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REVIEW

Fibre types in skeletal muscle: a personal account

S. Schiaffino[1,2,3]

1 Department of Biomedical Sciences, University of Padova, Padova, Italy

2 Venetian Institute of Molecular Medicine (VIMM), Padova, Italy

3 CNR Institute of Neuroscience, Padova, Italy

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Correspondence: S. Schiaffino, Venetian Institute of Molecular Medicine (VIMM), Via Orus 2, 35126 Padova, Italy.
E-mail: stefano.schiaffino@unipd.it

Abstract

Muscle performance is in part dictated by muscle fibre composition and a precise understanding of the genetic and acquired factors that determine the fibre type profile is important in sport science, but is also relevant to neuromuscular diseases and to metabolic diseases, such as type 2 diabetes. The dissection of the signalling pathways that determine or modulate the muscle fibre phenotype has thus potential clinical significance. In this brief review, I examine the evolution of the notion of muscle fibre types, discuss some aspects related to species differences, point at problems in the interpretation of transgenic and knockout models and show how *in vivo* transfection can be used to identify regulatory factors involved in fibre type diversification, focusing on the calcineurin-nuclear factor of activated T cells (NFAT) pathway.

Keywords calcineurin, muscle fibre types, myosin heavy chain, NFAT, signalling pathways.

The recognition that skeletal muscles are composed of fibre types which differ in structure, molecular composition and functional properties has contributed to our understanding of muscle physiology and plasticity, to the evaluation of muscle performance in sport science and to the diagnosis of neuromuscular diseases. More recently, the increasing appreciation of the role of skeletal muscle in metabolic diseases has brought the notion of muscle fibre types to the attention of a wider audience in clinical medicine. The diversification of muscle fibre types starts during embryonic stages independently of neural influence, but motor neurone activity and various hormones, in particular thyroid hormone, are able to modulate the fibre type profile during early postnatal development and in the adult. Significant progress has been made during the last several years in the identification of the signalling pathways that control muscle fibre types. The function of specific genes has been defined by gain- and loss-of-function approaches using transgenic and knockout mouse models. However, a limitation of these models is that the effects observed may be due to developmental changes caused by gene addition or deletion.

Here, I briefly review the evolution of the notion of muscle fibre types, discuss some aspects related to species differences, point at problems in the interpretation of transgenic and knockout models, and review some results from my laboratory using an alternative approach of *in vivo* transfection. This is by no means a comprehensive review but rather a personal account reflecting my own experience in this field. The signalling pathways involved in skeletal muscle remodelling have been recently reviewed (Bassel-Duby & Olson 2006). Here, I will exclusively focus on the calcineurin-NFAT pathway to illustrate the approach we have been using to validate the role of a specific pathway in muscle fibre type specification.

A brief history of muscle fibre types

The current notion of muscle fibre types emerged during the last 50 years and one can identify three phases in the evolution of this notion, each characterized by a prevailing paradigm (Table 1). When I started working on skeletal muscle around 1966–1967, the prevailing view, not much different from that first put forward by

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Table 1 Three stages in the evolution of the notion of skeletal muscle fibre types

Date	Prevailing paradigm	Selected milestones
Circa 1960–1967	Two muscle fibre types: Fast white muscle fibres Slow red muscle fibres	Enzyme histochemistry reveals reciprocal relation between glycolytic and oxidative enzymes in muscle fibres (Dubowitz & Pearse 1960) Cross-reinnervation of fast and slow cat muscles leads to partial conversion of their contractile properties (Buller *et al.* 1960b). This is due to nerve activity, as shown by electrical stimulation studies (Salmons & Vrbova 1969, Pette *et al.* 1973, Lomo *et al.* 1974) Myosin ATPase activity is higher in fast than in slow muscles and correlates with muscle speed of shortening (Barany 1967)
Circa 1967–1975	Three muscle fibre types: Slow type 1 Fast type 2A Fast type 2B	All motor units in rat EDL muscle have similar fast-twitch properties (Close 1967), but this muscle contains both SDH-rich and SDH-poor fibres (Schiaffino *et al.* 1970) Rat TA muscle contains fast-twitch fatigable units homogeneously composed of SDH-poor fibres and fast-twitch fatigue-resistant units composed of SDH-rich fibres (Edstrom & Kugelberg 1968) Mitochondria-rich and -poor fibres in rat fast muscles have a more richly developed SR, consistent with a fast twitch, than most fibres in slow soleus muscle (Schiaffino *et al.* 1970) Identification of type 1, 2A and 2B fibres by myosin ATPase histochemical staining (Guth & Samaha 1969, Brooke & Kaiser 1970) Motor units composed by type 1, 2A or 2B fibres identified in cat muscle (Burke *et al.* 1971) Fast glycolytic, fast oxidative glycolytic and slow oxidative fibres can be distinguished by enzyme biochemistry (Peter *et al.* 1972) Enzyme biochemistry on microdissected single human muscle fibres shows differences in SDH activity (type 1 > 2A > 2B) and PFK activity (type 2B > 2A > 1) (Essen *et al.* 1975)
Circa 1986–1991	Four muscle fibre types: Slow type 1 Fast type 2A Fast type 2X Fast type 2B	Monoclonal antibodies to MYHs distinguish four MYH isoforms, including type 2X, and four corresponding fibre types in rat muscle (Schiaffino *et al.* 1986, 1988, 1989) Electrophoretic separation of four MYH bands, including type 2D, by SDS-PAGE in rat muscle (Bar & Pette 1988, Termin *et al.* 1989) Identification of type 2X motor units (Larsson *et al.* 1991b) Single skinned fibre analyses reveal different speed of shortening of the four fibre types (Bottinelli *et al.* 1991, 1994) Identification of a specific 2X MYH gene and distribution of four MYH mRNAs in rat muscles by *in situ* hybridization (DeNardi *et al.* 1993) Human muscle fibres classified as type 2B by ATPase histochemistry contain 2X MYH (Smerdu *et al.* 1994, Ennion *et al.* 1995)

EDL, extensor digitorum longus (a fast-twitch muscle); PFK, phosphofructokinase; MYH, myosin heavy chain; SDH, succinate dehydrogenase; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SR, sarcoplasmic reticulum; TA, tibialis anterior (a fast-twitch muscle).

Ranvier in 1873 (Needham 1926), was that there are two major types of skeletal muscles and corresponding fibre types: (1) slow red muscles, composed of fibres rich in myoglobin and mitochondria, characterized by an oxidative metabolism and involved in continuous, tonic activity and (2) fast white muscles, composed of fibres poor in myoglobin and mitochondria, relying more on glycolytic metabolism and involved in phasic activity. Histochemical staining showed that there is a reciprocal relationship between glycolytic and oxidative enzymes in the two fibre populations (Dubowitz & Pearse 1960, Gauthier & Padykula 1966), and that, in addition to typical 'red' and 'white' fibres, most muscles contain fibres with intermediate properties. A major advance in this period was the demonstration that fast and slow muscles differ in myosin ATPase activity, which correlates with the speed of muscle shortening (Barany 1967). This crucial observation paved the way for the utilization of myosin as a standard marker of muscle fibre type (see below). The role of innervation in modulating muscle fibre type properties was demonstrated by cross-reinnervation experiments (Buller *et al.* 1960b); subsequent studies demonstrated that nerve electrical activity is the critical factor involved in

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nerve-dependent muscle remodelling, as shown by electrical stimulation of skeletal muscles either via nerve (Salmons & Vrbova 1969, Pette *et al.* 1973) or directly on denervated muscles (Lomo *et al.* 1974).

A second phase in this story started around the years 1967–1972 and led to the view that skeletal muscles contain in fact three major fibre types, the slow or type 1, the fast highly oxidative or type 2A and the fast weakly oxidative or type 2B. Several convergent lines of evidence using different approaches validated this new interpretation of muscle fibre types. One approach was the analysis of motor units pioneered by Close, who analysed the properties of motor units in fast extensor digitorum longus (EDL) and slow soleus muscles of the rat (Close 1967). He showed that nearly all EDL motor units displayed a similar fast twitch, although the force they exerted varied considerably. At that time I was studying the same rat muscles by enzyme histochemistry, using succinate dehydrogenase (SDH) and myoglobin staining, and by electron microscopy. The rat EDL is composed of fibres with different SDH staining and mitochondrial density. Thus, considering the results of Close and assuming that motor units are homogeneous in their fibre type composition, this muscle must contain at least two types of fast fibres, red fibres rich in myoglobin and mitochondria and white fibres with much reduced myoglobin and mitochondria content. This interpretation was confirmed by the finding that both mitochondria-rich and mitochondria-poor EDL fibres have a richly developed sarcoplasmic reticulum, which would be consistent with a rapid calcium release and uptake during the contraction–relaxation cycle, whereas slow soleus muscle fibres have a poorly developed sarcoplasmic reticulum (Schiaffino *et al.* 1970). At the same time, Edstrom and Kugelberg reported the results of a very elegant experiment in which, by using the glycogen depletion technique, it was possible for the first time to demonstrate that rat motor units are homogeneous in their fibre type composition, based on SDH staining, and that motor units composed of fibres with high oxidative enzyme content are as fast, in terms of contraction and relaxation time, as units composed of fibres with low oxidative enzyme content, but are more resistant to fatigue (Edstrom & Kugelberg 1968). The different force exerted by the two types of units was clearly a consequence of the smaller size of oxidative vs. non-oxidative fibres, as the number of fibres per unit was essentially similar. Two other approaches supported the existence of distinct fast fibre type populations. The first was the introduction of novel histochemical staining procedures for the demonstration of ATPase activity based on the calcium precipitation method, presumed to reflect actomyosin ATPase, following alkali and acid pre-incubation, that allowed to distinguish slow type 1 and fast type 2A and 2B fibres (Guth & Samaha 1969, Brooke & Kaiser 1970). Selective extraction procedures supported the notion that this ATPase reaction is really due to myosin enzymatic activity: ATPase was unchanged following detergent extraction, which abolished membrane-bound ATPases and SDH activity, but was abolished by hypertonic KCl treatment, a standard method to solubilize myosin, which leaves membrane-bound ATPases and SDH activity unaffected (Schiaffino & Bormioli 1973b). The type 1, 2A and 2B nomenclature, introduced by Brooke and Kaiser, became common and the ATPase staining procedure after acid pre-incubation was widely adopted as the standard method for muscle fibre typing in skeletal muscle. Motor unit studies in cat muscles confirmed that units composed by 2A fibres are as fast but more resistant to fatigue compared with units composed by 2B fibres (Burke *et al.* 1971). Finally, the existence of two fast fibre populations was supported by biochemical analyses. Using guinea-pig muscles with relatively pure fibre type composition and defined physiological properties, Peter *et al.* (1972) found that both fast white and fast red muscles are rich in glycolytic enzymes in spite of different oxidative enzyme content: this led to the classification of fast glycolytic, fast oxidative glycolytic and slow oxidative fibres. Subsequent direct analyses on single human muscle fibres showed that type 2B fibres have higher glycolytic enzyme activities compared with 2A fibres and 2A fibres higher than type 1 fibres (Essen *et al.* 1975).

The third phase of the fibre type story is characterized by the discovery in rodent muscle of another fast type myosin heavy chain (MYH), called 2X or 2D, with properties intermediate between the 2A and 2B MYH, and its localization in a distinct fibre type. This was the result of two independent approaches: the generation of monoclonal antibodies to MYHs (Schiaffino *et al.* 1986, 1988, 1989) and the electrophoretic separation of MYH bands by a modified sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) procedure (Bar & Pette 1988, Termin *et al.* 1989). It was shown that the two approaches recognize the same fibre type (LaFramboise *et al.* 1990), which has an intermediate oxidative metabolism in rodent muscles, though the intensity of SDH staining of 2X fibres is generally more similar to that of 2A than 2B fibres. Subsequent studies using the glycogen depletion technique identified a distinct type 2X motor unit in rat muscles with twitch properties similar to 2A and 2B fibres but fatigue index intermediate between them (Larsson *et al.* 1991b). An important result, based on correlated immunohistochemical and physiological analyses on single rat skinned fibres, was the finding that the maximal speed of shortening varies among type 2 fibres, 2B fibres being faster than 2X and 2X faster than 2A fibres (Bottinelli *et al.* 1991, 1994). This is in contrast with the finding

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that twitch properties are essentially identical among the three types of motor units (Close 1967, Edstrom & Kugelberg 1968, Larsson *et al.* 1991b) and is consistent with the notion that the twitch profile is to large extent dictated by sarcoplasmic reticulum properties, whereas the maximal velocity of shortening depends exclusively on myosin composition. The definitive demonstration of the existence of a distinct MYH type was the finding that MYH-2X has a distinct amino acid sequence and is coded by a distinct gene (DeNardi *et al.* 1993). *In situ* hybridization analysis, confirming previous immunohistochemical and single fibre analyses, showed that rat muscles contain a spectrum of fibre types, including hybrid fibres with preferential combinations of MYH transcripts, according to the sequence 1↔1-2A↔2A-↔2A-2X↔2X↔2X-2B↔2B (DeNardi *et al.* 1993). This fibre heterogeneity was previously demonstrated by MYH analyses at the protein level using immunohistochemical (Schiaffino *et al.* 1989, Gorza 1990) (Fig. 1) and single fibre analyses (see Bottinelli *et al.* 1991, Pette *et al.* 1999). Hybrid fibres are more frequent in the ageing skeletal muscle (Klitgaard *et al.* 1990b) and following endurance training (Klitgaard *et al.* 1990a), presumably in relation to fibre type conversion that takes place under these conditions. Motor units with mixed fibre type composition can also be observed in the ageing skeletal muscle (Larsson *et al.* 1991a). Single fibre studies also showed that even fibres with apparently pure MYH composition may vary in terms of metabolic enzyme complement (see Pette *et al.* 1999), adding a further degree of complexity to the notion of muscle fibre heterogeneity.

A final step in this fibre type story was the recognition that human muscles differ from rat and mouse muscles both in myosin and metabolic enzyme composition, but this is an important point that deserves a separate discussion.

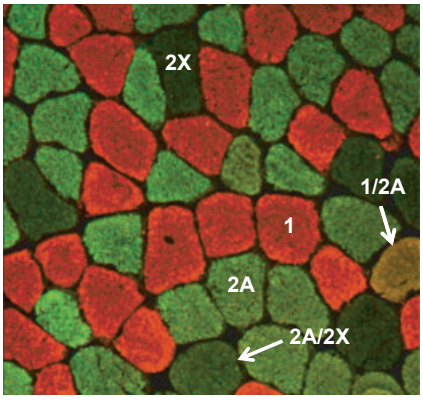

Figure 1 Fibre types in mouse skeletal muscle. Section of mouse soleus muscle stained with an antibody to MYH-slow, visualized in red, and an antibody to MYH-2A, visualized in green. In addition to type 1 and 2A fibres, note the presence of unstained fibres, corresponding to the minor proportion of type 2X fibres present in mouse soleus, and of hybrid 1-2A and 2A-2X fibres.

Human muscles are different from mouse muscles

When the ATPase histochemical staining after acid preincubation was applied to human skeletal muscle, three fibre types corresponding to type 1, 2A and 2B fibres were found to be present in human muscle like in mouse and rat muscles, the major difference being the much greater abundance of slow fibres in human compared with rodent muscle. Electrophoretic separation of MYHs likewise led to the identification of three MYH bands (Biral *et al.* 1988). These two approaches were extensively used to characterize fibre type and myosin changes in human skeletal muscle under a variety of physiological and pathological conditions.

With the discovery of type 2X/2D fibres in rodent muscle, the issue of human muscle fibre types was reconsidered and the surprise was to find that human fibres typed as 2B by ATPase staining contain in fact the human orthologue of rat MYH-2X, as determined by *in situ* hybridization using probes specific for the various MYH types (Smerdu *et al.* 1994) and PCR analysis of single muscle fibres (Ennion *et al.* 1995). The human MYH-2B gene is apparently expressed only in extraocular and laryngeal muscles (Andersen *et al.* 2000), a situation similar to that described for other large mammals. Not only do most human muscles contain a more restricted repertoire of MYH isoforms and corresponding hybrids due to the absence of the 2B component (1 ↔ 1-2A ↔ 2A ↔ 2A-2X ↔ 2X), but their relative proportions and metabolic properties are markedly different compared with mouse and rat muscles. Mouse muscles are predominantly composed of type 2B and 2X fibres, with 2A fibres representing a minor component and slow type 1 fibres being rare and mostly confined to some muscles, such as the soleus. In contrast, human muscles contain mostly type 1 and 2A fibres, 2X fibres being a relatively minor component in most individuals. The oxidative enzyme complement is likewise different between the two species, as shown by the more intense staining of mouse muscles in sections stained for SDH activity (Fig. 2a). The difference in staining intensity corresponds to well-known differences in mitochondrial content and oxygen consumption between muscles from small compared with large mammals (Hoppeler & Kayar 1988). In addition, whereas in human muscles the abundance of mitochondria and oxidative enzymes is greatest in type 1 fibres and lowest in 2X fibres, in mouse and rat muscles the oxidative potential is highest in 2A fibres and lowest in 2B fibres (Fig. 2b). Given these differences, one might

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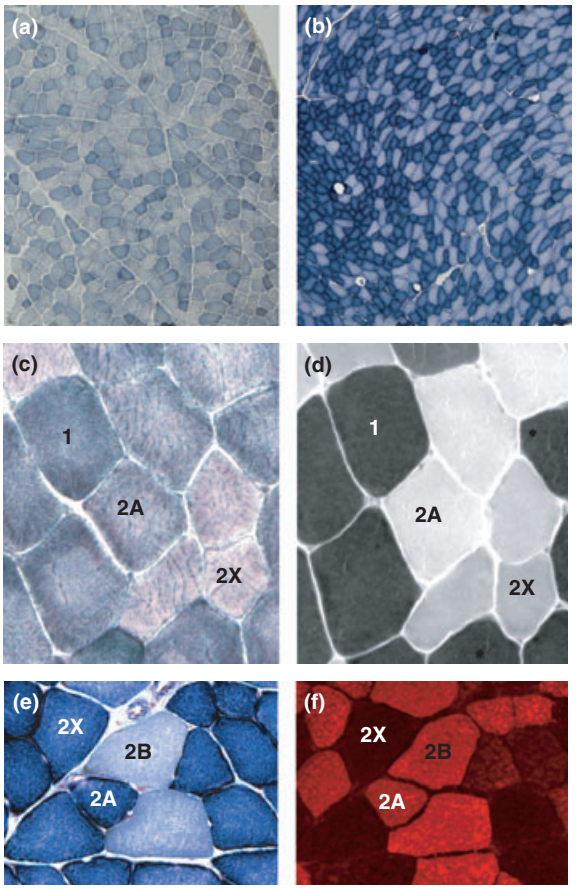

Figure 2 Differences between human and mouse skeletal muscles. (a, b) Sections of human vastus lateralis (a) and mouse tibialis anterior muscle (b) stained for succinate dehydrogenase (SDH) activity. Note weaker staining of human muscle. (c, d) Sections of human vastus lateralis stained for SDH and myosin ATPase after pre-incubation at pH 4.6. Note that 2X fibres show the weakest staining for SDH. (e, f) Sections of mouse tibialis anterior stained for SDH and with an antibody that reacts with all fibres except 2X fibres. Note that 2X fibres show intense staining for SDH.

conclude that the mouse skeletal muscle is not the best model for human muscle, and indeed it is important to keep in mind these species differences when one tries to extrapolate conclusions derived from studies on transgenic and knockout models to human conditions. The temptation to find clinical implications of transgenic mouse models has been stimulated by the recognition that the muscle fibre type profile has wide implications in many areas of biomedicine, in particular metabolic diseases. Let us consider just one example of erroneous interpretations when findings in mice are extrapolated to humans.

Insulin resistance, and the consequent risk of metabolic syndrome and type 2 diabetes, appears to be associated with mitochondrial disfunction and reduced oxidative enzyme content in skeletal muscle (Mootha *et al.* 2003, Patti *et al.* 2003, Lowell & Shulman 2005). The beneficial role of exercise in preventing insulin resistance and thus reducing the risk of diabetes might thus reflect the ability of exercise to promote mitochondrial biogenesis. The identification of the signalling pathways involved in the transcriptional regulation of muscle mitochondrial genes is thus a major objective in diabetes research, as it can offer insights into possible therapeutic interventions aimed at correcting the mitochondrial disfunction. The study of transgenic and knockout model can provide important information in this respect, provided one is aware of the differences between mouse and human muscle. For example, in a recent study an increased expression of the MYH-2X gene and of the corresponding predominantly oxidative type 2X fibres was detected in transgenic mice overexpressing PGC-1β in skeletal muscle (Arany *et al.* 2007). The authors discuss the implication of this finding for human diseases and conclude: '… these data have potential importance for the therapy of a number of muscular and neuromuscular diseases in humans. Many conditions accompanied by loss of physical mobility … involve a loss of oxidative fibres and their replacement with glycolytic fibres.... The identification of PGC-1β as a potential mediator of the development of oxidative type IIX fibres suggests new ways to modulate muscle fibre type in health and disease'. The problem here is that type 2X fibres, which are mostly oxidative in mouse muscles, are actually the least oxidative of all fibre types in human skeletal muscle.

Another problem which is usually overlooked when interpreting the results of fibre type changes in transgenic or knockout mice is the effect of the transgene on developing muscle, as discussed in the next section.

Postnatal changes in fibre types and plasticity of developing muscle

Significant changes in the fibre type profile take place in rodent skeletal muscle during the first weeks after birth, such as the down-regulation of embryonic and neonatal MYH and the upregulation of fast MYH-2A, -2X and -2B. Another change that takes place in mouse muscles during the first weeks of postnatal development is the progressive disappearance of type 1 fibres and MYH-slow from fast muscles (Whalen *et al.* 1984, Agbulut *et al.* 2003). For example, the neonatal mouse plantaris muscle contains a significant proportion of MYH-slow (about 6% at P7) that disappears almost completely in the adult muscle (Agbulut *et al.* 2003). It was reported that transgenic mice overexpressing constitutively active calcineurin contain an increased amount of type 1 fibres and MYH-slow, suggesting that calcineurin is able to induce a fast-to-slow switch (Naya *et al.* 2000). However, the increase is modest, for example the transgenic

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plantaris contains about 6% MYH-slow (Talmadge *et al.* 2004); curiously enough the same proportion is found in the normal neonatal muscle. It would be of interest to carry out a developmental analysis of these transgenic muscles. One might well discover that what takes place in these muscles is not a fast-to-slow switch, but rather a block of the slow-to-fast switch that occurs after birth in fast muscles with the maintenance of the slow fibres and slow myosin in the adult. The point I want to make here is that the transgene starts to act since early developmental stages (the exact timing depends on the promoter used to drive the transgene, but most promoters are active before birth), therefore the phenotype observed in the adult may be the result of effects of the transgene on developing not in adult muscle. This applies to other transgenic models in which an increase in type 1 fibres has been observed in adult mouse muscles, for example the PGC-1α transgenic mouse (Lin *et al.* 2002).

Another aspect to consider when interpreting the phenotype of a transgenic model is the fact that developing muscles are more plastic than adult muscles. For example, functional overload induced in the adult rat EDL muscle by removal of the synergist tibialis anterior was found to cause muscle hypertrophy but no significant change in the fibre type profile, as determined by histochemical reactions for myosin ATPase and SDH activity (Schiaffino & Bormioli 1973a). In contrast, a significant increase in type 1 fibres and a complete switch from type 2B to 2A/2X, with corresponding increase in SDH activity, was seen when overload was induced at birth (see also Schiaffino *et al.* 2007). Similar effects are found when comparing regenerating and adult muscle following manipulations aimed at inducing a fast-to-slow switch. For example, the regenerating rat EDL undergoes more rapid and extensive transformation in contractile properties, fibre type profile and MYH composition following cross-reinnervation or low frequency electrical stimulation compared with adult EDL (Donovan & Faulkner 1987, Erzen *et al.* 1999, Pette *et al.* 2002, Kalhovde *et al.* 2005). *In vivo* transfection experiments, to be discussed below, also point to a differential response of developing vs. adult muscle and to greater plasticity of regenerating muscle: for example, a constitutively active mutant of the transcription factor NFATc1, a target of calcineurin, is able to induce the expression of MYH slow in regenerating EDL but not in adult EDL (McCullagh *et al.* 2004).

The issue of whether the phenotype of a transgenic or knockout mouse model results from effects of the transgene on developing or adult muscle is not just a scientific curiosity, but has obvious practical implications, if one wants to draw from these experiments indications for the identification of therapeutic targets. Consider we aim to induce a fast-to-slow fibre type switch or a switch to a more oxidative fibre type profile as a way to prevent the transition from pre-diabetes to diabetes. To design appropriate interventions, we can use information derived from transgenic models with specific perturbations of a signalling pathway, but it would be better to know whether the transgene has the same effect when switched on in adult mice. This can be done using inducible transgenic models, in which a transgene is silent throughout development and is specifically switched-on in the adult animal; likewise, in inducible knockout models an endogenous gene is normally expressed during development and is knocked out in the adult. The effect of these gain- or loss-of-function perturbations can be quite different depending on the developmental stage.

A striking example of the different results of inducible vs. traditional knockouts concerns the role of the transcription factor *Pax7*. *Pax7* is essential for the survival and expansion of muscle progenitors, as *Pax7* null mice have no or very few satellite cells (Seale *et al.* 2000) and show severely compromised muscle regeneration (Oustanina *et al.* 2004, Kuang *et al.* 2006). As muscle regeneration is of clinical importance to muscular dystrophies and sport injuries, one could consider inducing *Pax7* expression to promote muscle regeneration. However, a recent study shows that unexpectedly, when *Pax7* is inactivated in adult mice using an inducible knockout approach, mutant satellite cells can proliferate and differentiate normally and muscle regeneration is not compromised even after a repeated round of injury (Lepper *et al.* 2009). This is not due to compensation by *Pax3* because double mutants show the same result. To determine when myogenic precursors become independent of *Pax7 in vivo*, the gene was inactivated at different time points: when *Pax7* was inactivated between P7-11, regeneration was severely compromised, but when inactivation was carried out at P14-18 and P21-25, regenerative capacity gradually increased. Thus there is a critical period of *Pax7* dependency during early postnatal development in the transition from muscle progenitor to adult stem-cell state. It is appropriate to quote the authors' own words to fully appreciate the implications of these observations: '... it was entirely unexpected that adult satellite cells require neither *Pax7* nor *Pax3* for muscle regeneration. We imagine that postnatal changes of muscle organization, mechanics and physiology demand stem cells to alter their transcriptional programme as a means to adapt to these challenges. *Changes in genetic requirement for muscle stem cells from embryonic to juvenile to adult stages elucidate the inadequacy of applying knowledge gained from developmental studies to adult stem-cell biology*' (italic added). One can easily apply the same argument to muscle fibre type biology.

Procedures for generating inducible transgenic or knockout mice are very complex, thus only few

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inducible models are available for skeletal muscle studies. In our laboratory we have taken an alternative approach to explore the effect of a transgene in the adult, namely the generation of 'transgenic muscle fibres' by intramuscular injection of plasmid DNA, as discussed in the next section.

Exploring the mechanisms that control muscle fibre type properties by *in vivo* transfection: the calcineurin-NFAT pathway

Intramuscular injection of plasmid DNA can be used for gene transfer in both regenerating and adult muscle, electroporation being required to obtain significant efficiency of transfection with adult skeletal muscle. An obvious advantage of this approach is that 'transgenic muscle fibres' can be obtained in a few days compared to the long time required to generate a transgenic mouse. On the other hand, a limitation of this approach is that the efficiency of transfection is variable and only a proportion of muscle fibres are transfected, although usually this proportion is quite substantial (Fig. 3a). Another problem is that injection and electroporation lead to focal damage, a problem that can be circumvented by using appropriate controls with irrelevant transgenes and analysing the muscles 1 week after transfection, when inflammatory changes have extinguished. The most important advantage of *in vivo* transfection is that the effect of a transgene is determined in the adult animal and transfected fibres can be compared with neighbouring non-transfected fibres within the same muscle. Finally, with this procedure only muscle fibres are transfected, not interstitial cells or satellite cells (Fig. 3b). The potential of this approach can be illustrated by studies aimed at exploring the role of the calcineurin-nuclear factor of activated T cells (NFAT) pathway in controlling muscle fibre type properties. Calcineurin is a protein phosphatase, which is activated by Ca^{2+}-calmodulin and dephosphorylates the transcription factors of the NFAT family causing their translocation from the cytoplasm to the nucleus and subsequent activation of target genes (Fig. 4a).

A role of calcineurin in promoting the slow gene programme in muscle fibres was first suggested by the group of R.S. Williams based on studies on cultured muscle cells and on the effect of cyclosporin A in adult rats (Chin *et al.* 1998) and was supported by the phenotype of a transgenic mouse overexpressing a constitutively active variant of calcineurin (Naya *et al.* 2000). However, all these approaches have some limitation: cultured muscle cells may not reflect the response of adult muscle fibres, cyclosporin A has additional effects unrelated to calcineurin inhibition and, as discussed above, there are problems in the interpretation of transgenic models. To explore the role of calcineurin and its downstream target, the transcription factor NFATc1, we started a series of *in vivo* transfection experiments using different types of transgenes, as summarized in Table 2 (Serrano *et al.* 2001, McCullagh *et al.* 2004, Tothova *et al.* 2006, Calabria *et al.* 2009). Some of these transgenes can be used to monitor calcineurin-NFAT activity, such as the NFATc1-GFP fusion protein which allows visualizing the translocation of NFAT to the nucleus when the pathway is activated. We found that NFATc1-GFP is mostly nuclear in the slow soleus muscle fibres but can be rapidly induced to translocate to the cytoplasm by denervation or even general anaesthesia; vice versa NFATc1-GFP is mostly cytoplasmic in the fast tibialis

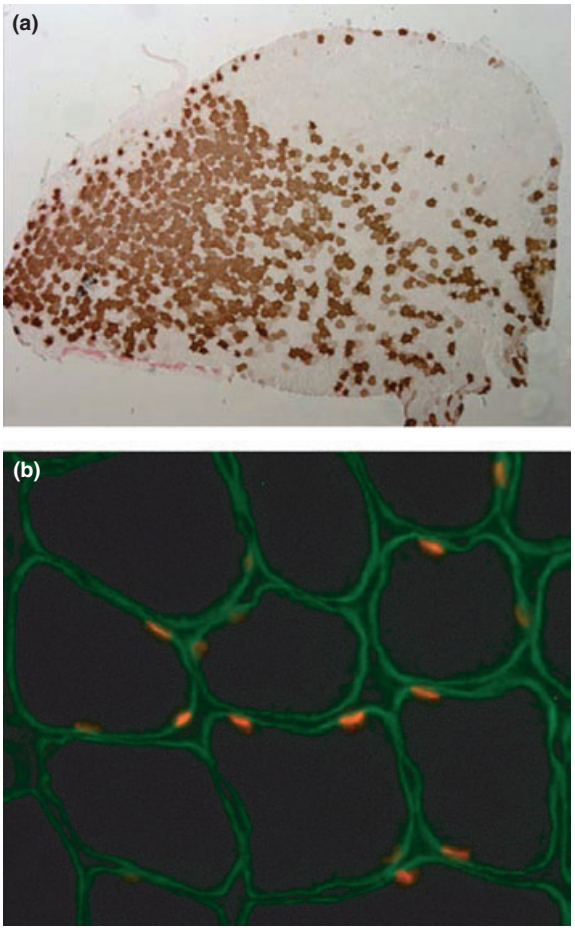

Figure 3 Gene transfer in adult skeletal muscle. (a) Rat extensor digitorum longus transfected *in vivo* with plasmid DNA coding for green fluorescence protein (GFP) by electroporation. The muscle was removed 1 week later and cryosections stained with anti-GFP antibody revealed with immunoperoxidase. (b) Rat soleus muscle transfected *in vivo* with DNA coding for a fusion protein of histone 2B linked to red fluorescence protein (RFP), section stained for dystrophin (green). Note that all nuclei expressing histone 2B-RFP are contained within the muscle fibre plasma membrane.

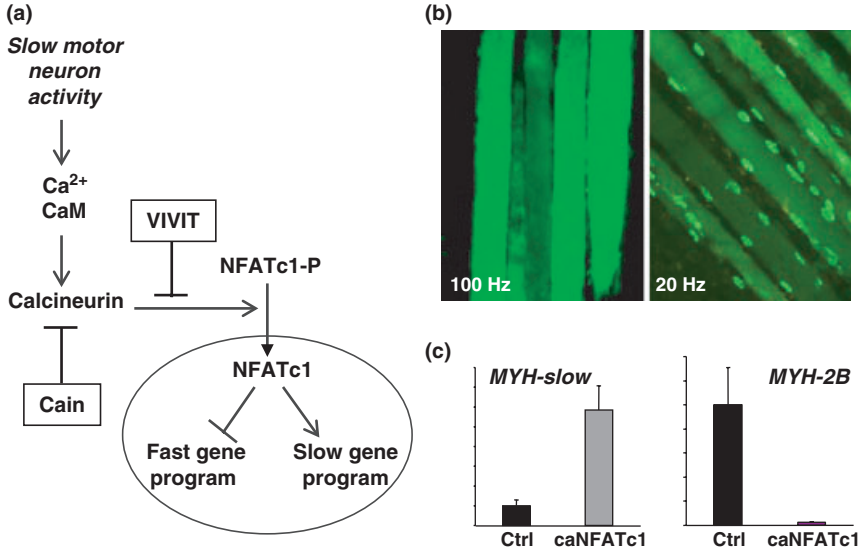

Figure 4 The calcineurin-NFAT pathway and fibre type specification. (a) Scheme of the signalling pathway connecting slow motor neurone activity via calcium changes to activation of calcineurin, dephosphorylation and nuclear translocation of NFATc1 and final upregulation of the slow gene programme and downregulation of the fast gene programme in muscle fibres. The inhibitors of this pathway, cain and VIVIT, are also indicated. (b) Rat tibialis anterior muscles were transfected with plasmid DNA coding for NFATc1-GFP fusion protein and 1 week later stimulated for 2 h via peroneus nerve either with a fast-type phasic 100 Hz impulse pattern (left) or with a slow-like tonic 20 Hz pattern (right). Note nuclear translocation of NFATc1-GFP induced by 20 Hz stimulation. (from Tothova *et al.* 2006). (c) Adult EDL muscles were co-transfected with constitutively active NFATc1 mutant (caNFATc1) and either MyHC-slow promoter linked to luciferase or MyHC-2B promoter-luciferase. Note that MyHC-slow promoter activity is increased, whereas MyHC-2B promoter activity is decreased, by caNFATc1. Luciferase activity is expressed as the percentage of that measured in muscles injected with the empty vector instead of caNFATc1 (from McCullagh *et al.* 2004).

anterior, but translocates to the nucleus following electrical stimulation with a slow-type not a fast-type pattern of impulses (Fig. 4b) (Tothova *et al.* 2006). To determine the transcriptional activation of NFAT target genes, we used NFAT sensors, made by concatamers of NFAT binding sites linked to reporter gene, and specific promoters, e.g. MYH promoters linked to reporter genes, which reflect the response of fibre type-specific genes to the activation of the calcineurin-NFAT pathway. Other transgenes consist of mutant variants which cause activation of the pathway, such as constitutively active NFATc1 mutant, or inhibition of the pathway, such as the NFAT inhibitory peptide VIVIT. By co-transfection experiments, we found that constitutively active NFATc1 upregulates a MYH-slow promoter but downregulates a MYH-2B promoter (Fig. 4c). In contrast, VIVIT blocks the activation of the MyHC-slow but not of the MyHC-2B promoter in adult soleus muscle. These different approaches support the notion that the calcineurin-NFAT pathway is involved in the activity-dependent induction of the slow gene programme in regenerating muscle and in the maintenance of this programme in adult slow muscles. In more recent studies, using transfection with plasmid vectors that generate siRNAs to specifically silence target genes, we examined the relative role of other NFAT family members, NFATc2, c3 and c4, which appear to be involved in the regulation of fast gene programs (Calabria *et al.* 2009).

The role of the calcineurin-NFAT pathway in muscle fibre type differentiation is supported by the study of a transgenic mouse line over-expressing the calcineurin inhibitor RCAN1 (alias MCIP1, alias DSCR1) (Oh *et al.* 2005). The soleus muscle of these mice contains the normal proportion of MYH-β/slow at birth, suggesting that calcineurin is not required for the embryonic expression of slow myosin, but slow myosin disappears completely during the first postnatal weeks. Buller *et al.* (1960a) first demonstrated that motor neurone silencing produced in the neonatal kitten by transection of the spinal cord and its isolation from afferent stimuli had virtually no effect on the development of the contractile properties of fast muscle, but prevented the slow muscle from developing its normal speed. As a result the slow muscle became practically as fast as the normal fast muscle. Subsequent studies showed that neonatal denervation does not interfere with the postnatal accumulation of fast-type MYHs in rodent muscles but causes the disappearance of slow myosin (Butler-Browne *et al.* 1982). Accordingly, the regenerating rat soleus undergoes the switch from embryonic and neonatal to adult fast MYHs even in the absence of innervation, whereas slow motor unit activity is required to induce slow myosin (Esser *et al.* 1993, Jerkovic *et al.* 1997, Kalhovde *et al.*

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Table 2 *In vivo* transfection experiments aimed at exploring the role of the calcineurin-NFAT pathway on muscle fibre type properties*

General aim	Specific function examined	Type of transgene	Results
Monitoring pathway	Nuclear translocation of NFAT	NFAT-GFP chimera	NFATc1-GFP is mostly nuclear in SOL fibres but translocates to the cytoplasm upon denervation, whereas it is mostly cytoplasmic in TA fibres but translocates to the nucleus upon low frequency stimulation (Tothova *et al.* 2006)
	Transcriptional activation of NFAT target genes	NFAT sensor† MYH gene promoter-reporter constructs	Transcriptional activity higher in SOL than TA, increased in TA by c.a.NFAT, decreased in SOL by denervation or by NFAT inhibition (McCullagh *et al.* 2004, Calabria *et al.* 2009)
Perturbing pathway: gain-of-function approaches	Activation of NFAT	c.a.NFATc1	Activates co-transfected MYH-slow promoter and inhibits fast MYH-2B promoter in adult muscles; induces expression of MYH-slow in regenerating not in adult TA (McCullagh *et al.* 2004)
Perturbing pathway: loss-of-function approaches	Inhibition of Cn	Cain (Cn inhibitor)	Blocks induction of MYH-slow in regenerating SOL (Serrano *et al.* 2001); blocks nuclear translocation of NFATc1-GFP in stimulated TA (Tothova *et al.* 2006)
	Inhibition of Cn–NFAT interaction	VIVIT (NFAT inhibitor)‡	Blocks induction of MYH *β*/slow in regenerating SOL (McCullagh *et al.* 2004)
	Inhibition of expression of specific NFATs	NFAT-specific siRNAs	Differential effects of different NFATs on various MYH promoters (Calabria *et al.* 2009)

c.a.NFAT, constitutively active nuclear factor of activated T cells (NFAT); Cn, calcineurin; siRNAs, small interfering RNAs; SOL, soleus muscle (a slow-twitch muscle); TA, tibialis anterior (a fast-twitch muscle); MYH, myosin heavy chain.
*Transfection experiments performed in adult or regenerating rat muscles.
†Artificial promoter made by concatamer of NFAT binding elements linked to luciferase.
‡The small peptide VIVIT blocks selectively the interaction of Cn with NFAT thus preventing the activation of NFAT by Cn; used as VIVIT-GFP fusion protein in transfection experiments.

2005). On the other hand, the early appearance of slow myosin and diversification of fast and slow fibres in embryonic mammalian muscle take place independently of neural influences (Condon *et al.* 1990). The mechanisms responsible for the embryonic activation of the slow gene programme remain to be determined. The transcription factor *Sox6* may be involved in this process based on the finding that slow myosin is widely expressed in foetal muscles of mutant mice lacking a functional *Sox6* gene, suggesting that in foetal muscle *Sox6* functions as a repressor of slow fibre type-specific genes (Hagiwara *et al.* 2005, 2007). A similar role of *Sox6* has been demonstrated in the development of muscle fibre types in the zebrafish (von Hofsten *et al.* 2008).

Conclusions and open issues

Some general conclusion can be drawn from the studies reviewed here and other related studies, but many questions are still unanswered.

(1) The signalling pathways responsible for the induction of the slow gene programme in the embryo are independent of nerve activity and differ from those involved in the maintenance of this programme in adult muscles.
(2) After birth the switch from the expression of embryonic and neonatal MYH to adult fast MYHs occurs independently of the neural influence; a similar default switch is observed in denervated regenerating muscle.
(3) During early postnatal development and in regenerating muscle, the gene programme is dependent on innervation and specifically on slow motor neurone activity. However, once established for some weeks after birth the slow gene programme is not easily erased from adult fibres and tends to persist for a long time. In humans, only long-term motor neurone silencing induced by spinal cord injury can lead to complete disappearance of slow myosin expression (Fig. 5). This suggests that

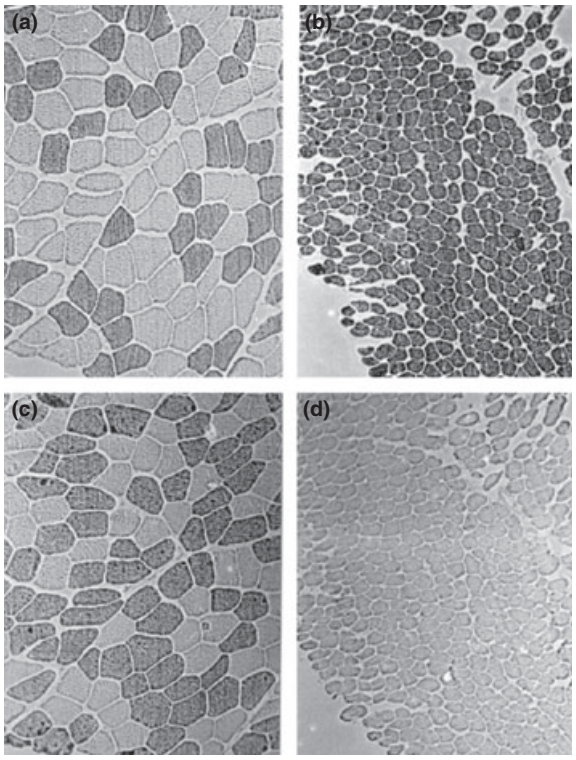

Figure 5 Fibre type changes in paralysed human skeletal muscle. Sections of normal (a, c) and paralysed (b, d) human skeletal muscle, 1 year after spinal cord injury. Sections stained with an antibody that reacts with MYH-2A and -2X (a, b) and an antibody to MYH-slow (c, d). Note complete switch to fast-type myosin in paralysed muscle.

transcriptional regulation may vary in myonuclei from neonatal to adult muscle fibres, not unlike what occurs with *Pax7* regulation in satellite cell nuclei. We have previously reported that a constitutively active NFATc1 is able to upregulate a MYH-β/slow promoter-reporter construct in both regenerating and adult fast muscles, though the corresponding protein is induced only in the regenerating muscle (McCullagh *et al.* 2004). It remains to be established whether the maturation of the fast muscle fibres is accompanied by epigenetic modifications at the MYH-β/slow gene locus that make this gene essentially inaccessible for transcription or whether gene expression is also controlled at the post-transcriptional level.

(4) The existence of intrinsic differences that limit the plasticity of adult muscles was first suggested by the incomplete transformation of fast and slow muscles after cross-reinnervation experiments. It was later shown that fast and slow muscles respond differently to the same electrical stimulation protocol, slow myosin being easily induced in slow but not fast muscles (Ausoni *et al.* 1990). More recent studies have demonstrated that also fast and slow muscles regenerating in the absence of the nerve respond differently to the same electrical stimulation protocol, slow myosin being more easily induced in slow compared with fast muscles (Kalhovde *et al.* 2005). These findings suggest the existence of intrinsic differences between satellite cells from fast and slow muscles, an issue that must be further explored. A related open question is whether there are intrinsic differences between different categories of fast fibres, independent of innervation. The existence of such differences is suggested by the following finding that (1) fast MYHs are upregulated after birth even in denervated muscle, as discussed above, (2) their expression occurs in very precise spatially defined patterns, e.g. 2B MYH is expressed in more superficial layers of fast leg muscles (DeNardi *et al.* 1993, Allen & Leinwand 2001), and (3) knockout of the MYH 2B or 2X genes leads to contractile disfunction and loss of fibres with only partial compensation by upregulation of other fast MYH genes, suggesting a unique role of different MYHs (Allen *et al.* 2000).

(5) The calcineurin-NFAT pathway is among the best characterized signalling pathways in mediating the effect of motor neurone activity on the muscle fibre transcriptional machinery. NFATc1 behaves as a slow motor neurone activity sensor and is involved in the maintenance of the slow fibre phenotype; other NFATs might contribute to the maintenance of the fast 2A, 2X and 2B phenotypes (Calabria *et al.* 2009). The relationship of the calcineurin-NFAT pathway with other signalling pathways, e.g. MEF2 and HDACs, which are also involved in the regulation of the muscle fibre type phenotype, is discussed elsewhere (Bassel-Duby & Olson 2006).

Note added in proof

We have recently identified two novel myosin heavy chains, coded by myosin genes MYH14, also called MYH7b, and MYH15, which are selectively expressed in specific fibers of extraocular muscles and muscle spindles (Rossi et al, 2010). The myosin coded by MYH14 (MYH7b) corresponds to the slow-tonic myosin previously defined by immunohistochemistry in mammalian extraocular muscles (Bormioli et al, 1979; Bormioli et al, 1980). This myosin is present in fibers that, like the slow-tonic fibers of amphibian and birds, respond to stimulation with a long lasting contracture rather than a twitch and have multiple ''en grappe'' innervation rather than the single ''en plaque'' motor endplate typical of both fast- and slow-twitch muscle fibres. The functional properties of the myosin coded by MYH15 have not been established.

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Conflict of interest

There is no conflict of interest.

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