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Novel/ancient myosins in mammalian skeletal muscles: MYH7B and MYH15

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Riassunto

La miosina è il motore molecolare responsabile della contrazione muscolare ed esiste in diverse forme che riflettono alcune proprietà del muscolo, come ad esempio la velocità di accorciamento e la forza di contrazione. Fino a dieci anni fa solo otto catene pesanti della miosina sarcomerica (MYH) erano note essere presenti nel muscolo scheletrico dei mammiferi, suddivise in due gruppi altamente conservati (Weiss *et al.*, 1999a; Weiss *et al.*, 1999b). Un gruppo si trova nel cromosoma umano 14 e codifica per le due miosine cardiache alpha e beta, l'ultima delle quali è espressa oltre che nel cuore anche nei muscoli lenti. Un altro gruppo si trova nel cromosoma umano 17 e codifica per le sei miosine scheletriche, che includono le isoforme veloci 2A-, 2X, 2B-MYH, la embrionale e la neonatale espresse nei muscoli nelle fasi dello sviluppo, e la MYH13, un'isoforma espressa esclusivamente nei muscoli extraoculari (EO). Il completamento del Progetto Genoma Umano, circa dieci anni fa, ha rivelato che oltre a questi due gruppi di miosine cardiache e scheletriche, il genoma umano contiene altri tre geni della catena pesante della miosina sarcomerica: *MYH7B* (chiamata anche *MYH14)*, *MYH15* e *MYH16*. La MYH16 è espressa nei muscoli masticatori dei carnivori e primati, ma è uno pseudogene negli umani (Stedman *et al.*, 2004). Tuttavia, nulla è noto circa l'espressione degli altri due geni: *MYH7B* e *MYH15*. Pertanto, ci siamo posti la domanda se questi due geni fossero espressi nei muscoli striati dei mammiferi.

Abbiamo constatato che ortologhi di *MYH7B* e *MYH15* sono presenti nelle rane e negli uccelli, e, rispettivamente, codificano per la miosina lenta di tipo 2 e la miosina ventricolare, mentre solo ortologhi della *MYH7B* sono presenti nei pesci. In tutte le specie il gene *MYH7B* contiene al suo interno un microRNA, il miR-499. In questo studio abbiamo dimostriamo che in topo e ratto i trascritti di MYH7B e miR-499 sono espressi nel cuore, nei muscoli lenti e EO, mentre la proteina MYH7B è presente esclusivamente in una sottopopolazione di fibre dei muscoli EO corrispondenti alle fibre *slow-tonic* e nelle fibre *nuclear bag* dei fusi neuromuscolari. Il trascritto di MYH15 è espresso esclusivamente nei muscoli EO e la proteina MYH15 è presente nella maggioranza delle fibre nello strato orbitale dei muscoli EO e nella regione extracapsulare delle fibre *bag* dei fusi neuromuscolari. Durante lo sviluppo, la MYH7B è presente come trascritto a bassi livelli nei muscoli scheletrici, cuore e muscoli EO, tuttavia dopo la nascita scompare limitando la sua espressione proteica solo nelle fibre *slow-tonic*. Al contrario, la MYH15 è assente durante lo sviluppo fetale ed embrionale, ed è presente solo dopo la nascita nello strato orbitale dei muscoli EO.

Abbiamo analizzato la storia evoluzionistica di queste miosine nei vertebrati e abbiamo dimostrato che la MYH15 subisce drastici cambiamenti nella struttura e funzione nei mammiferi rispetto ai vertebrati inferiori; infatti, lo stesso gene che codifica per una miosina espressa nel cuore ed usata nella contrazione cardiaca nelle rane, codifica per una miosina espressa nei muscoli EO ed utilizzata per il controllo del movimento degli occhi nei mammiferi; questo

fenomeno rappresenta un affascinante caso di *tinkerig* evoluzionistico (Jacob, 1977). Abbiamo dimostrato che l'altro gene, *MYH7B*, contiene un microRNA, il miR-499, che è conservato in tutte le classi di vertebrati. Abbiamo ipotizzato che questo microRNA possa essere coinvolto nella differenza di espressione tra il trascritto della MYH7B, abbondante nei muscoli lenti e cardiaco, e la rispettiva proteina, limitata in una sottopopolazione di fibre nei muscoli EO. Questa differenza tra il livello di espressione di trascritto e proteina rappresenta un caso unico nella famiglia delle miosine sarcomeriche. Infine, abbiamo dimostrato che queste due miosine subiscono importanti cambiamenti nei livelli di espressione nelle due settimane successive alla nascita, un periodo durante il quale l'esperienza visiva è necessaria per il corretto sviluppo del sistema sensoriale visivo ed oculomotorio. Inoltre, abbiamo dimostrato che queste miosine sono espresse anche nelle fibre intrafusali dei fusi neuromuscolari che costituiscono il sistema sensoriale propriocettivo dei muscoli scheletrici.

In conclusione, la caratterizzazione delle miosine MYH7B e MYH15 completa il quadro dell'espressione delle miosine sarcomeriche nei muscoli striati di mammifero, ridefinendo l'inventario di miosine coinvolte nell'architettura del sarcomero. Inoltre, abbiamo confermiamo definitivamente l'esistenza della miosina *slow-tonic* come prodotto di un gene distinto, fornendo le basi molecolari per lo studio della fisiologia di queste fibre nei mammiferi.

Abstract

Myosin, the molecular motor responsible for muscle contraction, exists in multiple forms which dictate muscle properties, such as shortening velocity and contractile force. Until ten years ago only eight sarcomeric myosin heavy chain (MYH) genes, associated into two highly conserved gene clusters, were known to be present in mammals (Weiss *et al.*, 1999a; Weiss *et al.*, 1999b). Two tandemly arrayed genes, located in human chromosome 14, code for the cardiac myosins, α- and β-MYH, the latter being also expressed in slow skeletal muscle. Another gene cluster, located in human chromosome 17, codes for the six skeletal myosins, including the adult fast 2A-, 2X- and 2B-MYH, the developmental embryonic and neonatal/perinatal isoforms, and MYH13, an isoform expressed specifically in extraocular (EO) muscles. The completion of the Human Genome Project almost ten years ago showed that the mammalian genome contains three additional genes coding for sarcomeric MYHs. One of these, *MYH16*, was shown to code for a myosin expressed in jaw muscles of carnivores and primates but is a pseudogene in humans (Stedman *et al.*, 2004). However nothing is known concerning the two other novel MYHs: MYH7B and MYH15. Therefore, we asked whether these two genes are expressed in mammalian striated muscles. We found that *MYH7B* and *MYH15* orthologs are present in frogs and birds, coding for chicken slow myosin 2 and ventricular MYH, respectively, whereas only *MYH7B* orthologs have been detected in fish. In all species the *MYH7B* gene contains a microRNA, miR-499. We report that in rat and mouse, MYH7B and miR-499 transcripts are detected in heart, slow muscles and extraocular (EO) muscles, whereas MYH7B protein is detected only in a minor fiber population in EO muscles, corresponding to slow-tonic fibers, and in bag fibers of muscle spindles. MYH15 transcripts are detected exclusively in EO muscles and the MYH15 protein is present in most fibers of the orbital layer of EO muscles and in the extracapsular region of nuclear bag fibers. During development, MYH7B is expressed at low levels in skeletal muscles, heart and all EO muscle fibers but disappears from most fibers, except the slow-tonic fibers, after birth. In contrast, MYH15 is absent in embryonic and fetal muscles and is first detected after birth in the orbital layer of EO muscles.

We trace the evolutionary history of these myosins in vertebrates and show that MYH15 undergoes drastic changes in structure and function in mammals compared to lower vertebrates, whereby the same gene codes for a myosin used to pump blood in frogs and birds or to control eye movements in mammals, thus providing a striking case of evolutionary tinkering (Jacob, 1977). We show that the other gene, *MYH7B*, contains a specific microRNA, miR-499, which is conserved in all vertebrate classes, and we suggest that this may account for the striking mismatch, unprecedented in sarcomeric myosins, between the expression of transcript, high in slow and cardiac muscles, and corresponding protein, restricted to slow-tonic fibers in EO muscles. Finally, we show that these novel myosins undergo striking changes during early postnatal development, namely during the critical period when visual experience is

required for the correct maturation of both sensory visual and oculomotor systems. In addition, we find that these myosins are also expressed in intrafusal fibers of muscle spindles, the proprioceptive sensory organs embedded in most skeletal muscles.

In conclusion, the characterization of the expression pattern of MYH7B and MYH15 provides a definitive picture of MYH expression in mammalian striated muscle, thus completing the inventory of MYH isoforms involved in sarcomeric architecture of skeletal muscles. We also establish the existence of slow-tonic MYH as a specific gene product, providing an unambiguous molecular basis to study the contractile properties of slow-tonic fibers in mammals.

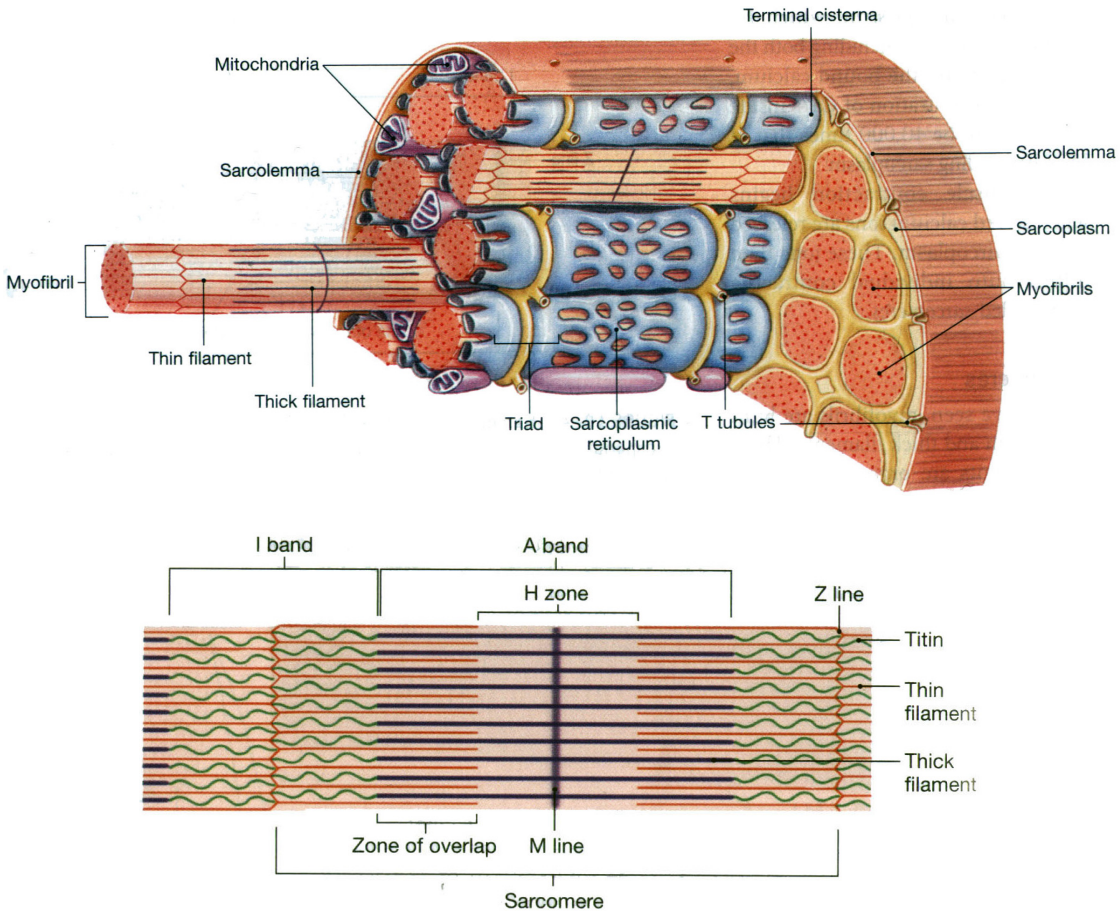
Introduction

1. Skeletal muscle fiber and myosin heavy chains

1.1 Molecular anatomy of the muscle

Mammalian skeletal muscles are composed by a variety of fiber types characterized by wide differences in their functional properties, such as force, speed of contraction and resistance to fatigue. These differences are mainly evident in the myosin heavy chain (MYH) isoform that each fiber express, however many other cellular structures are involved in this functional diversification, such as plasma membrane, sarcoplasmic reticulum channels and pumps, myofibrillar and cytoskeletal proteins, mitochondria and metabolic enzymes (Schiaffino & Reggiani, 1996).

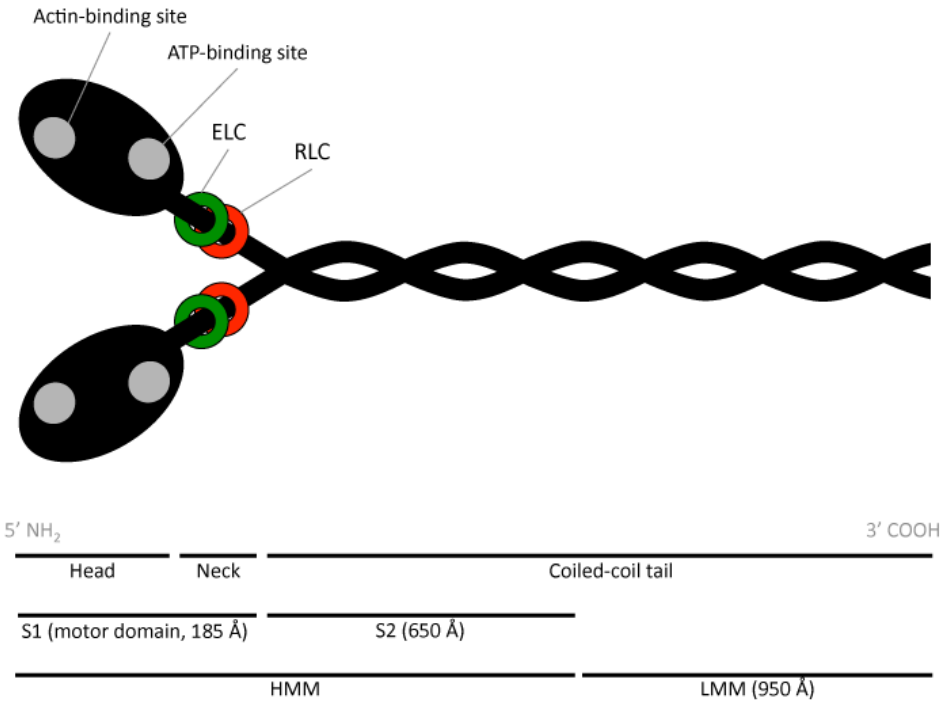
Fig. 1 Anatomical organization of skeletal muscle.



Representation of an entire skeletal muscle (top) and of the sarcomere (bottom).

The functional unit of the skeletal muscle is the sarcomere (Fig. 1) which is the responsible for skeletal muscle's striated appearance and the basic machinery necessary for muscle contraction. Together with other important proteins, the sarcomere is mostly composed by myosin and actin which act as molecular motors according to the sliding filaments theory. Sarcomeric myosin is an asymmetrically hexameric protein, consisting of four myosin light chains (MLC) and two myosin heavy chains (MYH) (Fig. 2). The myosin molecule can be divided into two functional domains: an NH_2-terminal globular head motor domain containing ATP- and actin-binding sites and a COOH-terminal helical coiled-coil rod domain having filament-forming ability. Two loop structures, loop 1 and loop 2, are located in the head motor domain at the ATP- and actin-binding sites, respectively; both loops function essentially in the motor activity, playing important roles in the speed of contraction properties of the myosin (Sweeney *et al.*, 1998).

Fig. 2 Representation of the class II myosin, or conventional myosin, and its subfragments



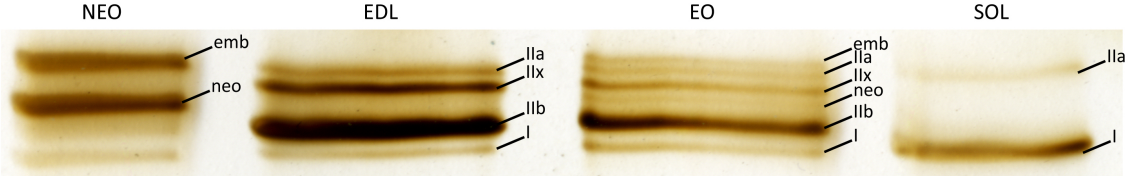
Myosin II can be proteolytically cleaved into heavy meromyosin (HMM) and light meromyosin (LMM) by α-chymotrypsin. Heavy meromyosin consists of the myosin head subfragment-1 domain (S1), its associated light chains (essential light chains and regulatory light chains), and the coiled-coil subfragment-2 domain. Light meromyosin consists of coiled-coil protein structure. The myosin S1-subfragment is produced by papain digestion of HMM.

1.2 Fiber type diversity and myosin isoforms: historical view

The muscle fiber phenotype can adapt to different physiological stimuli, such as hormonal influences, nutritional status, level of activity and nerve activation, the last being the major determinant. We now know that all these stimuli are somehow transformed into activation/ silencing of signaling pathways which finally dictate the muscle fiber profile (Bassel-Duby & Olson, 2006; Schiaffino *et al.*, 2007; Sandri, 2008). However, these observation are the results of more than one century of research in the muscle field.

The first observation on myofiber diversity comes from a very old report (Needham, 1926). At that time two major types of skeletal muscles were recognized: i) slow red muscles, composed of fibers rich in myoglobin and mitochondria, characterized by an oxidative metabolism and involved in continuous, tonic activity, and ii) fast white muscles, composed of fibers poor in myoglobin and mitochondria, relying more on glycolytic metabolism and involved in phasic activity. Later, it was demonstrated that the actin-activated ATPase activity of myosin correlates with speed of muscle shortening (Barany, 1967). On the subsequent years, between the 1967 and 1972, emerged a new scheme composed by three fiber types: the slow-twitch oxidative called type 1, one fast-twitch oxidative glycolytic called 2A and one fast-twitch glycolytic called 2B. These results were obtained with different lines of research: experiments on motor units physiology (Close, 1967; Edstrom & Kugelberg, 1968), electron microscopy (Schiaffino *et al.*, 1970), new procedures for myosin ATPase staining (Guth & Samaha, 1969; Brooke & Kaiser, 1970; Burke *et al.*, 1971) and biochemical analysis (Peter *et al.*, 1972). At the end of the '80, a new important contribution was provided with the discovery of a third fiber type among the fast group, called 2X, with properties intermediate between the 2A and 2B MYH. These findings were the results of two different approaches: use of monoclonal antibodies against the MYHs (Schiaffino *et al.*, 1986b; Schiaffino *et al.*, 1988; Schiaffino *et al.*, 1989) and improved electrophoretic procedure for MYH separation (Bar & Pette, 1988; Termin *et al.*, 1989). An example of MYH electrophoretic separation showing the presence of different bands corresponding to different MYHs is presented in Fig. 3. The existence of the 2X fiber was further

Fig. 3 Separation of myosin heavy chain isoform of rat muscles.



Myosins from different rat muscles resolved by SDS-PAGE using polyacrylamide gel containing high glycerol concentration which allows the separation of MYH isoforms (Mizunoya *et al.*, 2008). Note different distribution of MYHs in different muscles. NEO: neonatal hindlimb muscles; EDL: adult fast extensor digitorum longus muscle; EO: adult extraocular muscles; SOL: adult slow soleus muscle.

confirmed at gene level in the following years (DeNardi *et al.*, 1993). Thus, the final scheme describing the fiber type diversity includes one slow (type 1, very often also called β/slow or cardiac-β) and three fast types (2A, 2X, 2B), each being the product of a distinct gene (*MYH7*, *MYH2*, *MYH1* and *MYH4*, respectively). However, different fiber types can be coexpressed in the same muscle fiber, giving rise to hybrid fibers (Gorza, 1990; Pette & Staron, 1990; Bottinelli *et al.*, 1991; DeNardi *et al.*, 1993). Generally, this heterogeneity follows the sequence 1 ↔ 1/2A ↔ 2A ↔ 2A/2X ↔ 2X ↔ 2X/2B ↔ 2B. A final contribution to fiber type classification in mammals was provided with the finding that human limb muscles do not express the 2B MYH (Smerdu *et al.*, 1994), which is apparently restricted in a limited subpopulation of extraocular and laryngeal muscle fibers (Andersen *et al.*, 2000). Comparative analysis suggests that large mammals, including humans, have a greater proportion of type 1 and 2A fibers, and *vice versa*. Detailed description of mammalian fiber type can be found elsewhere (Schiaffino & Reggiani, 1996).

This short summary only included fiber types expressed in limb muscles; however, the variety of fiber type and myosin heavy chain isoform is wider if one also consider other muscles, such as the developing muscles, the cardiac muscles, the extraocular muscles and the intrafusal fibers of the muscle spindles. Although the presence of these other fiber types is limited and restricted to certain group of muscles, their biological significance cannot be ignored. During fetal/embryonic stages and during regeneration after injury, muscles express two myosin isoforms, the embryonic and the neonatal/perinatal as product of the *MYH3* and *MYH8* genes, respectively. Expression of these isoforms in physiological condition is progressively lost after birth or completion of the regeneration process; only under pathological conditions these isoforms may be expressed in adult stages where muscle regeneration occurs (e.g. dystrophies). The cardiac muscles, in addition to the β/slow, also express the α-cardiac isoform which has, from a physiological perspective, faster speed of contraction than the β/slow. The extraocular muscles express all the known fiber types, including an extraocular muscles-specific isoform called MYH13 (Wieczorek *et al.*, 1985; Sartore *et al.*, 1987). This group of muscles will be discussed in more details in the next section. In conclusion, up to the completion of the Human Genome Project in 2000, the spectrum of muscle fiber types and myosin heavy chains included eight genes and corresponding proteins, as summarized in Table 1. In the next session the new contribution provided by the Human Genome Project to the identification myosin heavy chain genes will be presented.

Table 1. Known fiber type and myosin heavy chain before the completion of the Human Genome Project (2000)

Gene	Protein
MYH1	Type 2X
MYH2	Type 2A
MYH3	Embryonic
MYH4	Type 2B
MYH6	α-cardiac
MYH7	Type 1 (β/slow or β-cardiac)
MYH8	Neonatal (also called perinatal)
MYH13	Extraocular specific

1.3 Why different myosin isoforms

Skeletal muscle accomplish the fundamental task to provide contraction, thus movement and force generation, but have also important roles in whole body metabolism and thermoregulation. One of the major peculiarity of the skeletal muscles is their great ability to adapt to different environmental tasks such that they can always be functionally efficient. The phenomenon is called skeletal muscle plasticity and is achieved by structural and molecular rearrangements of the myofibrillar proteins, mainly myosin. Exercise training is probably one of the best example of skeletal muscle plasticity. Endurance training promotes a shift of the myosin toward a slower profile following the patter 2B → 2X → 2A → 1 accompanied by the induction a more oxidative metabolism (mainly greater content of mitochondria and oxidative enzyme capacity). On the contrary, resistance training promotes the shift 1 → 2A → 2X → 2B and greater expression of glycolytic enzymes. In physiological condition, such as exercise, however, the myosin transition seems to have a limited range of adaptation, the variation being restricted to only one shift toward the closest profile (i.e. 2A to 1 or 2X, not 2A to 2B). Only studies on prolonged cross re-innervation have achieved wider range of myosin switching from fast to slow pattern and *vice versa* (Buller *et al.*, 1960; Pette & Vrbova, 1992)

This ability of the muscle to adapt to environmental stimuli confers them the advantage to be always highly effective in their tasks. The major functional differences, such as speed of contraction and resistance to fatigue are, in fact, reflected in the role each muscle fiber has. For example, postural muscle such as the soleus are mainly composed of type 1 slow, fatigue-resistant fibers; these fibers are recruited with long lasting trains (over minutes) at low frequency of firing (~20 Hz). Only muscles with these functional and structural properties can accomplish such “tonic” task. As opposite, fast fibers are recruited with short term trains (few seconds) at high discharge frequency (~100 Hz), leading to “phasic” movements such as sprinting or jumping.

Thus, the intrinsic existence of a wide spectrum of muscle fiber type plus their ability to adapt to environmental stimuli lead to highly efficient muscular system.

2. Human Genome Project revealed new myosin genes

Until ten years ago only eight sarcomeric myosin heavy chain (MYH) genes, associated into two highly conserved gene clusters, were known to be present in mammals (Weiss *et al.*, 1999a; Weiss *et al.*, 1999b). Two tandemly arrayed genes, located in human chromosome 14, code for the cardiac myosins, α- and β-MYH, the latter being also expressed in slow skeletal muscle. Another gene cluster, located in human chromosome 17, codes for the six skeletal myosins,

including the adult fast 2A-, 2X- and 2B-MYH, the developmental embryonic and neonatal/ perinatal isoforms, and another isoform expressed specifically in extraocular (EO) muscles, the MYH13. More recently, the completion of the human genome project led to the identification of three additional sarcomeric MYH genes: *MYH7B* (also called *MYH14*, see below Nomenclature) located on human chromosome 20, *MYH15* on chromosome 3 and *MYH16* on chromosome 7 (Berg *et al.*, 2001; Desjardins *et al.*, 2002). The three new sarcomeric MYH genes differ significantly in sequence, size and exon-intron organization from the other sarcomeric MYHs and appear to correspond to ancestral MYHs (Desjardins *et al.*, 2002; McGuigan *et al.*, 2004; Ikeda *et al.*, 2007). Orthologs of MYH7B are found in fish, Xenopus and chicken genome, the chicken ortholog coding for a slow-type MYH, called slow myosin 2 (SM2), whereas orthologs of MYH16 are not found in Xenopus or chicken but are present in fish and, surprisingly, in the ascidian, Ciona intestinalis (McGuigan *et al.*, 2004; Garriock *et al.*, 2005; Ikeda *et al.*, 2007; Nasipak & Kelley, 2008). MYH15 is the ortholog of Xenopus and chicken genes coding for ventricular MYH but no MYH15 ortholog was detected in fish (McGuigan *et al.*, 2004; Garriock *et al.*, 2005; Ikeda *et al.*, 2007).

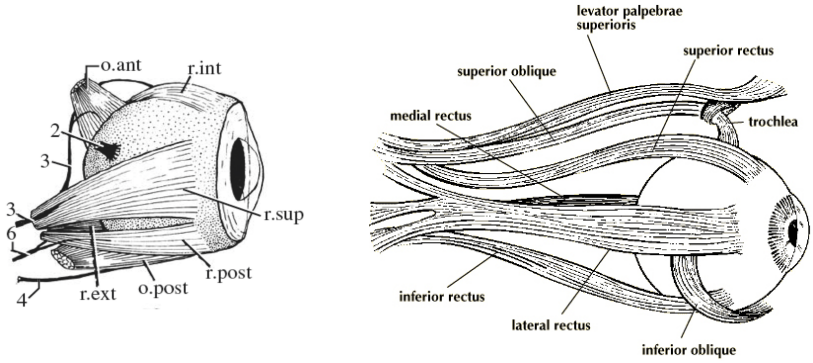
The expression pattern of these three MYH isoform genes has not been characterized in mammals except for MYH16. *MYH16* codes for a myosin expressed in jaw muscles of carnivores, primates and marsupials, but is a pseudogene in humans (Schachat & Briggs, 1999; Berg *et al.*, 2001; Qin *et al.*, 2002; Stedman *et al.*, 2004). MYH16 is often referred to as “superfast”, however a recent study shows that single cat and dog fibers containing exclusively this myosin isoform display a maximum shortening velocity similar to that of fast 2A fibers, namely intermediate between that of slow type 1 and fast 2B fibers, but exert more force than any other fiber type examined (Toniolo *et al.*, 2008). In contrast, no information is available about expression and function of mammalian MYH7B and MYH15. Several EST matches for MYH7B and MYH15 were detected in humans, but these were not from cardiac or skeletal muscles (Desjardins *et al.*, 2002). On the other hand, Garriock *et al.* (2005) failed to detect any entries corresponding to MYH15 transcripts in human and mouse EST databases and were unable to detect MYH15 mRNA using reverse transcriptase-polymerase chain reaction (RT-PCR) on RNA from adult mouse heart and suggested that MYH15 is probably a pseudogene in mammals. The issue is further confused by an early report (Nagase *et al.*, 2000) on a MYH cDNA clone (designated KIAA1512) isolated from a human fetal brain library, located on chromosome 20 and apparently corresponding to the sarcomeric MYH7B subsequently identified by Desjardins *et al.* (2002): transcripts of this gene were found to be expressed at high levels not only in human heart and skeletal muscle but also in brain and testis, and at lower but significant levels in ovary and kidney. The distribution of mammalian MYH7B transcripts is of special interest, in view of the finding that a specific micro-RNA, miR-499, is nestled within intron 19 of this gene in the mouse (van Rooij *et al.*, 2008a).

3. Anatomy and physiology of extraocular muscles

3.1 Anatomical organization of extraocular muscles

The extraocular (EO) muscles are a group of six muscles responsible for the movements of the eye ball. These are: medial rectus (MR), lateral rectus (LR), superior rectus (SR), inferior rectus (IR), superior oblique (SO), and inferior oblique (IO) (Fig 4 and 5). Remarkable is that all these six muscles are of very ancient origin and highly conserved throughout evolution (Fig. 4). In fact, the EO muscles "*appear in the lower vertebrates in essentially the same form as in man. Indeed their number and their nerve relations are the same in man as in the dogfish. Of the entire group*

Fig. 4 Comparison of extraocular muscles of lamprey and human



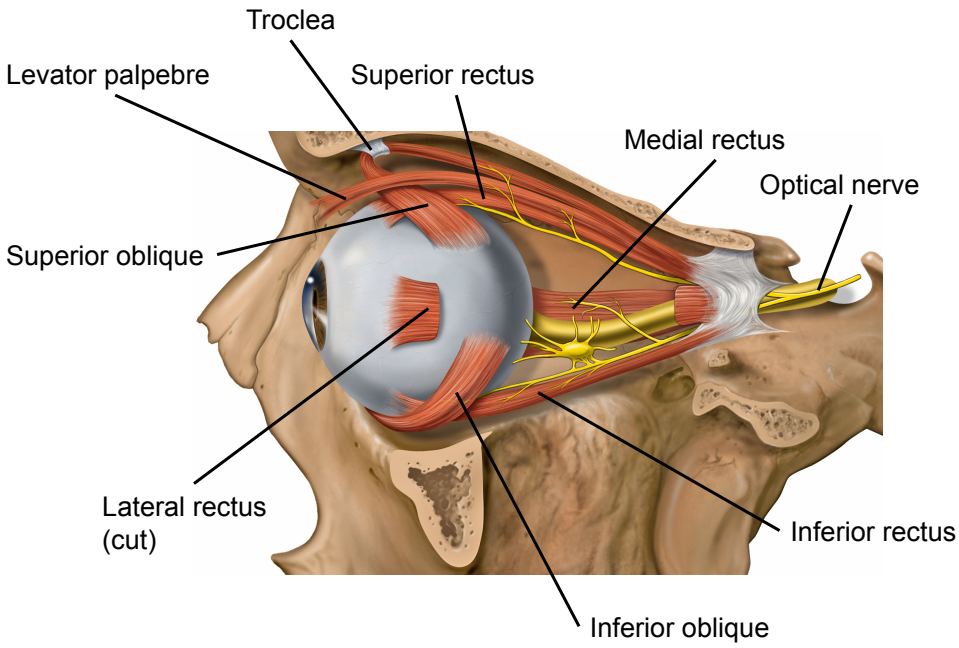
Graphical representation of the extraocular muscles of lamprey (left) and human (right). Illustrations not to scale (Young, 2008).

of eye muscles only the superior oblique shows a function change in the course of phylogeny. The direction of its pull is altered as the result of the development of the trochlear tendon" (Neal, 1918). In addition to the six EO muscles, most vertebrates, but not human, have an accessory ocular muscle involved in the protection of the cornea, the rectractor bulbi. Although anatomically very close to the six EO muscles, the rectractor bulbi has not to considered as one of the EO muscles. Also the levator palpebrae superioris, which elevates the eyelid, has not to be considered one of the EO muscles.

The EO muscles are considered very highly specialized muscles and are well known to be composed by all presently known striated myosin isoforms (Dietert, 1965; Bormioli *et al.*, 1979; Bormioli *et al.*, 1980; Wieczorek *et al.*, 1985; Sartore *et al.*, 1987; Pedrosa-Domellof *et al.*, 1992; Asmussen *et al.*, 1993; Lucas & Hoh, 1997; Briggs & Schachat, 2000; Pedrosa-Domellof *et al.*, 2000; Rubinstein & Hoh, 2000; Wasicky *et al.*, 2000; Porter *et al.*, 2001; Briggs & Schachat, 2002; Kjellgren *et al.*, 2003; Budak *et al.*, 2004; Khanna *et al.*, 2004; Fischer *et al.*, 2005; Lim *et al.*, 2006). Among these myosin isoforms, striking is the fact that EO muscles retain the

embryonic and perinatal isoforms in the adult stage, the α-cardiac isoform (elsewhere restricted only to cardiac muscle), the slow-tonic isoform and an EO muscles-specific isoform called MYH13. Moreover, fibers in the EO muscles are much smaller than any other body muscle fiber. For a detailed description of EO muscles fiber type distribution we suggest the review of Spencer and Porter (2005). One of the reason which may explain such broad myofiber diversity, a peculiarity exclusive of the EO muscles, is the different functional tasks they have to provide, *i.e.* from extremely fast saccadic movements to long lasting periods of fixation at a given position. Although the nature and extent of the functional diversity of the EO muscles is clear,

Fig. 5 Graphical representation of the extraocular muscles



Left human eye from lateral view. Note that the levator palpebrae has not to be considered an extraocular muscles.

the mechanisms or adaptations used by the EO muscles to achieve such functional diversity remain largely unknown. Another feature of the EO muscles is the fact that, contrary to the limb muscles, they are not innervated by somatic alpha-motoneurons from the spinal cord but instead by the cranial nerves, with very small motor units. More precisely, the lateral rectus by the cranial nerve VI, the superior oblique by the cranial nerve IV and the remaining EO muscles by the cranial nerve III.

The EO muscles are organized into two layers: an outer thin orbital layer (OL) facing the orbital bone and periorbita and a inner global layer (GL) facing the bulb. Generally, the OL fibers are smaller than those of the GL. The reason for the existence of a GL and an OL layer is still controversial and under debate. The emerging view supports the novel mechanism of the

"active pulley hypothesis" which correlates the distinctive properties of OL and GL with layer-specific division of functions. The GL extends the full muscle length, inserting into the sclera; on the contrary, the OL ends before the muscle becomes tendinous and it inserts into the pulley at approximately the globe equator in the Tenon's fascia. The pulleys are rings of dense collagen containing smooth muscle cells and elastin encircling the EO muscles. As a result of this complicated anatomical organization, the OL alters the direction of action of the GL fibers, whereas these last are the responsible for the eye movements (Demer *et al.*, 1995; Demer *et al.*, 2000; Lim *et al.*, 2007). More detailed description of the active pulley hypothesis can be found elsewhere (Miller, 2007).

3.2 Extraocular muscles in neuromuscular and metabolic diseases

EO muscles are also interesting from a clinical perspective since they respond to metabolic and neuromuscular diseases differently than other striated muscles. They are completely spared in Duchenne, limb girdle and congenital muscular dystrophies (reviewed in Spencer and Porter, 2005). Fascinating is the observation that in dystrophic mice, while the six EO muscles are completely spared, the retractor bulbi and levator palpebrae, which are closely related to but different from the EO muscles, show relevant pathological signs (Porter *et al.*, 1998). However, EO muscles are preferentially affected in oculopharyngeal muscular dystrophy, myasthenia gravis and mithcondrial myopaties (reviewed in Spencer and Porter, 2005). The elucidation of the mechanisms that predispose and/or protect the EO muscles may significantly contribute also to the clinical approach to these neuromuscular diseases.

4. Aim of the study

The aim of the present work is to describe the expression pattern of MYH7B and MYH15 in mammalian muscles. This study was originally motivated by the search for the gene coding for slow-tonic myosin that was previously identified in mammalian muscles (Bormioli *et al.*, 1979; Bormioli *et al.*, 1980) on the basis of the reactivity with an antibody specific for chicken slow anterior latissimus dorsi (ALD). Slow-tonic muscle fibers present in amphibian muscles respond to stimulation with a long lasting contracture rather than a twitch and are characterized by multiple "*en grappe*" innervation rather than the single "*en plaque*" motor endplate typical of twitch muscle fibers (Morgan & Proske, 1984). Slow-tonic fibers with similar properties are also present in the chicken slow ALD muscle and in mammalian EO muscles and muscle spindles

(Morgan & Proske, 1984). The presence of a distinct MYH in mammalian slow-tonic fibers was suggested by the finding that a polyclonal antibody raised against chicken ALD muscle myosin stained specifically multiply innervated fibers in human EO muscles and bag fibers in muscle spindles (Bormioli *et al.*, 1979; Bormioli *et al.*, 1980). Subsequent studies used monoclonal antibodies against chicken myosins to label as “slow-tonic” a number of fibers in EO muscles and spindles (Pedrosa & Thornell, 1990; Kucera *et al.*, 1992), however the existence of a distinct gene coding for mammalian slow-tonic myosin was never established.

Materials and Methods

1. Nomenclature

The MYH gene located in human chromosome 20 was named *MYH14* in several studies of sarcomeric MYHs (Desjardins *et al.*, 2002; McGuigan *et al.*, 2004; Ikeda *et al.*, 2007) (NCBI Entrez Gene ID: 311570). However, the same denomination *MYH14* was subsequently given to another gene located in chromosome 19 (Leal *et al.*, 2003) and coding for nonmuscle myosin IIC (Golomb *et al.*, 2004)(NCBI Entrez Gene ID: 79784). This was probably due to the fact that, even today, a Pubmed search for "MYH14" does not bring up the paper of Desjardins *et al.* To avoid confusion, the HUGO Nomenclature Committee termed *MYH7B* (full name: MYH7B cardiac muscle β) the gene located in human chromosome 20. However, this denomination appears wholly inappropriate, since it suggests a nonexistent relation of this gene with the *MYH7* gene coding for the MYH-β/slow expressed in mammalian slow-twitch muscle fibers (NCBI Entrez Gene ID: 4625). Given the wide use of the *MYH14* nomenclature for the sarcomeric myosin gene in the field of the muscle, it was recently suggested to name *MYH17* the gene *MYH14* on chromosome 19 coding for nonmuscle myosin IIC (Ikeda *et al.*, 2007). Although we support this proposal, the use of *MYH7B* in now widely accepted and a nomenclature change from *MYH7B* to *MYH14* is now not possible (see below). Therefore we also will use hereafter the term *MYH7B* for the human chromosome 20 sarcomeric MYH gene. Gene names are written in *italics* (capital for human) and protein names in normal (roman) characters throughout the text, according to a widely accepted use.

To elucidate the reason of the confusion on the nomenclature of the *MYH7B/MYH14* gene, we performed an accurate research on public databases and published papers. Unfortunately, we were not able to find an accurate report history of this gene, and therefore in our opinion the denomination "MYH7B cardiac muscle β" remains unfounded. The study of Nagase and colleagues in 2000 reported the sequences of 100 new human cDNA clones of previously unidentified genes and among them it was identified on the chromosome 20 the clone named KIAA1512 (Nagase *et al.*, 2000). This clone has later been found to correspond to a sarcomeric MYH gene (Berg *et al.*, 2001) and then to the gene *MYH7B/MYH14*, although it is nowhere specified when and which group described such correspondence. In 2001, as part of the Human Genome Project, the sequence of the human chromosome 20 was completed (Deloukas *et al.*, 2001). The *MYH7B* gene is reported here as one of the "*'know' genes, that is, those that are identical to known human complementary DNA or protein sequences (all known genes were in the LocusLink database, http://www.ncbi.nlm.nih.gov/LocusLink)*". However, there are no reports of any gene named *MYH7B* archived on any database, including NCBI, before this paper/date. Subsequently, on 2002 and 2004, two key studies specifically focusing on the evolution of the

sarcomeric myosin heavy chain family named the same gene *MYH14* (Desjardins *et al.*, 2002; McGuigan *et al.*, 2004). In rat and mouse the nomenclature given to this gene seems to derive from that used in human. In rat, the gene name *Myh7b* was deposited on January 2005 as "predicted", and then approved April 2008. In mouse, the gene name *Myh7b* was assigned on May 2005. We also enquired the OMIM (Online Mendelian Inheritance in Man) in order to get some more information; the OMIM scientific writer, dr. Patricia A. Hartz, confirmed us that "*the symbol MYH7B was generated by the people who create either the EntrezGene or GenBank databases and cannot be attributed to a published report.*" Moreover, she confirmed us that the *MYH7B* gene entry in EntrezGene entry was created on 16th January 2002 (*i.e.* later than the first report of Deloukas *et al.*, 2001). Recently, in december 2009 we contacted the HUGO coordinator dr. Elspeth Bruford asking for a revision of the gene nomenclature. She confirmed that the matter is indeed confusing and that it had already been under consideration some year ago; yet, she expressed the impossibility to change it now since it is used in different publication, therefore changing its name would bring even more confusion.
In conclusion, it has not been possible to trace the original source of the *MYH7B* designation, *i.e.* why in the report Deloukas *et al.* (2001) this gene was called as such. It is likely that the *MYH7B* symbol was given to this gene simply based on its sequence similarity with MYH7, which is higher compared to the other MYHs (see Table 6).

2. Bioinformatics: generation of syntenies of *MYH7B* and *MYH15* genes

Nucleotide sequences were identified and amino acid sequences were deduced using public databases. Accession numbers of MYH7B and MYH15 are reported on Table 2. Sequences were aligned using the BLAST program available at NCBI (www.ncbi.nlm.nih.gov/BLAST/). Physical maps were created using NCBI databases (www.ncbi.nlm.nih.gov/gene) to obtain the syntenies reported in Fig. 9. However, the Ensembl genome browser (www.ensembl.org/) and JGI database (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html) were used to determine the gene order of Xenopus tropicalis MYH genes (due to lack of completeness for this specie on NCBI). Additional data for Xenopus Tropicalis were obtained also using the Treefam database (http://www.treefam.org/) at the accession TF314375. Note that scaffold number we reported are in contrast to those reported by other (Nasipak & Kelley, 2008).
When available on the Entrez Gene page, the “Homology” option was used to confirm gene homology among species. Some genes, for example rat *Myh15*, are not identified as such in public databases at the time of this investigation. In this case, known genes located upstream and downstream of *MYH15* in the human genome were identified in the rat genome and genes in between were aligned to known putative orthologs using NCBI BLAST. Rat *Myh15* was found

to correspond to a gene called *RGD1565858* (see Table 2). A similar approach was also used for the identification of other genes, and for the generation of the syntenies.

Table 2. Gene ID of *MYH7B*, *MYH15* and miR-499 in different species *

Gene name	Database	Gene ID
Homo sapiens MYH7B	NCBI Entrez Gene	57644
Rattus norvegicus Myh7b	NCBI Entrez Gene	311570
Mus musculus Myh7b	NCBI Entrez Gene	668940
Gallus gallus SM2 (ortholog of MYH7B)	NCBI Entrez Gene	395279
Xenopus tropicalis ortholog of Myh7b	Ensembl Gene ID	ENSXETG00000007456
	Ensembl Transcript ID	ENSXETT00000016276
Danio rerio ortholog of Myh7b	NCBI Entrez Gene	795534
Homo sapiens MYH15	NCBI Entrez Gene	22989
Rattus norvegicus Myh15	NCBI Entrez Gene	303965
Mus musculus Myh15	NCBI Entrez Gene	667772
Gallus gallus vMYH (ortholog of MYH15)	NCBI Entrez Gene	395534
Xenopus tropicalis vMYH (ortholog of MYH15)	Ensembl	ENSXETG00000016136
	JGI Protein ID §	449348
Homo sapiens miR-499	NCBI Entrez Gene	574501
	miRBase	MI0003183
Rattus norvegicus miR-499	Ensembl	ENSRNOG00000036443
	miRBase	MI0003721
Mus musculus miR-499	NCBI Entrez Gene	735275
	miRBase	MI0004676
Gallus gallus miR-499	NCBI Entrez Gene	777908
	miRBase	MI0003710
Xenopus tropicalis miR-499	Ensembl	ENSXETG00000029201
	miRBase	MI0004885
Danio rerio miR-499	NCBI Entrez Gene	100033733
	miRBase	MI0004766

* MYH7B is also referred as MYH14 (see "Nomenclature" under Materials and Methods)
§ Available at: http://genome.jgi-psf.org/Xentr4/Xentr4.home.html

3. Muscle samples

Sprague Dawley rats weighing approximately 300 g and CD-1 mice of approximately 30 g were bred in-house in a conventional colony, housed in controlled conditions of 25°C, 50% relative humidity, and a 12-h light (0600-1800) and 12-h dark cycles, with water and food available *ad libitum*, at the Animal Facilities of the Venetian Institute of Molecular Medicine. Three months old (n = 12), embryonic day 16 (E16, n = 6), E20 rats (n = 6), three months old (n = 12) and E12.5 mice (n = 6) were used. Rats were killed by carbon dioxide (CO_2) gas asphyxiation, whereas mice were killed by cervical dislocation by appropriately trained staff. Muscles were quickly removed and frozen in liquid nitrogen-cooled isopentane and stored at –80°C. All experimental protocols were reviewed and supervised by the Veterinary Service and Animal Care Committee

of the University of Padova, in accordance with the D.Lgs. 116/92. Human vastus lateralis muscle biopsies were obtained from Neuromuscular Tissue Bank (NMTB) at the Regional Neuromuscular Center of the University of Padova approved by the Ethical Committee of the University of Padova in compliance with the Helsinki Declaration. The vastus lateralis samples used in this study were evaluated as normal by the pathologist based on morphological and histochemical analyses of muscle sections. Human extraocular muscle samples were obtained from the Veneto Eye Bank Foundation (FBOV; Mestre-Venezia, Italy) according to the ethical recommendations of the Italian Transplantation Law. Extraocular muscles were removed during the 6 hr *post-mortem*, rapidly frozen in isopentane chilled with liquid nitrogen and stored at -80°C until use. Human myocardial samples, obtained from cardiac surgery, were kindly donated by Professor C. Poggesi, University of Florence. The samples were immediately frozen in liquid nitrogen and stored at -80°C.

To test the expression of the Myh7b and Myh15 in early regenerating muscles we used the myotoxic drug bupivacaine (Marcaina, Astra) to induce acute damage and stimulate the consequent regeneration response. Muscle tissue injury was induced in rat soleus muscles by intramuscular injection, through a small cutaneous incision, of freshly made 0.5 mL of 0.5% bupivacaine in physiologic solution (NaCl 0.9%) previously sterilized with 0.2 µm filter.

4. Quantitative real time RT-PCR

RNA was extracted from frozen muscle samples using the Promega SV Total RNA Isolation System kit according to manufacturer's instructions. RNA quality was considered acceptable when the 260 to 280 nm absorbance ratio (OD260/OD280) measured with UV spectrophotometer was >1.8. Total RNA (400 ng) was reverse transcribed using SuperScript® III Reverse transcriptase (#18080, Invitrogen). Complementary DNA was analyzed by quantitative real-time RT-PCR using the QuantiTect SYBR Green PCR Kit (QIAGEN) on an ABI PRISM® 7000 Sequence Detection System apparatus (Applied Biosystems). PCR protocol consisted of a step at 50°C for 2 min, then at 95°C for 15 min, followed by 40 cycles at 94°C for 25 sec and 1 min at 58°C. Data were normalized to the expression of a housekeeping gene and for the efficiency of the primers through analysis of the standard curve, according to the Pfaffl method (Pfaffl, 2001).

Primers were designed using either Primer3 (http://frodo.wi.mit.edu/primer3/) or NCBI Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/). Whenever possible, primers were separated by one intron. Specificity of real-time RT-PCR products was further confirmed with agarose gel electrophoresis which confirmed that the bands were of the desired length and by analyzing the dissociation curve with ABI PRISM 7000 SDS software (Applied Biosystems). Primers used in

these experiments are reported in Table 3 (in some case, to confirm the results more primers for the same gene were used). Quantitative real-time RT-PCR on miR-499 was performed using the TaqMan® MicroRNA Assays kit (Part Number 4427975, AB Assay ID 001352, Applied Biosystems Inc) according to the manufacturer's protocol using RNA isolated with Trizol (Invitrogen). U6 snRNA was used for normalization of the miR-499 expression (Part Number 4427975, AB Assay ID 001973, Applied Biosystems Inc). Transcripts levels are expressed as the percentage of the tissue with the highest expression level.

Table 3. Primers used for quantitative real-time RT-PCR

Gene	NCBI Nucleotide	Forward (5'-3')	Reverse (5'-3')
Rat Myh15	XM_001064527	TTCCAGAGAAAAGAGCAACCTC	GGTCATAGTCATGCTTGGTTGA
Rat Myh7b	NM_001107794	GAGTGTGGAGCAGGTGGTATTT	GGACCCCAATGAAGAACTGA
		CAAGTAGAGGACCTGGAGTGCT	TCTTGTGTGGTGTCTGTCACTG
		TGCGAATGCCAAGTGCTCCTCA	TGGCTGTGCCGCAATCGAAA
Rat Myh7 (β/slow)	NM_017240.1	GTATGCTGGAGCTGATGCAC	GACACGGTCTGAAAGGATG
Rat TBP *	NM_001004198	TCAAACCCAGAATTGTTCTCC	AACTATGTGGTCTTCCTGAATCC
Mouse Myh15	XM_001002743	GAAGGAGTTTGAAATGGGTCAG	CTCTCTTTCCACCTTGGCTCTA
		AGACTGGAGAGAGGCATCAAAG	TGCTGTTTGTAACTCTGCACCT
Mouse Myh7b	NM_001085378	AGAGTGTGGAGCAGGTGGTATT	GGTCTGATTGATTCGAGAAACC
Mouse Myh7 (β/slow)	NM_080728.2	AGCAGGAGCTGATTGAGACC	TGTGATAGCCTTCTTGGCCT
Mouse Myh4 (2B)	NM_010855.2	ACAGACTAAAGTGAAAGCCTACAA	CACATTTTGTGATTTCTCCTGTCAC
Mouse Hprt1§	NM_013556.2	CTCATGGACTGATTATGGACAGGAC	GCAGGTCAGCAAAGAACTTATAGCC
Mouse β-Actin *	NM_007393	CAAACATCCCCCAAAGTTCTAC	TGAGGGACTTCCTGTAACCACT
Human MYH15	NM_014981.1	CCTCTCTCTCATGGTTCTGGAT	GGCCTGGAGTCATATTTTCAAG
		GCAACTGGAAGCAGATGGCACAGAA	ATTGACTTGGACAGGGCACCGACA
Human MYH7B	NM_020884.2	GCTTCGACTTACTGGAGGACAT	GTTGATGGTGACACAGAAGAGG
Human MYH7	NM_000257.2	TGCTCTGTGTCTTTCCCTGCTGCT	TCAAAAGGCCTGGTCTGCGCTT
Human β-Actin *	NM_001101.3	ACAATGAGCTGCGTGTGGCT	AGGGATAGCACAGCCTGGATAGCA
Chicken Myh7b (SM2)	NM_204587	ACGTTTTGGCAAGTTCATCC	GTGGTAGCTTCGCTCTTTGG
Chicken Myh15 (vMYH)	NM_204766	GGATGCCCTTCTTGTAATTCAG	GTTCCTTTCTCCTTGCTTCAGA
		TTCGGGCATACTAAGGTGTTCT	CTGAATTACAAGAAGGGCATCC
Chicken GAPDH *	NM_204305	TCTGGAGAAACCAGCCAAGTAT	CCATTGAAGTCACAGGAGACAA

* genes used as housekeeping
§ gene used as housekeeping to normalize adult vs embryonic mouse muscles

5. Myh7b and Myh15 antibodies

Polyclonal antibodies were raised against two peptides corresponding to the amino terminal sequences of rat Myh7b (residues 4 - 77) and Myh15 (residues 4 - 104) (PRIMM, Milano, Italy), which are markedly different from the same regions of cardiac and skeletal MYHs (Fig. 6). Antibodies were purified from rabbit serum by affinity chromatography on insolubilized immunogens. Reactivity of the antibodies was shown to be specific by the use of peptide-

saturated primary antibody, which demonstrated an absence of signal due to nonspecific binding of primary antibodies in tissue immunohistochemistry. The peptide saturation was obtained by incubation with an excess (ten times) of the peptide that corresponds to the epitope recognized by the antibody (the immunizing peptide) prior to standard immunohistochemistry protocol (Table 4). To further validate the specificity of our antibodies, we saturated the MYH7B peptide with the MYH15 immunizing peptide, and *vice versa*, and in these cases no neutralization of the primary antibody was found (Fig. 7).

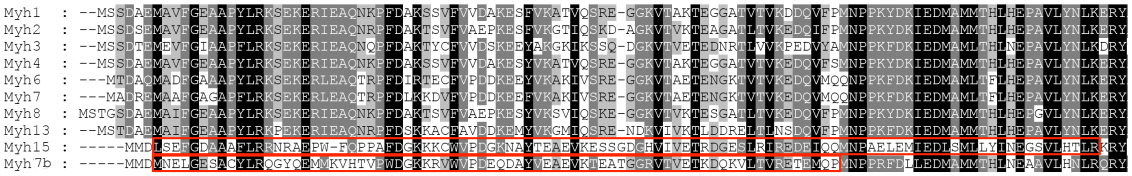
Table 4. Blocking with immunizing peptide

	[ab] µg/µL	working dilution	working concentration µg/µL	[immuniz. pept.] µg/µL	block with 10x [ab] µg/µL	immnuniz. pept. working dilution
MYH15	0,08	1:500	0,00016	2,48	0,0016	1:1550
MYH7B				0,80	0,0016	1:500
MYH7B	0,84	1:50	0,01680	0,80	0,168	1:4,76
MYH15				2,48	0,168	1:14,76

Amino acid sequence of the MYH7B antibody is: MNELGESACYLRQGYQEMMKVHTVP WDGKKRVWVPDEQDAYVEAEVKTEATGGRVTVETKDQKVLTVRETEMQP.

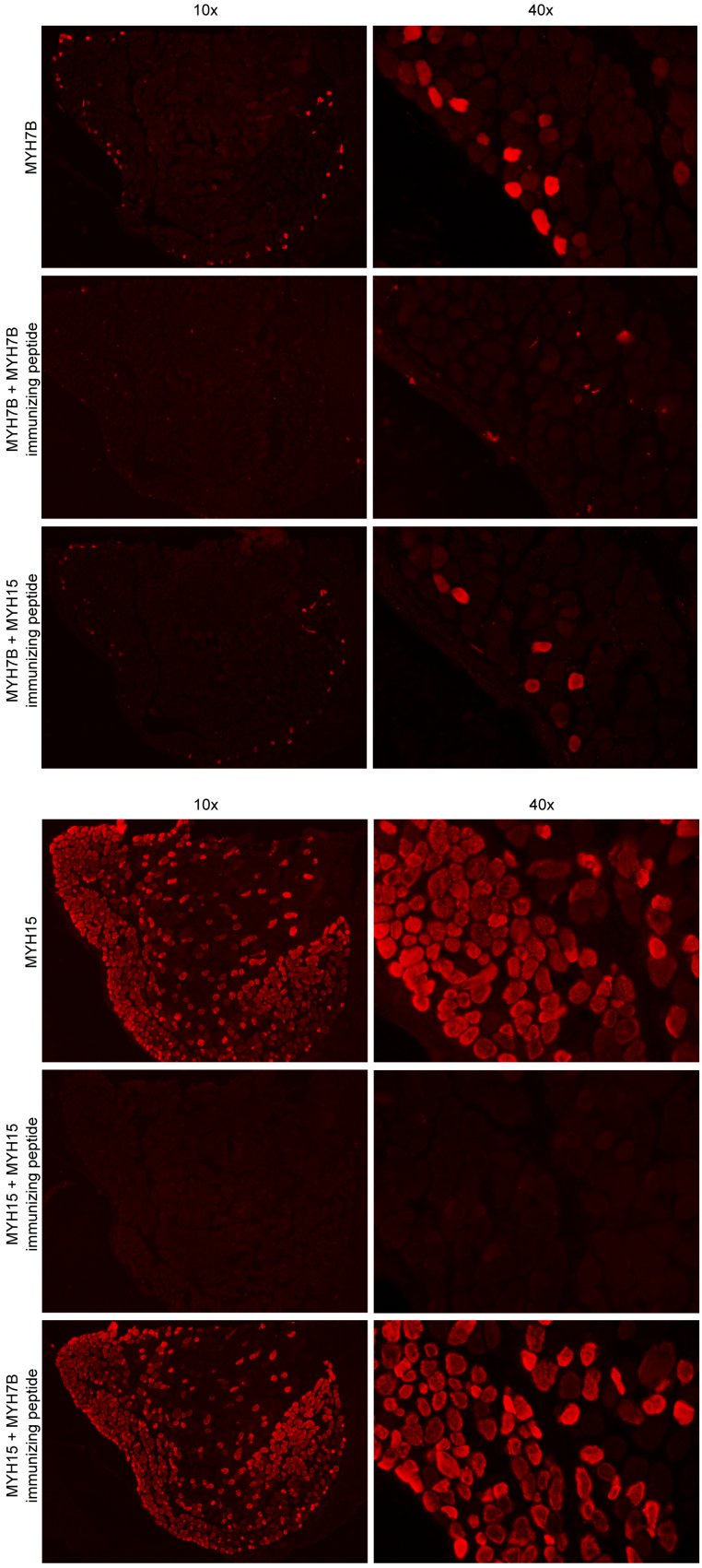
Amino acid sequence of the MYH15 antibody is: LSEFGDAAAFLRRNRAEPWFQPPAFDGKKK CWVPDGKNAYTEAEVKESSGDGHVIVETRDGESLRIREDEIQQMNPAELEMIEDLSMLLYINEG SVLHTLR

Fig. 6 Production of polyclonal antibodies against the rat Myh7b and Myh15



Alignment of amino terminal sequences of rat sarcomeric MYHs. Note that in this region the sequence of MYH7B and MYH15 is markedly different from that of the other cardiac and skeletal MYHs. The sequences boxed in red correspond to the peptides used for immunization. Alignment was performed using Gendoc software. Conserved sequences are highlighted according to the degree of conservation (black = 100%, dark grey ≥ 80%, light grey ≥ 60%, white < 60%).

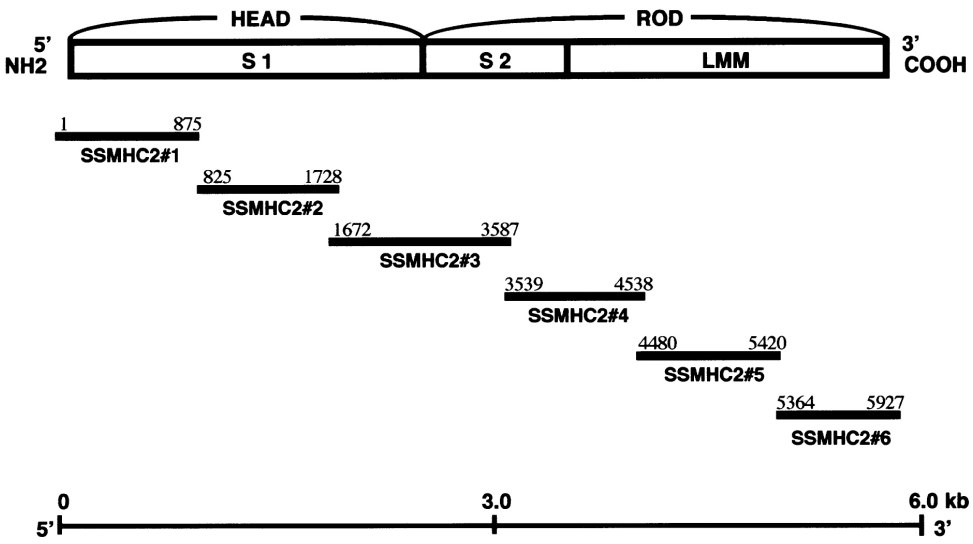
Fig. 7 Specificity of MYH7B and MYH15



5.1 Epitope mapping of S46 antibody

In our study we took great advantage by the use of the monoclonal S46 antibody developed by Prof Stockdale FE and purchased at the Developmental Studies Hybridoma Bank (DSHB). Although this antibody has been widely used and characterized (Miller *et al.*, 1985; Page *et al.*, 1992; Sokoloff *et al.*, 2007), we were not aware to which portion of the chick slow skeletal MHC2 (SM2, orthologous of the mammalian MYH7B) it is reactive to. We were interested to know whether our MYH7B antibody reacts or not with a sequence similar/equal to that recognized by the S46 monoclonal antibody, therefore we performed the epitope mapping.

Fig. 8 Chick myosin SM2 heavy chain cDNA clones



Relationship of the six chick myosin heavy chain cDNA clones (SSMHC2#1, SSMHC2#2, SSMHC2#3, SSMHC2#4, SSMHC2#5, and SSMHC2#6) against the domain map of myosin heavy chain (Machida *et al.*, 2002).

Clones of the chick SM2 were kindly donated by Rumiko Matsuoka (Machida *et al.*, 2002). HEK293 cells were transfected at 90% confluency with 4 µg DNA/well and 10 µL of Lipofectamine 2000 (Invitrogen). Cells where collected after 48 hours, lysed, quantified with Bradford assay and subjected to immunoblotting with anti-GFP and S46 antibodies.

6. Immunoblotting

Muscles were ground using mortar and pestle previously cooled with liquid nitrogen and then incubated 1 hour at 4°C in a pyrophosphate based solution to extract the myosins (Agbulut *et*

al., 1996), assayed by BCA protocol (23227, BCA Protein Assay Kit, Thermo Scientific) and then conserved at -80°C in 50% glycerol. Protein samples were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) for 90 minutes using 3-8% pre-cast gels at 120 V (NuPAGE® Novex 3-8% Tris-Acetate Midi Gel, Invitrogen). For separation of MYH isoforms we used polyacrylamide gel containing high glycerol concentration according to previously published protocol (Mizunoya *et al.*, 2008). Gels were transferred to a Invitrolon™ PVDF membrane (Invitrogen) according to manufacturer's instructions (1 hour and 30 minutes, 4°C, 50 V). Membrane was blocked for 1 hour at room temperature (RT) in 5% non-fat dry milk in 0.01% Tween in Tris-buffered saline (TBS-T) solution and directly incubated with primary Abs (1 hour at RT in the same solution). After extensive TBS-T washing the membranes were incubated with secondary anti-mouse IgG (Goat Anti-Mouse IgG (H+L)-HRP Conjugate, #170-6516, Bio-Rad Laboratories) or anti-rabbit IgG (Goat Anti-Rabbit IgG (H+L)-HRP Conjugate, #170-6515, Bio-Rad Laboratories), both at a concentration of 1:2000, with the same conditions as primary antibody. After extensive washing the membrane signal was detected by enhanced chemiluminescence (ECL) substrate (Thermo Scientific, SuperSignal West Pico Chemiluminescent Substrate, Prod # 34080) and visualized by exposure to radiographic film. The same antibodies used for immunofluorescence were diluted 10 times for immunoblotting. α-pan–actin (clone AC-40, # A3853, Sigma-Aldrich) diluted 1:10000 was used as loading control.

7. Immunofluorescence

Immunofluorescence was performed on 10 µm thick cryosections. Briefly, slides were rinsed in phosphate saline buffer (PBS), incubated 1 hour at 37°C with the primary antibody in 0.5% bovine serum albumin (BSA) in PBS, washed repeatedly with PBS and incubated 1 hour at 37°C with the secondary antibody in 0.5% BSA and 2% rat serum in PBS. Finally, slides were extensively washed in PBS and quickly rinsed with double distilled H_2O and coverslipped with mounting medium (Dako). Rat tissues, when to be incubated with anti-mouse secondary antibody, were incubated with 2% rat serum for 20 min at RT prior to primary antibody to block unspecific binding in the interstitium. Antibodies used are reported in Table 5. For EO muscles the rectus superior was used in most analyses, but similar results were obtained with other EO muscles. The polyclonal antibody raised against chicken ALD myosin, specifically reactive with multiply innervated slow-tonic fibers in mammalian EO muscles, was previously described (Bormioli *et al.*, 1979; Bormioli *et al.*, 1980). Monoclonal antibody S46, reactive with chicken slow myosin 1 and 2 (Miller *et al.*, 1985; Page *et al.*, 1992), was obtained from the Developmental Studies Hybridoma Bank (DSHB). A recent study showed that S46 is more specific for slow-tonic fibers in mammalian skeletal muscles than other anti-chicken myosin

antibodies, which show partial cross-reaction with the MYH-β/slow present in slow-twitch muscle fibers (Sokoloff *et al.*, 2007). Other antibodies used in the present study include mAbs BF-G6, specific for embryonic MYH (Schiaffino *et al.*, 1986a), BA-D5, specific for MYH-β/slow (Schiaffino *et al.*, 1989) and BF-13, which is specific for developing and adult fast MYHs, but in immunoblotting is unreactive with 2B- and Emb-MYH (our unpublished observations). Concentration used for the secondary antibodies were 1:2000. Products: anti-rabbit made in sheep Cy3 (Sigma C2306), anti-mouse made in goat Cy3 (Jackson ImmunoResearch).

Table 5. Antibodies used to determine myosin isoforms expression

	Dilution	Mono/polyclonal	Specificity	Provenience
BA.D5	1:1000	Monoclonal	Slow-twitch	Prof. Schiaffino
BF-G6	1:1000	Monoclonal	Embryonic	Prof. Schiaffino
S46	1:100	Monoclonal	Slow-tonic	DSHB Iowa Uni
α-ALD	1:4000	Polyclonal	Slow-tonic	Prof. Schiaffino
HV11	1:1000	Monoclonal	Ventricular	DSHB Iowa Uni
MYH7B	1:50	Polyclonal	Slow-tonic	Prof. Schiaffino
MYH15	1:500	Polyclonal	EOM orbital	Prof. Schiaffino
Alpha actinin sarcomeric	1:4000	Monoclonal	Z disk	Sigma (A7811)

8. *In vitro* motility assay with chicken myosins

We wanted to assess the physiological properties of the Myh7b and Myh15; however, we found that fibers expressing these two myosins also express other isoforms, thus making impossible to isolate pure Myh7b and Myh15 (see Results). Therefore we analyzed their chicken orthologs. To determine the functional properties of the chicken slow myosin heavy chain 2 (SM2) and the ventricular myosin heavy chain (vMYH), we performed the *in vitro* motility assay, which allows the measurement of the sliding velocity of the actin over pure molecules of myosin heavy chain. The anterior latissimus dorsi (ALD), posterior latissimus dorsi (PLD), the pectoralis major and the ventricle were collected from an adult/old chicken. We used these muscle since at this age the ALD is mostly composed of SM2 myosin, ortholog of the mammalian MYH7B, and the PLD of fast fibers. Ventricle was used since it is exclusively composed of vMYH, ortholog of the mammalian MYH15. Pectoralis major was used as reference for fast myosin (Hooft *et al.*, 2007). *In vitro* motility assay was performed as previously described (Canepari *et al.*, 2000).

9. Dark condition rearing

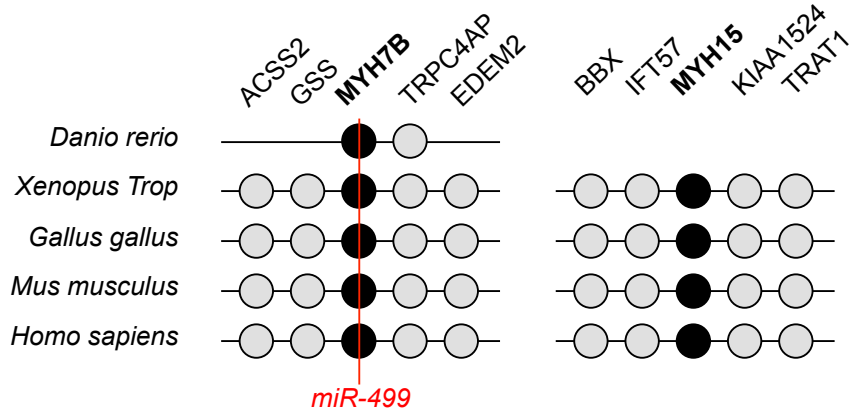
To evaluate whether visual experience and/or sensory deprivation play a role on the expression of Myh7b and Myh15, we reared rats in prolonged dark condition. Dark rearing condition was obtained putting pregnant females in a standard cage in a dark lightproof environment 7 days before estimated birth and litters were dark-reared until sacrificed. All manipulations were done with infrared viewers. Dark-reared rats were sacrificed at P45 and expression of Myh7b and Myh15 in EO muscles was analyzed by quantitative PCR, immunoblotting and immunofluorescence. Signal from immunoblotting was quantified by densitometry of scanned radiographic films using the software ImageJ (NIH); data were normalized to loading (Actin, Clone AC40, Sigma) and expressed as fold change.

Results

1. Comparative analyses of MYH7B and MYH15

Partial synteny analysis of sarcomeric MYH genes in vertebrates were previously reported (McGuigan *et al.*, 2004; Garriock *et al.*, 2005; Ikeda *et al.*, 2007). Using available data for

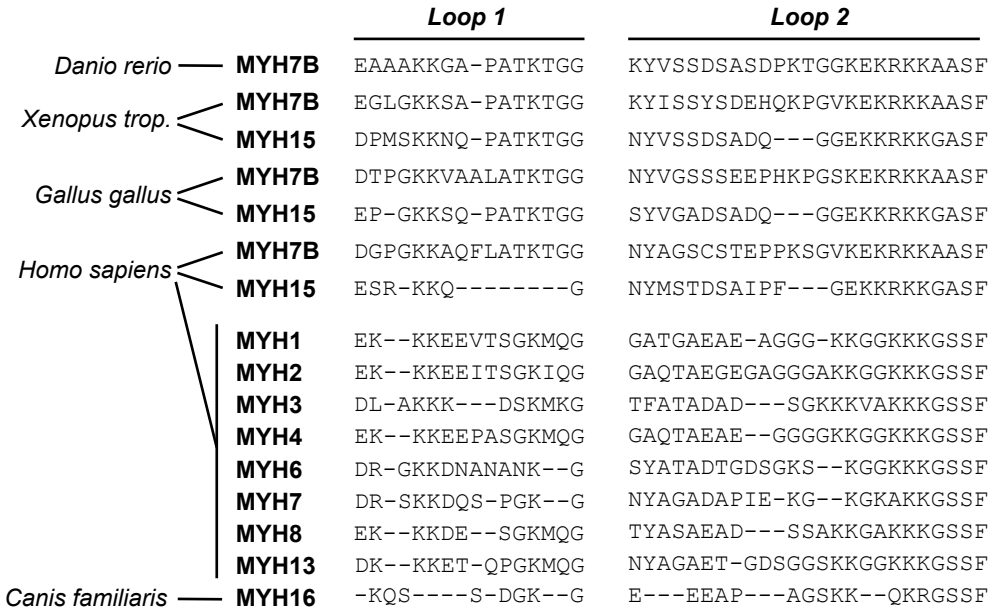
Fig. 9 Syntenies of MYH7B/14 and MYH15



Physical maps showing syntenic organization of MYH7B and MYH15 genes in fish, frog, chicken and mammals. The microRNA miR-499 is contained within the MYH7B gene in all species. Spacing of markers does not reflect actual scale. Gene organization information was obtained by NCBI and Ensembl Genome Browser databases (see Table 2 for gene ID).

different vertebrate classes, we re-examined the syntenic organization of *MYH7B* and *MYH15* genes and found that orthologs of mammalian *MYH7B* and *MYH15* genes are present in birds and frogs (Fig. 9). In contrast, as reported in previous studies (McGuigan *et al.*, 2004; Ikeda *et al.*, 2007), orthologs of *MYH7B*, but not of *MYH15*, genes were detected in the fish genome. Based on exon-intron analysis, it was suggested that a common precursor gave origin to the fish *Myh7b* and to the *MYH7B* and *MYH15* genes in other vertebrate classes (Ikeda *et al.*, 2007). We re-examined the structure of *MYH7B* and *MYH15* genes in different species and found additional evidence that supports this view but also points to a unique evolution of the mammalian *MYH15* gene. First, we noted that a conserved microRNA, miR-499, previously identified in mouse *Myh7b* gene (van Rooij *et al.*, 2008a), is contained within the *MYH7B* gene in all species (Fig. 9 and Table 2). Next, we focused on the structure of myosin loop 1, a surface loop which connects the 25 and 50 kDa domains in the myosin head and is located near the ATPase catalytic site. The sequence of loop 1 is known to vary between paralogous myosin isoforms, to be conserved between myosin orthologs and to affect some myosin properties,

Fig. 10 Loop 1 and Loop 2 of MYH7B and MYH15



Amino acid sequence of MYH7B and MYH15 loop 1 and loop 2 in different vertebrate species. The structure of the two surface loops in other human sarcomeric MYHs is also shown for comparison. The dog sequence is shown for MYH16, because human MYH16 is a pseudogene.

Tab 6. Alignment of human MYH proteins

	MYH1 IIX	MYH2 IIA	MYH3 Embryon	MYH4 IIB	MYH6 α-card	MYH7 β-slow	MYH8 Neonat	MYH13 MYH13	MYH7B slow-ton	MYH15 MYH15
MYH1	100%	94%	83%	94%	80%	80%	92%	81%	66%	58%
IIX	0%	97%	92%	97%	90%	91%	96%	90%	83%	79%
MYH2	94%	100%	84%	91%	80%	80%	92%	81%	67%	59%
IIA	97%	0%	92%	96%	90%	91%	96%	91%	83%	79%
MYH3	83%	84%	100%	83%	78%	78%	84%	78%	66%	59%
Embryonic	92%	92%	0%	92%	89%	89%	92%	90%	83%	78%
MYH4	94%	91%	83%	100%	80%	80%	90%	81%	66%	59%
IIB	97%	96%	92%	0%	90%	90%	96%	90%	83%	79%
MYH6	80%	80%	78%	80%	100%	92%	79%	76%	68%	61%
α-cardiac	90%	90%	89%	90%	0%	96%	90%	88%	85%	81%
MYH7	80%	80%	78%	80%	92%	100%	80%	76%	69%	61%
β-slow	91%	91%	89%	90%	96%	0%	90%	88%	85%	80%
MYH8	92%	92%	84%	90%	79%	80%	100%	81%	66%	59%
Neonatal	96%	96%	92%	96%	90%	90%	0%	90%	83%	79%
MYH13	81%	81%	78%	81%	76%	76%	81%	100%	65%	59%
	90%	91%	90%	90%	88%	88%	90%	0%	82%	79%
MYH7B	66%	67%	66%	66%	68%	69%	66%	65%	100%	60%
slow-tonic	83%	83%	83%	83%	85%	85%	83%	82%	0%	80%
MYH15	58%	59%	59%	59%	61%	61%	59%	59%	60%	100%
	79%	79%	78%	79%	81%	80%	79%	79%	80%	0%

Pecentages indicate value of identity (upper line) and similarity (lower line)

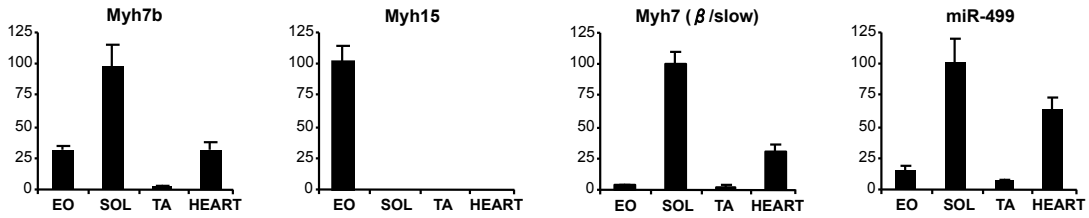
such as the rate of ADP release (Sweeney *et al.*, 1998). The loop 1 sequence of zebrafish Myh7b is similar to that of MYH7B in other species but also to that of Myh15 in Xenopus and chick, in particular a specific ATKTGG signature is present at the 3' end of loop 1 in all these myosins (Fig. 10). In contrast, a completely different sequence is present in mammalian MYH15, which is much shorter than all other sarcomeric MYHs. These findings are consistent with the view that vertebrate *MYH7B* and *MYH15* genes derive from a common precursor similar to fish *Myh7b*, but that the mammalian *MYH15* gene underwent a drastic structural remodeling possibly related to a divergent pattern of expression and function (see below). The divergent evolution of mammalian MYH15 is also apparent from sequence comparisons of the whole molecule: chicken Myh7b (SM2) and Myh15 (vMYH) proteins have a much higher degree of similarity between them (83% identity) compared to the corresponding mammalian MYHs (human MYH7B *vs* MYH15: 60% identity). The sequence of loop 2, another region of variability among MYH isoforms, shows a specific signature KEKRKKAASF at the 3' end, which is identical among the various species, and is slightly different from the corresponding MYH15 sequence, EKKRKKGASF, which is also identical among the various species (Fig. 10).

2. Expression of MYH7B and MYH15 in mammalian muscles at transcript level

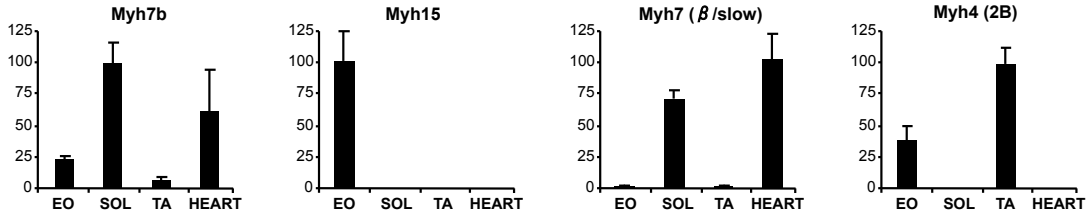
Quantitative RT-PCR analysis was used to examine the expression pattern of these two MYHs in mammalian muscles. Myh7b and miR-499 transcripts are present in rat heart, slow skeletal muscles and EO muscles but only at low levels in fast skeletal muscles (Fig. 11). In contrast, Myh15 transcripts are present at high levels in EO muscles but absent in fast and slow leg muscles and heart. The pattern of distribution of these MYH transcripts is similar in mouse muscles (Fig. 11) and is completely different to that of typical slow and fast MYH, such as the MYH7 gene, coding for MYH-β/slow, expressed in slow-twitch fibers, and MYH4 gene, expressed in MYH-2B fibers. In human skeletal muscle MYH7B transcripts are present in EO muscles but also in vastus lateralis and in both atrial and ventricular myocardium, whereas MYH15 transcripts are detectable exclusively in EO muscles with minimal traces in myocardial tissues (Fig. 11).

Fig. 11 Expression of *MYH7B* and *MYH15* in mammalian striated muscles at transcript level

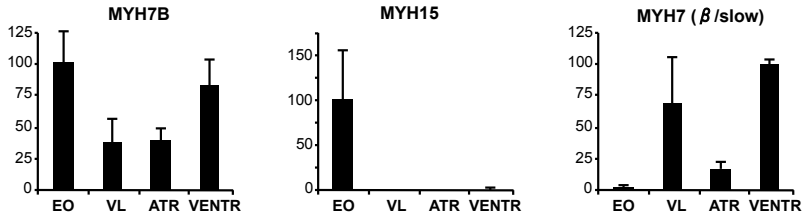
Rat



Mouse



Human

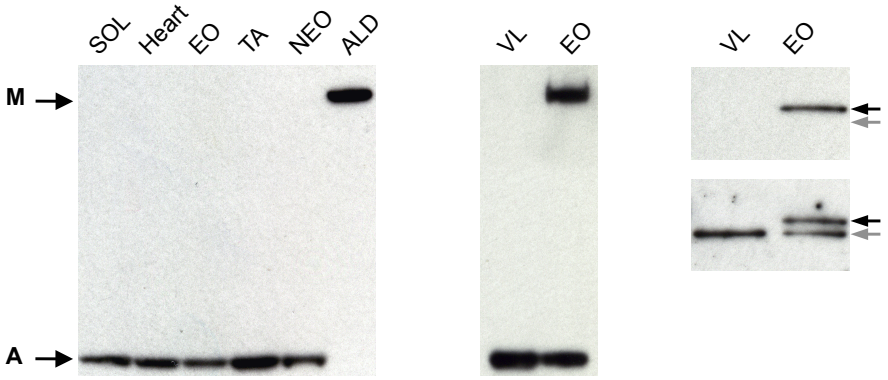


Quantitative RT-PCR analysis of different rat, mouse and human striated muscles with probes specific for *MYH7B* and *MYH15* gene transcripts. The expression pattern of *MYH7*, coding for β/slow MYH, and *MYH4*, coding for IIB MYH, are shown for comparison. Transcripts levels are normalized to housekeeping genes and expressed as the percentage of the tissue with the highest expression level. Note that the distribution of miR-499 is similar to that of *Myh7b*. EO: extraocular muscles; SOL: slow- twitch soleus muscle; TA: fast- twitch tibialis anterior muscle; VL: vastus lateralis; ATR: atrium; VENTR: ventricles. Data are mean ± SEM. Rat: n = 6 (*Myh7b* and *Myh15*), n = 3 (*Myh7* and mir-499); mouse: n = 6 (*Myh7b* and *Myh15*), n = 3 (*Myh4* and *Myh7*); human: n = 4 (*MYH7B*, *MYH15* and *MYH7*).

3. Expression of MYH7B at protein level

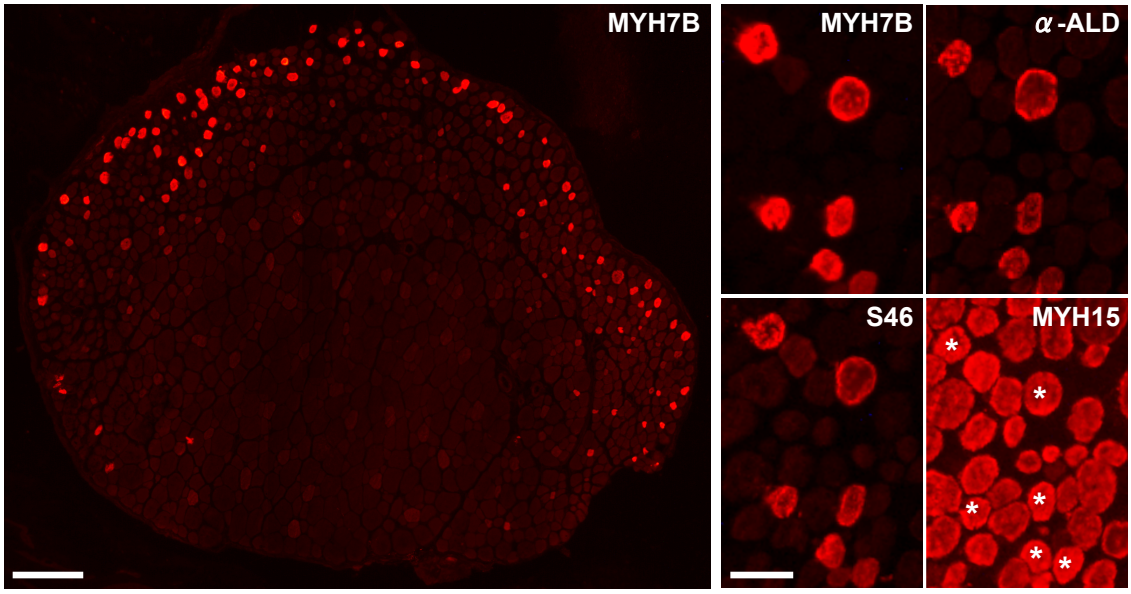
We examined the expression of MYH7B protein. Western blots show no reactivity with different rat or mouse muscles, but give a strong reaction with chicken anterior latissimus dorsi (ALD) MYH. In contrast, a specific reaction with MYH from human EO but not vastus lateralis muscles was easily detected (Fig. 12). Glycerol gel analysis shows that the MYH band recognized by anti-MYH7B antibody migrates slightly slower than human β/slow MYH (Fig. 12). By immunofluorescence, MYH7B is detected in a minor fiber population present in the orbital layer and occasional global layer fibers of rat EO muscles: these fibers correspond to slow-tonic fibers labeled by anti-ALD myosin and S46 antibodies and co-express MYH15 (Fig. 13). In rat hindlimb muscles anti-MYH7B antibody reacts with different intensities with the two bag fibers of muscle spindles, strongly with bag 2 and weakly with bag 1 fibers, a pattern similar to that obtained with anti-ALD (Fig. 14). In human EO muscles, MYH7B positive fibers are more numerous than in rat, which might explain the different results in Western blotting, and widespread in both orbital and global layers. MYH7B positive fibers correspond to slow-tonic fibers stained by anti-ALD (Fig. 15). Anti-MYH7B antibody stains specifically the sarcomere A-band in longitudinal sections of EO muscles (Fig. 16).

Fig. 12 Western blot with anti-MYH7B antibody



Distribution of MYH7B in adult muscles. (Left panel) Western blot analysis of homogenates from different rat striated muscles and chicken ALD muscle with anti-MYH7B antibody. The same blot was reacted with an antibody against α-actin to demonstrate equal loading of all lanes with the exception of ALD, that was about 50 times less loaded. (Middle panel) Western blot of homogenates from human EO and vastus lateralis (VL) muscles with anti-MYH7B antibody. (Right panel) Western blot with human muscle myosins separated in glycerol gels, blotted and reacted with antibodies against MYH7B (upper panel, black arrow), or MYH7B followed by anti-MYH-β/slow (lower panel, grey arrow). MYH7B (black arrow) corresponds to a band with lower electrophoretic mobility compared to MYH-β/slow (grey arrow). M, myosin heavy chain; A, Actin.

Fig. 13 Immunofluorescence with anti-MYH7B antibody in rat EO muscles

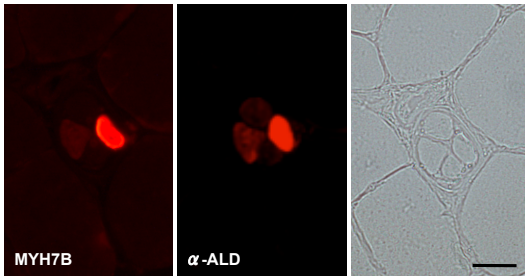


Transverse sections of rat EO muscle reacted with antibodies specific for MYH7B, showing few reactive fibers mostly localized in the orbital layer (left panel). These fibers correspond to slow-tonic fibers labeled by anti-ALD and S46 antibodies, as shown at high power in the right panels. Note that these fibers also co-express MYH15. Scale bar left panel, 100 µm; scale bar right panels, 25 µm.

Our results confirm for the first time that *MYH7B* gene codes for the slow-tonic MYH isoform. This evidence is based on the fact that our MYH7B antibody, made against a portion of the N-terminal of the rat Myh7b sequence, reacts exactly with the same fibers stained by two anti slow-tonic antibodies largely used in the past, the monoclonal S46 (Miller *et al.*, 1985; Page *et al.*, 1992; Sokoloff *et al.*, 2007) and the polyclonal chick anti-ALD (Bormioli *et al.*, 1979; Bormioli *et al.*, 1980) (see Fig. 13, 14, 15 and 23).

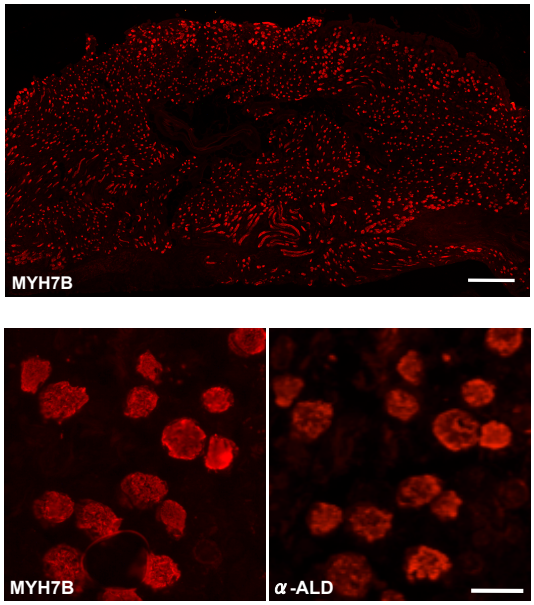
Through epitope mapping we found that the S46 antibody reacts specifically with the clone "SSMHC2#5" (see Fig. 8). Alignment of all rat myosin sequences with the SM2 sequence does not unambiguously reveal a region in the SM2 clone #5 (aa residues 1493 to 1807) particularly similar to the rat MYH7B and at the same time markedly different from the other rat MYHs. Therefore, we could only conclude that the S46 antibody recognizes a portion of the light meromyosin of the rod (see Fig. 2 and 8). This finding, although partially incomplete, might be of some interest since our MYH7B polyclonal antibody recognizes an epitope at the N-terminal of the protein (the myosin head). The fact that our MYH7B antibody has a distinct antigenic determinant enhances the strength of our results, having characterized the expression of the slow-tonic isoform in mammals with two markedly different antibodies, plus another polyclonal antibody, the anti-ALD.

Fig. 14 Immunofluorescence with anti-MYH7B antibody in rat muscle spindles



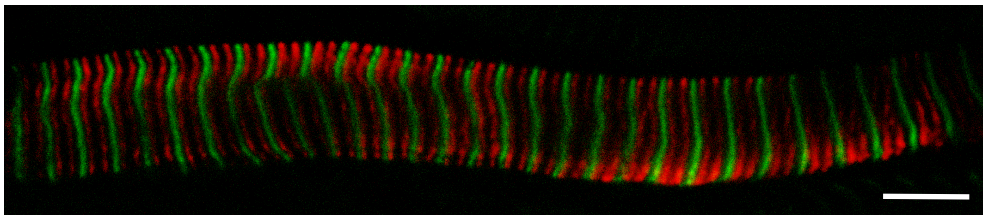
Serial transverse sections of rat soleus muscle showing a muscle spindle cut through the intracapsular region, stained with anti-MYH7B or anti-ALD or examined by phase contrast microscopy (right panel). Note similar staining pattern of the two antibodies with stronger reactivity in one of the two bag fibers, corresponding to bag 2 fiber. Also note that surrounding extrafusal fibers are unstained. Scale bar, 20 µm.

Fig. 15 Immunofluorescence with anti-MYH7B in human EO muscles



Top: transverse sections of human EO muscle reacted with antibodies specific for MYH7B, showing numerous reactive fibers. Scale bar, 500 µm.
Bottom: section of human EO muscle stained with anti-MYH7B and anti-ALD myosin antibodies. Note that MYH7B positive fibers correspond to slow-tonic fibers labeled by anti-ALD antibody. Scale bar, 50 µm.

Fig. 16 Staining of the striation with anti-MYH7B antibody

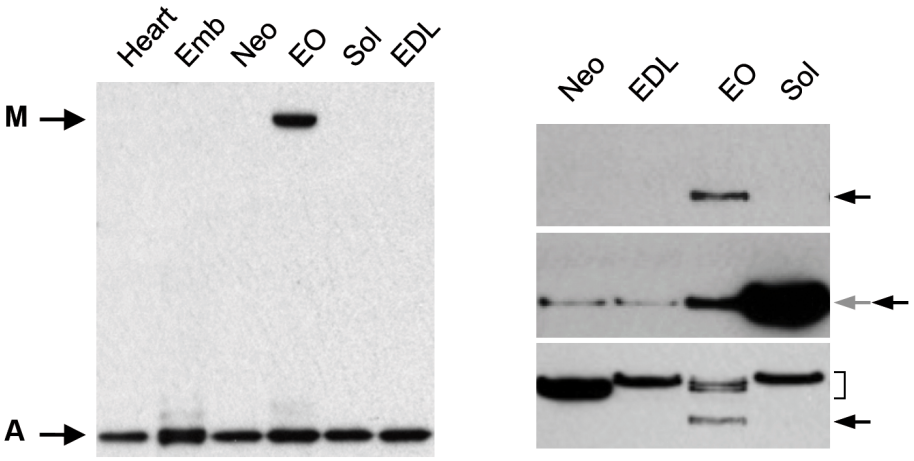


The positive fibers display a clear striation pattern corresponding to the A band (red), as shown by co-staining with an antibody against α-actinin that labels the Z disks (green). Scale bar, 5 µm.

4. Expression of MYH15 at protein level

By immunoblotting after standard polyacrylamide gel electrophoresis the antibody to MYH15 reacted selectively with MYH from rat EO muscles, whereas other striated muscles were completely unreactive (Fig. 17). Western blots of MYHs separated in polyacrylamide gel containing high glycerol concentration show that the MYH band recognized by anti-MYH15 comigrates with the high mobility β/slow MYH band present in slow muscles (Fig. 17). Analysis of serial transverse sections shows that MYH15 is present in almost all fibers of the orbital layer

Fig. 17 Western blot with anti-MYH15 antibody

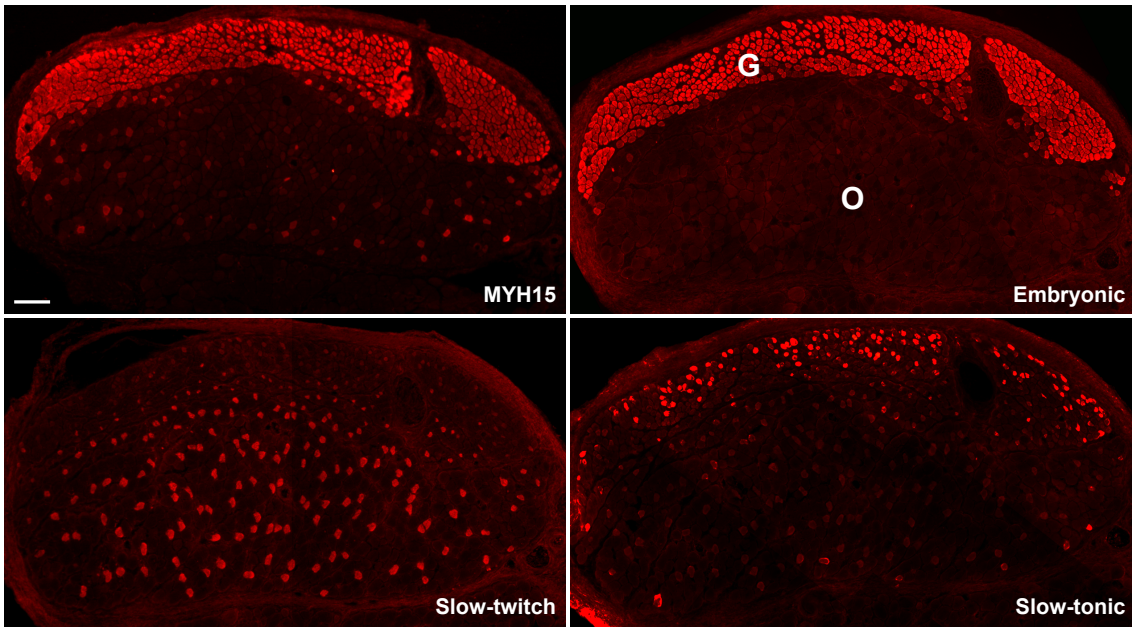


Western blot analysis of homogenates from different rat striated muscles with anti-MYH15 antibody. (Left panel) Note specific reaction of this antibody with MYH (M) from EO muscles. The same blot was reacted with an antibody against α-actin (A) to demonstrate equal loading of all lanes. (Right panel) Myosins from different rat muscles were separated by SDS-PAGE using glycerol gels, a procedure which allows separation of different MYH isoforms, blotted and reacted with antibodies against MYH15 (upper panel, black arrow), MYH15 followed by anti-β/slow MYH (middle panel, grey and black arrow), or MYH15 followed by an antibody reactive with fast 2A- and 2X-MYH and Neonatal MYH (lower panel, parenthesis). Note that MYH15 comigrates with MYH-β/slow. Emb: Embryonic day 20 (E20) hindlimb skeletal muscles; Neo: neonatal hindlimb muscles; SOL: adult slow soleus muscle; EDL: adult fast extensor digitorum longus muscle.

and in rare fibers of the global layer of EO muscles (Fig. 18). This pattern of distribution is very similar to that of embryonic MYH, although a number of EO muscle fibers were reactive for MYH15 but not embryonic MYH and *vice versa* (Fig. 19). In contrast, MYH15 distribution is completely different from that of slow-tonic MYH, detected by anti-chicken ALD myosin, which is present in a minor population of fibers present in both the orbital and global layer, with the tendency for a weaker intensity of staining in positive global fibers (Fig. 18). MYH15 is not detected in other cranial muscles, such as tongue and masseter (not shown). In fast and slow hindlimb muscles, anti-MYH15 stains exclusively rare small fibers, corresponding to the extracapsular polar regions of the nuclear bag fibers of muscle spindles, while both bag and chain intrafusal fibers are unreactive in the intracapsular region (Fig. 20). This staining pattern

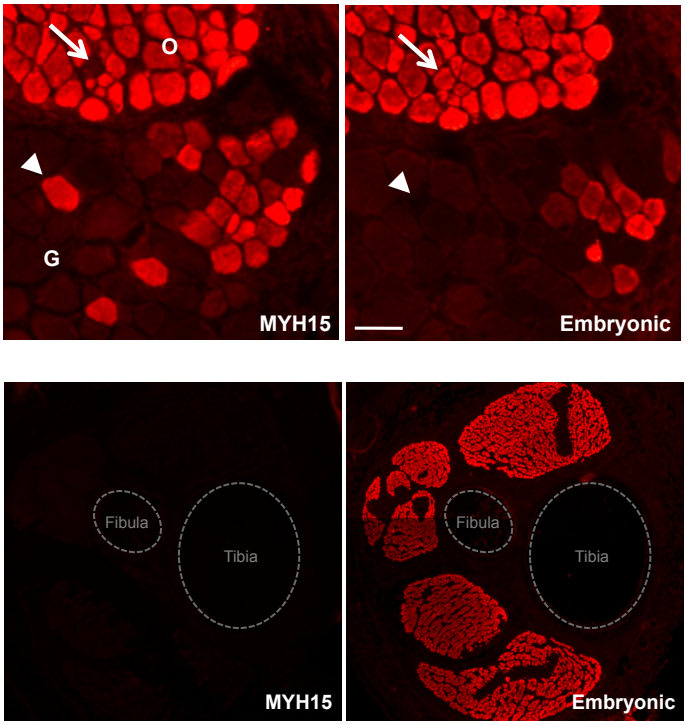
contrasts with that of embryonic MYH, present mostly in the chain fibers, and of slow-tonic MYH, which is also expressed in the equatorial region of bag fibers but not in chain fibers (Bormioli *et al.*, 1980) (see also (Walro & Kucera, 1999). MYH15 is a sarcomeric myosin, as shown by immunofluorescence staining of A bands in longitudinal sections of EO muscles (Fig. 21).

Fig. 18 Immunofluorescence with anti-MYH15 antibody in rat EO muscles



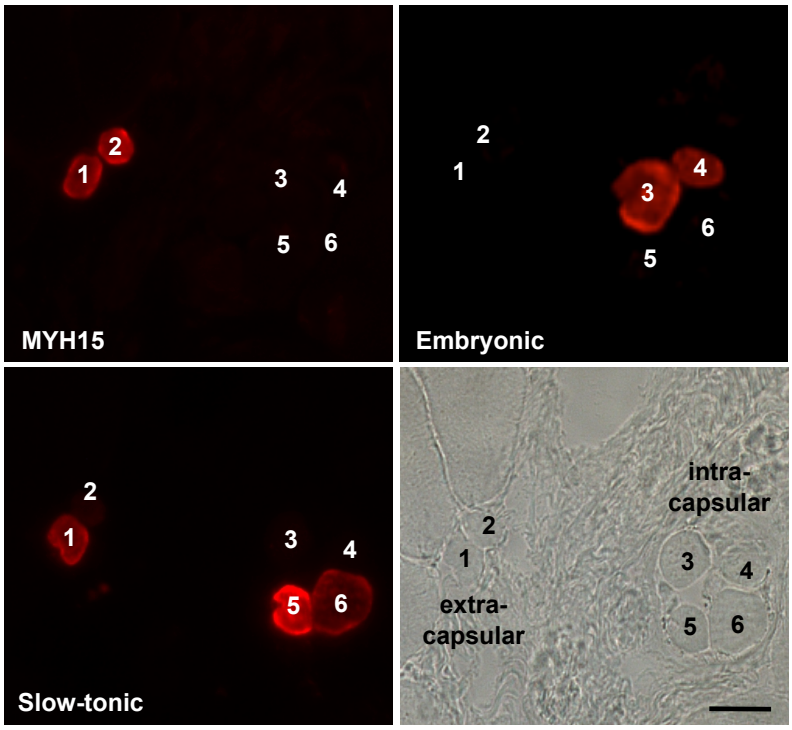
Serial transverse sections of rat EO muscle reacted with antibodies specific for MYH15, embryonic (BF-G6), slow-twitch (BA-D5) and slow-tonic (chicken ALD) MYHs. Note that MYH15 protein, like embryonic MYH, is expressed in most fibers of the orbital layer (O) and rare fibers of the global layer (G), whereas slow-tonic MYH is present in a very minor fiber population mostly localized in the orbital layer. Scale bar, 100 µm.

Fig. 19 Comparison of the MYH15 and Embryonic fibers



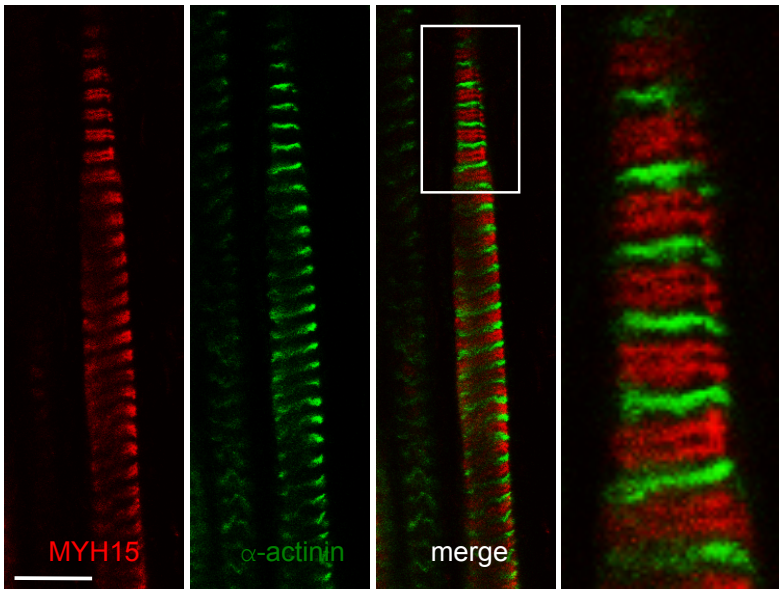
The pattern of expression of MYH15 in EO muscles is similar but not identical to that of embryonic MYH.
(Top panel) Serial transverse sections of rat EO muscle stained for MYH15 or embryonic MYH. An area at the boundary between the orbital (O) and global (G) layers is shown. Note that most fibers in the orbital layer stain for both MYH15 and embryonic MYH. However, some fibers react for MYH15 but not embryonic MYH (arrowhead) while other fibers stain for embryonic MYH but not MYH15 (arrow). Scale bar, 25 µm.
(Bottom panel) Serial transverse sections of rat E20 leg stained for MYH15 or embryonic MYH. Note no reactivity of the MYH15 antibody with embryonic fibers.

Fig. 20 Immunofluorescence with anti-MYH15 antibody in rat Soleus muscles



Serial transverse sections of rat soleus muscle showing two muscle spindles, one cut through the intracapsular region and one through the polar extracapsular region, stained with antibodies specific for MYH15, chicken ALD MYH (slow-tonic) and embryonic MYH or examined by phase contrast microscopy. Note selective expression of MYH15 in the polar region of the two bag intrafusal fibers (fibers 1 and 2), whereas embryonic MYH is expressed in the chain fibers (fibers 3 and 4) and slow tonic MYH is present in the intracapsular region of two bag fibers (fibers 5 and 6) and in the polar region of one bag fiber (fiber 1). Extrafusal fibers are unstained. Scale bar, 20 µm.

Fig. 21 Staining of the striation with anti-MYH15 antibody

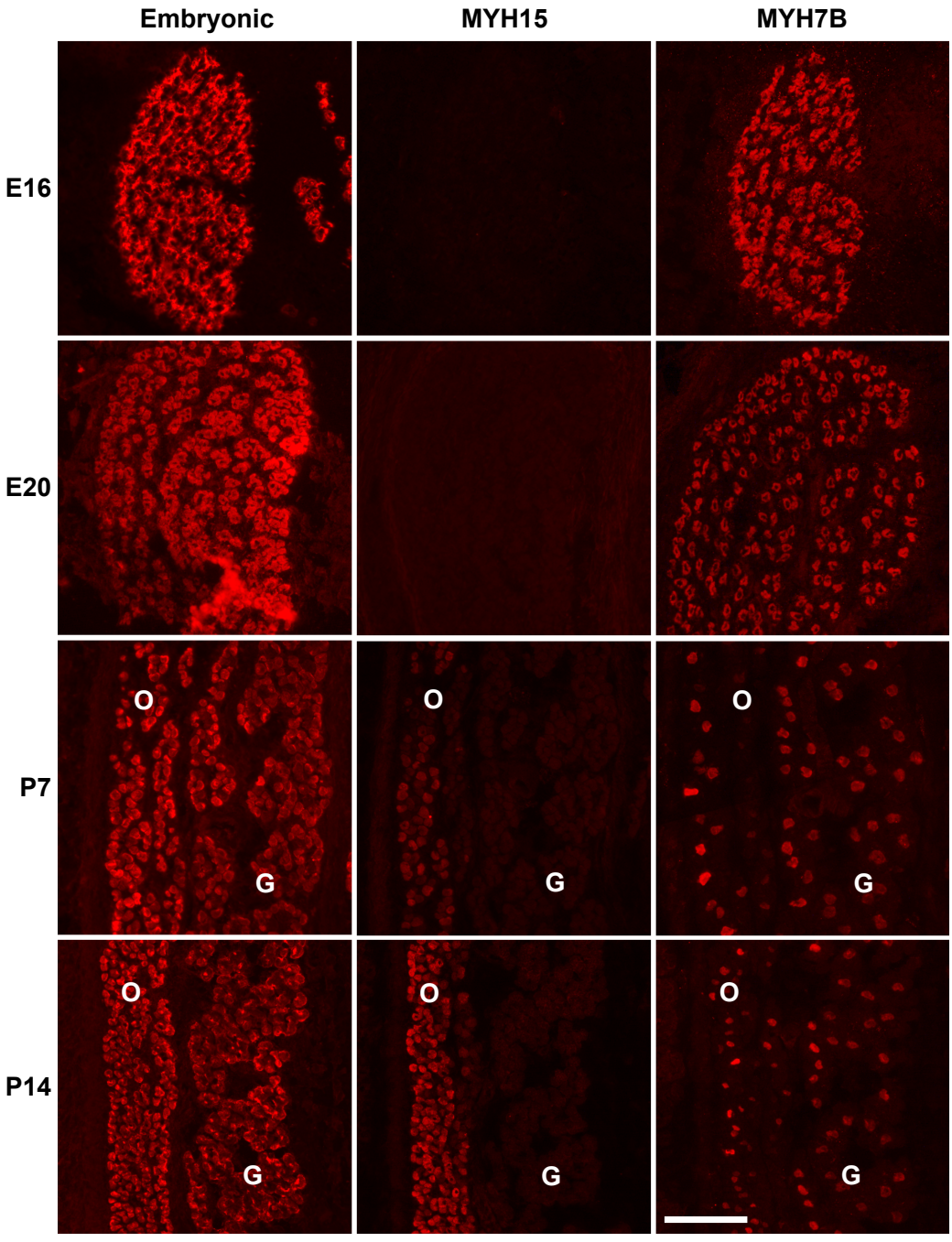


The positive fibers display a clear striation pattern corresponding to the A band (red), as shown by co-staining with an antibody against α-actinin that labels the Z disks (green). Scale bar, 10 µm.

5. Developmental expression of MYH7B and MYH15

During development, the pattern of expression of Myh15 is completely different from that of Myh7b. Myh15 is not detected by PCR in skeletal muscle or heart from embryonic day 12 (E12) mouse embryos (Fig. 24), nor by immunofluorescence in fetal and neonatal hindlimb muscles (not shown). MYH15 is also not found in fetal and neonatal EO muscles, being first detected at postnatal day 7 (P7) and at higher levels at P14 (Fig. 22). Since its first appearance at P7, MYH15 is exclusively present in the peripheral region of EO muscles, corresponding to the developing orbital layer (Fig. 22).

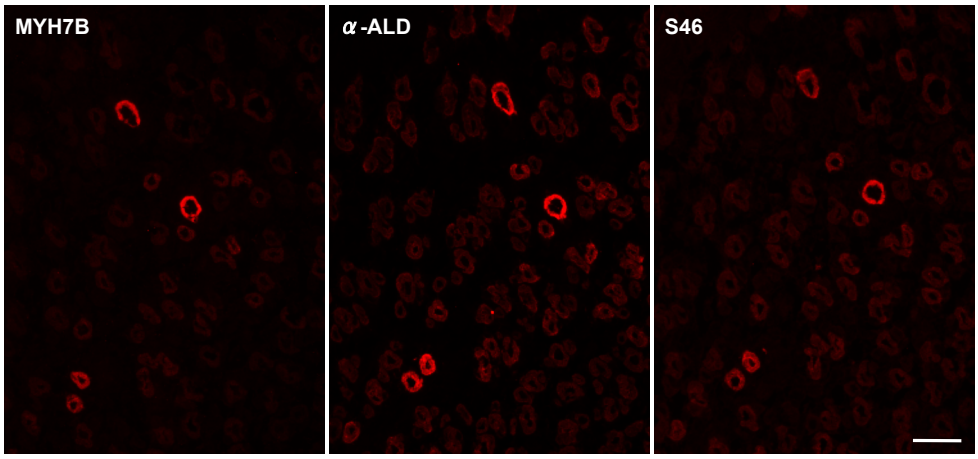
Fig. 22 Developmental expression of MYH7B and MYH15 in extraocular muscles



Serial sections of developing rat EO muscles stained for embryonic MYH (left panels), MYH15 (middle panels) or MYH7B (right panels). Note that MYH15 is undetectable in fetal muscles (E16 and E20), is barely visible in the orbital layer at postnatal day 7 (P7) and is clearly expressed at P14. In contrast, MYH7B is expressed in all fibers in fetal EO muscles and disappears in most fibers except the slow-tonic fibers during early postnatal stages. In panel Embryonic E16 is also detectable a portion of the retractor bulbi. Scale bar, 100 µm.

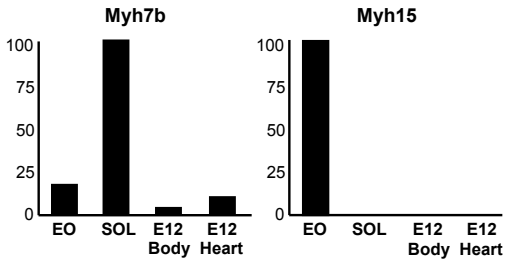
In contrast, Myh7b transcripts are detected by qPCR at low levels in skeletal muscle and heart from E12 mouse embryos (Fig. 24). In fetal and neonatal rat muscles, MYH7B protein is detected by immunofluorescence in the rare fibers, also stained by anti-ALD and S46 antibodies, destined to become the bag 2 fibers of muscle spindles (Fig. 23). MYH7B protein is expressed at high levels in all rat EO muscle fibers since E16 (Fig. 22 and 25) but disappears from most fibers, except the slow-tonic fibers, during the first two weeks after birth (Fig. 22).

Fig. 23 Expression of MYH7B in embryonic leg muscles



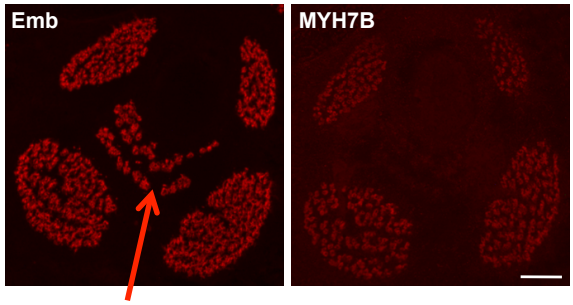
Section of E20 rat leg muscles stained with anti-MYH7B, anti-ALD and S46 antibodies. MYH7B protein is expressed in rare fibers, destined to become the bag2 fibers of muscle spindles. Scale bar, 40 µm.

Fig. 24 Expression of *Myh7b* and *Myh15* in embryonic tissues at transcript level



Myh15 transcripts are not detectable by RT-PCR in E12 mouse heart and body (rest of the body after heart removal) while Myh7b transcripts are detectable at low levels. Transcripts levels are normalized to housekeeping gene (HPRT) and expressed as the percentage of the tissue with the highest expression level.

Fig 25 Expression of MYH7B in E16 extraocular muscles

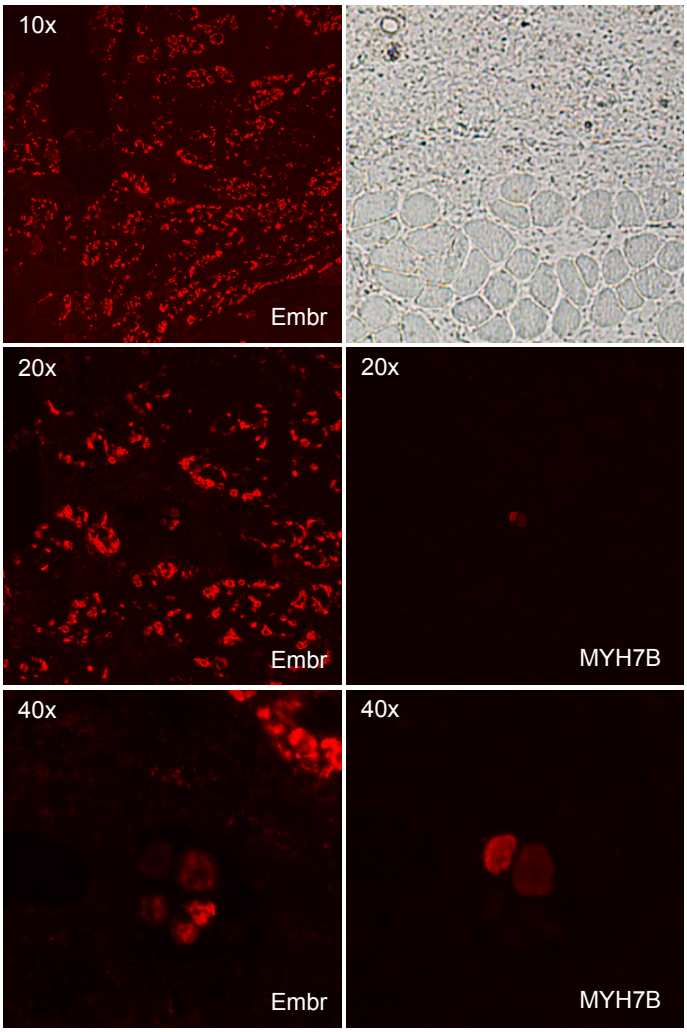


Serial sections of E16 rat EO muscles stained for embryonic MYH (leftl) or MYH7B (right). MYH7B is detected by immunofluorescence in four oculorotatory muscles present in the section, but not in the retractor bulbi muscle. Scale bar, 100 µm.

5.1 Expression of MYH7B and MYH15 in regenerating muscles

MYH7B and MYH15 proteins are not expressed in early regenerating muscles after 2 to 5 days post bupivacaine injection on rat soleus muscle (Fig. 26). It remains to be investigated whether these myosins are expressed in somites during development.

Fig. 26 Expression of MYH7B in regenerating muscles

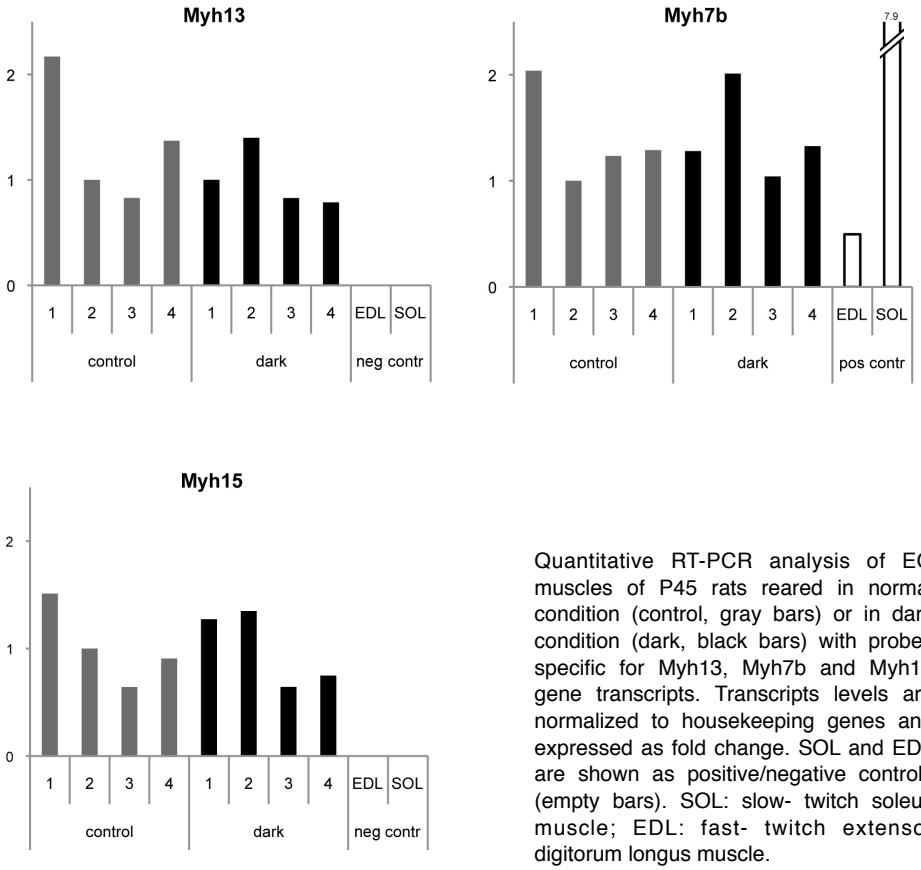


Serial rat SOL section stained with anti-embryonic (left) and anti-MYH7B (right) antibodies 3 days post bupivacaine injection. Note that none of the regenerating fibers are positive to the MYH7B antibody, with exception of one fiber into the muscle spindle. Same results were obtained at 2 and 5 days post bupivacaine injection, both for MYH7B and MYH15 (not shown). Top right panel: bright field section showing the effect of bupivacaine injection (top) compared to uninjected portion (bottom).

5.2 Expression of MYH7B and MYH15 in dark-rearing condition

We found that Myh7b and Myh15 undergo striking changes during early postnatal development, namely during the critical period when visual experience is required for the correct maturation of both sensory visual and oculomotor systems (see Results). To evaluate whether visual experience and/or sensory deprivation play a role on the expression of these myosins, we reared rats in prolonged dark condition and compared the expression level of transcript and

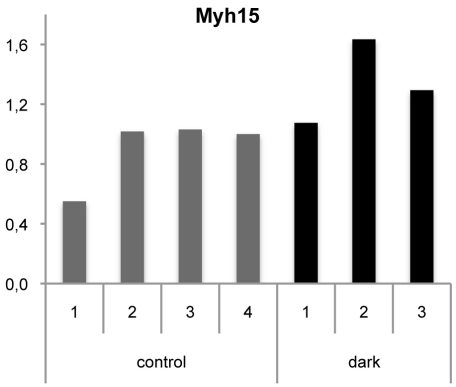
Fig. 27 Expression of Myh13, Myh7b and MYH15 in at transcript level in dark-reared rats



Quantitative RT-PCR analysis of EO muscles of P45 rats reared in normal condition (control, gray bars) or in dark condition (dark, black bars) with probes specific for Myh13, Myh7b and Myh15 gene transcripts. Transcripts levels are normalized to housekeeping genes and expressed as fold change. SOL and EDL are shown as positive/negative controls (empty bars). SOL: slow- twitch soleus muscle; EDL: fast- twitch extensor digitorum longus muscle.

protein with those of rats reared in normal condition, *i.e.*12-h light (0600-1800) and 12-h dark cycles. The possibility that the MYH composition of EO muscle is somehow sensitive to changes in visuomotor activity is supported by a previous study in which the Myh13, a myosin isoform EO muscles-specific, was down-regulated under visual deprivation condition (Brueckner & Porter, 1998). A following study, however, did not detected such down-regulation with microarray analysis (Cheng *et al.*, 2003). We examined by real time PCR the expression of

Fig. 28 Myh15 expression in dark-reared *vs* control rat EO muscles



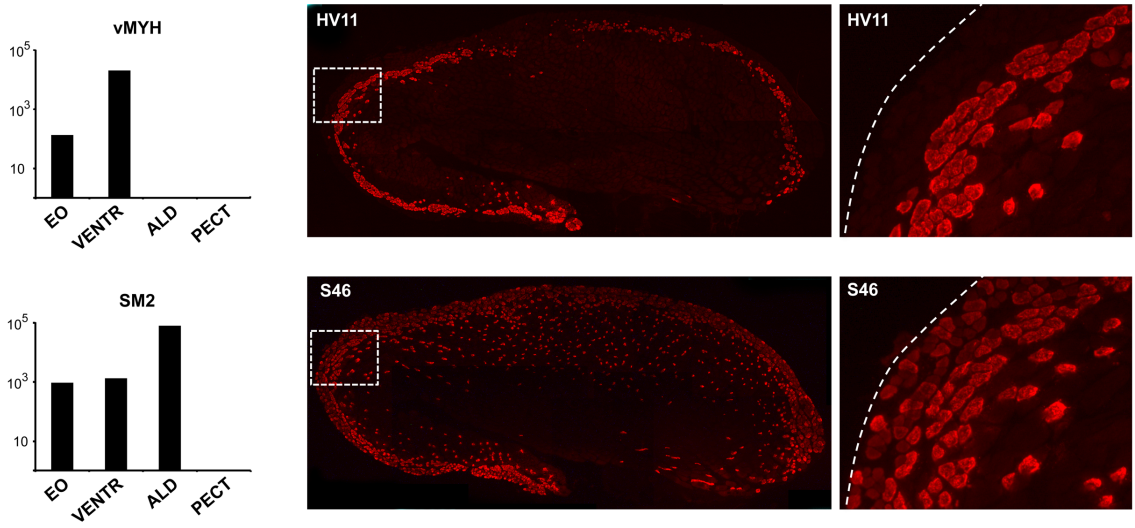
Densitometric quantification of Myh15 protein expression in control (gray bars) and dark-reared (black bars) P45 rat EO muscles. No significant differences were found between the two groups. Densitometric quantification was normalized to the level of actin and expressed as fold change.

Myh13, Myh7b and Myh15 transcripts in EO muscles of P45 dark-reared and control rats and found no evident differences between them (Fig. 27). At protein level, immunofluorescence against the embryonic, Myh7 and Myh15 protein (not shown) and immunoblotting against the Myh15 revealed no differences (Fig. 28). Densitometric quantification of immunoblot signal was possible only for Myh15 since for Myh13 and Myh7b we do not have specific antibodies.

6. Expression of MYH7B and MYH15 orthologs in chicken muscles

The specific pattern of expression of mammalian MYH7B and MYH15 genes in EO muscles contrasts with that of the orthologs avian genes, which are present in slow muscles and heart, respectively. However, the expression of these genes in chicken EO muscles was not previously investigated. Therefore we asked whether the chicken *ventricular MYH* (*vMYH*) gene, ortholog of mammalian *MYH15*, and *slow myosin 2 (SM2)*, ortholog of *MYH7B*, are expressed in chicken EO muscles. By RT-PCR, ventricular MYH transcripts are detected at high levels in ventricular myocardium and at lower but significant levels in EO muscles, but are undetectable in both adult slow (ALD) and fast (pectoralis) muscles (Fig. 29). This pattern differs from that of the *SM2*, which is expressed at much higher levels in the slow ALD muscle compared to EO muscles and is undetectable in pectoralis. Taking advantage of the availability of specific antibodies, we also examined the distribution of the corresponding proteins in chicken EO muscles. By immunofluorescence, chicken ventricular MYH is specifically expressed in a thin layer of peripheral fibers in the orbital portion of EO muscles, with a pattern of distribution overlapping only in part with that of the anti-slow myosin S46 antibody, which reacts with the slow myosins, SM2 and SM1 (Fig. 29).

Fig. 29 Expression of MYH7B and MYH15 in chicken



Distribution of MYH7B and MYH15 orthologs in chick EO muscles. (Left panel) RT-PCR analysis of transcripts of chick ventricular MYH, ortholog of mammalian MYH15 (upper panel) and SM2, ortholog of mammalian MYH7B (lower panel) in chick EO muscles, ventricle (VENTR), slow anterior latissimus dorsi muscle (ALD) and fast pectoralis muscle (PECT). Note log scale. (Middle and right panels) Transverse sections of chick EO muscle stained with monoclonal antibodies HV11, specific for ventricular MYH (upper panels), and S46, reactive with the slow myosins SM1 and SM2 (lower panels) seen at low magnification (left) and higher magnification (right). The outer margin of the muscle is outlined by a dotted line in the higher power figures. Note that both antibodies react mainly with peripheral fibers but S46 has a wider distribution, including fibers of the most external layer, which are not stained by HV11.

7. *In vitro* motility assay with chicken myosins

We wanted to assess the physiological properties of the Myh7b and Myh15; however, we found that fibers expressing these two myosins also express other isoforms, thus making impossible to isolate pure Myh7b and Myh15 (see Results). Therefore, we analyzed their chicken orthologs, slow myosin heavy chain 2 (SM2) and ventricular myosin heavy chain (vMYH).

Preliminary results on *in vitro* motility assay confirmed previous observations indicating that fibers of the ALD and ventricle are composed of slow myosins whereas those of the pectoralis and PLD are fast myosins (Dalla Libera *et al.*, 1979). *In vitro* motility assay was performed both on myosin extracted directly from the whole muscle as well as from single fibers. Table 7 summarizes our preliminary results. The *in vitro* sliding velocity of the pectoralis was found to significantly vary between the two types of myosin extraction (3.70 *vs* 2.10 µm/sec, for whole muscle and single fiber, respectively). We believe this result needs further confirmation, however, values of *in vitro* sliding velocity from myosin extracted from single fiber preparation are more similar to values already reported in the literature (Hooft *et al.*, 2007).

Table 7. *In vitro* motility assay in chicken muscles

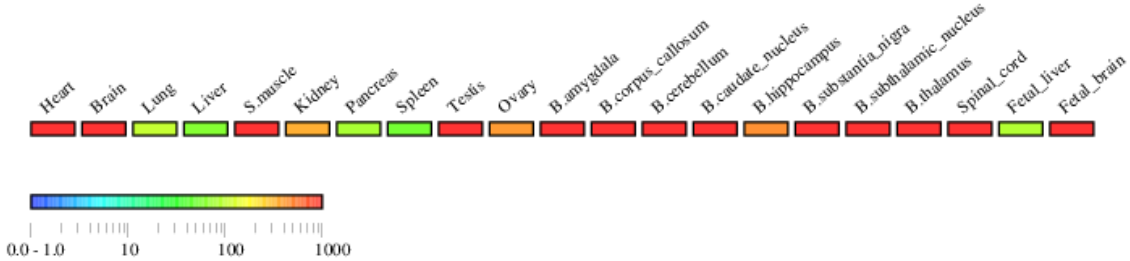
		in vitro sliding velocity (μm/sec)	# filaments
ALD	whole muscle	0.29 ± 0.05	7
	single fiber	0.29 ± 0.05	9
Ventricle	whole muscle	0.32 ± 0.06	3
PLD	whole muscle	2.06 ± 0.50	4
	single fiber	2.32 ± 0.42	9
PECT	whole muscle	3.70 ± 0.90	9
	single fiber	2.10 ± 0.50	18

Data are expressed as mean of the # of filaments examined ± stand. dev.

8. Expression of Myh7b in non muscle tissues

A previous study aimed to identify the coding sequences of unidentified human genes, as part of the sequencing project of human cDNAs which encode large proteins, originally reported the presence of a cDNA clone on human chromosome 20, named KIAA1512 (Entrez Gene NCBI AB040945) isolated from human adult and fetal brain cDNA libraries (Nagase *et al.*, 2000). This KIAA1512 cDNA clone has been subsequently identified as the gene coding for the myosin heavy chain MYH7B (Desjardins *et al.*, 2002). Surprisingly, in the original paper it is also reported that the KIAA1512 clone is expressed not only in human heart and skeletal muscle but also in brain and testis, and at lower but significant levels in ovary and kidney (Fig. 30).

Fig. 30 Expression profile of the KIAA1512 cDNA clone measured by RT-PCR-ELISA

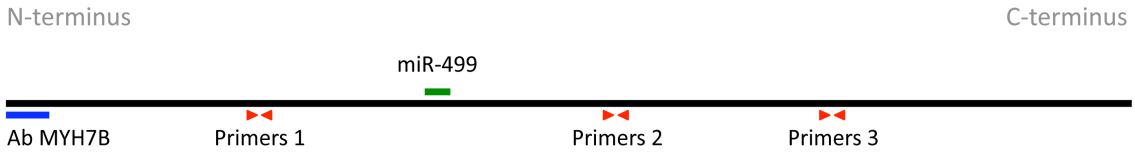


The expression profile evidences the unexpected finding that the KIAA1512 cDNA clone, later identified as the MYH7B gene, is expressed not only in the cardiac and skeletal muscles but also at relevant in non muscles tissues. Figure taken from http://www.kazusa.or.jp/huge/gfpage/KIAA1512/ (Nagase *et al.*, 2000).

We examined by real time PCR the expression profile of the Myh7b transcript on different rat muscle and non muscle tissues, such as the liver, brain, kidney and lung. We found the transcript expressed at significant levels in the kidneys, whereas in the other tissues it was

lower, yet not negligible (Fig. 32). We also examined the testis, although for this tissues we could not find a proper house keeping gene and therefore we could not include it with the other tissues. However, our preliminary data suggest that *Myh7b* is expressed at relevant level also in this sample. Three different sets of primers spanning the whole sequence of the *Myh7b* gene

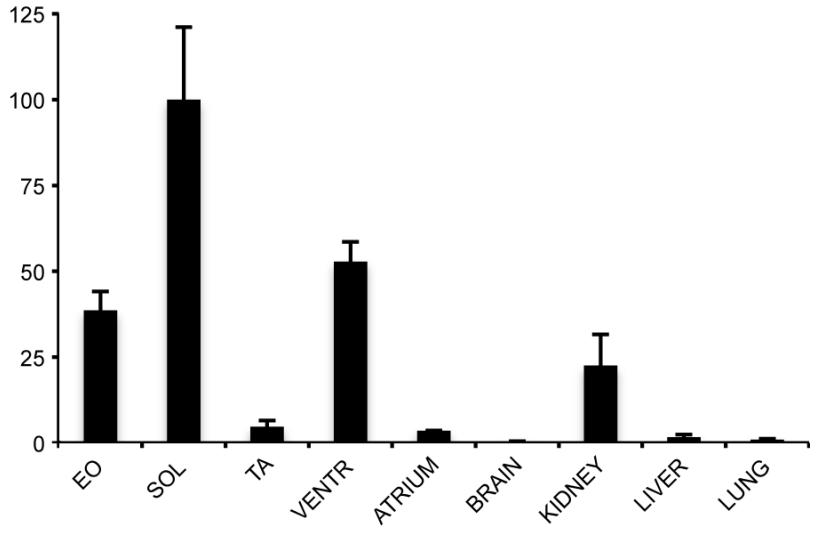
Fig. 31 Position of the sequences used to produce the MYH7B antibody and the primers for qPCR.



To confirm the results obtained with the qPCR on non muscle tissues we designed different sets of primer to cover the whole sequence of the MYH7B gene. Scheme reflects real scale.

(Fig. 31) were used to confirm these results, which were very consistent among them (in Fig. 32 only one set of primer is reported). Our data substantially confirm what it was previously reported by Nagase *et al*. (2000). However, our results indicate that the kidney is the non muscle tissues (among those we considered) with the highest expression of Myh7b transcript, whereas it was at lower, yet significant, level in the report of Nagase *et al*. (2000). Another incongruence we noticed regards the Myh7b transcript expression in the brain: low in our experiments, high in the report of Nagase *et al*. (2000).

Fig. 32 Expression of *Myh7b* in muscle and non muscles tissues at transcript level

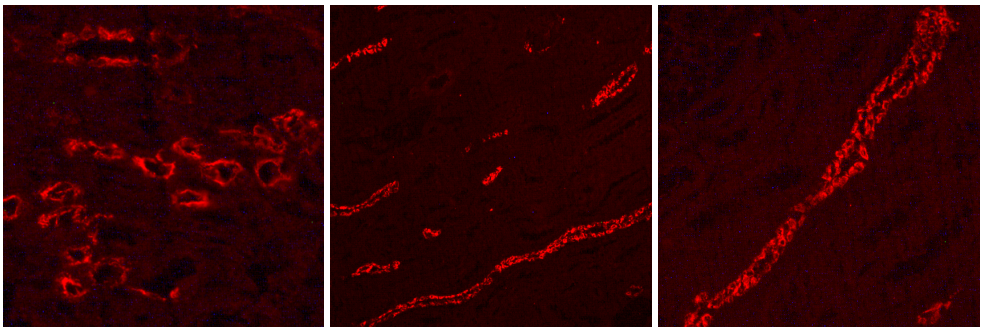


Quantitative RT-PCR analysis of different rat striated muscles and non muscle tissues with probes specific for *Myh7b*. Transcripts levels are normalized to housekeeping genes and expressed as the percentage of the tissue with the highest expression level. Comparable results were obtained with two other sets of primers (not shown). EO: extraocular muscles; SOL: slow-twitch soleus muscle; TA: fast- twitch tibialis anterior muscle; VENTR: ventricles. Data are mean ± SEM. $n = 3$.

We also started to examine the expression of the miR-499, which is embedded in the *MYH7B* gene. Our preliminary data suggest that the miR-499 is also expressed in non muscle tissues, with a pattern that parallels the MYH7B transcript.

Next, we examined the expression of the MYH7B at protein level in the same samples we used for the qPCR. By immunofluorescence, we found the protein only in the renal tubules of kidney (Fig. 33), while all other tissues were unstained (not shown). Reactivity of the Ab was shown to

Fig. 33 Immunofluorescence with anti-MYH7B antibody on rat kidney



Sagittal (left) and transverse (middle and left, two magnifications) sections of rat kidney. Note positive staining of some renal tubules.

be specific by the use of peptide-saturated primary antibody controls, which demonstrated an absence of signal due to non-specific binding of primary antibodies in kidney tissue immunohistochemistry (not shown). To further confirm these results, we took advantage of the availability of the two other antibodies specific for the slow-tonic isoform, S46 and anti-ALD. Unexpectedly, none of them reacts with the renal tubules. Moreover, no signal can be detected with the MYH7B antibody in immunoblotting at the molecular weight of the myosin (~220 kDa). Future investigation should focus on proteomic analysis which could reveal the presence of MYH7B at protein level in the renal tubules.

Discussion

The expression analysis of the two sarcomeric myosin heavy chains (MYHs) reported here, MYH7B and MYH15, completes the picture of sarcomeric MYH gene expression in mammalian striated muscles (Fig. 36). We find that *Myh7b* gene, the ortholog of chicken *slow myosin 2*, is detected in both heart and slow skeletal muscles at the transcript level, but only in the slow-tonic fibers of extraocular (EO) muscles and in bag fibers of muscle spindles at the protein level. *MYH7B* is, thus, the gene coding for the mammalian slow-tonic myosin, whose existence was previously postulated based on the reactivity of anti-chicken myosin antibodies but was never demonstrated. In contrast, *MYH15* gene, the ortholog of frog and chicken *ventricular MYH*, is expressed exclusively in a restricted region of EO muscles and in the extracapsular region of bag fibers of muscle spindles. The results of this study are of interest both with respect to MYH gene evolution in vertebrates and to the possible functional significance of these genes.

1. Evolutionary tinkering with sarcomeric myosin genes

It is of interest to compare the pattern of expression of mammalian *MYH15* gene with that of the ortholog *ventricular MYH* in frog and chicken (Table 8). The Xenopus ventricular MYH is expressed in both embryonic and adult heart and is never detected in skeletal muscle, with the exception of a weak transient expression in jaw muscles of stage 49 larvae (Garriock *et al.*, 2005; Nasipak & Kelley, 2008). Immunocytochemical studies showed that chicken ventricular MYH is expressed in embryonic and adult heart, as well as embryonic and regenerating but not adult skeletal muscle (Masaki & Yoshizaki, 1974; Cantini *et al.*, 1980; Gorza *et al.*, 1983; Sweeney *et al.*, 1984) and these findings were confirmed by analyses at the transcript level (Bisaha & Bader, 1991; Stewart *et al.*, 1991). However, our results indicate that this myosin is also expressed in peripheral fibers in adult chicken EO muscles (Fig. 29). The pattern of expression of MYH15 is still different in mammals, as this myosin is exclusively detected in adult EO muscles and in the extracapsular region of bag intrafusal fibers. This appears to be a striking case of evolutionary tinkering (Jacob, 1977), whereby a myosin gene expressed in the ventricle used to control cardiac pumping in amphibians is used to control eyeball movement in mammals. The change in functional specialization appears to correlate with major structural remodeling of the myosin molecule, including loop 1 structure: the sequence of Xenopus ventricular MYH is only 65% identical to mouse MYH15, whereas Xenopus atrial MYH and mouse α-MYH, which are both expressed in cardiac muscle and presumably have similar function, are 88% identical (Garriock *et al.*, 2005).

The evolution of the *MYH7B* gene is also remarkable and seems to parallel the evolution of the slow-tonic fibers, which represent a major slow fiber population in amphibian and avian skeletal

Table 8. Expression pattern of mammalian MYH7B and MYH15 genes and their orthologs in different vertebrate classes [a]

		Cardiac muscle		Skeletal muscle		Refs.
		Embryonic	Adult	Embryonic	Adult	
MYH7B	Torafugu	+ mRNA	+ mRNA	+ mRNA	+ mRNA	b
	Xenopus	nd	nd	nd	+ slow-tonic m., EO m.	c
	Chicken (SM2)	+ conduction tissue	+ conduction tissue	+ slow m. (minor component)	+ slow m. (major component), EO m.	d
	Mammals	+ mRNA, low levels	+ mRNA - protein	+ mRNA, low levels + protein, EO m.	+ mRNA, slow m. + protein, EO m.	e
MYH15	Xenopus (vMYH)	+ conduction tissue	+ (mRNA)	-	-	f
	Chicken (vMYH)	+ ventricle	+ ventricle	+	+ EO m.	g
	Mammals	-	-	-	+ EO m.	h

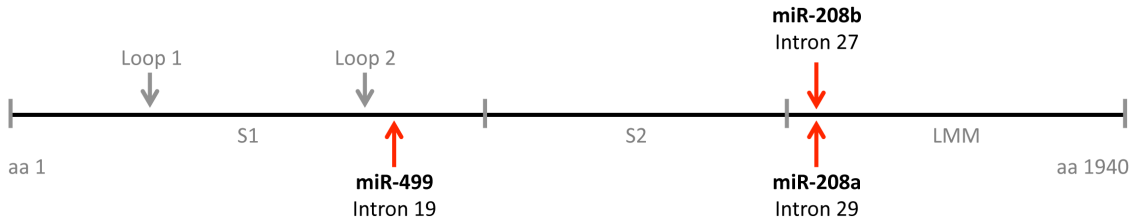
a Abbreviations: m., muscle fibers; nd, not determined; vMYH, ventricular MYH; SM2, slow myosin 2; EO: extraocular. The signs + (present) and - (absent) refer to analyses at the protein level unless indicated otherwise.
b (Ikeda *et al*., 2007)
c (Bormioli *et al*., 1980; Dieringer & Rowlerson, 1984)
d (Sartore *et al*., 1978; Matsuda *et al*., 1982; Gonzalez-Sanchez & Bader, 1985; Machida *et al*., 2002) & present study
e (Bormioli *et al*., 1979; Bormioli *et al*., 1980) & present study
f (Garriock *et al*., 2005)
g (Masaki & Yoshizaki, 1974; Gorza *et al*., 1983; Sweeney *et al*., 1984; Bisaha & Bader, 1991; Stewart *et al*., 1991) & present study
h present study

muscles, whereas they are confined to the EO muscles in mammals concomitantly with the emergence of slow-twitch fibers and β/slow-MYH as the major slow component. These findings validate the notion that two distinct myosins genes/proteins are present in slow-twitch and slow-tonic muscle fibers (Bormioli *et al*., 1980).

A recent paper hypothesized that vertebrate *MYH6* and *MYH7* derive from a common precursor, *MYH7B*, and that when this myosin gene duplicated, so too did its intronic microRNAs (van Rooij *et al*., 2009). We assume these observations are based on i) whole sequence analysis and ii) presence of microRNAs (miRNAs) in these sequences (miR-208a, -208b and -499) sharing a common seed. The similarity of MYH7B is in fact few points higher with MYH6 (α-cardiac) and MYH7 (β-slow) as compared to the other myosins (85% vs 83%); however, a previous study showed that percentage of similarity is not a good indicator since minimal amino acid differences even in highly similar ortholog sequences may correspond to profound differences in the functional properties (Canepari *et al*., 2000). Moreover, both MYH6 (α-cardiac) and MYH7 (β-slow) are more similar to the MYH4 (IIB) than to MYH7B (90% vs 85%); thus, it seems quite strange that if MYH6 and MYH7 were both the result of the duplication of the MYH7B, that they should now resemble more to a phylogenetically and functionally distinct myosin such as the MYH4 rather than their parent gene (MYH7B). Alternatively, after its duplication the MYH7B should have undergone a drastic remodeling of the sequence/function

and at the same time MYH6 and MYH7 an evolutionary action convergent toward the skeletal fast group. The common seed of the miRNAs expressed in the *MYH6*, *MYH7* and *MYH7B* (miR-208a, -208b and -499, respectively) seems indeed highly conserved, with only one mismatch out of 7 nucleotides; however, the remaining portion of 13 nucleotides presents as much as 7 mismatches. We analyzed the position of these miRNAs and their intronic sequences in the context of the whole sequence of the myosin. The miR-208a and miR-208b are located in different introns: 29 and 31, respectively (according to Van Rooij *et al*. 2009 the miR-208a is on intron 27 while according to miRBase, www.mirbase.org, is on intron 29). However, we analyzed the exons flanking these introns and found that those exons code exactly for the same amino acid sequence, more precisely for a portion of the myosin tail. On the contrary, we found that the exons flanking the intron 19, where the miR-499 is nestled, code for a portion of the S1 subfragment of the myosin (Fig. 34). Taken together these considerations

Fig. 34 Scheme reproducing the position of the miRNA-208a, -208b and -499 in the context of the whole myosin amino acid sequence.



Note the different position of the miR-499, located in an intron between two exons coding for the head portion, compared to the miR-208a and -208b, located in introns between two exons coding for the myosin rod. Scheme reflects real scale.

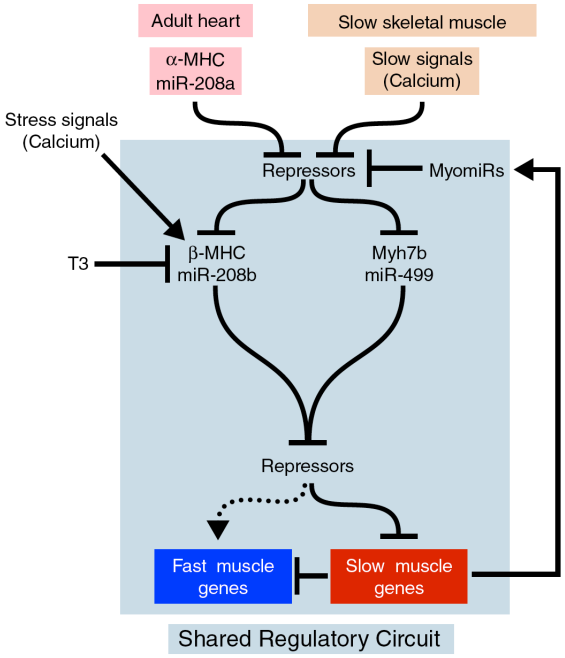
seem to suggest that MYH7B and MYH15 underwent independent evolution from the groups of cardiac and skeletal myosins (see Fig. 36). This view is validated by other published phylogenetic trees (Stewart *et al.*, 1991; Desjardins *et al.*, 2002; McGuigan *et al.*, 2004; Stedman *et al.*, 2004; Ikeda *et al.*, 2007) which support the existence of five distinct lines of evolution: *MYH16*, *MYH7B*, *MYH15*, cardiac group (*MYH6* and *MYH7*) and skeletal group (*MYH1*, *MYH2*, *MYH3*, *MYH4*, *MYH8*, *MYH13*).

2. New insight into fast and slow myosin gene regulation: the myomiRs network

Fast and slow myosin heavy chain expression can be modulated by various factors, hormonal and nutritional states and nerve activity. The signaling pathways regulating the myosin gene expression undergoes up and down regulation, therefore dictating the fiber phenotypes (Bassel-Duby & Olson, 2006; Schiaffino *et al.*, 2007; Sandri, 2008). More recently, there has been

emerging evidence for an additional regulatory mechanism of the myosin gene expression, *i.e.* a network of microRNAs (miRNA) (van Rooij *et al.*, 2007; van Rooij *et al.*, 2008a; van Rooij *et al.*, 2008b; McCarthy *et al.*, 2009; van Rooij *et al.*, 2009; Williams *et al.*, 2009). These miRNAs have been called myomiRs since they are located within and coexpressed with their corresponding myosin genes. Indeed, *MYH6* contains the miR-208a on the intron 29, *MYH7* contains the miR-208b on the intron 31 and *MYH7B* contains the miR-499 on the intron 19. miRNAs are single-stranded RNA molecules of 21-24 nucleotides in length which inhibit mRNA translation or promote mRNA degradation by annealing to complementary sequences in the 3' untranslated regions (UTRs) of target mRNAs (Bartel, 2004). A recent study demonstrated that myomiRs have a role on the regulation of the expression of the gene they are hosted and also of the gene they are signaling to; as a result, myomiRs modulate fast and slow myosin gene expression (van Rooij *et al.*, 2009). Thus, the emerging view is now that myosin genes not only encode the major contractile proteins of muscle, *i.e.* myosin heavy chain, but act more broadly to control muscle gene expression and performance through a network of intronic miRNAs. A comprehensive representation of this signaling network is presented on Fig. 35 (van Rooij *et al.*, 2009). It is of particular interest to understand these mechanisms since they could, al least in part, explain the discrepancy we found between the level of MYH7B transcript, high in slow and cardiac muscles, and the protein, restricted to a limited subpopulation in the EO muscles. The significance of this mismatch remains to be established, but it is tempting to speculate that the unique pattern of *MYH7B* gene regulation may be related to the presence within this gene of the miR-499, which is also expressed in fish cardiac and skeletal muscle (Kloosterman *et al.*, 2006) and might thus have maintained an evolutionary conserved function in all vertebrates. miR-499 has numerous potential targets, among which the transcription factor Sox6, Purβ and HP-1β. Interestingly, Sox6 has been shown to repress slow gene expression in both mouse (Hagiwara *et al.*, 2005; Hagiwara *et al.*, 2007) and zebrafish (von Hofsten *et al.*, 2008). Recently it

Fig. 35 Control of myosin gene expression by the myomiR network



has been shown that, indeed, the effect of miR-499 is partly mediated by the transcription factor Sox6 (van Rooij *et al*., 2009). Thus, by suppressing the expression of Sox6, the miR-499 in mouse enables the expression of slow MYH genes under the inhibitory influence of that repressor (and possibly some other repressors); accordingly, over-expression of miR-499 induces fast-to-slow shift in soleus. Conversely, in the absence of the miR-499, the expression of these repressors is enhanced, resulting in the inhibition of the slow gene program and other downstream targets with consequent up-regulation of the fast phenotype (van Rooij *et al*., 2009). In summary, the expression of *MYH7B* and miR-499 in slow but not fast muscles seems to play a role in the induction/maintenance of the slow phenotype by abrogating the Sox6-mediated repression of slow muscle-specific genes. van Rooij *et al*. (2009) also demonstrated that the expression of miR-499 and *MYH7B* were extinguished in hearts of miR-208a$^{-/-}$ mice, whereas they were unaffected in the soleus. This implies the existence of two distinct regulatory mechanisms: i) in cardiac miR-499 functions downstream of miR-208a (i.e. *Myh6* and miR-208a sit atop the hierarchy of regulatory steps), whereas ii) in skeletal muscles, where *Myh6* and miR-208a are absent, miR-499 functions independently of miR-208a. In light of these results, it would be of interest to investigate these regulatory mechanisms in the EO muscles since, although considered cardiac specific by van Rooij *et al*., the *Myh6* (and presumably its host miRNA, miR-208a) is also expressed in the EO muscles (Pedrosa-Domellof *et al*., 1992; Rushbrook *et al*., 1994; Lucas & Hoh, 2003; Budak *et al*., 2004). Moreover, while the MYH7B is never expressed at protein level in the heart, we demonstrated that it is expressed in the EO muscles, suggesting the presence of additional/different regulatory mechanisms in the EO muscles. There might also be some species differences since the presence of MYH7B at protein level in human EO muscles is much more abundant as compared to rat and mouse (see Fig. 13 and 15). van Rooij *et al*. also reported that Myh7b is highly expressed at transcript level in E15.5 mouse tongue, while in this tissue we did not detect expression at protein level in adult rat (not shown). This contrast is even more unexpected in view of the fact that Myh7b transcripts are high in slow and cardiac muscles and very low in fast muscles (our results and van Rooij *et al*., 2009), the tongue being predominantly composed by fast fibers, at least in postnatal stages (Agbulut *et al*., 1996; Agbulut *et al*., 2003). Alternatively, as we noticed for the rat EO muscles, it is possible that the Myh7b is expressed at high level during embryonic stages in the tongue, and then progressively lost during postnatal life. The expression profiles made by van Rooij *et al*. (2009) need further confirmation at protein level, which was done only for α/cardiac- and β/slow-MYH but not for MYH7B. In view of the mismatch we described between the mRNA and protein expression, data at protein level in miR-499 transgenic mice might significantly contribute to the elucidation of the regulatory mechanisms of this pathway.

In conclusion, the myomiRs network seems to play an important role on the regulation of the slow and fast gene program. Yet, this mechanism alone does not explain the wide mismatch

between the MYH7B transcript and protein we have reported here. Further research should focus on this aspect.

3. Functional significance of MYH7B and MYH15

It was previously suggested that MYH7B and MYH15 represent slow-type myosins based on sequence comparison of their motor domain which is more similar to that of slow than fast myosins (Desjardins *et al.*, 2002). This conclusion is consistent with the fact that mammalian MYH7B is orthologs to a chicken slow myosin (SM2), a major MYH component in the chicken slow anterior latissimus dorsi (ALD) muscle, both with respect to sequence similarity (McGuigan *et al.*, 2004) and synteny (our results), and is supported by the finding that MYH7B transcripts are expressed in cardiac and slow but not fast skeletal muscle. Finally, and more importantly, MYH7B is selectively expressed in multiply innervated EO fibers labeled by anti-ALD myosin antibody. The existence of a specific slow-tonic myosin in these fibers was first suggested based on the reactivity of a polyclonal antibody against chicken ALD muscle myosin (Bormioli *et al.*, 1979; Bormioli *et al.*, 1980) and later with different monoclonal anti-chicken myosin antibodies (Pedrosa & Thornell, 1990; Kucera *et al.*, 1992), many of which show partial cross-reactivity with the myosin β/slow present in slow-twitch fibers (Sokoloff *et al.*, 2007). Therefore it was never possible to rule out the possibility that this immunoreactivity could be due to post-translational modifications of another myosin, e.g. MYH-β/slow, rather than to a distinct novel isoform. Our results provide the definitive evidence that slow-tonic MYH indeed exists as a specific MYH isoform coded by a distinct gene.

Circumstantial evidence supports the notion that also MYH15 is a slow-type myosin. First, the MYH15 is orthologs to chicken ventricular MYH (vMYH) both with respect to sequence similarity (McGuigan *et al.*, 2004) and synteny (Garriock *et al.*, 2005), and it has been shown that ventricular MYH has a relatively low Ca^{2+}-ATPase activity, similar to that of the slow ALD muscle (Dalla Libera *et al.*, 1979).

To confirm these previous observations, *i.e.* that both chicken SM2 and vMYH are slow type, we performed *in vitro* motility assay with chicken muscles composed exclusively of these isoforms, ALD and ventricle, respectively. Our results confirmed that SM2 and vMYH are slow myosins, having an *in vitro* sliding velocity of 0.29 ± 0.05 and 0.32 ± 0.06 μm/sec, respectively. However, these results cannot be directly applied to their mammalian orthologs, in particular for the MYH15. In fact, MYH15 underwent a drastic sequence remodeling presumably related to a change in function, being a myosin used to pump the blood in frogs and birds and a myosin used to control eyeball movements in mammals. Xenopus and chicken vMYH (MYH15) share 94% similarity (87% identity), whereas chicken and human MYH15 share only 85% similarity

(70% identity). Moreover, we demonstrated that this remodeling is particularly evident in the loop 1 (see Fig. 10), which is a key regulator of the myosin speed of contraction (Sweeney *et al.*, 1998). Therefore, direct enzymatic and *in vitro* motility assays with pure mammalian myosins will be necessary to provide definitive evidence about the functional properties of MYH7B and MYH15. However, functional experiments on mammalian muscle fibers containing MYH7B or MYH15 is complicated by the fact that these myosins are always co-expressed with other myosins within the same muscle fibers, e.g. MYH15 is co-expressed with embryonic MYH in most orbital layer fibers and MYH7B is co-expressed with MYH15. Taking advantage of the specificity of our antibodies, we tried to immunoprecipitate both MYH7B and MYH15 from rat EO muscles. This approach could have answered two crucial points: i) determine whether these myosin tend to form heterodimers; ii) determine their physiological properties by *in vitro* motility assay. The possibility that different myosin isoforms form heterodimers has been previously reported (Dechesne *et al.*, 1987); although the physiological significance of this phenomenon as yet to be explained, these authors found that in the rat ventricle almost 30% of the myosin molecules are heterodimers composed by one subunit of β-MYH and one of α-MYH. Therefore, we could not exclude the possibility that also MYH7B and MYH15 form heterodimers. Unfortunately, we were unsuccessful in our preliminary immunoprecipitation experiments. Recently, a new technique has been proposed which permits, by adenoviruses infection, the expression of selected myosin isoforms in C_2C_{12} myotubes. The *in vitro* over-expression of these recombinant proteins gives the possibility to do physiological analyses, such as the *in vitro* motility assay (Resnicow *et al.*, 2009). It could be of interest to apply this technique to the MYH7B and MYH15 and establish their functional properties.

The selective expression of MYH15 in the orbital layer of EO muscles is consistent with the existence of major anatomical and physiological differences between orbital and global layers. It is now recognized that while global layer fibers insert directly on the globe, orbital layer fibers insert into pulleys, rings of dense collagen containing smooth muscle cells and elastin encircling EO muscles (Demer *et al.*, 1995; Demer *et al.*, 2000; Lim *et al.*, 2007). According to the active pulley hypothesis, the orbital layer is predominantly involved in adjusting the pulley position, whereas the global layer is responsible for eye rotation. Analysis of gene expression profiles revealed that a large number of genes are differentially expressed in orbital versus global layer fibers (Budak *et al.*, 2004; Khanna *et al.*, 2004). However, *MYH15*, which was not identified in those studies, appears to be the only gene uniquely expressed in the orbital layer of EO muscles.

The new evidence confirming the different roles of the orbital and global layers (Demer *et al.*, 1995; Demer *et al.*, 2000; Lim *et al.*, 2007), points to the importance of understanding their myosin composition. In particular, this aspects is of interest in view of some apparent differences among species. First, chicken EO muscles appear to have two orbital layers (in addition to the global layer): a thin external portion composed of SM2 fibers, and one more

internal thin portion composed of both SM2 and vMYH (orthologs of MYH7B and MYH15, respectively; Fig. 29); such orbital layer distinction was also reported in rabbit (Lucas & Hoh, 2003). Second, human EO muscles seem not to have a clear distinction between global and orbital layer, as suggested by our results (Fig. 15) and other reports (Stirn Kranjc *et al.*, 2009). However, this is contrast to what reported by others who could identify on human EO muscles three distinct layers: global, orbital and a more external layer called marginal zone (Wasicky *et al.*, 2000; Wicke *et al.*, 2007), thus reflecting the three layers noticed in chick by us and in rabbit by others (Lucas & Hoh, 2003). Also, there might be a longitudinal variation in the thickness of orbital layer such that at its extremities it is less pronounced and less detectable (our unpublished observation, not shown). An antibody against the human MYH15, a unique marker of orbital layer in rat and mouse, could significantly contribute to the distinction between global and orbital layers in human EO muscles.

4. Developmental expression of MYH7B and MYH15

We demonstrate that the MYH15 is not an embryonic muscle marker, since it is not expressed in embryonic and fetal muscles and is first detected after birth at postnatal day 7 (P7) in the orbital layer of EO muscles and at higher levels at P14. This result shows that the the orbital layer maturation begins approximately two to three weeks earlier that previously thought (Cheng *et al.*, 2004; Spencer & Porter, 2005). The MYH15 developmental pattern is opposite to that of MYH7B, which is widely expressed in EO muscles since early developmental stages (E16) but is down-regulated in most fibers, except the rare slow-tonic fibers, during the first two weeks after birth. These early postnatal stages correspond in part to the critical period when visual experience is required for the correct maturation of both sensory visual and oculomotor system (Wiesel & Hubel, 1963; Fagiolini *et al.*, 1994). The development of the visual afferents that drive eye movements proceeds under the regulation of well-established activity-dependent mechanisms. The visual cortex of mammals is anatomically and physiologically immature at birth, with key properties such as binocularity and depth perception developing during a species-specific postnatal window. This process depends on the visual experience obtained during an early period of plasticity known as the critical period (Wiesel & Hubel, 1963). Alterations in sensory experience disrupt the maturation of neural connections, especially if they occur during the critical period, whereas perturbations occurring either before or after this period have less chronic consequences for visual development. A previous study showed that the EO-specific myosin, MYH13, was down-regulated in rats reared in dark condition (binocular deprivation) from birth to P45, whereas it was not affected in monocular deprivation condition (Brueckner & Porter, 1998). These authors concluded that, because only specialized MYH

isoforms in EO muscles were affected by visual and vestibular insults, it may be that epigenetic influences, such as integrated signals from these two sensory systems, are vital in defining the EO muscles phenotype. However, the magnitude of such Myh13 down-regulation seems minimal, and a subsequent study from the same group failed to detect such down-regulation using microarray analysis (Cheng *et al.*, 2003). We examined the mRNA expression of Myh13, Myh7b and Myh15 in rats dark-reared from birth to P45 and found no evident differences as compared to the control group (Fig. 27). We also examined the MYH15 protein expression by immunoblotting (Fig. 28) and the embryonic, MYH7B and MYH15 protein expression by immunofluorescence (not shown) and found no differences between the two groups. Therefore, our data seem to suggest that disruption of an important epigenetic influence, *i.e.* visually driven eye movements, does not induce changes in the myosin phenotype of the EO muscles. One alternative possibility is that visual deprivation during the critical period only slightly modulates the expression profile of these three “specialized” myosins (Myh13, Myh7b and Myh15) and that such changes are not detectable with the techniques we have used. In this hypothesis, however, the functional significance of such minor changes remains arguable.

Expression of chicken SM2 and ventricular MYH during early embryonic stages has been investigated in the past. These studies suggest that the neural tube, the embryo's precursor of the central nervous system and motor innervation, is required for the primary and secondary myotube differentiation into adult myofibers expressing the SM2 and ventricular MYH isoforms (Lefeuvre *et al.*, 1996; DiMario & Stockdale, 1997; Sacks *et al.*, 2003). In light of these observations, it will be of interest to understand whether such mechanism (motor innervation) is crucial also for the expression of the mammalian orthologs MYH7B and MYH15 during embryonic and postnatal stages. To date, there is only one report where the expression of Myh7b has been investigated and *in situ* hybridization revealed expression of Myh7b transcript in heart as early as at 10.5 and also in somites at E12.5 (van Rooij *et al.*, 2009). In contrast, no information is presently available for the Myh15, although our results seem to suggest that it is never expressed during embryonic stages (Fig. 24) or regenerating muscles. Further research should be focused on Myh7b and Myh15 expression during early embryonic stages.

5. Expression of Myh7b in non muscle tissues

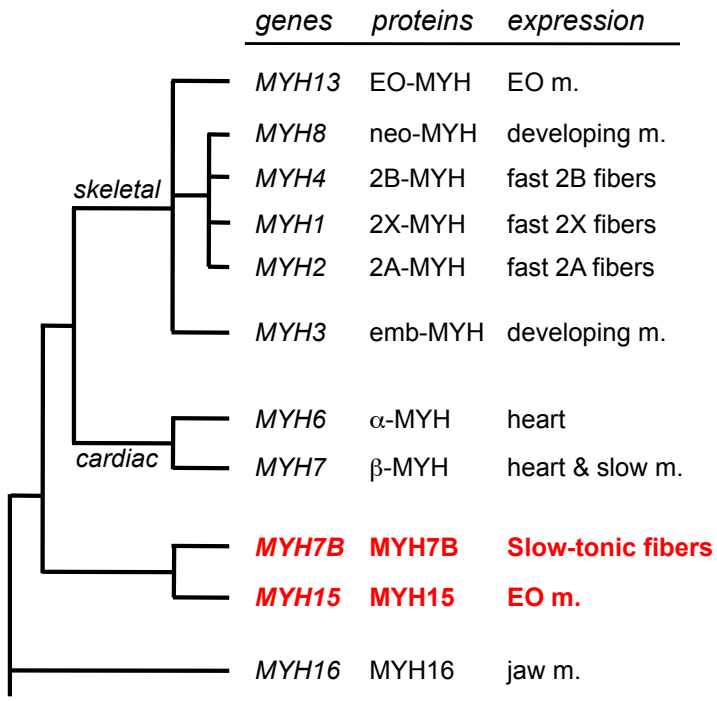
It is of particular interest the finding that MYH7B transcript is expressed in non muscle tissues, especially in the kidney. It is difficult to speculate on the significance of sarcomeric myosin expression in non muscle tissues. One possibility is that Myh7b is expressed widely, yet with tissues differences, at transcript level but confined to minor subpopulation of muscles fiber at protein level. This would imply the presence of a potent post-transcriptional regulatory

mechanism which has not been investigated yet. miR-499 may also been involved in this, since according to our preliminary results it reflects the expression profile seen for the *Myh7b*. If this was the case, the evolutionary/physiological advantage of such post-transcriptional mechanism remains to be explained. However, expression of class II sarcomeric myosin heavy chain at gene and protein level has already been reported in some tissues, such as lung (Rice & Leinwand, 2003), kidney (Schaart *et al*., 1991; Mayer & Leinwand, 1997) and liver (Ogata *et al*., 1993; Mayer & Leinwand, 1997; van der Ven & Furst, 1998). In these studies, the expression of sarcomeric myosin heavy chains is localized in the myofibroblasts of these tissues. Myofibroblasts are cells that share morphological and functional features of muscle and nonmuscle cells. Cultured BHK cells (kidney myofibroblasts) were found to express up to six MYH isoforms (embryonic, perinatal, IIa, IIx, IIb, and β/slow), whereas cultured liver stellate cells and cultured lung myofibroblasts to express only a subset (perinatal, IIa, IIx and embryonic IIa, IIx, respectively)(Mayer & Leinwand, 1997; Rice & Leinwand, 2003). Among other functions, thanks to their unique contractile properties, myofibroblasts are thought to control liver blood flow and kidney glomerular filtration rate by changing the capillary surface area and to generate tensile forces in wound healing and pathological contracture (Tomasek *et al*., 2002). Mayer and Leinwad (1997) speculated that these unique contractile properties of the myofibroblasts are provided by sarcomeric motor molecules, mainly myosin heavy chains. Further research is needed to elucidate the expression pattern of sarcomeric myosin heavy chains in non muscle tissues as well as their functional role.

Conclusions

The findings reported in the present study complete the inventory of the myosin heavy chain isoforms which act as molecular motors of the contraction in mammalian skeletal muscle fibers (Fig. 36). In particular, the present results provide an unambiguous and definitive identification of the myosin isoform expressed in slow-tonic fibers (MYH7B) and identify a novel and unexpected myosin isoform present in the orbital layer of extraocular muscles (MYH15). Such identification represents the basis for a thorough functional characterization.

Fig. 36



Abbreviations

ALD, chicken anterior latissumus dorsi;

ATR, atrium;

EDL, extensor digitorum longus;

Emb, embryonic;

E16, embryonic day 16

EO, extraocular;

GL, global layer;

mRNA, messenger RNA;

miRNA, microRNA;

miR-499, micro-RNA 499;

MYH, myosin heavy chain;

Neo, neonatal;

OL, orbital layer;

P7, postnatal day 7

qPCR, quantitative polymerase chain reaction;

PECT, chicken pectoralis major;

RT-PCR, reverse transcriptase-polymerase chain reaction;

SOL, soleus;

TA, tibialis anterior;

VENTR, ventricle;

VL, vastus lateralis.

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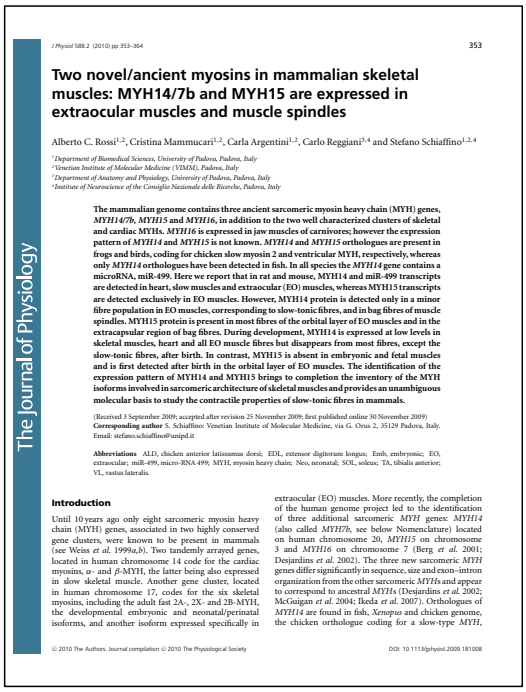

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Two novel/ancient myosins in mammalian skeletal muscles: MYH14/7b and MYH15 are expressed in extraocular muscles and muscle spindles

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The mammalian genome contains three ancient sarcomeric myosin heavy chain (MYH) genes, *MYH14/7b*, *MYH15* and *MYH16*, in addition to the two well characterized clusters of skeletal and cardiac MYHs. *MYH16* is expressed in jaw muscles of carnivores; however the expression pattern of *MYH14* and *MYH15* is not known. *MYH14* and *MYH15* orthologues are present in frogs and birds, coding for chicken slow myosin 2 and ventricular MYH, respectively, whereas only *MYH14* orthologues have been detected in fish. In all species the *MYH14* gene contains a microRNA, miR-499. Here we report that in rat and mouse, MYH14 and miR-499 transcripts are detected in heart, slow muscles and extraocular (EO) muscles, whereas MYH15 transcripts are detected exclusively in EO muscles. However, MYH14 protein is detected only in a minor fibre population in EO muscles, corresponding to slow-tonic fibres, and in bag fibres of muscle spindles. MYH15 protein is present in most fibres of the orbital layer of EO muscles and in the extracapsular region of bag fibres. During development, MYH14 is expressed at low levels in skeletal muscles, heart and all EO muscle fibres but disappears from most fibres, except the slow-tonic fibres, after birth. In contrast, MYH15 is absent in embryonic and fetal muscles and is first detected after birth in the orbital layer of EO muscles. The identification of the expression pattern of MYH14 and MYH15 brings to completion the inventory of the MYH isoforms involved in sarcomeric architecture of skeletal muscles and provides an unambiguous molecular basis to study the contractile properties of slow-tonic fibres in mammals.

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Abbreviations ALD, chicken anterior latissumus dorsi; EDL, extensor digitorum longus; Emb, embryonic; EO, extraocular; miR-499, micro-RNA 499; MYH, myosin heavy chain; Neo, neonatal; SOL, soleus; TA, tibialis anterior; VL, vastus lateralis.

Introduction

Until 10 years ago only eight sarcomeric myosin heavy chain (MYH) genes, associated in two highly conserved gene clusters, were known to be present in mammals (see Weiss *et al.* 1999*a*,*b*). Two tandemly arrayed genes, located in human chromosome 14 code for the cardiac myosins, α- and β-MYH, the latter being also expressed in slow skeletal muscle. Another gene cluster, located in human chromosome 17, codes for the six skeletal myosins, including the adult fast 2A-, 2X- and 2B-MYH, the developmental embryonic and neonatal/perinatal isoforms, and another isoform expressed specifically in extraocular (EO) muscles. More recently, the completion of the human genome project led to the identification of three additional sarcomeric *MYH* genes: *MYH14* (also called *MYH7b*, see below Nomenclature) located on human chromosome 20, *MYH15* on chromosome 3 and *MYH16* on chromosome 7 (Berg *et al.* 2001; Desjardins *et al.* 2002). The three new sarcomeric *MYH* genes differ significantly in sequence, size and exon–intron organization from the other sarcomeric *MYH*s and appear to correspond to ancestral *MYH*s (Desjardins *et al.* 2002; McGuigan *et al.* 2004; Ikeda *et al.* 2007). Orthologues of *MYH14* are found in fish, *Xenopus* and chicken genome, the chicken orthologue coding for a slow-type *MYH*,

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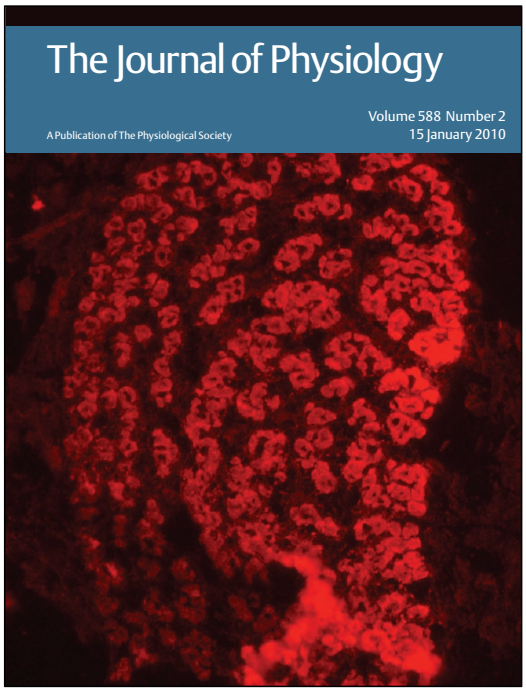

Ai miei genitori,
con infinita gratitudine

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