Review

DIVERSITY AND PLASTICITY OF VERTEBRATE SKELETAL MUSCLE: INSIGHTS FROM HYBRID FIBRES

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ABSTRACT

It is now generally accepted that hybrid skeletal muscle fibres are not experimental artefacts, but complex molecular systems that expand the functional repertoire of the muscle to which they belong. The purpose of this review is to highlight the *cognitive value* of hybrid fibres by discussing several insights into skeletal muscle biology produced by studies using hybrid fibres and/or muscles containing hybrid fibres. There is strong evidence that hybrid fibres can be used as indicators of muscle remodeling and specialization. Also, there is increasing evidence that hybrid fibres are suitable for investigating issues related to (i) the co-expression of different myosin heavy chain (MHC) isoforms and their assembly in the sarcomeric structure, (ii) the operation of the muscle cell as a multinuclear system, (iii) the tightness of the relationship between MHC isoform expression and expression of other polymorphic muscle proteins, (iv) the tightness of the relationship between MHC isoform expression and various contractile parameters, and (v) the extent of the neural input into defining the molecular and functional phenotype of skeletal muscle cells. It is predicted that, when used together with imaginatively designed methods, the hybrid fibres will further our (still limited) understanding of the regulation of muscle gene expression in multinuclear cells and of the interactions of gene products within and across different intracellular signalling pathways.

Key words: Denervation, MHC isoform, MHC polymorphism, myosin heavy chain, TnC, troponin C

INTRODUCTION

Since their introduction at the beginning of the 1990s (see for example Staron and Pette [35]), the terms 'pure' and 'hybrid' fibres have been used to describe and distinguish muscle cells expressing one (pure) or several (hybrid*) myosin heavy chain (MHC) isoforms+ (see Table 1 for a list of the MHC isoforms commonly detected in mammalian skeletal muscle fibres). It is worth noting that reports of muscle fibres displaying mixed functional characteristics preceded those of fibres containing two or more MHC isoforms. Thus, Shamarina [32] described, as early as 1963, a population of slow (tonic) fibres from frog iliofibularis and rectus abdominis muscles that could generate propagating action potentials similar to those produced by twitch fibres. In the next three decades, Serratrice *et al.* [31] identified a substantial number of human masseter fibres producing intermediate staining patterns for myofibrillar ATPase, Lännergren [15] showed compelling evidence of *Xenopus* iliofibularis muscle fibres displaying both tonic and twitch membrane and contractile characteristics, and Wilson and Stephenson [38] reported that rat soleus muscle contains fibres with mixed fast- and slow-twitch Sr^{2+}-activation properties. Interestingly, however, it was only after the discovery of the polymorphic nature

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* Hybrid (or chimeric) myofibres, produced by the fusion of transplanted myoblasts with host muscles undergoing regeneration (see for example Huard *et al.* [12]), are not included in this review.

+ Myosin heavy chains are the two largest (~ 230 kDa each) subunits of myosin II, the hexameric molecular motor of skeletal muscle. Together they form an elongated molecule, which contains, at the C-terminus, an α-helical coiled-coil tail domain and at the N-terminus a motor/head domain. The motor/head domain includes the ATP-binding/ATPase active site and the actin-binding site. It is worth noting that, while the amino acid sequences of the mammalian MHC isoforms, which are encoded by a multigene family (isogenes), are highly homologous, there are several regions (one being localized near the ATP- and actin-binding sites) that display significant sequence divergence (see review by Schiaffino and Reggiani [30]).

of myofibrillar proteins (particularly myosin) and of the fibres displaying more than one MHC isoform that the topic *diversity and plasticity of skeletal muscle* became a cognitive area in its own right.

In a review article published 5 years ago [36], I argued that hybrid fibres are more common than generally acknowledged in the literature and suggested that these fibres may be useful for gaining further insights into the structure and function of skeletal muscle. Here I propose to validate this suggestion by presenting a number of insights into muscle biology that have emerged from experiments involving single hybrid fibres and/or whole muscles containing a large proportion of hybrid fibres. Details of the methods that proved, so far, to be the most effective for studying hybrid fibres and some of the current views regarding the functional significance of hybrids can be found in several reviews (e.g. Pette and Staron [25]; Stephenson [36]) and research articles (e.g. Caiozzo *et al.* [5]).

An increase in the proportion of hybrid fibres may be used as an indicator that skeletal muscle undergoes a structural change

In the last decade there has been an abundance of studies concerned with the changes in the proportion of hybrid fibres associated with certain physiological (e.g. maturation, aging, training), pathological (e.g. hypothyroidism) or experimental (e.g. drugs, overloading, manipulation of innervation pattern) conditions (reviewed by Pette [23]; Pette and Staron, [25,27,35]). With few exceptions (e.g. Parcell *et al.* [21]; Williamson *et al.* [37]), these studies show an increase in the proportion of hybrid fibres in transforming muscles (i.e. muscles undergoing structural and functional change). Conversely, an increase in the proportion of hybrid fibres associated with a given pathological condition could be regarded as an indication of structural remodeling, even if the muscle appears to be only mildly affected functionally. For example, Gosker *et al.* [11] reported that the vastus lateralis (VL) muscle of patients with chronic obstructive pulmonary disease (COPD), particularly those with emphysema, displays a decrease in the proportion of type I fibres and a marked increase in the proportion of hybrid fibres. Following the line of reasoning developed above, one could conclude that, in these patients, VL muscle is undergoing a process of structural and functional remodeling rather than of specific type I fibre loss and use this conclusion as a basis for designing an appropriate exercise-based therapy program.

Hybrid fibres may enable 'normal' (non-transforming) muscles to perform very specialized mechanical activities

As hybrid fibres were initially detected in transforming skeletal muscles, they have been regarded as 'transient' entities that occur as part of a programmed process of molecular and functional adaptation to a new set of conditions (see review by Pette [23]). However, results obtained from different laboratories show that a large proportion of hybrid fibres can be found also in muscles of adult rat [3,5], toad [17,18] and *Xenopus* [2], which have not been subjected to conditions inducing fibre transformation. To date, the largest proportions (> 80%) of 'stable' hybrid fibres have been found in

Table 1. List of myosin heavy chain isoforms that are more commonly detected in extrafusal mammalian skeletal muscle fibres (modified from Pette and Staron [26])

MHC isoform (type)	Type of muscle fibres**/muscle (abbreviated nomenclature) in which they are expressed
MHCI or MHCIβ (slow-twitch)	Fibre type I (I) and cardiac muscle
MHCIIa (fast-twitch)	Fibre type IIA (IIA)
MHCIId/x (fast-twitch)	Fibre type IID/X (IID; IID/X)
MHCIIb (fast-twitch)	Fibre type IIB (IIB)
$MHC_{eo\ or\ IIL}$	Extraocular and laryngeal muscle fibres
MHC_{m}	Masticatory muscle fibres
MHC_{ton} (slow-tonic)	Fibres from specialized adult muscles (see Table 2)
MHC-emb* (developmental)	Hybrid fibres from immature or specialized muscles (see Table 2)
MHC-neo* (developmental)	Hybrid fibres from immature or specialized muscles (see Table 2)
MHCα-card	Fibres from specialized muscles (see Table 2) and cardiac muscle

* **emb**, embryonic; **neo**, neonatal. ** MHC isoforms listed in the left column are also detected in hybrid fibres.

muscles performing highly complex activities, such as the laryngeal, mylohyoid and stapedius muscles (see Table 2). This would suggest that, while retaining the organization and function of various intracellular compartments characteristic of all skeletal muscle fibres, regardless of the number of MHC isoforms co-expressed, hybrid fibres may expand the functional repertoire of a muscle thereby enabling it to perform highly specialized motor tasks. Muscles such as the laryngeal, mylohyoid and stapedius muscles are not easy to access or dissect, but their high content in hybrid fibres should make them extremely valuable for studies of muscle cell polymorphism.

Issues related to the coexistence of multiple MHC isoforms in hybrid fibres

Patterns of co-expression

In a recent review on the molecular regulation of skeletal muscle fibre types, Spangenburg and Booth [34] point out the difficulties encountered when investigating the mechanisms of gene regulation in type IIA fibres because these fibres often contain both slow and fast isoforms of a protein (in this case myosin light chain 1). The molecular complexity of hybrid fibres may explain, therefore, why they have not been used so far in investigations of the regulatory mechanisms of MHC isogene expression in skeletal muscle.

There are, however, several interesting findings regarding the pattern of MHC isoform co-expression in hybrid fibres that deserve mention here. Thus, not all mathematically possible combinations of MHC isoforms have been detected in mammalian hybrid fibres. Based on results obtained from many experiments with transforming muscles, Pette and colleagues suggested that in mammalian skeletal muscle, MHC isoform co-expression is constrained by the so-called 'nearest-neighbour rule' (for recent reviews see Pette [23]; Pette *et al.* [24]). According to this rule, the transition of MHC expression, from the slowest (with respect to speed of shortening) isoform (MHCI) to the fastest (MHCIIb) isoform, or vice-versa, occurs as a multi-step process (I↔ I/IIa ↔ IIa ↔IIa/IId ↔ IId ↔ IId/IIb ↔ IIb), such that hybrid fibres can co-express only combinations of near neighbouring MHC isoforms (e.g. I + IIa; IIa + IId; I + IIa + IId). One should note, however, that pools of fibres that are inconsistent with the 'nearest neighbour rule' (e.g. I/IID fibres) have been found in some normal mammalian muscles, such as rat diaphragm [3,5,20], plantaris, rectus femoris and tibialis anterior. [5]. A model of MHC isoform expression that includes these 'atypical' hybrid fibres as well as hybrid fibres co-expressing three or more isoforms is yet to be developed.

Interestingly, some MHC isoforms have been detected only in combination with other MHC isoforms. For example, embryonic and neonatal MHC isoforms (MHC-emb and MHC-neo), which are expressed in the late embryonic and early postnatal diaphragm, were displayed only by fibres co-expressing MHCI and/or MHC IIa isoforms [5]. Another MHC isoform that was not found singularly expressed is the anuran tonic MHC. A survey of 412 rectus abdominis muscle fibres from adult and juvenile cane toads [17] and 201 iliofibularis muscle fibres from adult *Xenopus laevis,* [2] produced only tonic-twitch hybrids expressing the tonic MHC isoform in combination with one or more twitch MHC isoforms.

Patterns of assembly

How do different MHC isoforms co-expressed in hybrid skeletal muscle fibres assemble: do they form homodimers and/or heterodimers? To my knowledge, there are no studies using hybrid fibres to address this specific question. However, some information on the pattern of dimerization of different MHC isoforms comes from several studies on the developing avian skeletal muscle, in which were detected both neonatal and adult MHC isoforms. The data obtained, for example, by Lowey *et al.* [16], using immunoelectron microscopy, immunoaffinity chromatography and monoclonal antibodies against adult and neonatal avian MHC isoforms, support the idea that these MHC isoforms form primarily homodimers, with only a small population (~10%) of heterodimers being detected. In discussing these results, the authors relate the pattern of dimerization of different MHC isoforms to their degree of sequence homology, with only highly homologous isoforms (such as the α-cardiac and β-cardiac MHC isoforms) being more likely to form thermodynamically stable heterodimers.

It is worth noting another point regarding the organization of the two different MHC isoforms that are co-expressed in a hybrid fibre. Based on their observation (see next section) that the two different MHC isoforms are uniformly distributed along the

length of the fibre, Sieck and Prakash [33] suggest that they must be arranged in a parallel fashion. This is because an arrangement in series of the cross-bridges associated with the two different isoforms, and therefore cycling at different rates, would result in sarcomere inhomogeneity and altered force transmission through the length of the fibre, neither of which have been observed experimentally.

MHC polymorphism and multiple nuclei

The question whether isogenes encoding the MHC isoforms co-expressed in a hybrid fibre are located in each myonucleus or in different myonuclei is not trivial. Indeed, this question is very much on the minds of molecular myologists, but the results obtained so far, largely with hybrid fibres, lend equal support to each of these two possibilities. Thus, there are the data of Peuker and Pette [27] showing non-uniform distribution of MHCIIb and MHCIId mRNAs along IIB/D hybrids from normal rabbit muscle and the data of Edman *et al.* [9] showing segmental differences in MHC isoform expression and ATPase activity in frog skeletal muscle fibres. Together, these data would support, as discussed by Caiozzo *et al.* [5], a localized expression of different MHC isoforms. However, there are also the data of Sieck and Prakash [33], showing uniform distribution of MHCI and MHCIIa isoforms in type I/IIA hybrids from rat diaphragm muscles, 2 weeks post-denervation. As stated by the authors, these data are consistent with a non-localized, uniform expression of MHC isoforms. One possible explanation for these conflicting sets of results is that they were obtained with two different pools of MHC hybrids, one comprising 'stable' hybrids from normal muscles [9,27], the other comprising transient hybrids generated as part of the denervation-induced remodeling of the muscle [33]. To date there has been no systematic comparison of 'stable' and 'transient' hybrid fibres with respect to the MHC isoform distribution along the fibre.

MHC isoform expression is related to the expression of other myofibrillar proteins and to several functional indicators of mechanical performance

The experimental strategy of choice for addressing this issue involves determination of one or several functional parameters (such as ATPase activity, speed of shortening, kinetics of stretch-induced force response, isometric force) of a single fibre segment, which is then subjected to microelectrophoretic analyses of MHC and other myofibrillar protein isoform composition. If the fibre examined expresses two or more MHC isoforms in quantifiable proportions, one can establish the correlation between the proportions of MHC isoforms co-expressed in the fibre, the estimated proportions of other myofibrillar protein isoforms and/or the value of the functional parameter(s) of interest.

Recently, we used this strategy and a population of 59 pure and hybrid fibres from adult rat diaphragm muscle to examine the relationship between the molecular expression of MHC isoform type and the molecular and functional expression (indicated by the sensitivity to Sr^{2+} of the contractile system) of Troponin C (TnC) in mammalian skeletal muscle [20]. In the graph shown in Figure 1, are plotted the percentage of slow type MHC isoform present in each fibre (x-axis), the percentage of slow twitch-cardiac TnC isoform (*, y-axis on right) and the percentage of the slow-type Sr^{2+} sensitivity component (w_1), the functional indicator of the slow twitch-cardiac TnC isoform (○, y-axis on left). Based on this graph one would expect that MHC isoform-based hybrid fibres that contain slow and fast MHC isoform in ratios falling within the range 20-80% are likely to contain both slow- and fast-twitch TnC isoforms and to produce complex Sr^{2+} activation curves composed of a high and a low Sr^{2+}-sensitivity components. In other words, this graph

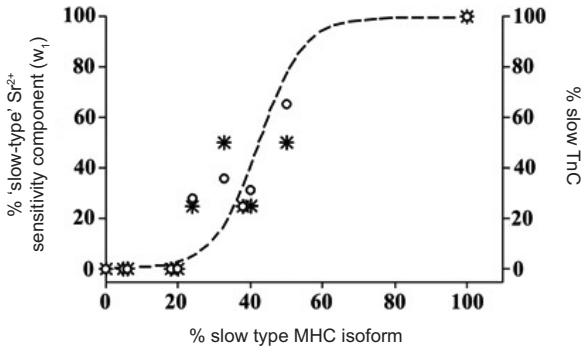

Figure 1. Relationship between the proportion of TnC-s, estimated electrophoretically (*, *y*-axis on *right*), the proportion of the slow-type Sr^{2+} sensitivity component (w_1) of the force-pSr curve (○, *y*-axis on *left*), and the proportion of slow MHC isoform present in each fiber (*x*-axis). The sigmoidal curve indicating the trend followed by the data was generated using the nonlinear regression (variable slope) option provided by Graphpad Prism. Reproduced with permission from O'Connell *et al.* [19]

provides the basis for predicting either the sensitivity to Sr^{2+} of the contractile apparatus, the TnC isoform composition, or the MHC isoform type expressed in mammalian muscle fibres when only one of these three parameters is determined experimentally.

Knowledge of the quantitative relationship between the MHC isoform composition of fibres and their functional parameters, obtainable, as shown above from studies on hybrid fibres, provides also further insights into the role played by MHCs in various contractile processes. This point is well illustrated in a study by Galler *et al.* [10] in which the authors discuss the relationship between MHC isoforms co-expressed in a fibre and its maximum unloaded speed of shortening (V_{max}) or stretch activation kinetics. More specifically, Galler *et al.* [10] used the data produced by a series of physiological studies on the contractile properties of mammalian MHC hybrid fibres to argue that (i) V_{max} is determined not only by MHC isoform expression, but also by the expression of other myofibrillar proteins, such as the myosin light chains and (ii) MHC isoform expression is the main determinant of events involved in the stretch-induced force response produced by a fibre. The tight correlation between stretch activation kinetics and MHC isoform expression found by Galler and colleagues with the rat muscle fibres has been validated also for mouse, rabbit, human [1] and *Xenopus* [2] muscles.

The molecular form of TnC determines fibre type-differences with respect to the sensitivity to Sr^{2+} of contractile processes

Strontium ion (Sr^{2+}) is a non-physiological activator of muscle contraction, which has been used for the last three decades, mostly by muscle physiologists, to distinguish fast- and slow-twitch fibres in studies of mammalian muscle contractility using single fibre preparations. In a survey of the relevant literature we noted a lack of consensus regarding the role played by the TnC isoforms in fibre type differences with respect to the sensitivity to Sr^{2+} of contractile processes in skeletal muscle. Thus, some researchers (e.g. Yamamoto [40]) associated these differences with the molecular species of TnC present in fast- and slow-twitch fibres, while others (e.g. Kerrick *et al.* [13]) related them to myofibrillar protein-protein interactions associated with the activation process rather than to the molecular type of TnC. Using a large population of hybrid (and pure) fibres from the rat diaphragm, many of which co-expressed the slow and fast TnC isoforms, a rapid electrophoretic method developed in our laboratory, which allows the unequivocal identification of TnC isoforms in single fibre segments [19] and the method for measuring isometric contractile characteristics described in detail in Bortolotto *et al.* [3,4], we produced compelling evidence that skeletal muscle fibre-type differences in the sensitivity to Sr^{2+} of contractile activation processes are determined primarily by differences in TnC isoform composition [20].

Anuran skeletal muscle expresses not only a twitch TnC isoform, but also a slow-tonic TnC isoform

According to the prevalent view (see review by Schiaffino and Reggiani [30]), mammalian striated muscles express two molecular forms of TnC, one in the fast-twitch muscle and the other in slow-twitch and cardiac muscles. Anuran skeletal muscles (and mammalian muscles performing highly specialized functions; see Table 2) contain, in addition to twitch fibres, slow-tonic fibres, which produce slow, graded mechanical responses to graded membrane depolarization. Anuran slow-tonic fibres have been shown to differ from twitch fibres with respect to many structural and functional characteristics of the intracellular compartments known to play key roles in the process of Ca^{2+} regulation of the contractile events [29]. To our surprise, none of the relevant reports to date mention anything about the molecular form of TnC expressed in anuran slow-tonic muscle. To investigate the possibility that anuran slow-tonic muscle fibres contain a TnC isoform that differs from that expressed by twitch fibres, we used the strategy described in the previous section, i.e. we examined the TnC isoform composition and isometric contractile characteristics of a large number of hybrid and pure fibres from cane toad rectus abdominis muscle [18]. This muscle had been shown by us earlier [17] to contain a mixture of pure twitch, hybrid twitch and hybrid twitch-tonic fibres. The main finding of the study by O'Connell *et al.* [18] was that striated muscles of the cane toad contain not one, but two TnC isoforms, one present in hybrid fibres displaying the tonic MHC isoform and in cardiac muscle and the other being present in all fibres expressing only twitch MHC isoforms, regardless of their number and identity.

Table 2. Examples of highly specialized muscles containing a large proportion of hybrid fibres

Muscle (species)	Function	MHC isoforms expressed	% hybrid fibres	Pattern of MHC isoform co-expression	Method of fibre typing used (reference)
Laryngeal muscles: (mammalian) thyroarytenoid (TA), lateral cricoarytenoid (LCA), interarytenoid (IA), posterior cricoarytenoid (PCA), crycoarytenoid (CT)	Perform motor tasks related to airway protection, respiration and phonation	**I**, **IIa**, **IId**, **IIb eo/IIL**, **ton**, **neo**, **emb**	eg. 85% (rat TA)	IIa+IId; IId+IIb; IIb+IIL; I+IIa+IId; IIa+IIb+IId; IIa+IIb+IIL; I+IIa+IId+IIb+IIL	Single fibre SDS-PAGE (Wu *et al.* [39])
Mylohyoid muscle (MH;adult human) anterior MH posterior MH	Performs motor tasks needed to displace and stabilize the mandible and hyoid bones during chewing, lapping, licking (anterior MH), swallowing, respiration and phonation (posterior MH)	**I**, **IIa**, **IId**, **ton, neo, emb**	87%	I+IIa; IIa+IId; I+ton; IId+neo; IIa+emb; IIa+neo; I+ton+IIa; IIa+neo+emb	Immunohistochemistry (Ren *et al.* [28])
Stapedius muscle (adult rat)	Stiffens the middle year bone chain thereby protecting the auditory receptors in the inner ear from injury by sudden or long standing high levels of intense noise (> 80 dB)	α **-card**, **I**, **IIa**, **IIb**, **IId**	100%	I+IIb; IIa+IIb+IId; I+IIa+IIb I+IIa+IIb+IId; α+I+IIa+IIb; α+I+IIa+IIb+IId;	Immunohistochemistry (Dammeijer *et al.* [8])

The contractile apparatus in fibres containing the slow-tonic-cardiac TnC isoform was found to have a significantly higher sensitivity to both Ca^{2+} and Sr^{2+} and to produce contractile responses over a wider range of activating ion concentrations than that in fibres containing the twitch TnC isoform.

Not only neural factors but also the intrinsic properties of the muscle fiber determine the molecular and functional phenotype of hind limb muscle cells

In a recently completed study (Patterson *et al.* [22]), we used a single fibre approach and two skeletal muscles that do not overlap in their fibre type composition (extensor digitorum longus, EDL and soleus muscles of adult Long-Evans Hooded rat) to test the hypothesis that on denervation, when the neural input is removed, the mature fibres from the two muscles undergo a process of molecular remodeling, converging to *different* fiber phenotypes. This hypothesis was formulated based on the data of Kalhovde *et al.* [14] showing that denervated EDL and soleus stimulated with the same slow stimulus pattern expressed slow and fast MHC isoforms in different proportions, which led to the interpretation that the satellite cells in the EDL and soleus muscles from which the regenerated muscles originate, have intrinsically different properties.

The results of our study [22] showed that 50 days post-denervation, there was still no overlap in the fibre types present in the EDL and soleus muscles. A large proportion (> 75% of the fibres) in the two muscles became hybrid with respect to MHC isoform composition, with the EDL fibres co-expressing MHCIIb and MHCIId isoforms and the soleus fibres co-expressing MHCI and MHCIIa isoforms. The two populations of hybrid fibres (IIB/D_{EDL} and I/IIA_{soleus}) displayed different properties with respect to Ca^{2+}-activation of the contractile system and sarcoplasmic reticulum (SR) Ca^{2+} handling, but not with respect to the maximum specific force produced. These results explain the incomplete fibre

transformation revealed earlier [6,7], but not further explored, by studies involving cross-innervation of rat EDL and soleus muscles.

CONCLUDING REMARKS

It is now generally accepted that hybrid skeletal muscle fibres, first described less than three decades ago, are not experimental artefacts, but complex molecular systems. In this mini-review I turned the spotlight on the cognitive rather than physiological significance of hybrid fibres, by discussing several insights into skeletal muscle biology produced so far by studies using hybrid fibres and/or muscles containing hybrid fibres.

The first and most important piece of information provided by hybrid fibres refers to *the extent* of the molecular diversity and plasticity of skeletal muscles. As many studies show, subtle structural and molecular differences between different muscles of the same organism, between homologous muscles from different species or from different strains for the same species, and between different fibre types in the same muscle often translate into meaningful functional differences and even into differences with respect to responsiveness to pathological conditions. A second major cognitive role played by hybrid fibres is that of indicators of muscle remodeling. Their use, as such, has revealed, on the one hand, the variety of conditions that are accompanied by structural and functional muscle remodeling and, on the other hand, the remarkable ability of skeletal muscle to adapt to changing conditions and functional demands. It is not difficult to imagine that, when used together with imaginatively designed methods, the hybrid fibres will further our understanding of the regulation of muscle gene expression in multinuclear cells and of interactions of gene products within and across different intracellular signalling pathways.

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