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Review

Developmental organization of skeletal muscle fiber types and the motor unit

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ABSTRACT

Structural specification of skeletal muscle is not visually obvious compared to more dramatic examples such as the heart and brain. Though its functional subdivision is not clear to the naked eye, skeletal muscle is a complex and highly organized organ which orchestrates the movement of the body. Skeletal muscles are formed by the assembly of many myofibers with distinct properties, generally classified as slow-twitch and fast-twitch fibers. The physiological distinctions between specific muscle fiber types are determined by expression of fiber type-specific contractile protein isoforms (e.g. myosin heavy chain) and metabolic enzyme profiles that support the mechanistic requirement. Each mammalian skeletal muscle is a heterogeneous bundle of different types of myofibers, allowing the same muscle to respond to a wide spectrum of physiological activities. A working unit that controls the movement of skeletal muscle is the motor unit. The motor unit consists of a motor neuron and myofibers of similar functional properties, i.e. a slow type motor neuron innervates slow and more oxidative myofibers whereas a fast type motor neuron innervates fast and more glycolytic myofibers. This functional coupling of the motor unit is established during late gestation to early postnatal stages in mammals. Recent studies utilizing genetically engineered animal models have revealed many regulatory factors involved in muscle fiber type specification and the hierarchical organization of motor neurons. The molecular mechanisms governing the functional matching of motor neurons and skeletal myofibers, however, still remain poorly understood. This short review summarizes the representative studies which revealed the regulatory networks responsible for achieving the organized development of skeletal muscles and motor neurons and discusses the prospect of identifying the mechanisms establishing the motor unit during mammalian development.

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1. Introduction

Skeletal muscle is a highly plastic organ adapting its size, contractile properties and metabolic capacity in response to different types of mechanical needs (e.g. posture, long distance running, and lifting) or to the lack thereof (e.g. sedentary life style, bed rest, microgravity environment). The heterogeneity and physiological plasticity of skeletal muscle holds a key for accomplishing complex tasks. Adult mammalian skeletal muscle is composed of bundles of mixed myofibers of different contractile characteristics and metabolic capacity, i.e. slow oxidative, fast oxidative-glycolytic, and fast glycolytic fiber types. What unifies this mixed assortment into a working organization is the motor unit, which consists of a singular type of motor neuron (slow tonic or fast phasic) and a group of muscle fibers of closely related physiological functions [1]. This ordered structure is the building block for the functional versatility of muscle to operate in various

physiological needs. When the same kind of physical task is continued for long enough time, for example, training for long distance running, skeletal muscle is capable of shifting its fiber type compositions to operate at the best of its ability. It has been reported that leg muscles of the elite marathon runners have a higher ratio of fatigue-resistant slow oxidative fibers to fatigue-sensitive fast glycolytic fibers compared to untrained individuals [2]. These observations exemplify the functional versatility of the motor unit as a whole.

In this review, the molecular mechanisms for the development of different types of skeletal muscle fibers and their assembly into motor units will be discussed using classic papers and recent experimental data obtained using embryonic manipulation and genetic engineering of model organisms. Of note, the motor neurons being discussed here are alpha motor neurons innervating force-generating extrafusal muscle fibers, the overwhelming majority of the skeletal muscle mass. Discussions regarding other types of motor neurons, gamma motor neurons innervating intrafusal muscle fibers (constituting the stretch-sensor mechanism) and beta motor neurons innervating both extrafusal and intrafusal muscle can be found elsewhere [3, 4].

2. Specification of skeletal muscle phenotypes

As mentioned above, the physiological properties (slow vs. fast) are matched between a motor neuron and the skeletal muscle fibers it innervates (motor unit homogeneity). When and how this functional matching is achieved has not yet been clarified. The observation that embryonic/fetal skeletal muscle acquires a basic spatial distribution of slow/fast type fibers in the absence of innervation [5] indicates the presence of muscle-intrinsic mechanisms which form a blue print of muscle fiber distribution during prenatal development. Studies investigating initial innervation patterns of individual motor neurons to developing myofibers in the prenatal period suggest that single motor neurons initially contact a mixed population of muscle fiber types in an apparently random manner [6]. In the postnatal period, however, motor units rapidly obtain functional homogeneity, and in cases of rats and mice, within 1-2 weeks after birth motor units become markedly homogeneous [7-9]. These observations indicate that the functional matching between myofibers and motor neurons is a multi-step process. Below, prenatal and early postnatal differentiation of skeletal muscle fiber type will be discussed with a focus on the muscle-intrinsic mechanisms, followed by skeletal muscle fiber type plasticity in adult where muscle shows its remarkable flexibility in responding to activity-driven stimuli.

2.1. Fiber type differentiation in prenatal skeletal muscle and initial motor neuron-myofiber contact

In vertebrates, skeletal muscles in the body are derived from muscle progenitor cells (myoblasts) which originate from the somites [10]. The progressive myogenic differentiation (from myoblasts to post-mitotic myocytes, and eventually to multinucleated myofibers) is directed by the MyoD family of myogenic factors (MRFs), specifically, Myf5, MyoD, Myogenin and MRF4 [11, 12]. Each MRF exhibits distinct expression patterns as myogenesis progresses, regulated by a combination of other transcription factors and signaling molecules which display spatio-temporally specific expression during development [10-13]. These regulatory factors are not muscle specific, but their expression patterns specify the timing and location of MRF gene expression, which then initiates the cascade of myogenesis at the right time and the right place. To illustrate this point, limb muscle formation is used here as an example.

For the differentiation of limb muscle, multiple transcription factors are known to initiate myogenesis by activating Myf5 expression. It has been shown that the transcription factor Pax3 plays a role in commitment of mesodermal stem cells to myogenic precursors as well as in delamination of the precursors for migrating from the somites to the limb [10, 14-16]. Pax3 activates transcription of both Myf5 and c-met, which dictate myogenic lineage commitment and delamination of myoblasts from the lateral edge of the somite, respectively [17, 18]. The loss of a functional Pax3 protein (e.g. mouse *Splotch* mutations), results in a massive loss of limb musculature [15, 18, 19]. Additionally, the transcription factors Six1 and Six4, along with their cofactors Eyes absent proteins (Eya) [20], likewise function as transcriptional activators for Myf5 during limb myogenesis [21, 22]. Simultaneous inactivation of either Six1 and Six4 or Eya1 and Eya2 in mice also leads to a significant loss of limb and trunk muscles [22, 23]. Migration of myoblasts to the limb is controlled

by another transcription factor, the homeobox transcription factor Lbx1 [24]. In the absence of a functional Lbx1 gene, the limb musculature suffers an extensive loss because of the failed migration of muscle precursors into the limb [24]. These examples demonstrate that muscle development is an amalgamation of finely structured orders established throughout the body axes which regulate MRF expression and dictate myoblast migration in a highly controlled manner. The factors listed above, Pax3, Six1/4, Eya1/2, and Lbx1, are a small sampling of a large number of proteins that are part of the regulatory network orchestrating MRF expression during limb development. More in-depth reviews on this topic can be found here [10, 11].

Although MRF expression is critical for specification of the myogenic lineage, MRF expression by itself does not appear to play a determining role in the formation of specific myofiber types (slow/fast); rather, fiber type of embryonic muscle in the limb is regulated by temporally regulated migration of the myogenic precursors and specification of these cells by a combination of regulatory factors, some intrinsic to muscle and some presented by the environment [25, 26]. Migration of myogenic precursors from the somites to the limb and subsequent myofiber differentiation occur in three sequentially appearing and slightly overlapping waves during the prenatal and early postnatal periods [27, 28]. Corresponding to this sequence, three classes of myogenic precursors, embryonic myoblasts, fetal myoblasts and satellite cells are identified in higher vertebrates [27-30]. The first wave, termed primary myogenesis, generates primary myofibers committed to originally differentiate into slow myofibers (Fig. 1) [31, 32]. The second wave, termed secondary myogenesis, generates secondary myofibers whose default state is fast myofiber [33]; however, fetal myoblasts generated during secondary myogenesis are capable of fusing with primary myofibers and are also receptive to the environmental cues, adapting their final fiber types according to the surrounding local environment (Fig. 1) [34, 35]. Primary myogenesis and secondary myogenesis occur in mice around embryonic days 11-15 and day 15 to birth [36, 37], and in humans during gestation weeks 6-8 and 8-18 [38]. Satellite cells, which facilitate postnatal growth, remodeling

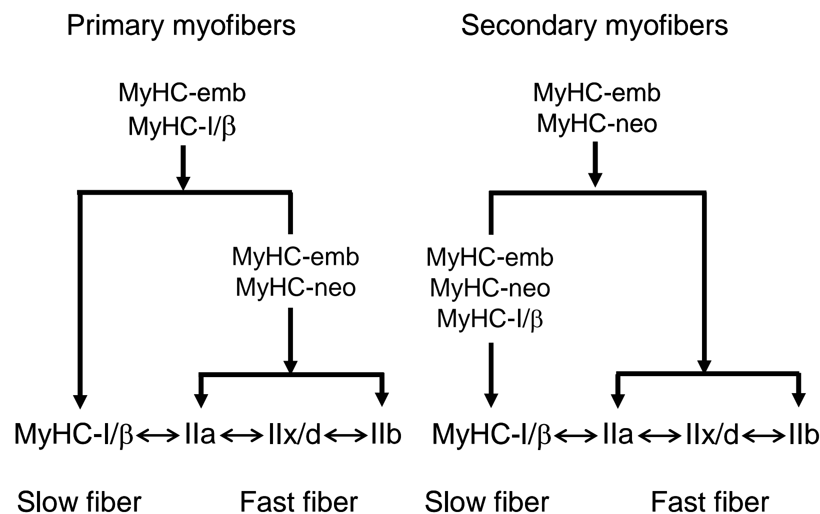

Fig. 1. Muscle fiber type specification during development and muscle fiber plasticity defined by MyHC isoform expression. Fiber type specification during muscle development, which is classified as temporal transition in MyHC isoform expression, is indicated by vertical arrows. Fiber type plasticity defined by the shift in MyHC isoform expression in adult skeletal muscle is indicated by horizontal bi-directional arrows on the bottom. MyHC isoform expression patterns shown in this figure are based on the data obtained using rodent skeletal muscles. MyHC-emb: embryonic myosin heavy chain (Myh3). MyHC-neo: neonatal fast myosin heavy chain (Myh9). MyHC-I/β: slow myosin heavy chain-β (Myh7). MyHC-IIa, IIx/d, and IIb: adult fast myosin heavy chain isoforms (Myh2, Myh1, and Myh4).

and regeneration of adult skeletal muscle, are generated in the last wave and afterward [39].

During the primary and early secondary myogenesis, the influence of the motor neuron input is not yet critical in determining muscle fiber types and characteristic distributions of slow and fast myofibers within each muscle group. Experiments utilizing neurotoxin-induced denervation in prenatal rat hindlimb muscle demonstrated that fiber type differentiation and their characteristic distribution in prenatal muscles advanced normally even in the absence of innervation, indicating the independence of initial differentiation and distribution of muscle fiber types from motor neurons [5]. It should be noted that in these experiments muscle mass was reduced, showing that motor neurons elicit a trophic effect on muscle. In a study investigating the formation of motor units in prenatal rat intercostal muscle, it has been shown that during the fetal period, single neurons are found to initially contact both primary and secondary myofibers [6]. Additionally, it has been observed that almost all single muscle fibers are contacted by multiple motor neurons at birth [40, 41]. These results indicate that motor units developing during prenatal and neonatal stages are highly heterogeneous, and motor neurons in these early stages do not yet play a resolute role in specification of muscle fiber types. This nerve-independent muscle differentiation, however, lasts just briefly and from the late fetal through postnatal stages, motor neurons elicit an increasingly significant influence on differentiation of muscle fibers [42-45].

In summary, formation of the motor unit proceeds as follows: commencing in the prenatal stage with the specification of skeletal myofiber types in the absence of or with very limited neural input and forming heterogeneous motor units of mixed motor neuron and myofiber phenotypes in the prenatal period. Homogeneous motor units are then formed by rapid elimination of unmatched synapses during the early postnatal period as described below.

2.2. Postnatal differentiation of skeletal muscle: establishment of fiber type specificity and motor unit homogeneity

Mature skeletal muscle consists of mixed fiber types, most simply classified as slow and fast fibers, displaying a range of contractile speeds and metabolic properties [2, 46, 47]. The metabolic capacity of myofiber is tightly coupled with its contractile speed, which is dictated by the ATPase activity of myosin heavy chain (MyHC) [48]. Oxidative myofibers contain more slowly contracting MyHC isoforms and a higher amount of mitochondria, and thus are fatigue-resistant and optimized for supporting long durations of contractile demands. Glycolytic myofibers, on the other hand, contain less mitochondria than oxidative fibers and express faster MyHC isoforms, and thus are fatigue-sensitive and geared towards bursts of quick contractile activities [2, 46, 47]. Skeletal muscle fiber type differentiation has been intensively investigated using rodents as a model. Classification of skeletal muscle fiber type is typically defined by expression of specific myosin heavy chain isoforms. In adult rodent skeletal muscle, four isoforms of MyHC proteins, from the slowest to fastest, MyHC-I/β (Myh7), MyHC-IIa (Myh2), MyHC-IIx/d (Myh1), and MyHC-IIb (Myh4) are expressed (Fig. 1) [49]. In the prenatal slow and fast myofibers discussed above, MyHC-emb (Myh3) is expressed along with MyHC-I/β or MyHC-neo (Myh9), respectively (Fig. 1) [5, 31]. After birth, expression of both developmental MyHC isoforms (embryonic and neonatal) is lost during the functional maturation of skeletal muscle [50]. In adult mouse hindlimb skeletal muscle, except for the soleus, more than 95% of muscle mass is composed of fast MyHC-II isoform expressing fibers [51]. This high composition of fast fibers is characteristic of small mammals [47], whereas in a larger mammal like humans, more than 50% of fibers in the calf muscle are composed of slow MyHC-I/β expressing myofibers [52].

In early postnatal mouse muscle, downregulation and elimination of the developmental MyHC isoforms (embryonic and neonatal) and upregulation of the adult fast MyHC-II isoforms occur in the first few weeks [51]. This period coincides with the developmental changes in neuromuscular junctions. As described above, neuromuscular junctions in prenatal and neonatal muscle are still immature with highly heterogeneous and polyneuronal innervations [6, 40, 41]. In 1-2 weeks after birth, however, functionally unmatched or polyneuronal muscle innervation is eliminated and motor units become substantially more homogeneous, establishing the slow/fast phenotypic match between myofibers and innervating motor neurons [7-9, 53, 54]. Though there is some nerve-independent regulation of fiber

type-specific genes in skeletal muscle [55], the influence of motor neurons on mature skeletal muscle fiber phenotypes is well established during early postnatal development [56, 57].

After homogeneity in the motor unit is established, does skeletal muscle completely lose regulatory control over its fiber type differentiation? Not necessarily. In recent years, transcription factors that directly regulate transcription of fiber type-specific genes have been identified, enabling for the first time the remodeling of skeletal muscle fiber type at the molecular level using genetically engineered mice. The obtained data suggest that muscle retains the ability to change fiber types in adult despite mature innervation. These transcription factors include peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) and Six1/4, which are activators of slow fiber and fast fiber-specific genes, respectively [58, 59], and Sox6, a suppressor of slow fiber-specific genes [60-62]. The transcription factors which directly regulate transcription of fiber type-specific genes have been overexpressed as a transgene or inactivated by targeted gene mutagenesis specifically in skeletal muscle in order to induce a significant change in fiber type composition. For example, it has been shown that overexpression of PGC-1α driven by the muscle creatine kinase promoter induces an increase in oxidative metabolism and slow myofiber-specific gene expression [58]. Using PGC-1α transgenic mice, it was also tested whether the increase in slow-oxidative fibers in the muscle would induce phenotypic changes in the corresponding innervating motor neurons. In these mice, presynaptic gene expression, morphology and electrophysiological properties of innervating motor neurons displayed changes towards slow-tonic motor neuron type [63, 64]. Importantly, PGC-1α driven slow muscle phenotype was able to convert the phenotype of motor neurons even after completion of synaptic elimination and formation of homogeneous motor units (assessed by expression of the presynaptic vesicle protein SV2a, a newly identified marker for motor neurons innervating MyHC-I/β or IIa expressing myofibers) [64]. These reports indicate that retrograde signals from skeletal muscle transmitted to innervating motor neurons also play a significant role in postnatal remodeling of motor units, a departure from the long-held view that postnatal motor neuron firing patters are the sole influence on the innervated muscle fiber types [65].

Could remodeling of motor units be induced in knockout of either Six1/4 or Sox6 in adult skeletal muscle? It seems very likely so. For one, forced expression of Six1 and its cofactor Eya1 in adult skeletal muscle using electroporation led to transcriptional activation of fast contractile proteins and glycolytic enzyme genes [23]. In addition, it has recently been shown that disrupting transcriptional control by Six1 or Sox6 in adult skeletal muscle leads to an increase in slow fibers. Six1 upregulates expression of a long intergenic non-coding RNA (lincRNA) which activates fast fiber-specific genes and represses slow fiber-specific genes [66]. Electroporation of shRNA against this lincRNA to adult mouse fast muscle induced upregulation of slow fiber-specific genes and downregulation of fast fiber-specific genes two weeks after electroporation [59]. In our laboratory, we have induced skeletal muscle-specific knockout of Sox6 (a transcriptional suppressor of slow fiber-specific genes) in adult mice (7-8 weeks old) by using a muscle-specific tamoxifen inducible Cre gene (human skeletal α-actin promoter driven MerCreMer) [67]. Hindlimb skeletal muscle was collected 3, 4, or 5 weeks after tamoxifen injection. In all ages, originally fast, pale-colored skeletal muscles in the limb (e.g. tibialis anterior, extensor digitrum longus) became more red (more oxidative) and expressed significantly higher levels of slow fiber type-specific genes compared to control mice (Hagiwara, unpublished data). Importantly, the observed shift towards the slow-oxidative fiber phenotype achieved by suppressing Six1 or Sox6 activity in adult muscle indicates that even after the formation of neuromuscular junctions and the motor units have completed, fiber phenotype of skeletal muscle can still be changed by altering expression of the key regulatory factors. The next critical question to be answered is what happens on the presynaptic side of neuromuscular junction and motor neuron phenotype in these fiber type-shifted adult muscles. Will motor neuron adapt to the altered muscle fiber type and convert to a matching physiological phenotype? The answer is yet to come, but the genetically engineered mice discussed here provide excellent models to further investigate the plasticity of adult motor units and motor neuron phenotypes.

3. Specification of motor neurons and neuromuscular junctions

Depending on whether innervating to slow or fast-twitch muscle, motor neurons display distinctively different firing patterns. Contiguous electromyography (EMG) recording from individual motor units in the fast muscle (extensor digitorum longus; EDL) and slow muscle (soleus) in the rat hindlimb led to an identification of three classes of motor units: slow and fatigue resistant (S), fast and fatigue resistant (FR) and fast and easily fatigued (FF) [1]. The S motor unit is characterized by a low frequency firing pattern (~20 Hz) which is relatively uniform and sustained, and thus is generally termed "tonic"; whereas the FF motor unit is characterized by high frequency firing (70-90 Hz) in short duration and very modest activity, and thus is termed "phasic" [1, 68]. The FR motor unit falls in between the S and FF motor units, and is characterized by medium to high frequency firing (50-80 Hz) with a significantly longer duration than FF and medium activity [1]. The S, FR and FF motor units are considered to consist of myofibers expressing MyHC-I, IIA and IIx/d, and IIb, respectively [1, 47] (Fig. 2). Since each skeletal muscle (e.g. EDL or soleus) contains a mixture of fiber types, motor neurons need to discriminate amongst this physiological diversity of myofibers when innervating in order to form homogeneous motor units. As discussed below, the molecular mechanisms regulating this final physiological matching between motor neurons and muscle fibers are currently not yet understood.

Motor neurons in the FF motor units have relatively larger cell bodies and neuromuscular junctions (NMJ), and physiologically show faster axonal conduction velocity and higher input conductance, require significantly greater currents for activation, and demonstrate shorter after-hyperpolarization durations

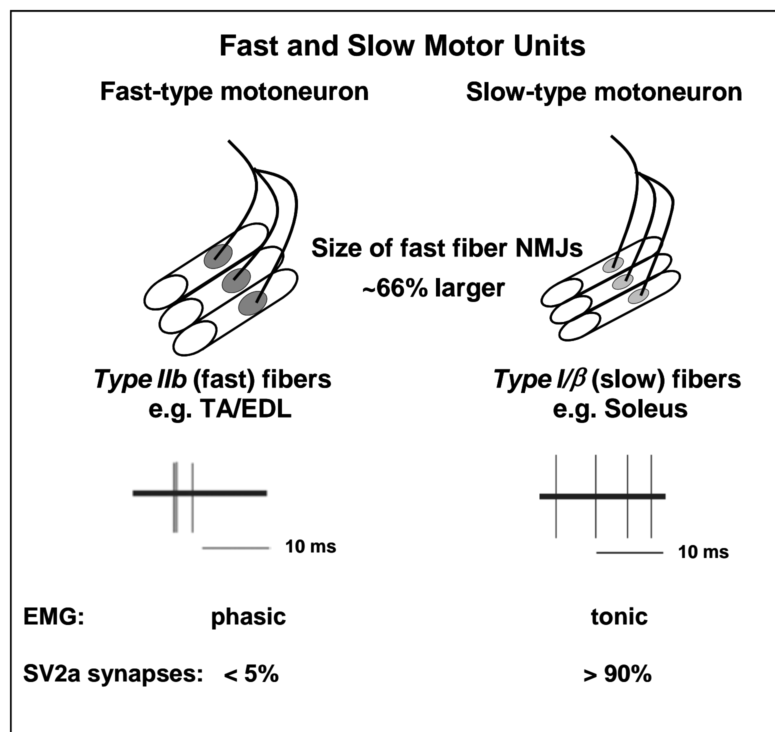

Fig. 2. Phenotypes of motoneurons in fast and slow motor units. Brief summary of morphological, electrophysiological and biochemical phenotypes of motor neurons innervating to fast (MyHC-IIb expressing myofibers) and slow (MyHC-I/β expressing myofibers) is shown. The presynaptic vesicle protein SV2a has been identified as a marker for motor neurons innervating MyHC-I/β or MyHC-IIa expressing myofibers [64]. Difference in the neuromuscular junction sizes between fast and slow fibers has been previously described [73].

than those in the S motor units [69-74]. In contrast to the well-established electrophysiological differences between fast and slow motor neurons, molecular markers that delineate different types of motor neurons are still very limited. The paucity of the molecular markers specifying fast and slow type motor neurons makes it challenging to investigate how motor unit homogeneity is established at the molecular level. However, it is evident that the development of motor neurons also follows a highly structured order according to the spatial cues established throughout the body axes, which are determined by combined expression of transcription factors, signaling molecules, growth factors, and cell surface proteins. Here, the hierarchical organization of motor neurons and the neuromuscular matching of physiological properties, which is the basis for the motor unit homogeneity, will be discussed.

3.1. The hierarchical organization of motor neurons

Motor neuron cell bodies are located at the ventral horn of the spinal cord. There are at least three levels of hierarchical organization for the locations of motor neuron cell bodies. First, the motor neuron cell bodies are organized into four columns (motor columns), contiguously or non-contiguously running in parallel along the rostral-caudal axis [75]. Motor neurons in different motor columns project axons to topographically different body muscles as targets, e.g., dorsal axial muscle, intercostal and abdominal wall muscles, limb muscle, and so on. For example, forelimb and hindlimb muscles are innervated by motor neurons located in the lateral motor columns (LMC) that are positioned only at the brachial and lumber levels, respectively [43, 76]. Second, in each motor column, motor neurons innervating to a specific target muscle (e.g. gastrocnemius in the hindlimb) are localized in close proximity, forming a confined structure termed motor pool [43, 76]. Third, in each motor pool, motor neurons are further subdivided into those innervating slow-twitch muscle fibers or those innervating fast-twitch muscle fibers [43]. Research into the molecular cues that bestow hierarchical organizations to motor neurons identified several key signaling molecules and transcription factors. To illustrate this process, I will focus on the development of motor neurons innervating the limb muscles.

Development of the lateral motor columns (LMC) that innervate forelimb and hindlimb muscles are regulated by combinatorial expression of transcription factors. The forelimb level LMC and hindlimb level LMC are demarcated by the expression of specific Hox genes, Hox6 and Hox10, respectively, whose expression is induced by the fine-tuned rostrocaudal gradient of fibroblast growth factor (FGF) activity combined with a gradient of retinoic acid signaling at the forelimb level and Gdf11 (a family of TGF-β, also known as BMP11) signaling at the hindlimb level [77, 78]. Within the LMC, motor neurons are further divided into the medial and lateral LMC neurons, whose identity is specified by the combined expression of another family of transcription factors, LIM homeobox proteins; the medial LMC motor neurons innervating the ventral side of limb muscles express islet-1 and islet-2 and the lateral LMC motor neurons innervating to the dorsal side of limb muscle express Lim-1 (also known as Lhx1) and islet-2 [79, 80].

Within the medial and lateral LMC subdivision, motor neurons are further compartmentalized into motor pools, which innervate a single limb muscle. It has been suggested that muscle-derived cues play an important role in inducing yet another family of transcription factors, ETS transcription factors, which define motor pools within the LMC [81]. Two EST transcription factors, PEA3 and ER81, specify motor pools by their expression in conjunction with LIM homeobox proteins described above [82, 83].

Motor neurons innervating slow muscle fibers and fast muscle fibers coexist in the same motor pool [43]. Whether characteristic positions of motor neurons within a motor pool influence their innervation to specific muscle fiber types was investigated using chicken embryos. Unlike mammalian skeletal muscles where fast and slow muscle fibers coexist, in chickens some muscles are uniformly fast, while other muscles have a clear division between fast and slow muscle fiber containing regions. This fiber type development in chicken embryo is muscle-autonomous and does not require innervation [84]. Taking advantage of this anatomical feature of chicken muscle, retrograde staining of motor neuron cell bodies was conducted by injecting dye to the fast or slow muscle fiber regions in embryonic limb muscle; the results have indicated that the positions of motor neuron cell bodies within motor pools have no clear correlation

with innervation to particular muscle fiber types [85]. Currently, transcription factors, secreted factors or receptors required for establishing the homogeneous matching of motor neuron and muscle fiber properties (slow vs fast) are not identified.

Though it is still limited, a few molecular markers specific to slow or fast motor neurons have been identified. Based on gene expression profiling using the ventral side of the spinal cord of one week-old mice, it has been identified that slow motor neurons express estrogen-related receptor β (Esrrβ), whereas fast motor neurons express calcitonin/calcitonin-related polypeptide α (Calca) and a membrane protein gene Chondrolectin (Chodl) [86]. Among them, Calca protein expression in fast motor neurons has been previously reported based on immunohistochemistry [87, 88]. Currently, the regulatory mechanisms of slow and fast motor neuron selective expression of these genes are unknown. Elucidating transcriptional regulation of motor neuron type-specific genes may uncover a family of transcription factors and signaling molecules that specify slow and fast motor neurons during development.

4. Conclusion

The development of motor units in mammals are regulated by highly organized hierarchies of multiple transcription factors and signaling molecules that together establish precise connections between motor neurons and skeletal muscle fibers. Identities of these regulatory molecules have started to be uncovered in recent years, but detailed molecular instructions for building motor units still remain elusive. In addition to how slow and fast motor neurons find their way to their right target muscle fibers, questions remain regarding the roles of retrograde regulation elicited from muscle in specification of slow and fast motor neuron types [63, 64]. The new knowledge to be gained in the field in the future will aid us in understanding the mechanisms regulating the development of motor circuitry as well as diseases of selective motor neuron and muscle fiber degeneration.

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CONFLICT OF INTEREST STATEMENT

None.

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