression of muscle fiber proteins associated with distinct MyHC isoform expression (i.e., fiber type) is likely to involve interactions between microRNAs and myogenic transcription factors. Removing neural influence or lowering thyroid hormone levels among several other conditions result in co-expression of MyHC isoforms and blurring of fiber type distinctions. Thus, it is obvious that maintenance of MyHC isoform expression and thus fiber type is an active and coordinated process that involves interaction with neural and hormonal influences. However, it is possible that expression of proteins associated with different fiber types will be more dynamic and respond to specific conditions in different ways that would not affect fiber type classification based on MyHC isoform expression. Thus, coordinated expression of proteins within muscle fibers may involve different microRNAs/myogenic transcription factor pathways.

A number of recent studies have employed microarray techniques to explore differences in gene expression primarily across muscles or within the same muscle across conditions. In these studies, fiber type differences are largely ignored. For example, it has been reported that the expression of nearly 725 genes differ between diaphragm and limb muscles in the mouse⁶². However, the muscles studied have mixed fiber type composition and varying activation and loading patterns, such that differences in gene expression are expected. Moreover it has been shown that at the whole muscle level, the correlation between mRNA and protein expression is often poor. For example, changes in MyHC isoform mRNA and protein expression in the diaphragm muscle during postnatal development (a period of rapid fiber growth)⁶³ and following unilateral denervation¹⁸ are not correlated. Both of these conditions are blurred by the lack of clear fiber type distinctions. Moreover both protein synthesis and degradation may be affected by these conditions, thereby affecting protein content. Recently developed techniques, e.g., Fluidigm, permit focused analysis of multigene expression in small volume samples, and thus offer opportunities to explore coordinated gene expression within single, type-identified muscle fibers. New proteomic approaches with tandem mass spectrometry permit measurements of very small amounts of protein that may allow analysis of expression of multiple proteins in single muscle fibers that are associated with the more abundant contractile proteins including MyHC isoforms.

Protein synthesis and degradation—Different types of muscle fibers vary in the concentration of MyHC and other contractile proteins. For example, the concentration of MyHC_{2X} and MyHC_{2B} in type IIx and IIb fibers in the rat diaphragm muscle is 2-3 fold

higher than that of MyHC_{slow} and MyHC_{2a} in type I and IIa fibers⁴⁴. Skeletal muscle proteins are in a constant state of flux between ongoing synthesis and degradation. At a global level, this balance determines whether there is a net gain or loss of muscle mass in response to common perturbations including exercise, disuse, or disease. Although adaptations may share a common outcome, the underlying mechanisms may differ. Indeed, the specific regulatory pathways involved in protein balance are not clear for many physiological states. Clearly, the molecular pathways that regulate protein synthesis and degradation both at a global and single protein level are extremely complex and interdependent (Figure 10), in part due to the dynamic nature of signaling cascades and the interrelation of individual pathways and proteins⁶⁴. It is beyond the scope of this review to provide an extensive summary of all pathways involved, but the key players in myosin protein balance are included here.

Gene expression profiling studies have examined several different models of muscle atrophy, including cancer cachexia, diabetes, fasting, chronic renal failure or denervation. These studies have identified a group of genes described as atrophy-related genes, or atrogenes^{65, 66}. For instance, two atrogenes known as atrogin-1/MAFbx and MuRF1 are upregulated during denervation-induced atrophy and are involved in increasing protein degradation through the ubiquitin-proteasome system. Building on such findings, total muscle protein synthesis and degradation rates were examined following unilateral phrenic nerve denervation and associated ipsilateral diaphragm muscle paralysis (Figure 10). Both MyHC gene transcription and translation change after denervation; however, posttranslational changes such as increased protein degradation and protein synthesis are primarily responsible for decreased MyHC protein expression^{18, 67}. Following denervation, the rate of protein degradation increases in a time-dependent manner and correlates with an increase in total protein ubiquitination. The rate of protein synthesis as measured by tyrosine incorporation also increases in a time-dependent manner after denervation at the whole muscle level, and correlates with changes in expression of pathways involved in protein synthesis (e.g., mTOR, Akt)⁶⁸. The upstream and downstream pathways of specific atrogenes are not completely known. In addition, we know very little about fiber type differences in expression of proteins involved in synthesis and degradation pathways. A recent study⁶⁹ examined protein synthesis rates in type-identified fibers in cross-section using a nonradiative technique (*in vivo* – immunohistochemical – surface sensing of translation; IV-IHC-SUnSET). Using this technique, these authors reported that type IIx and IIb fibers in murine hindlimb muscles have a lower rate of protein synthesis compared to type I fibers. Thus, protein degradation rates must also be lower in type IIx and IIb fibers in order to maintain fiber size and high contractile protein content. Future studies should expand on these observations.

Conclusion

The complexity of skeletal muscle physiology is simplified by fiber type classification. Protein expression underlies a variety of fiber type functional differences including neuromuscular transmission, excitation-contraction coupling, cycling of cross-bridges and ATP consumption. Thus, fiber type classification serves as a valuable organizing and simplifying principle for systems biology approaches to explore the complex interactions between the major proteins involved in muscle force generation and contraction. Unfortunately, very little is known about the coordination of gene regulation and protein expression in single multinucleated muscle fibers regardless of type. Obviously, gene and protein expression becomes disordinated under pathophysiological conditions as evidenced by MyHC co-expression and the blurring of fiber type distinctions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Four motor unit types - slow (type S), fast-twitch fatigue resistant (type FR), fast-twitch fatigue intermediate (type FInt), and fast-twitch fatigable (type FF) - are classified based on the contractile and fatigue properties of the innervated muscle fibers. Each motor unit type comprises a specific fiber that expresses a single myosin heavy chain (MyHC) isoform. Adapted from ¹¹, used with permission

FIGURE 2.

A) Muscle fibers comprise myofibrils that contain repeating arrangements of sarcomeres, which give muscle its striated appearance as seen by transmission electron microscopy. The primary components of the sarcomere are thick and thin filaments, which interact by cross-bridge formation and slide past each other during muscle contraction. **B)** The crystalline organization of myosin (red) and actin (yellow) filaments creating a myofilament lattice is clearly seen in an electron micrograph of a muscle fiber cross section.

FIGURE 3.

Muscle force generation depends on muscle fiber length. Underlying the force-length relationship of muscle is the overlap of thick (red and black) and thin (yellow) filaments within a sarcomere that determines the number of cross-bridges that can form during muscle activation.

FIGURE 4.

A) Representative confocal image of neuromuscular junction (NMJ) showing motor axons down to the presynaptic terminal (immunoreactivity for neurofilamin - red), motor endplates (cholinergic receptors fluorescently labeled using α -bungarotoxin - green) and muscle fibers (labeled with an antibody specific to the MyHC_{2B} isoform - blue). **B)** Electron micrograph showing the preand postsynaptic elements of a NMJ. Adapted from ³¹, used with permission. **C)** Electrophysiological recording of spontaneous miniature end-plate potentials (mEPPs) and an evoked EPP response. The presynaptic terminal was visualized by uptake of a styryl dye FM4-64. **D)** Neuromuscular transmission failure induced by repetitive nerve stimulation is reflected by the difference in muscle force generated by nerve stimulation compared to that induced by periodic (every 15 s) direct muscle stimulation. Adapted from ⁷⁰, used with permission.

FIGURE 5.

A) A nerve action potential initiates a transient increase in myoplasmic Ca^{2+} concentration that precedes the force response of a muscle fiber. **B)** The force- Ca^{2+} relationship is shifted leftward (increased Ca^{2+} sensitivity) in type I fibers ($MyHC_{slow}$) compared to type IIa ($MyHC_{2A}$), IIx ($MyHC_{2X}$) and type IIb ($MyHC_{2B}$) fibers. Adapted from ³⁹, used with permission.

FIGURE 6.

During muscle fiber activation, Ca^{2+} released from the sarcoplasmic reticulum binds to troponin C initiating a conformational change in the troponin/tropomyosin complex on the thin filament allowing binding of the myosin head to actin.

FIGURE 7.

A) Cross-bridge cycle between bound and unbound states with apparent rate constants for cross-bridge attachment (f_{app}) and detachment (g_{app}). Force depends on the number of available myosin heads per half-sarcomere (n), the fraction of cross-bridges in the strongly-bound state (α_{fs}), and the average force generated per cross-bridge (F). The ATP consumption rate during cross-bridge cycling occurs throughout the muscle fiber and thus also depends on the number of half-sarcomeres in series (b). Adapted from ⁵, used with permission. **B)** Cross-bridge cycling rate can be estimated by the rate constant for force redevelopment after rapid release (all cross-bridges broken) and restretch of a single permeabilized muscle fiber (k_{tr}) and varies across fibers expressing different MyHC isoforms. **C)** f_{app} can be estimated by the rate of force development resulting from rapid flash-photolytic release of caged Ca^{2+} and activation of the muscle fiber. **D)** g_{app} can be estimated by rapid removal of Ca^{2+} following flash-photolytic release of a caged Ca^{2+} chelator and measuring the rate of force relaxation. Both f_{app} and g_{app} are faster in fibers expressing MyHC_{2X} compared to those expressing MyHC_{slow}.

FIGURE 8.

A) Experimentally, the rate of ATP consumption in single permeabilized muscle fibers can be measured based on NADH fluorescence extinction using a stop flow technique in which ATP hydrolysis is coupled with the reduction of NADH to NAD. **B)** The force-velocity of shortening and force-power output relationships of a skeletal muscle fiber. ATP consumption rate (indicated by arrows) of a muscle fiber varies with force and velocity of shortening, and peaks at maximal power output. Adapted from ⁴⁰, used with permission.

FIGURE 9.

A) Confocal image of a single diaphragm muscle fiber clearly showing sarcomeres (membrane stained with RH414 - red) and myonuclei (stained with propidium iodide - green). **B)** Representative real-time RT PCR amplification curves for various MyHC isoforms. Adapted from ⁶³, used with permission. **C)** Representative electrophoretic determination of MyHC isoform expression in single rat diaphragm muscle fibers. The concentration of MyHC extracted from single fibers was compared to known concentrations of MyHC. Adapted from ⁴⁷, used with permission.