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REVIEW

Mixing it up: the biological significance of hybrid skeletal muscle fibers

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ABSTRACT

Skeletal muscle fibers are classified according to the myosin heavy chain (MHC) isoforms and other myofibrillar proteins expressed within these cells. In addition to 'pure' fibers expressing single MHC isoforms, many fibers are 'hybrids' that co-express two or more different isoforms of MHC or other myofibrillar proteins. Although hybrid fibers have been recognized by muscle biologists for more than three decades, uncertainty persists about their prevalence in normal muscles, their role in fiber-type transitions, and what they might tell us about fiber-type regulation at the cellular and molecular levels. This Review summarizes current knowledge on the relative abundance of hybrid fibers in a variety of muscles from different species. Data from more than 150 muscles from 39 species demonstrate that hybrid fibers are common, frequently representing 25% or more of the fibers in normal muscles. Hybrid fibers appear to have two main roles: (1) they function as intermediates during the fiber-type transitions associated with skeletal muscle development, adaptation to exercise and aging; and (2) they provide a functional continuum of fiber phenotypes, as they possess physiological properties that are intermediate to those of pure fiber types. One aspect of hybrid fibers that is not widely recognized is that fiber-type asymmetries – such as dramatic differences in the MHC composition along the length of single fibers – appear to be a common aspect of many fibers. The final section of this Review examines the possible role of differential activities of nuclei in different myonuclear domains in establishing fiber-type asymmetries.

KEY WORDS: Hybrid muscle fiber, Muscle plasticity, Skeletal muscle

Introduction

The principle defining feature of different muscle fiber types is the myosin heavy chain (MHC) isoform present (Pette, 2006; Reggiani et al., 2000; Schiaffino and Reggiani, 2011). In mammals, 11 different sarcomeric MHC genes have been identified, and four of these are commonly expressed in adult limb musculature (Schiaffino and Reggiani, 2011). These different MHC isoforms provide the range of contractile properties required for the diverse movements employed by animals. As techniques to identify fiber types improved, it became clear that many fibers do not fit neatly into one type or another, but are 'hybrids' co-expressing two or more types of MHC (Pette, 2006; Pette et al., 1999; Schiaffino, 2010) (see Box 1). A number of excellent reviews provide detailed accounts of skeletal muscle fiber types, their molecular organization, and the cellular and molecular regulation of muscle phenotype (Blaauw et al., 2013; Haddad et al., 2006; Pette and Staron, 2000, 2001; Punkt, 2002; Schiaffino and Reggiani, 2011); however, there has been relatively little attention paid to the role of hybrid muscle fibers in skeletal muscles, and many important observations are currently fragmented among papers that have not been widely recognized.

The recognition of hybrid fibers as common components of skeletal muscles presents a number of important questions. How common are hybrid fibers? What function do hybrid fibers serve in normal muscles? What role do hybrid fibers play during fiber-type transitions? There is no precise functional principle for predicting the abundance of hybrid fibers in a particular muscle, but one hypothesis is that these intermediate fiber types fulfill the requirement of a range of contractile output from muscles. Hybrid fibers also appear to play a role in mediating the fiber-type transitions that occur during processes such as development and aging.

Almost 20 years ago, G. M. M. Stephenson published a review focused on hybrid skeletal muscle fibers (Stephenson, 2001). Her review tackled the questions outlined above and highlighted a number of research questions that were likely to benefit from the study of hybrid skeletal muscles fibers. While moderate progress has been made on certain problems outlined in her review, many of Stephenson's questions continue to provide fertile ground for investigating key principles of skeletal muscle biology. This Review begins with an overview of the prevalence of hybrid fibers in different muscles, and discusses how hybrid fibers are identified. Hybrid fibers are important during a variety of fiber-type transitions, and the Review will examine their significance during development, exercise, disuse and aging. Next, the importance of hybrid fibers as physiological intermediates along a continuum of fiber types is considered. Finally, the Review discusses fiber-type asymmetries and what they may reveal about the cellular and molecular regulation of muscle genes.

The prevalence of hybrid fibers

Hybrid fibers are common components of normal muscles, although their prevalence varies hugely: they comprise 0–100% of fibers in muscles examined thus far, and the precise proportion of hybrid fibers is highly dependent upon the specific muscle examined (Fig. 1; Table S1). In muscles sampled to date, the mean proportion of hybrid fibers is more than 25% (Fig. 1); however, the relative proportion of hybrid fibers can vary significantly even within different regions of the same muscle (Table S1; for example, see range of values for different muscles from the rat or dog) (Kernell, 1998; Wang and Kernell, 2001). Currently, ∼50% of all available data are from just five species: mouse, rat, rabbit, dog and human (Fig. 2). In these mammals, the proportion of hybrid fibers in different muscles also varies from 0 to 100%, with median values of ∼20–40%. Although the current data are heavily skewed towards these mammalian muscles, there is information on the relative proportions of hybrid fibers from 39 species, representing mammals, reptiles, amphibians and invertebrates (Table S1). In the amphibian muscles studied, hybrid fibers make up at least 48% of the fiber types. The

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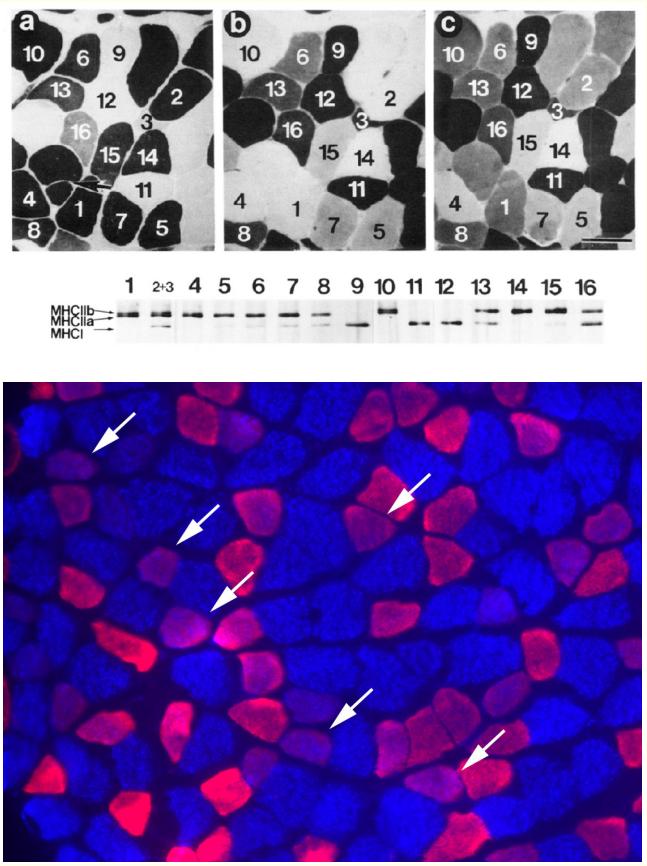
Box 1. Identifying muscle fiber types



First-hand accounts of the history of muscle fiber-type identification, including the recognition of hybrid fibers, are provided by Pette (Pette, 2006; Pette et al., 1999) and Schiaffino (2010, 2018). Almost 150 years ago, Ranvier reported that skeletal muscles exhibit differences in color that are correlated with their speed of contraction (Ranvier, 1873, 1874; Close, 1972). By the 1950s and 1960s, skeletal muscle biologists had developed histochemical techniques that helped to define these fiber types more precisely. In particular, myofibrillar ATPase histochemistry allowed fiber types to be defined based on their rate of ATP hydrolysis. Studies by Barany (1967) demonstrated a direct, functional linkage between ATP hydrolysis rate and muscle shortening velocity among diverse muscles.

During the 1980s, techniques to resolve myosin heavy chain (MHC) isoforms using SDS-PAGE gels improved, allowing more precise definition of muscle fibers according to the MHC isoforms expressed (Pette et al., 1999). When combined with sophisticated mechanical measurements from single muscle fibers, this allowed MHC isoform composition to be directly compared with physiological parameters like shortening velocity and force production (Reiser et al., 1985). Pairing ATPase histochemistry with the microdissection of fibers and applying SDS-PAGE cemented the connection between fiber-type histochemistry and the MHC isoforms present (Staron and Hikida, 1992; Staron and Pette, 1986). The upper panel of the figure shows a cross-section of human muscle, with numbers corresponding to those for MHC content shown below. Double protein bands from single fibers demark hybrid fibers. Reprinted with permission from Staron and Hikida (1992).

Subsequently, full-length sequences encoding MHC isoforms were obtained, and their chromosomal locations for humans and mice were revealed (Weiss et al., 1999a,b). Researchers discovered that the IIB MHC in humans and other mammals was identical to the IIX isoform in mice and rats (Denardi et al., 1993; Smerdu et al., 1994).

Another powerful tool for the study of skeletal muscle fiber types has been the development of monoclonal antibodies specific for MHC isoforms (Schiaffino, 2018). The lower panel of the figure shows an image of rat soleus, with arrows indicating hybrid fibers (image from Medler lab). Immunohistochemistry of muscle sections combined with western blotting provides a high degree of precision in identifying skeletal muscle fiber types generally, but is especially significant for the identification of hybrid fibers. Currently, single-fiber SDS-PAGE and immunohistochemistry have become the standard approaches for identifying hybrid fibers.

intermediate contractile properties of these hybrid fibers help to provide a continuum of shortening speeds in amphibian muscles (Andruchova et al., 2006). In the garter snake, there appears to be a discrete separation of hybrid fibers and pure fiber types among muscles – the intermediate slow fibers always express a combination of fast and slow MHCs, whereas fast and slow tonic fibers each express a single fast or slow MHC, respectively (Wilkinson et al., 1991). In ghost crabs, anaerobic single fibers of the leg extensor and flexor muscles always express two different MHC isoforms in very close to 50:50 proportions (Perry et al., 2009). In the more aerobic regions of the same muscles, a third MHC isoform is expressed in varying proportions (Perry et al., 2009). Overall, the take-home point is that the relative abundance of hybrid fibers is highly variable among muscles, but that hybrid fibers are common.

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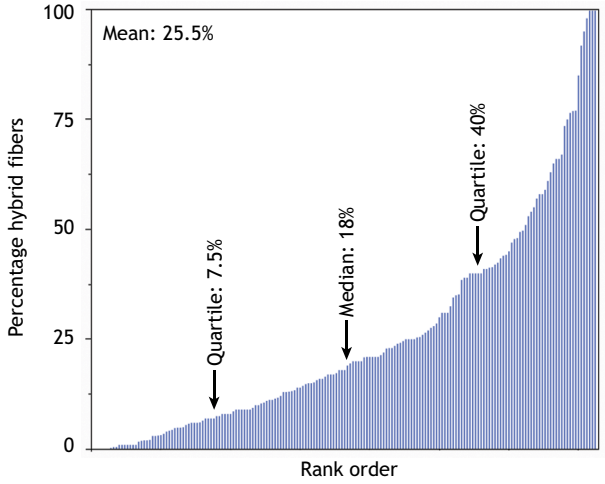

Fig. 1. Relative abundance of hybrid fibers in different muscles rank ordered according to hybrid fiber abundance. The proportion of hybrid fibers from 184 muscle samples from the 39 species listed in Table S1. The hybrid fiber composition of these muscles ranged from 0 to 100%, with a mean proportion of 25.5%. Half of the muscles possessed >18% hybrid fibers. The lower 25th quartile of muscles contained 7.5% or fewer hybrid fibers, whereas the 75th quartile comprised 40% or more hybrid fibers.

It is hypothesized that intermediate fiber types allow a muscle to achieve a greater range of contractile function. For example, extraocular and laryngeal muscles often possess large proportions of hybrid fibers expressing diverse combinations of MHC isoforms, and these muscles perform diverse contractions, from slow tonic to fast twitch (Bicer and Reiser, 2009; Hoh, 2005; McLoon et al., 2011; Porter, 2002; Rubinstein and Hoh, 2000; Wu et al., 1998, 2000a,b,c; Zhou et al., 2010). In some limb muscles, like the plantaris of rodents, hybrid fibers are abundant, and a variety of MHC combinations are represented (Table S1; Caiozzo et al., 2003; DeNies et al., 2014). By comparison, predominantly fast-twitch glycolytic muscles, like the 'white' regions of the vastus lateralis or vastus intermedius, comprise almost 100% type IIB fibers (Table S1; Caiozzo et al., 2003; Glaser et al., 2010), whereas the slow soleus is primarily composed of pure type I fibers, with type I/IIA hybrid fibers representing only 5–15% of the fibers in mouse, rat and human muscles (Table S1; Brummer et al., 2013; Caiozzo et al., 2003; DeNies et al., 2014; Glaser et al., 2010; Luden et al., 2012). Hypothetically, the plantaris, with its hybrid fibers, may generate more intermediate contractile patterns than fast 'white' muscles or the slow soleus.

Similar functional specialization also exists among certain species. For example, many felid species are ambush predators that depend upon explosive sprinting over short distances (Kohn et al., 2011a), whereas dogs and their canid relatives have evolved into highly aerobic endurance hunters (Acevedo and Rivero, 2006; Poole and Erickson, 2011). Of course, canids also rely upon bursts of speed, so their muscular performance may be more varied than that of felids. Accordingly, many of the dog limb muscles studied to date comprise 20–40% hybrid fibers (Acevedo and Rivero, 2006; Fig. 2; Table S1), whereas the muscles sampled from the lion and caracal contain large proportions of pure IIX fibers, and hybrid fibers account for <1% of fiber types (Kohn et al., 2011a). Likewise, several southern African prey species exhibit a preponderance of type IIX fibers, but relatively few hybrid fibers (Curry et al., 2012; Kohn, 2014). These trends are consistent with the hypothesis that

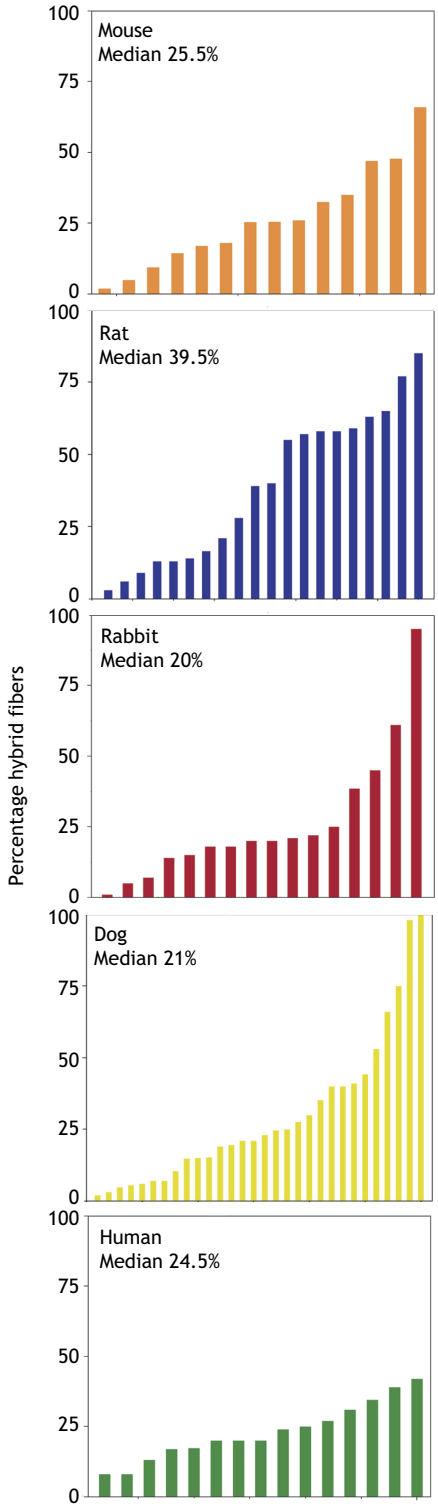

Fig. 2. Relative abundance of hybrid fibers in the muscles of mouse, rat, rabbit, dog and human. The percentage of hybrid fibers from various muscles is rank ordered according to hybrid fiber abundance. The relative proportions in the muscles of these intensively studied species are similar to those observed in Fig. 1 for all muscles. Overall, the median proportion of hybrid fibers ranges from 20% to 39.5%. Values for specific muscles can be found in Table S1.

muscles specialized for either explosive power output or slow sustained force production tend to have fewer hybrid fibers than muscles with more varied functional roles. However, these patterns

are largely anecdotal, and further study is needed to better understand the principles underlying hybrid fiber abundance.

Parameters used to classify fiber types

As discussed above, the MHC isoform is the main feature that defines muscle fiber type. The MHC protein comprises a head region (that contains both the ATP-binding region and an actin-binding region) as well as a rod region that facilitates polymerization into thick filaments (Schiaffino and Reggiani, 2011). Functional myosins consist of two MHC molecules and a total of four myosin light chains (MLCs). All MHC isoforms generate approximately the same magnitude of force, but shortening velocity among isoforms for a particular animal spans a range that varies in magnitude by up to 10-fold (Medler, 2002; Schiaffino and Reggiani, 2011). In mammals, the speed of shortening increases in the order MHC I<MHC IIA<MHC IIX<MHC IIB (Schiaffino and Reggiani, 2011). Myosin isoforms also determine the economy of force generation, with slower isoforms producing force for lower cost (Pette, 2006).

Metabolic capacity is another parameter that has been used to classify skeletal muscles. Fibers have often been classified as fast glycolytic (FG), fast oxidative glycolytic (FOG) or slow oxidative (SO) (Pette, 2006; Schiaffino, 2010; Schiaffino and Reggiani, 2011). Generally, fibers at the fastest end of the MHC-based classification are more glycolytic–anaerobic, whereas slower fibers tend to possess greater oxidative–aerobic potential. A reciprocal relationship typically exists between the relative degree of glycolytic–anaerobic versus oxidative–aerobic properties of muscle fibers. However, metabolic capacity within fibers exists along a continuum, even within single MHC-based fiber types (Punkt, 2002; Curry et al., 2012), and significant differences exist among species with respect to the correspondence between MHC isoform and metabolic capacity. For example, type I fibers are generally the most oxidative–aerobic fiber types in humans, whereas type IIA fibers hold this position in rodents (Schiaffino, 2010; Bloemberg and Quadrilatero, 2012). Wild African mammals possess a preponderance of type IIX fibers, yet these fast muscles also exhibit high levels of oxidative potential (Kohn et al., 2011b; Curry et al., 2012). Over time, the usage of a metabolic capacity-based system for identifying fiber types has declined and the MHC-based fiber-type classification scheme has become more standard (Schiaffino, 2010; Schiaffino and Reggiani, 2011).

It should be noted that, in addition to MHC isoforms, alternative isoforms of other myofibrillar proteins also characterize different fiber types (Medler and Mykles, 2015; Schiaffino and Reggiani, 2011). Significant correlations among different myofibrillar isoforms and other muscle parameters, like metabolic capacity, are discussed in greater detail below (see 'Hybrid fibers as points along a continuum').

Sarcomere length is another parameter that defines muscle fiber type, particularly in invertebrates. Sarcomere length in vertebrates is relatively constant, being in the range 2.5–3 µm at rest. Among animals more broadly, this uniformity in sarcomere length is not the rule. The body muscles of many invertebrates are composed of fibers with sarcomere lengths that range from 2 to >10 µm (Medler and Mykles, 2015; Paniagua et al., 1996). Sarcomere dimension is more than a descriptive morphological parameter, as muscle shortening velocity and force production are significantly influenced by sarcomere length (Huxley and Niedergerke, 1954; Josephson, 1975). Fibers with short sarcomeres contract with greater speed than those composed of longer sarcomeres. However, muscles with longer sarcomeres generate greater force. Although fibers possessing different sarcomere lengths are not necessarily thought of as being hybrid fibers, in principle, the continuum of sarcomere lengths provides the same range of contractile properties as the relative ratios of MHC isoforms. In invertebrates, sarcomere length acts as a functional parameter to affect shortening speed (Medler and Mykles, 2015; Shaffer and Kier, 2012, 2016).

Role of hybrid fibers in fiber-type transitions

Hybrid fibers are frequently interpreted as signifying transitional states in skeletal muscles; for example, during development, aging or exercise adaptation. Although hybrid fibers may play a central role in many fiber-type transitions, understanding the context of the specific study is vital to interpreting their role. For example, a hybrid I/IIA fiber subjected to endurance exercise may transform into a pure type I fiber (I/IIA→I), whereas a IIA fiber under the same conditions might become a I/IIA hybrid (IIA→I/IIA). Whether exercise elicits an increase or a decrease in the proportion of hybrid fibers ultimately depends on the starting composition of the muscle (Table 1). Moreover, hybrid fibers typically represent a stable component of 'normal' muscles from a variety of species (Figs 1 and 2; Table S1).

Nevertheless, as many fiber-type transitions proceed, the proportions of hybrid fibers are frequently in flux. The following sections focus on the role of hybrid fibers under a variety of conditions which promote changes in fiber type. In addition to the conditions discussed below, many other factors including thyroid hormones, androgens, β-agonists, experimental electrical stimulation, cancer and others conditions can have a significant impact on muscle fiber types (Blaauw et al., 2013).

Development

The prominence of hybrid fibers during development is a general feature of skeletal muscles in diverse groups of animals. In mammals and birds, developmental MHC isoforms are expressed early in myogenesis and are exchanged for adult isoforms as development proceeds (Bandman, 1992; Schiaffino et al., 2015). The development of claw closer muscles in decapod crustaceans provides another dramatic example of fiber-type changes (Mykles, 1997; Mykles and Medler, 2015). In lobsters, both claws comprise a mixture of fast and slow fibers up until the fourth molt. At this critical period of development, the claw that is used more frequently begins to differentiate into a crusher claw invested with slow muscle fibers. The contralateral claw is destined to differentiate into a cutter claw, primarily controlled by fast fibers. Thus, a fast-to-slow fiber-type progression occurs in the crusher claw, whereas a slow-to-fast transition occurs in the cutter (Costello and Lang, 1979; Govind and Lang, 1978; Lang et al., 1977a,b; Mykles and Medler, 2015). During these developmental phenotypic transitions, hybrid fibers transiently co-expressing multiple MHC and other myofibrillar isoforms are prominent. As the expression of myofibrillar isoforms changes, there is a period during which hybrid fibers containing different isoforms are present.

In birds, expression of a neonatal MHC isoform is exchanged for adult isoforms during post-hatch development (Bandman, 1992; Bandman and Rosser, 2000). However, residual expression of the neonatal isoform persists at the tapered ends of adult bird muscles (Rosser and Bandman, 2003; Rosser et al., 1995). In mammals, an embryonic MHC is first expressed early in development, and is sequentially replaced by a neonatal MHC and then adult MHC isoforms (Butler-Browne and Whalen, 1984; Denardi et al., 1993; Schiaffino et al., 2015). Hybrid fibers containing the neonatal MHC along with different combinations of adult isoforms are prominent in neonatal mammals, but typically only persist for a few weeks

Table 1. Effects of exercise on hybrid fiber proportions

Reference	Subjects	Type of training	Effects on % hybrid fibers	Comments
Longitudinal studies				
Andersen et al., 1994	Male sprinters	12 weeks sprint training	>50% decline	Increase in type IIA; decrease in type I
Trappe et al., 2000	Older men	12 weeks resistance	>40% decline	Increase in type I fibers
Williamson et al., 2000	Older men	12 weeks resistance	>60% decline	Increase in type I fibers
Williamson et al., 2001	Men/women	12 weeks resistance	>50% decline	Mostly toward IIA fibers
Parcell et al., 2003	Women	14 weeks resistance	No change	
Parcell et al., 2005	Men	8 weeks sprint cycle	No change	
Trappe et al., 2006	Men/women	16 weeks+marathon	∼50% decline	Increase in type I fibers
Malisoux et al., 2006	Men	8 weeks plyometric	No change	Increase in IIA fibers
Rietbroek et al., 2007	Horse	0.5–3 years jumping	No change	Developmental shift IIA/IIX→IIA fibers
Rivero et al., 2007	Horse	6 months conditioning	23% increase in IIA/IIX fibers	Decrease in IIX; increase in IIA
Glaser et al., 2010	Mouse	6 weeks treadmill running	No change	Increased capillary density
Luden et al., 2012	Men/women	13 weeks+marathon	∼50% decline in IIA/IIX fibers in VL	Increase in type I-containing fibers
Luden et al., 2012	Men/women	13 weeks+marathon	No change in soleus fibers	
Cross-sectional studies				
Klitgaard et al., 1990a	Men	BB versus control	∼2× fewer IIA/IIX hybrid fibers in BB	>IIA fibers in BB than controls
Klitgaard et al., 1990c	Men	Endurance trained versus control	More I/IIA; fewer IIA/IIX hybrid fibers	Trained had more type I fibers
Harber et al., 2002	Men	Runners versus control	Fewer in competitive runners	Dose response with running distance
Kohn et al., 2007	Men	Runners versus control	Fewer in competitive runners	Dose response with running distance
Kesidis et al., 2008	Men	Body building versus control	∼2× more I/IIA hybrid fibers in BB	>IIA fibers in BB than controls
Bathgate et al., 2018	Male twins	30 years endurance	10× fewer hybrid fibers with training	94% type I fibers in endurance trained
Serrano et al., 2019	Men/women	Olympic weightlifters	11% total hybrid fibers (range: 1–37%)	High proportions of IIA fibers (∼67%)

VL, vastus lateralis; BB, body building.

(Agbulut et al., 2003; Di Maso et al., 2000). Some level of developmental MHC expression may persist in adult muscles, including in extraocular and laryngeal muscles (Schiaffino et al., 2015), as well as in some instances following injury and regeneration (D'Antona et al., 2003, 2006; Blaauw et al., 2013).

Eventually, the neonatal MHC essentially disappears from maturing fibers, but hybrid fibers containing multiple MHCs persist. However, the precise combinations of these isoforms vary among specific muscles and among species. For example, cat soleus follows a transition to slow fibers, whereas faster fibers of the tibialis anterior consist of different hybrid combinations (Unguez et al., 2000). In the developing rat plantaris, an enormous range of fiber types are present during neonatal development, and many of the hybrid fibers persist well into adulthood (Di Maso et al., 2000). In mouse muscles containing large proportions of IIX/IIB hybrids, there is variability during maturation: some of these hybrid fibers transition into type IIX fibers, some into type IIB and some persist as hybrid fibers (Brummer et al., 2013). Although the precise fiber-type transitions and their timing vary among different species – and also vary with other factors like activity and hormone levels (Schiaffino et al., 2015) – a role for hybrid fibers as transient fiber types seems to be a consistent feature during development.

The transition to adult myofibrillar isoforms coincides with increased motor activity at the time of weaning, and thyroid hormones also play a critical role in this transition (Di Maso et al., 2000; Štrbenc et al., 2006; Wu et al., 2000a). Continued fiber-type transitions may occur over a period of months or years, depending on the species. In lobsters, co-expression of fast and slow MHC isoforms in the claw muscles occurs through several molt stages and the fiber-type transition may take up to 2 years to reach completion (Medler et al., 2007). Even fully differentiated claw muscles co-express varying levels of both slow and fast MHC at the level of mRNA (Medler et al., 2004, 2007; Medler and Mykles, 2003). In the rat soleus, hybrid fibers containing type IIA MHC gradually transition into pure type I fibers over a period of weeks to months (Wigston and English, 1992; Larson et al., 2019). In horses, the relative proportion of type IIA/IIX hybrid fibers declines and the number of pure IIA fibers increases over a period of 3–4 years (Rietbroek et al., 2007; Yamano et al., 2005).

Exercise

Exercise has a major impact on skeletal muscle phenotype. Generally, almost any type of exercise or activity appears to drive skeletal muscles toward slower fiber types (Blaauw et al., 2013). The effects of exercise on fiber type have been investigated using longitudinal studies, where muscle biopsies are used to collect fibers from subjects before and after an exercise intervention. Several of these studies have demonstrated that exercise intervention can significantly affect the proportion of hybrid fibers over a period of weeks (see Table 1). For example, 12 weeks of resistance training in previously untrained college-aged men and women results in significant reductions in the number of hybrid fibers in the vastus lateralis (Williamson et al., 2001). In both men and women, the reduction in hybrid fibers corresponds to a significant increase in pure type IIA fibers (Williamson et al., 2001). A similar pattern was observed in the vastus lateralis of older men in studies with a similar experimental design (Trappe et al., 2000; Williamson et al., 2000). However, the older subjects also exhibit an increase in pure type I fibers that was not observed in the younger groups. Marathon training also reduces the proportion of hybrid fibers, but with a predominant shift towards the slower type I fibers, rather than type IIA fibers, which increase with resistance training (Trappe et al., 2006). In a subsequent study with a similar experimental design, a significant reduction in IIA/IIX hybrids was observed in the vastus lateralis (12% to 6%), but no changes in fiber type were detected in the slower soleus muscle, and no other significant fiber-type changes were detected (Luden et al., 2012). Similar patterns are observed in the skeletal muscles of horses as a result of training (reviewed by Rivero and Hill, 2016). The most common hybrid fibers in horse muscles are IIA/IIX fibers, which generally constitute ∼10–25% of fibers in the gluteus medius (Table S1). Training typically causes an adaptive shift in fiber type in the direction IIX→IIA/IIX→IIA (Rivero and Hill, 2016).

Collectively, these studies indicate that, with exercise, the proportion of hybrid fibers declines, as they transform primarily into pure type I (with endurance training) or type IIA fibers (with resistance training). This shift in fiber type away from IIX expression towards slower fiber types is consistent with global changes in MHC content as determined from whole-muscle homogenates with training (Adams et al., 1993; Staron et al., 1994).

By contrast, a number of studies have failed to detect any changes in fiber type following exercise. For example, fiber-type composition in elite female track athletes participating in short sprint-type events does not differ from that of age-matched sedentary subjects (Parcell et al., 2003). Eight weeks of cycle sprint training of college-aged men results in a reduction in the proportion of IIX fibers and a concomitant increase in IIA fibers, but without any significant change in the proportion of hybrid fibers (Parcell et al., 2005). Young adult mice trained on a treadmill for 6 weeks experience increased muscle mass and capillary density, but no changes in fiber-type proportions (Glaser et al., 2010). Consistent with these patterns, training for show jumping in horses results in significant changes in several muscle performance parameters but no changes in muscle fiber-type proportions (Rietbroek et al., 2007). It seems likely that the training volume or intensity in these examples did not reach the threshold required to drive fiber-type changes. Indeed, a study investigating training intensity and duration in race horses revealed that adaptive changes in the muscles are proportional to the volume and intensity of exercise (Rivero et al., 2007). In particular, the observed type IIX→IIA shift is more dependent upon the intensity of training than the duration (Rivero et al., 2007).

Cross-sectional studies of different athletes report similar adaptive responses to those observed in longitudinal studies (Table 1). For example, highly trained cross-country skiers possess significantly higher proportions of type I and I/IIA hybrids than moderately active and sedentary subjects (Klitgaard et al., 1990c). Similarly, competitive male distance runners exhibit significantly higher proportions of type I fibers (~75%), but significantly lower proportions of hybrid fibers (6%) when compared with mid-distance athletes (54% type I; 13% hybrid fibers), or with recreational runners (56% type I, 23% hybrid fibers) (Harber et al., 2002). The major differences in hybrid fibers occur principally in the IIA/IIX fibers, which are non-existent in the distance runners. A similar pattern was reported in another study of competitive male distance runners: this study showed that athletes possess a greater proportion of type I fibers (49%) and a lower proportion of hybrid fibers (10.5%) when compared with non-runners (33% type I, 19% hybrid fibers) (Kohn et al., 2007). Moreover, when runners are divided into groups of longer versus shorter race distances, the skew towards more type I fibers and fewer hybrid fibers is more pronounced (55% type I fibers and <10% hybrid fibers in the longer-distance athletes). Interestingly, the proportion of type IIA/IIX hybrids is inversely correlated with training volume, whereas the proportion of type I/IIA hybrids is positively correlated with the amount of training (Kohn et al., 2007). In a study comparing elite body builders with physical education students, the muscles of the body builders were found to possess significantly more type IIA and I/IIA hybrids (39% and 19%, respectively) than those of the students (31% and 9%) (Kesidis et al., 2008). These results are consistent with an earlier study that reported a greater proportion of IIA fibers and lower proportions of IIA/IIX fibers of body builders versus controls (Klitgaard et al., 1990a). A recent case study compared multiple skeletal muscle parameters of identical twins, one of whom trained for endurance races for 30 years, compared with his brother, who had been sedentary. This study revealed that the proportion of hybrid fibers was 10 times greater in the sedentary brother, but the trained sibling possessed more than 90% type I fibers (Bathgate et al., 2018). Taken together, these observational studies are consistent with the pattern that physical exercise broadly leads to a shift towards slower fiber types, but the precise changes that occur depend upon the type of training.

Disuse

Disuse can take a range of forms, including simple inactivity, space flight (Fitts et al., 2000), bed rest (Andersen et al., 1999a; Borina et al., 2010; Gallagher et al., 2005), spinal cord injuries (Talmadge, 2000; Talmadge et al., 1995) and animal hibernation (Cotton, 2016; Riley et al., 2018). Collectively, these various types of muscle disuse drive the transformation towards faster fiber types (Blaauw et al., 2013; Talmadge, 2000). This general shift toward faster fiber types means that the proportion of hybrid fibers frequently increases, as existing slow fibers begin to change their expression to fast MHC isoforms. For example, spinal cord transection in rats elicits a dramatic shift towards hybrid fibers that is detected within a few days of injury, and this transition continues for up to a year (Talmadge et al., 1995, 1999). During space flight and in other anti-gravity models, there is a rapid reduction in slow fiber types, particularly in weight-bearing muscles, and an increase in hybrid fibers expressing fast MHCs that occurs within days of unloading (Fitts et al., 2000; Shenkman, 2016). Humans experience a global shift towards faster MHC isoform expression and a dramatic increase in the proportion of hybrid fibers after 35 days (Borina et al., 2010) and 84 days (Gallagher et al., 2005) of bed rest, respectively. In addition to the more common I/IIA and IIA/IIX hybrid types, the study by Gallagher et al. (2005) showed that bed rest also resulted in an increase in the proportion of fibers co-expressing all three isoforms: I, IIA and IIX. In another study, no changes in fiber type were observed at the protein level, but a significant shift towards faster MHC isoforms was detected at the mRNA level following 37 days of bed rest (Andersen et al., 1999a). Thus, the duration of inactivity or unloading affects the degree to which fiber-type transformations occur.

Skeletal muscles of hibernators are resistant to the dramatic atrophy and fiber-type shifts observed in humans during bed rest (Cotton, 2016). However, there is evidence that their muscles may experience a shift towards the expression of faster MHC isoforms. This is evident from a recent study of the soleus muscles of hibernating bears, in which the proportion of hybrid fibers co-expressing MHC I and IIA increased from 2% in the summer to 24% during hibernation (sampled in February and March) (Table S1; Riley et al., 2018). Most of these fibers appear to contain mostly slow MHC, suggesting that the inactivity inherent in hibernation is associated with an upregulation of the expression of fast MHC isoforms within these slow fibers.

Aging

Muscles of the elderly commonly exhibit multiple dysfunctional changes that characterize a condition referred to as sarcopenia (Larsson et al., 2019). Alterations of muscle phenotype in sarcopenia can be complex, but typically include severe atrophy and shifting muscle fiber types. A number of studies have reported that hybrid fibers become more prevalent in the muscles of the elderly (Andersen et al., 1999b; D'Antona et al., 2003; Klitgaard et al., 1990b). In these studies, muscles of older individuals (~69–88 years) frequently possess >50% hybrid fibers, and these proportions are significantly greater than in younger controls (~30 years). In the study by D'Antona et al. (2003), elderly immobilized muscles exhibit an even greater elevation in hybrid fibers, particularly in the proportion of type IIA/IIX hybrids (30%). Consistent with the higher proportions of

hybrid fibers associated with increasing age in human studies, the muscles of senescent rats (35 months) possess higher proportions of hybrid fibers than those of young adult animals (7–10 months) (Carter et al., 2010). Interestingly, in that study, fiber types that were predominantly fast or slow both displayed co-expression of the non-dominant MHC isoform within single fibers taken from the older muscles (Carter et al., 2010).

One of the reported effects of aging on skeletal muscle is a process of denervation, followed by re-innervation from the collaterals of adjacent motor neurons (Lexell et al., 1988). A non-random pattern of fiber-type clumping in histological sections has been interpreted as evidence of this process. A common interpretation of the high prevalence of hybrid fibers in aging muscle is that these re-innervated fibers are responding to conflicting signals between the original fiber type and the neural signals from the new motor neuron (Andersen et al., 1999b; Purves-Smith et al., 2014). It has also been reported that hybrid fibers co-expressing type I and IIA MHCs have been mis-classified as type I fibers in elderly individuals (Purves-Smith et al., 2014).

Although the evidence for an increase in the proportion of hybrid fibers in some older people seems compelling, it is not clear that these hybrid fibers are an inevitable symptom of aging muscles. First, hybrid fibers frequently represent a normal component of healthy muscle (Figs 1 and 2; Table S1). Second, studies of exercise interventions in the elderly report similar responses to training as their younger counterparts. For example, a longitudinal study of elderly subjects (average age 74 years old) showed a significant decline in hybrid and type IIX fiber proportions following 12 weeks of resistance training (Williamson et al., 2000). Furthermore, the hybrid content of the muscles from these subjects was only about 30% before training, which is not substantially higher than that typical of younger subjects (Table S1). Following the training period, the proportion of hybrid fibers was reduced to ∼11% (Williamson et al., 2000).

Data on hybrid fibers has also been collected from masters athletes – athletes who compete against age-matched competitors beginning at ages 35–40 years. A cross-sectional study of masters track athletes revealed that older inactive men (∼79 years old) exhibit a relatively large proportion of IIA/IIX hybrids (>20%) compared with age-matched world-class masters track athletes (∼5%) (Power et al., 2016). But other than possessing significantly fewer type IIX fibers, the fiber-type proportions of the masters athletes were not different from those of younger men (Power et al., 2016). Collectively, these results illustrate that the inactivity associated with aging, rather than age per se, may underlie the increased proportion of hybrid fibers observed in older individuals. Other studies have failed to identify any differences in the proportions of hybrid fibers between young and old subjects (Gueugneau et al., 2015; Trappe et al., 2003). In contrast to the work on humans, a study of a non-human primate, the green vervet monkey, actually reported fewer hybrid fibers in the muscles of older individuals (51%) when compared with their younger counterparts (67%) (Feng et al., 2012). The older monkeys in that study were aged 21–26 years, which corresponds to humans in their 70s (Feng et al., 2012). Overall, it seems that an increase in hybrid fibers is a characteristic of some aging muscles, but other factors, including specific age, level of physical activity, illness and other variables, determine the precise proportion of hybrid fibers.

Hybrid fibers as points along a continuum

As mentioned above, hybrid fibers exhibit properties that are intermediate to those of pure fibers expressing single MHC isoforms. This principle was originally reported in seminal studies demonstrating a direct correlation between MHC isoform content and the physiological properties of muscles (Reiser et al., 1988a,b, 1985). Subsequent studies have confirmed that hybrid fibers exhibit intermediate shortening velocities, and in hybrid fibers with different proportions of MHC isoforms, the precise shortening velocity is proportional to the relative amount of these isoforms (Andruchov et al., 2004; Andruchova et al., 2006; Larsson and Moss, 1993). It is now generally understood that a ‘continuum’ of fiber types exists (Fig. 3A,B; Caiozzo, 2002; Pette, 2006; Pette and Staron, 2000; Brummer et al., 2013; DeNies et al., 2014; Medler et al., 2004; Zhang et al., 2010). Indeed, hybrid fibers exhibit contractile properties that provide for a continuum of shortening velocities and energetics (Andruchova et al., 2006; Conjard et al.,

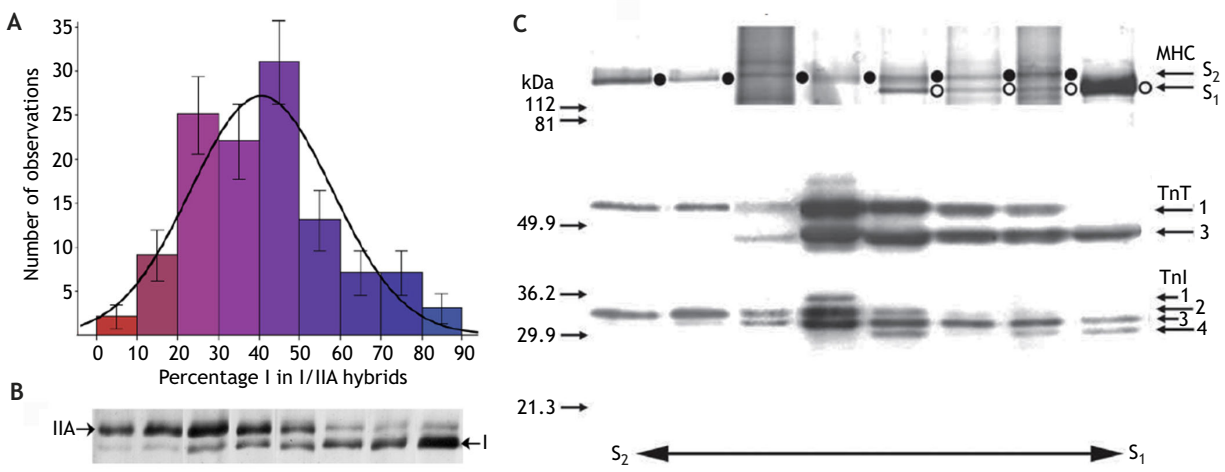

Fig. 3. Continuum of hybrid fiber types. (A) Relative proportion of myosin heavy chain (MHC) I and IIA within single fibers from mouse soleus muscles. The ratios of the two MHC proteins within single fibers were normally distributed, with the majority of fibers possessing similar amounts of each isoform. (B) Representative SDS-PAGE gels of I/IIA hybrids ordered from IIA-dominant hybrid fibers (left, corresponding to red bars in the graph in A) to I-dominant hybrid fibers (right, blue bars in A). (C) A continuum between slow, S_2 fibers and S_1 fibers is evident in lobster superficial abdominal extensor and flexor muscles. These fibers are defined not only by MHC isoforms but also by isoforms of troponin T (TnT) and troponin I (TnI). The MHC isoforms were identified by SDS-PAGE gels, whereas TnT and TnI panels show western blots. A and B are from Denies et al. (2014) with permission; C is from Medler et al. (2004) with permission.

1998; Greaser et al., 1988; Pette, 2006; Pette and Staron, 2000; Reiser et al., 1985; Staron and Pette, 1993).

Pette and colleagues also recognized that hybrid fibers are typically composed of 'nearest neighbor' MHC isoforms, i.e. those isoforms that are closest to one another in terms of shortening velocities (Pette, 2006; Pette and Staron, 1997). Although fibers co-expressing MHC isoforms outside of this pattern are sometimes observed, the large majority of hybrid fibers reported in the literature do conform to this nearest neighbor pattern (Pette, 2006). The precise hybrid fibers comprising a particular muscle depend upon the body size and the specific muscle fiber types most prevalent in a species (see Table S1). For example, in the muscles of small mammals like mice and rats, the large majority of hybrid fibers are IIX/IIB hybrids, consistent with the preponderance of fast fibers in these small animals. In humans, most hybrid fibers are either I/IIA towards the slower end of the continuum, or IIA/IIX hybrids at the faster end. In horses, nearly all of the hybrid fibers identified have been IIA/IIX, and these swift runners also have significant proportions of 'pure' IIA and IIX fibers. The role of hybrid fibers as physiological intermediates does not contradict their important role in fiber-type transitions, but we should recognize that hybrid fibers are also major constituents of phenotypically stable muscles (Figs 1 and 2; Table S1).

Muscle fiber properties are defined by the MHC isoforms that directly determine muscle shortening velocity, power output and other important aspects of function. However, these myosin motors work in conjunction with other essential parameters, including other myofibrillar proteins, metabolic enzymes, sarcoplasmic reticular proteins and structural parameters (Pette, 2006; Schiaffino and Reggiani, 2011; Stephenson, 2001). Skeletal muscles in decapod crustaceans provide a good illustration of hybrid fibers that are defined by assemblages of alternative isoforms of MHC, MLC, troponins, tropomyosin and other myofibrillar proteins (Medler and Mykles, 2015; Mykles, 1985, 1997). Lobster skeletal muscles comprise fibers classified as fast, slow phasic (S_1) and slow tonic (S_2). Each fiber type is characterized by alternative isoforms of MHC, tropomyosin, actin, troponin T, troponin I, troponin C and MLC. The expression of these myofibrillar isoforms is fiber-type dependent, but significant overlap exists among different fibers (Medler et al., 2004; Medler and Mykles, 2003). In lobster tail muscles, superficial extensors and flexors are composed of individual S_1 and S_2 muscle fibers (Medler et al., 2004). Many of these fibers are hybrid fibers, co-expressing varying proportions of S_1 and S_2 MHC isoforms and other associated myofibrillar isoforms at both the protein and mRNA levels (Medler et al., 2004). Expression levels of the alternative isoforms of MHC, troponin I, troponin T and tropomyosin myofibrillar proteins are correlated across a continuum of fiber types, ranging from 'pure' S_1 to 'pure' S_2 fibers (Fig. 3C) (Medler et al., 2004). Similar correlations in the expression levels of the isoforms of a variety of myofibrillar proteins exist in fast and S_1 fiber types within the lobster claw closer muscle (Medler and Mykles, 2003), in the leg muscles of ghost crabs (Perry et al., 2009) and in the muscles of crayfish (LaFramboise et al., 2000; Mykles et al., 2002).

The significant correlations in skeletal muscle parameters observed in these single fibers are typical of mammalian muscle fibers as well. Rat soleus fibers transforming from type IIA to type I exhibit graded levels of MLC isoforms in conjunction with their intermediate levels of MHC isoforms (Stevens et al., 2004). Combined immunohistochemistry and metabolic histochemistry demonstrate a continuum of fiber types in mouse, rat and human muscles (Bloemberg and Quadrilatero, 2012). Rivero and colleagues have demonstrated that there is significant interdependence among contractile, metabolic and morphological properties within single fibers from several mammalian species, including dog (Acevedo and Rivero, 2006), pig (Quiroz-Rothe and Rivero, 2004), llama (Graziotti et al., 2001), goat (Arguello et al., 2001), cow (Moreno-Sánchez et al., 2008) and horse (Quiroz-Rothe and Rivero, 2001). Each of these examples confirms that the expression levels of MHC isoforms are significantly correlated with other aspects of muscle cell physiology, including myofibrillar ATPase activity, metabolic capacity, capillary density and SERCA isoform content.

Fiber-type asymmetries

Given that hybrid fibers by definition contain two or more fiber type-specific isoforms of a given muscle protein, an essential question is: how are these proteins arranged spatially within a single cell? When considering MHC, a variety of patterns of regional expression are possible. Different isoforms of MHC might be incorporated into the same thick filaments, they could be segregated among adjacent myofibrils within the same fiber, or they could be located at different segments along a fiber (Figs 4 and 5). Each of these patterns has been reported for a variety of muscles from

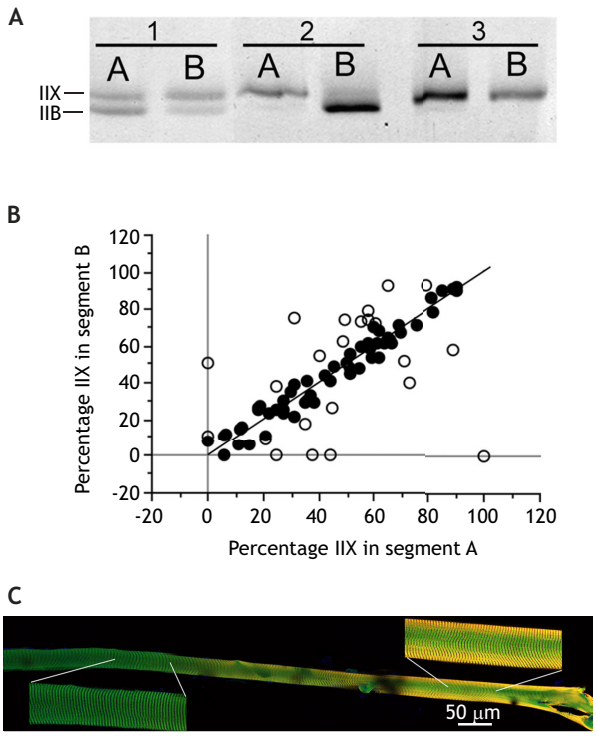

Fig. 4. Asymmetries in MHC isoform content along the length of single hybrid fibers in mouse muscle. (A) Pairs of fiber segments (A and B) from single IIX/IIB hybrids possess different amounts of each MHC isoform. In some fibers, the two segments express slightly different amounts of the two isoforms (fiber 1). Some fibers express totally different isoforms in different segments of the same fibers (fiber 2: segment A is IIX, while segment B is IIB). Fiber 3 is a 'pure' type IIX fiber in both segments. (B) The correlation between pairs of fiber segments. About 30% of IIX/IIB hybrids possess different proportions of the IIX and IIB isoforms (open circles). (C) A single mouse I/IIA hybrid fiber dissected from the soleus. Type IIA MHC was labeled with monoclonal antibody A4.74 (green), whereas MHC I was labeled with monoclonal antibody 4.84 (red). Yellow labeling represents co-expression of the two isoforms. This single fiber is a type IIA fiber on the left, but gradually changes to a I/IIA hybrid fiber towards the right. Insets show the indicated regions at higher magnification. A and B are from Zhang et al. (2010) with permission; C is from the Medler lab.

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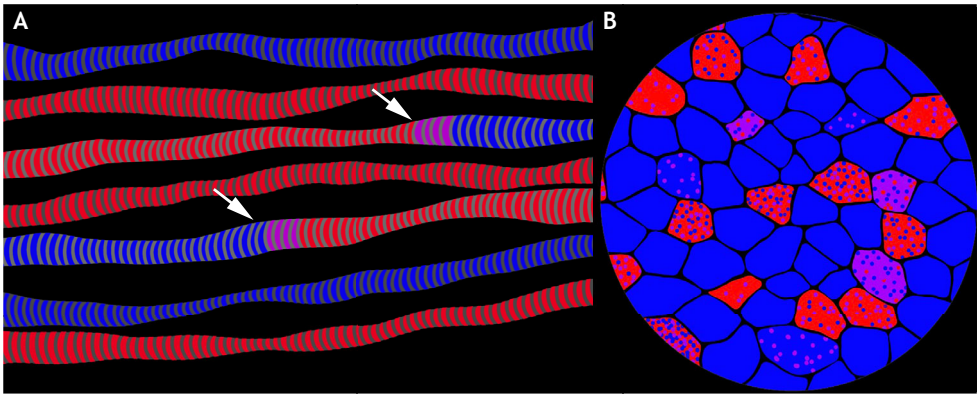

Fig. 5. Types of fiber-type asymmetries in hybrid skeletal fibers. (A) Depiction of longitudinal differences in MHC content. Two different MHC isoforms are indicated as red or blue. Regions of purple indicate co-expression within the same thick filaments. Arrows indicate two fibers that possess differences in MHC isoform content along their length. (B) Depiction of a muscle cross-section, where fibers possessing different MHC isoforms are segregated among different myofibrils within individual muscle fibers. Again, red and blue correspond to distinct MHC isoforms and purple denotes co-expression of the two isoforms within the same myofibril. Each of these patterns has been documented in different studies listed in Table 2.

multiple species (Table 2). Although most of the reported asymmetries in the distribution of fiber type-specific proteins involve MHC isoforms that are expressed within different regions of the fiber, it should be noted that some studies have also reported non-uniform expression of MLC isoforms (Lutz et al., 2001) and isoforms of troponin T (Nakada et al., 2000, 1997). The prevalence of regional differences in muscle protein expression is currently unknown, but this is an important issue to address.

In many instances, authors reporting fiber-type asymmetries attribute these patterns to experimental conditions, including motor neuron transplantation (Salviati et al., 1986), electrical stimulation (Staron and Pette, 1987), denervation (Schiaffino et al., 1988), muscle transplantation (Yao et al., 1994), aging (Andersen, 2003) or even diabetes (Snow et al., 2005). Although these conditions may well have contributed to the observed fiber-type asymmetries, dramatic regional differences in MHC expression are also

Table 2. Fiber-type asymmetries within single fibers

Species	Muscle	Condition	Pattern identified	Reference
Frog	TA	Control	Differences in shortening velocity of single-fiber segments	Edman et al., 1985
Rat	Soleus	Nerve transplant	Ectopic fast expression of MHC	Salviati et al., 1986
Rabbit	TA	Electrical stimulation	Longitudinal differences in MHC	Staron and Pette, 1987
Frog	TA	Control	Differences in MHC content among single-fiber segments	Edman et al., 1988
Rat	TA/soleus	Denervation	Regional expression of developmental MHCs	Schiaffino et al., 1988
Rat	Extraocular	Control	Regional differences in MHC expression	Jacoby et al., 1989
Cat	Intrafusal	Control	Regional differences in MHC expression	Kucera and Walro, 1989
Chicken	Pectoralis	Development	MHC_{emb} and MHC_{neo} in single thick filaments	Taylor and Bandman, 1989
Chicken	Pectoralis	Development	Myofibrils containing MHC_{emb} and MHC_{neo} present	Gauthier, 1990
Quail	Pectoralis	Stretch	Non-uniform fast MHC along fiber length	Alway, 1993
Chicken	Intrafusal	Control	Longitudinal differences in MHC isoforms	Maier, 1994
Chicken	Pectoralis	Muscle transplant	Slow TnT isoforms within single fast fibers	Yao et al., 1994
Rat	Soleus	Control	Longitudinal differences in MHC isoforms	Sakuma et al., 1995
Chicken	Pectoral	Control	MHC_{neo} restricted to tapered ends of fibers	Rosser et al., 1995
Chicken	ALD/Rhomb.	Control	Fast and slow TnT isoforms in single fibers	Nakada et al., 1997
Rabbit	TA	Control	Differences in MHC among fiber segments	Peuker and Pette, 1997
Pigeon	Pectoralis	Control	MHC_{neo} in tapered ends of fibers	Bartnik et al., 1999
Chicken	Biceps femoris	Control	Breast and leg TnT isoforms in single fibers	Nakada et al., 2000
Rat	Extraocular	Development	Longitudinal differences in MHC_{emb} and MHC_{EO}	Rubinstein and Hoh, 2000
Frog	TA	Control	Longitudinal differences in MHC and MLC content	Lutz et al., 2001
Human	Intrafusal	Control	Complex arrangements of four MHC isoforms	Liu et al., 2002
Human	VL	Elderly	Abrupt changes in MHC isoforms longitudinally	Andersen, 2003
Rabbit	Extraocular	Control	Longitudinal differences in MHC_{emb} and MHC_{EO}	Lucas and Hoh, 2003
Rat	Extraocular	Development	Longitudinal differences in MHC_{emb} and MHC_{EO}	Rubinstein et al., 2004
Rat	Soleus	Diabetes	Longitudinal differences in MHC along fiber segments	Snow et al., 2005
Mouse	TA and BR	Control	Differences in IIX and IIB MHC among fiber segments	Zhang et al., 2010
Rabbit	Extraocular	Control	Longitudinal differences in MHC isoforms	Mcloon et al., 2011
Mouse	TA and BR	Postnatal	Differences in IIX and IIB between fiber middle and ends	Brummer et al., 2013
Rabbit	Jaw/gastric	Control	Longitudinal differences in MHC I and IIA	Korfage et al., 2015
Rat	EDL	Control	Longitudinal differences in adult MHC	Zhang and Gould, 2017
Rabbit	Extraocular	Control	Longitudinal differences in MHC isoform expression	Lucas et al., 2018
Rat	Soleus	Postnatal	Myofibrils containing MHC IIA and I present	Larson et al., 2019

MHC, myosin heavy chain; TA, tibialis anterior; ALD, anterior latissimus dorsi; Rhomb., rhomboideus; VL, vastus lateralis; BR, brachioradialis; MHC_{emb}, embryonic MHC; MHC_{EO}, extraocular MHC; MHC_{neo}, neonatal MHC.

commonly observed in normal, control muscles (Table 2). For example, Peuker and Pette (1997) reported that one-third of the fibers from the rabbit tibialis anterior muscle exhibited significant differences in MHC isoform among segments of the same fibers. Consistent with this pattern, fibers from mouse tibialis anterior and brachioradialis muscles frequently show differences in the amounts of IIX and IIB MHC in single hybrid fibers (Brummer et al., 2013; Zhang et al., 2010). Approximately one-third of the hybrid fibers from these normal mouse muscles exhibit significant differences in the proportions of IIX and IIB between segments of the same fiber (Fig. 4) (Zhang et al., 2010). Other more recent studies have reported similar patterns in normal mammalian muscles (Korfage et al., 2015; Zhang and Gould, 2017).

In the pectoral muscles of birds, adult fibers express developmental MHC isoforms in their tapered ends, and this graded MHC expression is influenced by the activity of the motor neuron connected to the fiber (Rosser and Bandman, 2003). MHC isoform expression may also be influenced by innervation in extraocular and intrafusal muscle fibers (Maier, 1994; Rubinstein et al., 2004). Given the large number of studies reporting fiber-type asymmetries in normal muscles, it seems likely that major differences in regional protein expression within single fibers may be an under-recognized general feature of skeletal muscles.

One of the most dramatic patterns of MHC and other myofibrillar isoform distribution is regional differences in protein content along the length of single fibers (Alway, 1993; Andersen, 2003; Bartnik et al., 1999; Brummer et al., 2013; Edman et al., 1988; Korfage et al., 2015; Larson et al., 2019; Lucas and Hoh, 2003; Lucas et al., 2018; Lutz et al., 2001; Maier, 1994; McLoon et al., 2011; Peuker and Pette, 1997; Rosser et al., 1995; Rubinstein and Hoh, 2000; Rubinstein et al., 2004; Sakuma et al., 1995; Snow et al., 2005; Staron and Pette, 1987; Zhang and Gould, 2017; Zhang et al., 2010). Such regional differences are consistent with the fact that frog muscle fibers exhibit major differences in shortening velocity in different segments from a single fiber (Edman et al., 1985). Subsequent studies confirmed that these fibers exhibit differences in MHC and MLC content along their lengths (Edman et al., 1988; Lutz et al., 2001). These fiber-type asymmetries may have important functional implications for *in vivo* muscle performance (Ahn et al., 2018, 2003; Edman et al., 1988, 1985). For example, several recent studies have focused on spatial scale and structural heterogeneity in the function of skeletal muscles (Lichtwark et al., 2018; Moo et al., 2016; Williams and Holt, 2018). Researchers focusing on these functional aspects of skeletal muscle organization should be alert to the possibility that differences in the contractile properties along the length of a muscle fiber may play a significant role in these patterns.

Myonuclear domains and fiber-type asymmetries

Skeletal muscle fibers are very large cells formed by the fusion of hundreds of myoblasts during development (Allen et al., 1999; White et al., 2010). In mature fibers, satellite cells fuse with the fiber in response to wear and tear, and their nuclei become active determinants of muscle phenotype (Zammit et al., 2006; Bruusgaard et al., 2010; Gundersen, 2016). In addition, myonuclei from satellite cells may continually join with uninjured adult muscle fibers throughout life, especially in certain muscles including the diaphragm and extraocular muscles (Keefe et al., 2015; McLoon and Wirtschafter, 2002, 2003; McLoon et al., 2004; Pawlikowski et al., 2015). The resultant fibers contain hundreds of myonuclei, each controlling a limited volume of the fiber, referred to as a 'myonuclear domain'. Myonuclear domain size in mammalian muscle fibers ranges in volume from 20 to 100 μm^3 (Liu et al., 2009). Several authors have proposed that regional differences in MHC isoforms within hybrid fibers could be the result of differential expression among the myonuclei within these fibers (Brummer et al., 2013; McLoon et al., 2011; Stephenson, 2001; Zhang and Gould, 2017; Zhang et al., 2010). Indeed, the activity of multiple myonuclei within single fibers may differ significantly through time and space. For example, Newlands et al. (1998) reported that transcriptional activity was stochastic and occurred in pulses among the myonuclei of single fibers.

A mechanism that may explain regional differences in gene expression within single fibers is that transcription factors can differentially affect gene expression among myonuclei within the same fiber. For example, the NFAT family of transcription factors function as neural activity sensors, linking excitation to transcriptional activity (Gundersen, 2011; Schiaffino and Reggiani, 2011; Schiaffino et al., 2007). The four isoforms of NFAT identified in skeletal muscles have fiber type-specific effects on transcriptional activity (Abbott et al., 1998; Calabria et al., 2009; McCullagh et al., 2004). For example, binding of NFATc1 is sufficient to induce slow MHC expression and inhibit the expression of fast MHC IIB (McCullagh et al., 2004). By contrast, NFATc4 appears to be involved in the default expression of fast MHC IIX and IIB (Calabri et al., 2009). Before these transcription factors can affect gene expression, they must be translocated into nuclei. Abbott et al. (1998) demonstrated that distinct populations of myonuclei within the same cultured myotubes differ in their capacity to import specific NFAT isoforms. More recently, a study from the same research group has discovered that nuclear import within single fibers exhibits significant variability among closely spaced myonuclei (Cutler et al., 2018). The interaction between selective nuclear import processes and specific isoforms of NFAT provides a plausible mechanism for explaining regional differences in fiber type-specific expression among myonuclear domains within single fibers. Of course, NFAT is just one of several transcription factors that might be involved here; a number of other important transcription factors (e.g. MEF2, myogenin, MyoD) should also be considered for possible differential effects on transcriptional activity among myonuclei within single muscle fibers.

Conclusions and future directions

Hybrid fibers are common components of normal skeletal muscles. Although the number of studies reporting hybrid fibers continues to increase, the currently available data are highly skewed towards only a few mammalian species. The relative proportion of hybrid fibers varies significantly among muscles within an individual, as well as among different species. New data from multiple muscles representing diverse animals are needed to more fully understand the prevalence of hybrid fibers.

Although an increase in hybrid fibers may be associated with fiber-type transitions during muscle development, disuse or aging, the presence of hybrid fibers is not always an indicator of fiber-type transition. Indeed, many types of exercise actually lead to a decrease in the proportion of hybrid fibers (Table 1). Hybrid fibers typically possess contractile properties that are intermediate to those of fibers expressing single MHC isoforms. The idea that hybrid fibers provide intermediate fiber types that ultimately offer a continuum of available fiber types is broadly consistent with the available data.

The spatial distribution of MHC and other myofibrillar isoforms within single muscle fibers requires further investigation. Muscle fibers are unusual in possessing hundreds of nuclei within a single cell. How closely coordinated these myonuclei are in their expression remains uncertain, but in some circumstances, it is

clear that the nuclei operate with a level of independence. Hybrid fibers from a variety of experimental contexts should provide unique opportunities for investigating expression patterns under different conditions. For example, hybrid fibers may provide useful models for investigating the interactions between NFAT and other transcription factors with different myonuclei.

Finally, researchers should be alert to the possibility that MHC content may vary along the length of single fibers and that these patterns may be missed when studying ∼10 µm muscle sections. The precise effects of such regional differences in fiber type are uncertain, but variability in contractile velocity along the length of a fiber seems to suggest important functional consequences.

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Competing interests

The author declares no competing or financial interests.

Supplementary information

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