Myonuclear Organization and Regulation of Muscle Contraction in Single Muscle Fibres

Effects of Ageing, Gender, Species, Endocrine Factors and Muscle Size

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Abstract

The skeletal muscle fibre is a syncitium where each myonuclei regulates the gene products in a finite volume of cytoplasm i.e., the myonuclear domain (MND). A novel image analysis algorithm applied to confocal images, analyzing MND size and myonuclear spatial distribution in 3-dimensions in single skeletal muscle fibres has been used in this project. The goal was to explore the modulation of myonuclei count and MND size in response to muscle adaptation processes. The effects of ageing, gender, hormones, muscle hypertrophy and body size were investigated on MND size.

A strong linear relationship was found between MND size and body size in the muscle fibres from mammals representing a 100,000-fold difference in body size. Independent of species, MND size was highly dependent on MyHC isoform type and mitochondrial contents of skeletal muscle fibres. In hypertrophic mice, a significant effect of MND size on specific force and myosin content was observed. This effect was muscle fibre type-specific and shows that the bigger MNDs in fast-twitch EDL muscle fibres are optimally tuned for force production while smaller MNDs in slow-twitch soleus muscle fibres have a much more dynamic range of hypertrophy without functional compromise. This indicates a critical volume individual myonuclei can support efficiently for a proportional gain in muscle fibre force and size. In human muscle fibres, spatial organization of myonuclei was affected by both ageing and MyHC isoform expression. In fibres expressing type I MyHC isoform, an increased MND size variability and myonuclear aggregates were observed in old age although average MND size was unchanged. In contrast, in type IIa fibres, the average MND size was smaller reflecting smaller size of muscle fibres. Those changes may influence the transcriptional activity per myonucleus and/or local cooperatively of myonuclei in a gender and muscle fibre-type specific manner.

Finally, hormone replacement therapy was shown to negate menopause-related functional impairment in skeletal muscle fibres. The positive effect on force was due to quantitative effect in fibres expressing fast myosin isoform while the effect was both quantitative and qualitative in fibres expressing slow myosin isoform. The effect on MND size was fibre type dependent and was achieved by significantly reducing domain size in slow- but not the fast-twitch muscle fibres.

Together, our data suggest that modulation of myonuclei count and MND size is a mechanism contributing to remodelling of skeletal muscle in muscle adaptation process. These findings should be considered when developing therapeutic approaches towards restoring muscle mass and strength in muscle wasting conditions.

Keywords: single muscle cells, muscle nuclei, specific force, species, hypertrophy, mammals, ageing, gender

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Dedication

To my Abbu
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CSA</td>
<td>Cross sectional area</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6 Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>EDL</td>
<td>Extensor digitorum longus</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>GEC</td>
<td>General elliptical cylinder</td>
</tr>
<tr>
<td>GM</td>
<td>Gluteus medius</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-Like growth factor 1</td>
</tr>
<tr>
<td>LDE</td>
<td>Long digital extensor</td>
</tr>
<tr>
<td>MND</td>
<td>Myonuclear domain</td>
</tr>
<tr>
<td>MyHC</td>
<td>Myosin heavy chain</td>
</tr>
<tr>
<td>MyLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>MZ</td>
<td>Monozygous</td>
</tr>
<tr>
<td>NN</td>
<td>Nearest neighbour</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modifications</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>$V_0$</td>
<td>Maximum velocity of unloaded shortening</td>
</tr>
</tbody>
</table>
Introduction

The skeletal muscle fibre

The muscle fibre is the force generating unit in skeletal muscle. It is composed of myofibrils which constitute about $2/3^{rd}$ of the muscle fibre volume (Hoppeler & Fluck 2002) and a muscle fibre 50µm in diameter contains up to 2000 myofibrils in parallel. Each myofibril is 1-2µm in diameter and separated from neighbouring myofibrils by mitochondria, sarcoplasmic reticula and transverse tubules.

Myofibrils contain sarcomeres, the functional unit which gives muscle fibre its characteristic striated appearance due to alternance of light and dark bands. The light, isotropic, I-band corresponds to the presence of actin filaments that are inserted into the Z-disk in the centre of each I-band. The dark, anisotropic A-band contains myosin filaments. In the resting state, the actin and myosin filaments partially overlap each other in an inter-digitating manner and the central region of the A-band without the filament overlap constitutes the relatively pale H-zone.

The sarcomere is composed of thick and thin filaments along with titin and nebulin. The thick filaments mainly contain myosin and myosin binding proteins and form the M-region in the middle of the H-zone (Schiaffino & Reggiani 1996). Thin filaments contain actin, troponin and tropomyosin and other actin binding proteins. It provides myosin molecules with an ordered series of binding sites for attachment (Au 2004). Titin filament spans from the Z-disk to the centre of the sarcomere, i.e., the M line (Schiaffino & Reggiani 1996). It has a role in maintaining sarcomeric integrity (Horowits & Podolsky 1987) and also contributes to muscle passive tension (Linke et al. 1998). Nebulin acts as a thin filament ‘ruler’ specifying the precise length of the actin filament (McElhinny et al. 2003). The two key contractile proteins in the sarcomere are myosin and actin.

Myosin

Myosin is the dominating skeletal muscle protein and is believed to be the mechanical motor that converts free energy driven by ATP hydrolysis into mechanical work. A complete myosin molecule is ~150nm long and consists of two globular heads and a tail. The globular heads are often referred to as the S1 fragment. Each head consists of a myosin heavy chain (MyHC)
subunit of approximately 220kD and two light chains (MyLC) attached to it. The globular head consists of three parts with molecular weights of 50 kD, 25 kD and 20 kD, respectively. The two light chains are attached to the 20 kD moiety while the 50 kD segment has a site for attachment of actin as well as a pocket for binding and hydrolyzing ATP (Vibert 1988). The globular heads and the neck to which they are attached constitute the cross-bridge. X-ray crystallography and electron microscopy of the myosin head reveal a cleft through the 50 kD segment, dividing it into an upper and a lower domain (Fig.1)(Rayment et al. 1993). The width of the cleft is controlled by its interaction with the ATP. Opening of the cleft weakens the bond between the 50 kD moiety and actin while closure of the cleft strengthens it.

The two light chains attached to the myosin head are termed regulatory and essential MyLC respectively. They have a molecular weight of approximately 20 kD each and give structural stability to the cross-bridge. The regulatory light chains decrease Ca^{2+} sensitivity of the contractile proteins (Metzger & Moss 1992) while essential light chains have a modulatory influence on maximal velocity of shortening (V_0) in rats and rabbits (Greaser et al. 1988; Sweeney et al. 1988). On the other hand, this modulatory influence of essential MyLC isoform has not been confirmed in muscle fibres from old rats and humans (Li & Larsson 1996; Larsson et al. 1997a) suggesting that other modulators, such as thin filament proteins may have a role in regulation of contractile speed.

Fig.1. Ribbon diagram (A, in red) and schematic diagram (B) of the structure of myosin head.
Actin

Skeletal muscle actin is 5-10 nm wide, 42kD protein composed of a double helical strand of individual actin molecules (Au 2004). Actin has a 2-fold slower turnover rate compared to myosin heavy chain (Martin 1981). During muscle contraction, the actin filament is exposed to myosin heads that then appear as arrowhead-like structures along the filament length.

Muscle Contraction

Individual myofibrils are surrounded by an elaborated meshwork of channels called the sarcoplasmic reticulum (SR) that release Ca\(^{2+}\) around the myofibrils through ryanodine receptors (RyR). These channels appear anchored to a set of sarcoplasmic invaginations called T-tubules which conduct action potential leading to the release of Ca\(^{2+}\) from the SR. In the relaxed state, the myosin-binding site on actin is covered by tropomyosin. When calcium is released into the cytosol, it binds to troponin C which then undergoes conformational changes exposing the binding sites on actin by displacing tropomyosin which initiates contraction and actomyosin interactions (Squire 1975). The actomyosin interaction is initiated by a weak electrostatic bond between positively charged residues on the surface of the catalytic domain of the myosin head and the negatively charged residue on the surface of actin. Subsequently, actin-driven ATP hydrolysis leads to a strong binding state corresponding to a lever arm movement of the myosin head (the power stroke) (Reconditi et al. 2003). This is followed by release of ADP; and with replacement by ATP, the S1 segment dissociates from actin. The myosin filament remains stationary during the contraction with each cross-bridge acting as a force generator. The number of cross-bridges and the force generated per cross bridge determines the amount of force generated by the whole muscle fibre (McDonald & Fitts 1995; Martyn et al. 2007).

After the contraction phase is over, Ca\(^{2+}\) is removed from the cytosol by ATP-driven sarcoplasmic reticulum Ca\(^{2+}\) pumps (SERCA) (Toyoshima et al. 2000). Two isoforms of SERCA are known. SERCA 1 is found in fast-twitch fibres while SERCA 2 is restricted to slow-twitch fibres. The density of Ca\(^{2+}\) pumps is higher in fast- than slow-twitch fibres.

The duration of a cross-bridge cycle depends on temperature, MyHC isoform and velocity of contraction, and can vary from less than 1 ms to 50 ms. The myosin head remains bound to the actin filament in the strong-bound state for less than 50% of this time. The power stroke of fast-twitch MyHC isoform is a very brief event, probably lasting 2-10 ms during an isometric contraction. Fast-twitch fibres are called glycolytic, due to the small number of mitochondria while slow-twitch fibres are called oxidative.
due to large number of mitochondria. Mitochondrial density also determines the endurance capacity of the muscle fibre (Fig. 2).

<table>
<thead>
<tr>
<th>MyHC isoform type</th>
<th>Cross-sectional area</th>
<th>Shortening velocity</th>
<th>Mitochondrial density</th>
<th>Endurance</th>
<th>Myonuclear density</th>
<th>MND size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow-twitch (oxidative)</td>
<td>small</td>
<td>slow</td>
<td>high</td>
<td>high</td>
<td>high</td>
<td>small</td>
</tr>
<tr>
<td>Fast-twitch (glycolytic)</td>
<td>large</td>
<td>fast</td>
<td>low</td>
<td>low</td>
<td>low</td>
<td>large</td>
</tr>
</tbody>
</table>

Fig.2. Properties of the muscle fibres expressing slow- and fast-twitch MyHC isoforms. There are exceptions to this general design.

Regulation of muscle contraction

The membrane-permeabilized single muscle fibre preparation has contributed significantly to our understanding of regulation of muscle contraction. This single fibre technique allows examination of contractile proteins function under controlled physiological conditions and with an intact filament lattice but free of confounding factors related to connective tissues or protein heterogeneity between cells of multicellular preparations. It also allows selective removal of proteins such as myosin light chains (Moss et al. 1995) and troponin C (Zot & Potter 1982). On the other hand, possible confounding factors include pH, sarcomere length, temperature, ionic strength, Mg\(^{2+}\) and phosphorylation of regulatory MyLC (Gordon et al. 2000).

Maximum velocity of unloaded shortening (V\(_0\)) is an important parameter of muscle contractility. Studies from skinned fibres show that V\(_0\) is regulated by MyHC isoform expression and actin-activated ATPase activity (Greaser et al. 1988; Schluter & Fitts 1994). Ten different MyHCs have so far been identified in mammalian skeletal muscle. They include embryonic, foetal, β/slow (type I), α-cardiac, slow-tonic, type IIa, type IIx (IIId), IIb, a super-fast extra ocular myosin isoform (MyHC EOM) and another super-fast contracting type in jaw closing muscles of cats and dogs (Schiaffino & Reggiani 1996). Four of these isoforms (type I, IIa, IIx, Iib) are expressed in rats and mice hind limb muscles while three (type I, IIa, IIx) are expressed in human trunk and limb muscles. Shortening velocity is slowest in the fibres expressing the type I MyHC isoform and it increase in the order IIa<IIx<Iib (Larsson & Moss 1993; Bottinelli et al. 1994; Li & Larsson 1996). An approximate 3-fold difference is observed in the shortening velocity between the slowest type I fibres and fastest type IIb fibres in rat (Li & Larsson 1996) and 4-5 fold in rabbits (Sweeney et al. 1988). In humans, the difference
between type I and type IIx fibres contraction velocity is approximately 10-fold (Bottinelli et al. 1996).

Hybrid fibres are frequently observed in adult skeletal muscle, i.e. fibres representing multiple MyHC isoforms. An increased number of hybrid fibres is reported in relation to ageing and during transformation induced by physical activity and change in hormonal status (Schiaffino & Reggiani 2011). In muscle fibres expressing two MyHC isoforms, the slower of the two MyHCs has a disproportionately stronger influence on $V_0$ (Larsson & Moss 1993; Li & Larsson 1996).

In contrast to shortening velocity, muscle force generating capacity shows a less strong dependence on MyHC isoform type and the number of cross-bridges and force produced by each cross-bridge play a more important role (Schiaffino & Reggiani 1996). Nevertheless, a weak relationship has been described between specific force and fibre type with fast-twitch fibres having higher specific force (Medler 2002). This difference is attributed to relatively higher myofibrillar volume in fast glycolytic fibres compared to slow oxidative fibres (Lindstedt et al. 1998). On the other hand, no significant difference in specific force was observed between fibres expressing type I and type IIa MyHC isoforms from rats (Larsson et al. 1993), mice, humans and rhinoceros (Marx et al. 2006). Recently we showed significantly higher force generation capacity of fast (type IIa) than slow (type I) myosin extracted from single human muscle fiber segments (Li & Larsson 2010).

Effects of ageing

Ageing is associated with a constant decrease in strength of approximately 1-3% per year (Kallman et al. 1990; Frontera et al. 2000) primarily attributed to motor unit loss and an altered endocrine activity. Women have an additional 15% loss due to menopause (Kallman et al. 1990; Phillips et al. 1993) leading to accelerated muscle weakness. This results in qualitative and quantitative changes at the single muscle fibre level. A decrease in number of both slow- and fast-twitch muscle fibres (Lexell et al. 1988) along with a preferential atrophy of fast-twitch fibres (Tomonaga 1977; Larsson et al. 1978) has been reported. An ageing-related decline in the force generating capacity of single muscle fibres is observed in type I and type II fibres (Larsson et al. 1997b) due to a reduced fraction of myosin heads in the strong binding state (Lowe et al. 2001; Lowe et al. 2002). Thus, a reduction in the fraction of myosin heads in the strongly bound state and decreased myosin concentration in old age may result in the reduced specific force observed in skinned single fibre segments. In human muscle fibres, a strong correlation exists between the relative myosin content and specific force suggesting that a reduced number of cross-bridges is a major contributor to lower specific force in old age (D'Antona et al. 2003). An age-related slowing of the contractile speed ($V_0$) (Larsson et al. 1997a; D'Antona et al. 2003).
2003; D’Antona et al. 2007) has generally been reported, even though not always confirmed (Trappe et al. 2003). Post-translational modification (PTM) of the molecular motor protein myosin has been suggested to be related to the decrease in contraction velocity (Mooradian & Wong 1991; Hook et al. 2001; Ramamurthy et al. 2001) since myosin is a potential target for PTM due to its slow turnover rate and additional decrease in turnover rate in aged muscle (Balagopal et al. 1997).

Hormone replacement therapy (HRT) has been extensively used to partially counteract the deleterious effects on muscles in post-menopausal women (Skelton et al. 1999; Sipila et al. 2001). However, the beneficial effects of HRT on muscle function are still in debate, mainly due to experimental limitations such as genetic and lifestyle difference between HRT users and non-users (Taaffe et al. 1995; Bassey 1997). A unique population of postmenopausal monozygous twins discordant for HRT eliminates the potential of genetics and minimizes environmental differences between users and nonusers, i.e., extracting out HRT effects (Ronkainen et al. 2009; Finni et al. 2011). The results from these studies favour a positive impact of HRT on muscle performance supported by a strong relationship between muscle function and HRT usage.

The cellular and molecular mechanisms underlying the in-vivo improvement in response to HRT use remain unclear. It has been speculated that HRT can improve muscle function directly by affecting the acto-myosin interaction (Phillips et al. 1993; Lowe et al. 2010) and/or enhancing satellite cell activation, attenuating exercise-induced muscle damage and creating a pro-anabolic environment in muscles of post-menopausal women (Enns et al. 2008; Dieli-Conwright et al. 2009). While some or all the above factors may influence muscle function, little is known about estrogens effects at the cell/motor protein levels and myonuclear organization in human muscle fibres.

Myonuclei and myonuclear domain

Skeletal muscle is a highly ordered organ composed of the largest cells in the body that can be several centimetres long. In order to support a very large cytoplasm volume, skeletal muscle fibres are one of the few truly multinucleated cells. A muscle fibre can have hundreds to thousands of myonuclei sharing the same cytoplasm (Fig. 3). Myonuclei are typically located at the periphery of the muscle fibre just beneath the sarcolemma, are in a post-mitotic state and are incapable of further division. Myonuclei are believed to occupy fixed non-randomly distributed positions which optimize the transport distances for cellular proteins (Bruusgaard et al. 2003; Bruusgaard et al. 2006).
Information on individual myonuclei shape and spatial distribution is rare and is mainly collected from rats (Tseng et al. 1994) and mice (Bruusgaard et al. 2003; Bruusgaard et al. 2006). A strict fibre type dependent shape has been reported in mouse muscle with nuclei from fast-twitch EDL fibres having elliptical shapes aligned to the long axis of the fibre while nuclei from slow-twitch soleus fibres have a more rounded appearance (Bruusgaard et al. 2003). A more ordered positioning of nuclei has been reported in muscle fibres expressing fast-twitch (type II) MyHC isoforms while nuclei in slow-twitch (type I) fibres have a more random appearance. Such patterns of nuclear positioning may facilitate inter-nuclear communication for regulation and co-ordination of protein expression. As such many factors have a say in determining the positioning of myonuclei in the muscle fibre including blood vessels, the intermediate filament desmin (Ralston et al. 2006) and microtubuli (Bruusgaard et al. 2006). Not all the nuclei are active at the same time and transcription occurs in a pulsatile manner (Newlands et al. 1998). Furthermore, nuclei may have specified functions depending on their location in the muscle fibre e.g., nuclei at the myotendineus junction and motor end plate where specific proteins are required (Moscoso et al. 1995; Crosbie et al. 1999).

Fig. 3. Single muscle fibre (A) and myonuclei with various shapes (B). Scale bar = 25µm (fibre); 3µm (nuclei).

Each nucleus controls transcriptional activity in the surrounding volume of cytoplasm, i.e., the myonuclear domain (MND) (Landing et al. 1974; Pavlath et al. 1989). Myonuclear domain size varies along the length of the
muscle fibre (Rosser et al. 2002). The number, spatial distribution and transcriptional activity per myonuclear domain are prime determinants of muscle fibre size and strength (Favier et al. 2008). It is generally believed that a fine relationship is maintained between myonuclei number and fibre size in most conditions of muscle hypertrophy (Kadi et al. 2005; Mackey et al. 2007) and atrophy (Allen et al. 1997; Alway & Siu 2008). However, several reports demonstrate a less stringent relationship between myonuclei count and fibre size, i.e., muscle fibre hypertrophy has been reported without satellite cell activation (McCarthy & Esser 2007) and atrophy accompanied by an increase, decrease or unchanged number of nuclei (Allen et al. 1995; Allen et al. 1996; Kasper & Xun 1996; Gundersen & Bruusgaard 2008) (Fig. 4). Moreover, the distribution of myonuclei may also contribute to the functional output by optimizing transport distances (Bruusgaard et al. 2006).

Fig. 4. Changes in MND size with muscle fibre hypertrophy and atrophy.

Role of satellite cells
Satellite cells are myoblasts located between the sarcolemma and the basement membrane of the muscle fibre (Mauro 1961). In humans, satellite cell nuclei are slightly smaller than muscle nuclei and are mostly in a quiescent state (Watkins & Cullen 1986). Satellite cells are responsible for myonuclear addition during post-natal muscle growth. In addition, their proliferation can be evoked following acute injury (Bischoff & Heintz 1994)
and in response to muscle overuse and increased tension (Hawke & Garry 2001). Satellite cell proliferation and myonuclear addition lag behind myofibre growth suggesting that the initial phase of hypertrophy is characterized by enhanced transcription per myonucleus (Chen et al. 2002) leading to a slight expansion of MNDs. This is followed by fusion of satellite cells thus re-establishing the muscle fibre DNA to cytoplasmic volume ratio (Rosenblatt et al. 1994; Roy et al. 1999). This also supports the concept of a MND ‘ceiling’ (Petrella et al. 2006) discussed later in this thesis.

Effect of fibre type

MND size appears to be related to muscle fibre MyHC isoform type with slow-twitch fibres having a smaller MND size than fast-twitch fibres (Fig. 2). Thus, an increase in MND size is recorded from type I to type IIa to IIx and IIb fibres (Tseng et al. 1994; Allen et al. 1999; Roy et al. 1999). The same phenomenon has been observed in muscle fibres from different species including rat (Atherton & James 1980), rabbit (Burleigh 1977) and chickens (Matthew & Moore 1987). These differences in MND size have been attributed to the higher oxidative capacity in slow-twitch compared to fast-twitch fibres since MND size is inversely related to oxidative capacity of the muscle fibre (Tseng et al. 1994). This notion is further supported by a recent observation that the MND size is larger in type II fibres from the superficial glycolytic region of the rat plantaris muscle than in type II fibres from deep oxidative region (van der Meer et al. 2011b). The smaller domains in oxidative fibres have been attributed to a higher rate of protein turnover (Edgerton & Roy 1991) and accordingly a higher demand for mRNA transcription.

Effects of increase in muscle size

Effect of body size

Body size has a prime role in assigning the functional demand to the skeletal muscle. Muscles from small mammals have a faster phenotype than muscle from large mammals. For instance, mice muscle predominantly contains type IIb and IIx fibres with type IIa and type I fibres being rare and are mainly confined to slow-twitch muscles. In contrast, human limb muscles mainly contain type I and type IIa fibres. Likewise, the muscles fibres from smaller mammals have much a higher mitochondrial content than muscle fibres from large mammals (Howald et al. 1985; Mathieu-Costello et al. 2002). In addition, muscle fibre CSA is smaller in small mammals compared to large
mammals. This implies that the body size is relevant for energy metabolism and functional demand in mammals.

A. V. Hill (1950) proposed that contractile properties of skeletal muscle differ between species owing to differences in body mass and limb length, since animals of similar body shape and gate characteristics move at a similar velocity independent of body size. The mechanism behind regulation of muscle contraction has been studied in different species at the muscle fibre and motor protein level and a strong scaling effect of shortening velocity and limb length has been recorded (Andruchov et al. 2004; Bicer & Reiser 2004; Marx et al. 2006; Bicer & Reiser 2007).

Given these differences and knowing that the MND size is influenced by MyHC isoform type, mitochondrial contents and fibre size, there is a need to assess how the modulation of MND size scales with body size in different muscle fibre types.

Hypertrophy

Hypertrophy of the skeletal muscle has been linked with myonuclear accretion in rat (Roy et al. 1999), cat (Allen et al. 1995) and humans (Petrella et al. 2006). This is supported by attenuation (Rosenblatt & Parry 1993; Lowe & Alway 1999) or prevention (Rosenblatt et al. 1994; Phelan & Gonyea 1997) of hypertrophy by ‘gamma-irradiation’ which block satellite cell division. However some amount of hypertrophy is possible without the addition of myonuclei (Snow 1990; Kadi et al. 2004b). A MND ceiling size of 2000 μm² has been proposed in human muscle beyond which myonuclear addition is mandatory for hypertrophy (Petrella et al. 2006). This probably reflects that a myonucleus is not working at its maximum capacity under normal conditions and has the potential to enhance its transcriptional activity to support a larger MND size. However, the relationship between transcriptional ability and functional outcome has not been explored and there is, accordingly a strong need to relate MND size to functional capacity in hypertrophic muscle fibres since muscle hypertrophy is not necessarily accompanied by a proportional gain in force (Amthor et al. 2007; Watt et al. 2010).

Compounds which trigger such changes in myonuclei number and muscle size are multiple, including myostatin and Insulin like Growth factor-1 (Welle et al. 2007; Duan et al. 2010; Huang et al. 2011). Both have strikingly dissimilar effects on specific force whereas mice overexpressing IGF-1 exhibit maintained specific force (Del Prete et al. 2008) but not myostatin deficient mice (Mendias et al. 2006). IGF-1 is a peptide hormone with anabolic action on muscle growth (Barton et al. 2010). A mouse model has been developed where a tissue-restricted transgene encodes a locally acting isoform of IGF-1 in skeletal muscle (Musaro et al. 2001). These hypertrophic mice are characterized by maintained specific force (Gonzalez
et al. 2003; Del Prete et al. 2008; Colombini et al. 2009). A combination of satellite cell activation and increased protein synthesis has been proposed to induce hypertrophy in these muscles (Barton-Davis et al. 1999).

Myostatin is a member of the transforming growth factor-β (TGF-β) superfamily of cytokines and is a negative regulator of skeletal muscle mass. Deletion of the myostatin gene or post-natal inhibition of myostatin results in a significant increase in muscle mass (McPherron et al. 1997; Gentry et al. 2011). However, in contrast to IGF-1 mediated hypertrophy, a gain in force is not proportionally related to a gain in size resulting in a decreased specific force in myostatin deficient mice (Mendias et al. 2006; Amthor et al. 2007). Moreover, satellite cells seem to have no role in this kind of hypertrophy (Amthor et al. 2009).

Effects of decrease in muscle size

Effects of ageing

Ageing is associated with impaired adaptability of myonuclei as shown by a decrease in number (Kadi et al. 2004a) and activity (Kadi et al. 2004b) of satellite cells. Thus, myonuclei count determined from muscle cross-sections shows an increased (Kadi et al. 2004a) or unchanged (Manta et al. 1987) myonuclear number with ageing in human muscle. The same observations of an increased (Brooks et al. 2009; van der Meer et al. 2011a) or unchanged (Sultan et al. 2001; Gallegly et al. 2004) myonuclei number has been reported in rats. Part of these discrepancies may be due to erroneous counting of nuclei in muscle transverse sections (Brack et al. 2005) due to an altered shape and elongation of myonuclei associated with ageing (Brack et al. 2005; Bruusgaard et al. 2006). In the two reports that analyzed myonuclear density in isolated single muscle fibres, a decrease in nuclei count per muscle fibre length was observed in old mice (Brack et al. 2005; Bruusgaard et al. 2006). In contrast, information from human muscle is based on cross-sections and are not corrected for fibre types (Kadi et al. 2004a; Kadi et al. 2004b) which may give rise to erroneous conclusions, since slow-twitch muscle fibres have smaller MND size than fast-twitch fibres (Allen et al. 1997; Bruusgaard et al. 2003) along with ageing-related fibre-type transitions (Degens & Alway 2006) and a preferential atrophy of type II fibres (Larsson et al. 1978).
Aims of the present investigations

General Aims

The general objective of this thesis was to study the role of modulation of myonuclei in relation to change in cell size, functional capacity and MyHC isoform type using a recently developed novel algorithm applied to confocal single muscle fibre images.

The thesis can be divided into three parts. The first part (Paper I) addresses the influence of body size on myonuclear organization in relation to various MyHC isoforms. The second part (Paper II) investigates the role of myonuclei to determine the functional capacity of single fibres in hypertrophic muscle. The third part (Paper III, IV) aims at investigating ageing-related effects on myonuclear organization and MND size and how hormone replacement therapy affects these changes and the decrement in contractile function in post menopausal monozygous twins discordant for HRT.

Specific Aims

1. To study the effects of species and body size on the spatial arrangement of myonuclei in relation to different types of MyHC isoform (I).
2. To explore the effects of myonuclei and MND size on regulation of muscle contraction in single fibres from hypertrophic muscle (II).
3. To quantify gender- and fibre-type related variations in spatial organization of myonuclei during ageing and to determine if hormone replacement therapy can counter these changes and contractile dysfunction in post-menopausal twins (III, IV)

Materials and Methods

Animal and Human Subjects (I, II, III, IV)

The effect of body size on myonuclear organization (I) was studied in 6 species which include, in order of increasing body mass (with strain, age and body mass given in parenthesis), are as follows: mouse (C57BL/6J, 6 months, 25 g), rat (Sprague-Dawley, 6 months, 450 g), humans (21 years, 80 kg), pig (Swedish Landrace, 4 years, 200 kg), horse (Quarter horse, 1 year, 400 kg) and rhinoceros (white, 26 years, 2500 kg). All subjects were young adults, of either sex and with no history of musculoskeletal disorder except rhinoceros. The middle aged rhinoceros had a history of chronic pedodermatitis though the condition was under clinical control at the time the biopsy was taken. The pigs were bred for many years for meat.
production. The study was approved by ethical committee on human and animal research at Uppsala University, Uppsala, Sweden and the Karolinska Institute, Stockholm, Sweden and by the Institutional Review Board and the Institutional Animal Care and Review Board and Use Committee at the Pennsylvania State University, University Park, PA, USA.

The study on contractile recordings and myonuclear organization (II) was carried out in six month old, C57BL/6J mice. Four mice were included in each group (control, Mstn−/− and mIgf1+/+). The study conducted in accordance with the University of Pennsylvania Animal Care and User Committee.

Eight young men (21-27 years), nine old men (72-96 years), six young women (24-32 years) and nine old women (65-96 years) volunteered for the ageing study (III) on myonuclear organization. All subjects were healthy sedentary with no history of locomotor, metabolic or neuromuscular disorder. The study was approved by the ethical committees at the Karolinska Institute, Stockholm and Pennsylvania State University, University Park, PA, USA.

The study for effects of hormone replacement therapy (IV) was carried out in six monozygous twin pairs, postmenopausal and discordant for HRT. The mean age of the 12 subjects was 56.6 ± 1.3 years (range 55-59 years) while the mean duration of HRT use was 7.8 ± 4.3 years (range 4-16 years). Estradiol and progesterone were the effective agents of pills in three twin pairs while estradiol alone in the other three twin pairs. Subjects had no musculoskeletal disease or other contra-indications for the participation in the study. The study was approved by the ethical committee of the Central Finland Health Care District.

Muscle Biopsy (I, II, III, IV)

In the mice, the soleus and extensor digitorum longus (EDL) muscles were removed either immediately after the animals were killed by cervical dislocation (I) or after sedation with ketamine/xylazine mixture followed by euthanizing (II). In the rat (I), the soleus, gastrocnemius (GN) and EDL muscles were removed immediately after the animals were killed by removal of the heart in anesthetized animals. Muscle biopsy from horse (I) was obtained by percutaneous chonchotome method, with sedation and local anaesthesia from the gluteus medius (GN) and long digital extensor (LDE) muscles. In the pigs, muscle biopsies from the soleus and the gluteus were obtained immediately after the animals were killed in a commercial slaughterhouse. In the rhinoceros (I) biopsies were taken from the hamstring muscle group using the percutaneous chonchotome method after the animals were anesthetized.

In the human subjects (I, III, IV), the percutaneous chonchotome method was used under local anaesthesia to obtain biopsies from the right vastus
lateralis muscle. All the biopsy takings were done with prior understanding and consent of the subjects.

Each biopsy specimen typically contained 200-800 muscle fibres and weighed from 10-120 mg. The biopsies were placed in relaxing solution at 4 °C and dissected free of connective tissue and fat. From each biopsy specimen, 4-10 bundles of ~50 muscle fibres were dissected and tied with surgical silk to glass capillaries at slightly stretched lengths (110%). The muscle fibre bundles were chemically skinned for 24 h in skinning solution containing 50% (v/v) glycerol at 4 °C and stored at -20 °C for use within three weeks. Alternatively, bundles were cryoprotected (Frontera & Larsson 1997) for long term storage at -180 °C before use. For this purpose, bundles were freed from glass capillaries and snap frozen with isopentane chilled with liquid nitrogen. On the day before the experiment, a bundle was transferred to 2.0 M sucrose solution and passed through solutions of decreasing sucrose concentration (1.5, 1.0, 0.5 M) before placement in skinning solution at -20 °C to be used within three weeks.

Skinning is a chemical procedure to permeabilize sarcolemma of skeletal muscle fibres. For this purpose, glycerol is used to disrupt all membrane-bound structures in the muscle fibre including sarcolemma, T-tubules and sarcoplasmic reticulum while sparing the contractile apparatus. This allows the direct investigation of actin-myosin interaction outside the influence of neural factors, connective tissues, orientation of fibre and regulatory proteins of sarcolemma. Since buffering capacity of Ca2+, Mg2+, ATP and EGTA can be rigorously adjusted (Fabiato 1988), a controlled experimental condition is created to obtain reliable data concerning contractile recordings from single skinned muscle fibres.

Single fibre contractile recordings (II, IV)

The experimental procedure has been described in detail elsewhere (Moss 1979). Briefly, fibres were bathed in a relaxing solution for 30 minutes prior to the experiment, leaving an average fibre segment length of 1.60 ± 0.20 mm (mean ± SD, range 1.00-2.00 mm) exposed to the solution between the connectors to a direct-current torque motor (Model 300H, Cambridge Technology, Inc.) and a force transducer (Model 403, Cambridge Technology, Inc., USA). The apparatus was mounted on the stage of an inverted microscope (Zeiss Axiovert-35, Carl Zeiss, Oberkochen, Germany). Sarcomere length was adjusted to 2.79±0.01 (range 2.71-2.85) for human fibres or 2.65±0.05 (range 2.60-2.70) in mice fibres, by adjusting overall segment length. The sarcomere length, segment width and length between the connectors were measured using a high-speed video analysis system (model 901A, HVSL, Aurora scientific). The fibre sarcomere length during the experiment was measured routinely during maximum activation. Fibre depth was measured by recording the vertical displacement of the
microscope nosepiece while focusing on the top and bottom of the fibre segment. Fibre cross-sectional area (CSA) was measured from depth and diameter assuming an elliptical circumference and corrected for 20% swelling that is known to occur during skinning procedure (Moss 1979). Every width and depth value represented the mean of three different measurements. Specific tension was calculated as maximum tension normalized to cross-sectional area (P₀/CSA).

Once steady-state isometric force was reached, small-amplitude sinusoidal changes in length (ΔL: ± 0.2% of fibre length), were applied at 500 Hz at one end of the fibre (Martyn et al. 2007). The resultant force response (ΔF) was measured, and the mean of 20 consecutive readings of ΔL and ΔF was used to determine stiffness. The actual elastic modulus (E) was calculated as the difference between E in activating solutions and resting E measured in the same segment in the relaxing solution. E was determined as follows (McDonald & Fitts 1995):

\[ E = \frac{\Delta F}{\Delta L} \times \frac{\text{fibre length}}{\text{CSA}} \]

Relaxing and activating solutions contained (in mM): 4 MgATP, 1 free Mg²⁺, 20 imidazole, 7 EGTA, 14.5 creatine phosphate and sufficient KCl to adjust the ionic strength to 180. The free Ca²⁺ concentrations, expressed as pCa (-log [Ca²⁺]) were 10⁻⁹ M (relaxing solution) and 10⁻⁴.5 M for activating solution. The apparent stability constants for Ca²⁺-EGTA were corrected for temperature and ionic strength (Fabiato 1988). The computer program of Fabiato (Fabiato 1988) was used to calculate the concentration of each metal, ligand and metal-ligand complex. Immediately preceding each activation, the fibre was immersed for 10-20 s in a solution with a reduced Ca²⁺-EGTA buffering capacity (Moisescu & Thieleczek 1978). The solution was identical to the relaxing solution except that EGTA was reduced to 0.5 mM, which resulted in more rapid attainment of steady tension during subsequent activation and helped to preserve the regularity of cross-striations during activation.

The maximum velocity of unloaded shortening (V₀) was measured by the slack-test procedure (Fig. 5) (Edman 1979) at 15 °C. When a steady tension was reached in the activating solution (pCa 4.5) slacks (ΔL) of amplitude between 7-13% of the fibre segment between the connectors were rapidly (1-2 ms) applied. The time (Δt) required to take up the imposed slack was measured from the onset of length step to the beginning of tension redevelopment. The fibre was re-extended after each slack while relaxed in order to minimize non-uniformity of sarcomeres. V₀ was calculated as the slope for a straight regression line, using at least four measurements. Maximum active tension (P₀) was calculated as the difference between total tension in the activating solution (pCa 4.5) and the resting tension measured in the same segment while in the relaxing solution. Fibres were excluded
from analysis if $r^2$ for the fitted line was less than 0.96, if $P_0$ changed more than 10% from the first to the final activation, or if SL during isometric tension development changed by more than 0.10 µm compared with SL when the fibre was relaxed, according to established acceptance criteria (Moss 1979).

Fig.5. Single muscle fibre contractile recordings. (a) Plot of slack-test data, i.e. length step ($\Delta L$) vs. Duration ($\Delta t$) of unloaded shortening ($r^2 = 0.96$). (b) and (c) show the seven superimposed length and force records respectively. (d) Original time-base recording of tension at maximum level of Ca$^{2+}$ activation.

**Fluorescent labelling of the fibres (I, II, III, IV)**

Twelve to fifteen skinned single fibre segments from each individual biopsy were dissected free from the muscle bundle. Each fibre was attached to the connectors of an experimental device, leaving a length of approximately 1-2 mm of the fibre segment exposed to the media in an incubation chamber. The chamber was created on a borosilicate cover glass 24 × 50 mm (VWR International) with the help of a hydrophobic marker (Daido Sangyo Co. Ltd., Tokyo, Japan). The incubation chamber was filled with approximately 50 µL relaxing solution. The skinned fibre segment was attached on both ends parallel to the floor of the incubation chamber. Connectors similar to the ones originally described by (Moss 1979) were used. The connectors
were attached to 3D manipulators allowing orientation of the segment and adjustment of the sarcomere length. During fibre preparation and attachment, care was taken to avoid any contact between the fibres and the underlying cover glass. The fibres were incubated for 10 min in 0.1% Triton X-100 (Sigma Aldrich Chemie GmbH, Munich, Germany) in relaxing solution. After a brief wash in relaxing solution, the fibres were incubated for 45 min in rhodamine phalloidin (1:200; Invitrogen, Molecular probes, OR, USA) in relaxing solution containing protease inhibitors (1:1000, P 8340 Protease Inhibitor Cocktail; Sigma-Aldrich Inc, Stockholm, Sweden). The fibres were again washed 3 × 5 min in relaxing solution before incubating for 5 min with 4,6-diamino-2-phenylindol (DAPI) (1:1000 in relaxing solution; Invitrogen, Molecular probes), followed by a final wash for 5 min in relaxing solution prior to data acquisition. All incubation steps and fibre measurements were performed at room temperature.

Satellite cells (III)

Satellite cells were identified in three muscle fibres from two young men (21 and 31 years), two old men (71 and 78 years) and one old women (89 years) and were incubated for 1 h with the Pax7 primary antibody (1:10; DSHB, Iowa City, IA, USA) and rhodamine phalloidin (1:200; Invitrogen, Molecular probes), diluted in relax solution containing protease inhibitors (1:1000; P 8340 Protease Inhibitor Cocktail, Sigma-Aldrich Inc). The Pax7 primary antibody was visualized using an Alexa-488-conjugated secondary antibody (DAKO, Denmark). The fibres were subsequently washed 2 × 5 min in relaxing solution before incubating for 5 min with 4,6-diamino-2-phenylindol (DAPI) (1:1000 in relaxing solution; Invitrogen, Molecular probes), followed by a final wash for 5 min in relaxing solution prior to data acquisition.

Laminin staining (III)

Laminin are a network of proteins in the basal lamina which were stained to differentiate non-muscle nuclei from myonuclei and satellite cells. Twenty muscle fibres from a young man (22 years old) and an old man (78 years old) were permeabilized for 10 min with 0.1% (v/v) Triton X-100 in relax solution. The fibres were incubated for 1 h with primary antibody PC128 (1:1000; The binding site Ltd, Birmingham, UK) at 37 °C followed by washing (2 × 5 min) in relax solution and another incubation in secondary antibody (1:100, donkey anti-sheep CY5; Invitrogen) for 1 h at 37 °C and in the dark. Both antibodies were diluted in relax solution containing protease inhibitors (1:1000, P 8340 Protease Inhibitor Cocktail; Sigma-Aldrich Inc). The secondary antibody was conjugated with Alexa flour 647 for visualization of PC128. The fibres were again washed 2 × 5 min in relaxing
solution before incubating for 5 min with 4,6-diamino-2-phenylindol (DAPI) (1:1000 in relaxing solution; Invitrogen, Molecular probes), followed by a final wash for 5 min in relaxing solution prior to imaging. Fibre segments were scanned and analyzed in 3D to visualize the exact location of nuclei in relation to laminin signal. At the end of the experiment, the fibres were placed in SDS sample buffer for subsequent electrophoretic separation of MyHC isoforms.

Image acquisition (I, II, III, IV)
The experimental chamber was mounted on the stage of a Zeiss confocal microscope (LSM510 Meta; Zeiss, Jena, Germany) equipped with a Plan-Neofluar 20×/0.5 objective. By adjusting the overall fibre length, the sarcomere length was set to 2.74 ± 0.11 µm (humans) or 2.79 ± 0.01 µm (mice), corresponding to the optimal sarcomere length for force generation (Burkholder & Lieber 2001). The length of each fibre segment captured was 450 µm. Three-dimensional stacks were created by collecting consecutive images of the whole fibre length, from the top to the bottom of the fibre. For detection of nuclei by DAPI, the laser line 405 nm was used with HFT 404/514 and BP filter 420-480 nm. The phalloidin-rhodamine-labelled actin was detected by laser line 543 nm, HFT 488/543 and LP 550 nm emission filter. The satellite cells stained by pax-7 were detected using laser line 488 nm, NFT 490, BP 505-530 nm emission filter. The basal lamina stained by PC128 antibody was visualized by using a laser line 633 nm, HFT 488/543/633 and LP 650 nm emission filter. The optical slice and interval was set to 4.3 µm for all detection channels. The data was collected at a depth of 8 bit. Three-dimensional (3D) image volumes were reconstructed using the LSM 510 Meta Zeiss software (Carl Zeiss, Jena, Germany). The final proportions for the reconstructed image elements were set to 0.45 × 0.45 × 0.90 µm³ to standardize the subsequent data analysis. The 3D spatial coordinates of each nucleus were detected automatically by means of Imaris (version 5.7.2) graphical software and then controlled manually. Myonuclear number was determined by counting all the myonuclei in the stack for the region analyzed and converting them into myonuclei per millimetre. Easily recognized blood vessels and connective tissue nuclei with 3D coordinates outside the cell volume were not taken into account. Approximately 15% of all fibres were discarded from the analysis, because of suspected damages of the myonuclei or inconsistent fluorescent signal. The cytoplasmic volume and size of the MNDs were determined by using custom-made software, developed at the Centre for Image Analysis, Uppsala University and Swedish University of Agricultural Sciences.
The fibre was modelled through digital image analysis of the 3D fibre image volume. A linear model based on a general elliptical cylinder (GEC) was developed, which allowed for a robust segmentation of the fibre, and a parametric representation of the surface (III, Fig. 9A). The GEC, together with the nuclei centroids, was used to calculate the volumes of the MNDs, the cross-sectional area (CSA) of the fibre and the shared surface between the MNDs, and fibre (III, Fig. 9B). The fibre CSA was also determined manually by measuring the thickness and the width of the fibre by using built-in Zeiss software. By assuming an elliptical shape of the fibre, the fibre volume was calculated using the fibre length determined from the image. The volume values differed by 1–3% between the two methods. The 3D coordinates for every nucleus centre were controlled and, when needed, adjusted manually in the Imaris 5.7.2 software. A nearest neighbour (NN) measurement was also used to assess the level of myonuclei organization.

The GEC fibre model is constructed in a multi-step approach so that any excess anisotropic signal from the fibre can be compensated for. First, the dimensions of the fibre volume are arranged so that the $y$-axis is oriented along the length of the fibre, i.e., browsing through the fibre cross-sections are equal to browsing in the $y$-axis. Secondly, the image elements in the volume (voxels) are merged into cubic voxels of dimension $0.9 \times 0.9 \times 0.9 \text{ m}^3$, by averaging together four adjacent voxels, and their intensities, for each $xy$-plane. A linear model of a general fibre is applied to the image data. The modelling represents a segmentation of the fibre from the background and allows for parametric representation of the fibre surface. The modelling is carried out by first creating a weight volume $G$ to weight the original image intensities, before the parameters representing a fibre model are extracted from the weighted fibre volume. The parameters needed to represent the GEC is the centre points at each end of the fibre, the lengths of major and minor axis for an ellipse at each end of the fibre, and the angle between the major axes and the $x$-axes at each end of the fibre (III, Fig. 10A).

In the first step, the fibre volume is traversed slice-wise along the $y$-axis, and a fuzzy c-means (FCM) (Bezdek 1981) thresholding is applied to every $xz$-slice individually. FCM classifies all grey level pixels, in every slice individually, into two clusters representing foreground (fibre), and background (III, Fig. 10B). The two clusters are then separated by a threshold in between. The pixels in the current $xz$-slice, with grey levels above the threshold, are considered to contain information about the stained...
muscle fibre, and the excess anisotropic signal. The main reason why FCM is applied slice-wise along the $y$-axis is twofold. First, the FCM method always requires an input signal, which is the case along the fibre. Secondly, the signal variation for a subset of adjacent slices is low, even though a single slice may contain low signal intensity. Thus, the FCM output should be similar, providing robustness to the model. Unfortunately, the foreground cluster will also include pixels that are not part of the fibre but are part of the signal artefact. For each segmented fibre slice region, a centre point, major axis length, minor axis length and orientation of the major axes (in relation to the $x$-axis) are calculated. The $z$-positions of the centre points are likely to be overestimated, with regard to the signal artefacts along the $z$-axis. The five arrays (of length $y$) with the centre point $c$ (coordinates in three dimensions), the major ($a$) and minor ($b$) axes (lengths), and the orientation $\theta$ (angle) are each fitted with a line in the least square sense. The five linear parameter arrays describe a linear model of the fibre, and the artefact. An ellipse centred on the centre point $c$ is defined by,

$$\frac{(x-c_x)^2}{a^2} + \frac{(z-c_z)^2}{b^2} = 1,$$

where a rotation of $\theta$ degrees around the centre of the ellipse is a separate operation. To counter the effect of the artefacts in the $z$-direction, the initial linear model is used to create a normally distributed weight volume $G$ of the interior and exterior of the fibre (III, Fig. 10C). Volume $G$ is then used to weigh the grey levels of the original fibre volume, with the intention to lower the effect of the artefacts in the $z$-direction. To accomplish this, $G$ is shifted down, in the $z$-direction, by $\delta z$ voxels. A single slice $G_y$ of the model $G$ before rotation is defined as,

$$G_y(x, y) = \exp \left[ -\frac{1}{2} \left( \frac{(x - c_{xy})^2}{a_y^2} + \frac{(z - c_{yz} - \delta_z)^2}{b_y^2} \right) \right],$$

where $(x, z)$ are the pixel coordinates in slice $y$, $c_{xy}$ and $c_{yz}$ are the $x$ and $z$ coordinates, respectively, of the centre points of $G_y$, $a_y$ is the length of the major axis in slice $y$, and $b_y$ is the length of the minor axis in slice $y$. Every $G_y$ slice is then rotated according to the angle given by first degree polynomial approximated orientation of the major axes, in relation to the $x$-axis.

In the second step, the grey levels (normalized to $[0, 1]$) in the original fibre volume are weighted with $G$, element by element. Finally, the FCM method is applied to the product. The pixels belonging to the fibre get a
membership value closer to 1 (one), and pixels belonging to the background get a membership value closer to 0 (zero). The output from the FCM method is segmented into fibre and background by thresholding the slice at threshold in between the two clusters. From the pixels determined to belong to the fibre, the centre points, major and minor axes, and the orientation of the major axes in relation to the x-axis are again calculated. The extracted values of the five parameters, for all slices, are again fitted with lines in a least squares sense. The final model, i.e., the GEC, is then generated from these parameter values (III, Fig. 10D). The GEC is stored as a binary volume with the same size as the down-sampled original fibre volume, where voxels with the value 1 (one) belong to fibre, and voxels with the value 0 (zero) belong to the background. The GEC is then used together with the predetermined nuclei positions to calculate high level features.

Feature extraction

An MND is defined as all voxels within the fibre that are closest to an individual nucleus. For every nucleus, a MND is calculated by starting at the centre points of each nucleus, and calculating the distance to the surrounding voxels using a distance transform (DT) with an Euclidean metric (Friedman et al., 1997). The value in each voxel in the DT-volume contains the distance to the closest nucleus. The DT-volume is then segmented using a watershed algorithm (Vincent et al. 1991) that partitions the DT-volume into labelled regions, i.e., the MNDs. The volume containing the MNDs (MND) is made up of voxels with integer values (labels) denoting which nuclei a particular voxel has been assigned. The background outside the fibre is assigned the label 0 (zero). The MNDs within the MND that share one, or more, sides with the borders of the image volume are identified and their labels are stored separately. The individual volumes of the MNDs are calculated by counting all voxels for each label, except for the background, and disregarding the labels indicating that the domain is in contact with the image volume. The resulting array elements are multiplied with the volume of a voxel \((0.9 \times 0.9 \times 0.9 \, \text{m}^3)\) before interpretation. The MNDs that touch the border of the image volume are not counted, because they most likely are overestimated because of the lack of knowledge of the positions of nuclei outside the image volume.

The contact area between an individual MND and the surface of the fibre is calculated by using weighted surface elements (Lindblad 2005) to avoid overestimation of the surface measurement. The algorithm measures the surface areas shared between the fibre and the MNDs (Axelsson et al. 2008). Again, domains that are in contact with the border of the image volume are disregarded, and the measured surfaces are multiplied with the voxel side area \(0.9 \times 0.9 \, \text{m}^2\).
The cross-section of the GEC varies along the length of the fibre. An average cross-section for each image volume is calculated by dividing the volume of the GEC with the length of the fibre volume along the y-axis. The measured average cross-sectional area is multiplied with the voxel side area $0.9 \times 0.9 \, \mu m^2$.

The set of distances from every nucleus in an image volume to its nearest neighbour is calculated using the manually extracted coordinates (in 3D) from the nuclei volume image. The resulting array contains the distance values to nearest neighbours and the measured values are multiplied with the voxel side length 0.9 µm.

Proximity ligation assay (II)

A proximity ligation assay was performed to quantify myosin using the Duolink in situ PLA kit (Olink Bioscience, Uppsala) according to the manufacturer’s instructions. Muscle’s cross-sections from control, $Mstn^{-/-}$ and $mIgf1^{+/+}$ mice were either fixed on the same glass slide or on different glass slides using 2 % paraformaldehyde. MF-20 (1: 5, DSHB, Iowa, USA) was used as primary antibody. Slides were examined with a confocal microscope (LSM510Meta; Zeiss, Jena, Germany) under a X40 objective and the analysis of the fluorescent signal was performed using Imaris software (version 5.7.2). Assessment of pixel counts was done after applying a constant threshold and selectivity value. Three X40 fields were randomly chosen for analysis and averaged per condition examining three different samples separately. Whether the sections were on the same slide or not did not yield any different results.

Enzyme Histochemistry (II)

Frozen samples from the soleus muscles from all three groups were cut perpendicular to the longitudinal axis of the muscle fibres into 10µm cross-sections with a cryotome (2800 Frigo-cut E, Reichert-Jung GmbH, Heidelberg, Germany) at -20 °C. The sections were stained for the demonstration of myofibrillar ATPase activity after alkaline (10.3) and acid (4.5) pre-incubations and classified into type I, IIA and IIB fibres. Succinate dehydrogenase (SDH) staining was performed as described elsewhere (Dubowitz 1985).

SDS-PAGE (I, II, III, IV)

The MyHC composition was determined after the mechanical measurements (II, IV) and confocal scanning (I, II, III, IV) by 6% SDS-PAGE. The total acrylamide and bis concentrations were 4% (w/v) in the stacking gel and 6% in the running gel, and the gel matrix included 30% glycerol. The
ammonium persulphate concentrations were 0.04% and 0.029% in the stacking and separation gels, respectively, and the gel solutions were degassed (<100 millitorr) for 15 minutes before casting. Polymerization was activated by adding TEMED to the stacking (0.1%) and separation gels (0.07%). Sample loads were kept small to improve the resolution of the MyHC bands and electrophoresis was performed at 120 V for 22-24 h with a Tris-glycine electrode buffer (pH 8.3) at 15 °C (SE 600 vertical slab gel unit, Hoefer Scientific Instruments, USA). For details, see (Larsson et al. 1993).

Homogenate electrophoresis (II, IV)
The MyHC contents of the biopsy samples (II, IV) were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described elsewhere (Andersen & Aagaard 2000) with slight modification. For the analysis, 10-15 cryosections (10 µm) from each biopsy were placed into 700 µL of lysine buffer containing 10% (vol/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol, 2.3% (mass/vol) SDS in 62.5 mM Tris HCl buffer (pH 6.8) and heated for 10 min at 60 °C. A small amount of the muscle extracts (3-10 µL) was loaded into each lane of the SDS-PAGE gel system consisting of stacking gel with 3% acrylamide and separating gel with 6% acrylamide and 30% glycerol. The gels were run on an electrophoresis device (Bio-Rad protean II xi Cell) at 4 °C at a constant voltage of 70 V for 42 h. Following the run, the gels were fixed for 24 h in 5% acetic acid and 50% methanol, stained using coomassie blue, and destained in 7.5% acetic acid and 5% methanol overnight or until the background was clean. In the stained gel, distinct protein bands could be separated and identified as MyHC I, IIa, IIb, IIX (mice, II) or I, IIa, IIX (humans, IV) isoforms according to their migration characteristics. The relative proportion of each MyHC isoform in a biopsy sample was determined using a densitometric system (Cream 1D, Kem-En-Tec aps, Copenhagen, Denmark).

Statistical analysis (I, II, III, IV)
Means, standard errors of mean (SEM) and standard deviations (SD) were calculated from individual values by using standard procedures. Data normality was tested according to Kolmogorov-Smirnov test. When the normality test failed, the Mann-Whitney rank sum test was used to test for the difference. The relationship between MND size and body mass was described by log-log plot (I). The equation \( y = ax^b \) was used to describe these relationships, where the exponent of the equation, \( b \), was the scaling factor (I). Two-way analysis of variance (ANOVA) was used to test for the effects of the ageing and gender (III) as well as of CSA, MND size and force measurements (II) across different groups. Whenever a significant difference
was found, Holm-Sidak *post-hoc* test was used for multiple pair wise comparisons (II, III). Linear regression analysis was carried out across individual fibres pooled into groups based on MND size and CSA (II) or gender, fibre type and age (III). Student’s paired-sample *t*-test was used to compare fibre CSA, MND and contractile measurements between mean values of subjects pooled together according to HRT status (IV). Statistical significance was set at *p* < 0.05 for all analysis.
Results and Comments

Age and physical characteristics (I, II, III, IV)

The animals and human subjects (I, II) were young adults and free of musculoskeletal disorders, except the rhinoceros which was middle-aged and had a history of chronic pedodermatitis which was clinically controlled.

In study III, height and body weights for young and old sedentary men and women (III, Tab. 1) had a significant age × gender interaction (p < 0.05), i.e., men were taller and heavier than women irrespective age, young men and women were taller than old while old women were heavier than young women.

In study IV, the mean age of the participants in twin study (IV) was 56.6 ± 1.3 years (range, 55-59 years). No difference was found in physical activity, smoking behaviour or alcohol use between the HRT users and non-user twins.

Comments: Young subjects were chosen (I, II) to avoid the influence of ageing on muscle (Prochniewicz et al. 2005; Degens 2007) and myonuclei (Bruusgaard et al. 2006). The life style characteristics of subjects (III, IV) were matched to avoid the influence of confounding factors.

Accepted fibres (I, II, III, IV)

Approximately 15% of all muscle fibres stained for confocal imaging (I, II, III, IV) were excluded from analysis due to suspected damage/loss of myonuclei, inconsistent fluorescent signal and/or easily recognizable blood vessels and connective tissue nuclei on the muscle fibre surface.

For contractile recordings (II, IV) the fibres were discarded if they failed to meet the acceptance criteria (Moss 1979). In general, fast-twitch fibres were over-represented among discarded fibres and these observations were independent of hypertrophic stimulus (II) or HRT status (IV).

Comments: Special care was taken to avoid fibre contact with the incubation chamber, in order to preserve the myonuclear arrangement and number. Fibres with suspicion of non-muscle nuclei were not included since they can give rise to errors in nuclei count.

The criteria originally used by Moss (Moss 1979) was used to determine which contractile recordings should be included in analysis.
Single muscle fibre size and MyHC isoform expression (I, II, III, IV)

Muscle fibre volume was dependent on body size and a significant linear log-log relationship was observed between fibre volume and body size, with smaller species having the smaller muscle fibre volume (I, Tab. 2). Some deviations from the scaling effect were also observed, such as the largest muscle fibre volumes were observed in pig fibres expressing type I and type IIa MyHC isoforms despite a body size smaller than horses and rhinoceroses.

In hypertrophic mice (II), both experimental groups showed a significant increase (p < 0.05) in CSA in EDL fibres and type IIa fibres from soleus when compared to control (II, Tab 2). The hypertrophy was more prominent in muscle fibres expressing the IIa MyHC isoform from soleus (65 and 45% in Mstn−/− and mIgf1+/− mice, respectively) than IIb fibres from EDL (34 and 39% in Mstn−/− and mIgf1+/− mice, respectively).

In human muscle fibres (III), CSA of the type I fibres showed a significant increase (p < 0.001) in old men (23%) and old women (38%) while type IIa fibres showed a significant decrease (p < 0.001) in old men (31%) and old women (15%) compared to young men and women, respectively. This resulted in an ageing-related decrease (p < 0.01) in type IIa/I muscle fibre area ratio independent of genders. HRT had no effect on single fibre CSA or relative CSA in muscle fibres expressing slow or fast MyHC isoforms in twin sisters (IV).

Muscle fibres expressing type I, IIa, I/IIa, IIax, IIxb and IIb MyHC isoforms were isolated from the six species (I, Tab 1). The relative distribution of different MyHC expression shows that the smallest mammals had a faster phenotype than large mammals, with a predominance of fast-twitch fibres. In contrast, rhinoceros and horse primarily contained muscle fibres expressing the type I and type IIa MyHC isoforms. In Mstn−/− and mIgf1+/− mice, a trend towards a more fast phenotype was observed than in the controls (II, Tab. 3), but this difference failed to reach statistical significance.

In human muscle an ageing-related increase (p < 0.05) in the relative number of fibres expressing the type I MyHC isoform was observed in both men and women (III) while HRT had no significant effect on the relative content of MyHC isoforms (IV).

Comments: All single fibre CSA recordings were done at a fixed sarcomere length optimal for force generation (Burkholder & Lieber 2001). This has an advantage over CSA measurements from cross-sections where sarcomere length and CSA can be highly variable leading to erroneous measurements (Larsson & Skogsberg 1988). The major disadvantage in CSA measurements from single fibres is the small sample size. Muscle fibre CSA was measured assuming an elliptical circumference and was corrected for 20% swelling known to occur during the skinnng procedure (Moss 1979).
Single muscle fibre contractile properties (II, IV)

In the mice EDL, absolute force ($P_0$) did not differ significantly in $Mstn^{-/-}$ and $mIgf1^{+/+}$ mice when compared to controls (II, Fig. 5). $P_0$ normalized to single fibre CSA (specific tension) was lower ($p < 0.05$) in $Mstn^{-/-}$ mice but not in $mIgf1^{+/+}$ mice. In the soleus, muscle fibres expressing the type I MyHC isoform from both experimental groups had a similar $P_0$ when compared to controls. While the hypertrophic muscle fibres expressing the type IIa MyHC isoform had a 55% and 48% higher ($p < 0.05$) $P_0$ in $Mstn^{-/-}$ and $mIgf1^{+/+}$ mice, respectively, but the specific force was unchanged when compared to controls. A 40% lower ($p < 0.05$) stiffness value was recorded in EDL fibres from $Mstn^{-/-}$ mice while the stiffness values in soleus muscle fibres did not differ between control and experimental groups (II, Fig. 5). A 23% reduction in motor protein myosin content was observed in EDL muscle from $Mstn^{-/-}$ mice while no difference in myosin content was observed in EDL muscle from $mIgf1^{+/+}$ mice and soleus muscle from both experimental groups when compared to controls.

Specific force was higher in the HRT user group in muscle fibres expressing type I (~27%, $p < 0.05$) and type IIa (~23%, $p < 0.05$) MyHC isoforms (IV, Fig. 3A). In the muscle fibres expressing the type I MyHC isoform, the slightly higher stiffness (~13%) in the HRT user group was not statistically significant suggesting that the force per cross-bridge is higher in the HRT user group. On the other hand, in muscle fibres expressing the type IIa MyHC isoform, the ~17% increase in stiffness suggests that an increase in the number of cross-bridges contributes primarily to the higher specific force in the HRT user group (IV, Fig. 3B). Maximum velocity of unloaded shortening did not differ between HRT user and non-user groups.

Comments: Stiffness recordings represent an index of number of strongly attached cross-bridges in series (Regnier et al. 2004) and were performed once steady-state isometric tension was reached. A strict acceptance criteria (Moss 1979) was applied while doing contractile recordings and the fibres not meeting the criteria were discarded from analysis.

Phenotypical observations (I, II, III, IV)

Myonuclei were presented with large variations in the spatial organization and shapes, and were shown to be dependent on MyHC isoform expression and species type. The smallest mammal, i.e., mice, presented with predominantly elliptical shaped nuclei in muscle fibres expressing fast-twitch MyHC isoform with the long axis of nuclei typically aligned with the long axis of the fibre (I, Fig. 4 & II, Fig. 1). On the other hand, in muscle fibres expressing the slow-twitch MyHC isoform myonuclei with a rounder appearance dominated (II, Fig. 1), although both shapes of myonuclei were
observed in the same fibre segment independent of MyHC isoform type. Pig muscle fibres presented with long thin nuclei compared to smaller mammals while most nuclei from horse muscle fibres had a round appearance (I, Fig. 4).

In humans, myonuclei were typically rounded or elliptical independent of age, gender, fibre type and HRT status (III, Fig. 2 & IV, Fig. 1). Old age was associated with deviations from the rounded or elliptical shape in some, but not all fibres, with myonuclei presenting indentations of the nuclear envelop (III, Fig. 1C). Aggregates of nuclei were observed in the elderly, irrespective of gender and fibre type, but not in the young (III, Fig. 2) leading to an increase in myonuclear domain size variability in old age (III, Fig. 6).

In the hypertrophic mice (II), internal nuclei constituted 10% of all nuclei and 40% of nuclei in muscle fibres expressing the type IIb MyHC isoform in EDL muscle fibres from mIgf1+/+ mice. Internal nuclei, on the other hand, were rare in soleus muscle fibres and in EDL muscle fibres from control and Mstn−/− mice. In human muscle fibres (III, IV) occurrence of internal nuclei was rare and it is premature to relate them to age, gender, MyHC isoform type or HRT status due to paucity of internal nuclei.

Comments: In order to rule out the possibility of non-muscle nuclei being erroneously counted as myonuclei, a 3D immunolabelling of muscle fibre was performed for nuclei and basal lamina (III). All labelled nuclei in young and old subjects were myonuclei, i.e., they were all inside the basal lamina of the muscle fibre. Satellite cells were rarely observed in single muscle fibre segments (I, III) and it is highly unlikely that they would influence observations based on myonuclei appearance and count.

Myonuclei count and MND size (I, II, III, IV)

Body size and species had a significant effect (p < 0.001) on myonuclei count per unit fibre length in muscle fibres expressing type I and type IIa MyHC isoforms (I, Tab. 2). Pigs always had the highest myonuclei number (p < 0.001) in type I and type IIa fibres, while mouse had the lowest myonuclei count per unit fibre length. On the other hand, hypertrophy resulted in an increased myonuclei count per unit fibre length only in EDL muscle fibres from mIgf1+/+ mice while EDL muscle fibres from Mstn−/− mice and soleus fibres from both experimental groups had no change in myonuclei count in both experimental groups when compared to controls (II, Tab. 2).

In human muscle fibres (III) expressing the type I MyHC isoform, an increased number of myonuclei was observed in elderly irrespective gender (p < 0.001). Muscle fibres expressing type IIa MyHC isoforms, on the other hand, had a significant ageing and gender effect, i.e., old women had an increased number of myonuclei compared with young women (III, Fig. 3).
HRT status had no effect on myonuclei count per unit length in type I and type IIa muscle fibres from HRT discordant post-menopausal twins (IV).

In all species (I), MND size was dependent on MyHC isoform expression, i.e., the smallest MNDs were observed in type I and type IIa fibres which are characterized by their high endurance and mitochondrial activity (I, Tab. 2). MND size was scaled to body mass in log-log plots and a strong relationship was found between the two in muscle fibres expressing type I ($r = 0.84$, $p < 0.001$) and type IIa ($r = 0.91$, $p < 0.001$) MyHC isoforms. Some deviations from the scaling effect were seen, i.e., type IIa fibres from pig had larger MNDs than in horse and rhinoceros despite the fact that pig had a smaller body size. However, it must be noted that pigs were bred for meat production with an influence on muscle size.

In hypertrophic mice (II), an unchanged MND size was preserved in EDL muscle fibres from $mlgf1^{+/+}$ mice, the only fibre type population showing a significant increase in myonuclei number per unit length with hypertrophy (II, Tab. 2). In contrast, EDL muscle fibres from $Mstn^{-/-}$ mice and type IIa soleus muscle fibres from both experimental groups showed a significant increase ($p < 0.05$) in MND size as a result of hypertrophic fibres without a change in myonuclei count.

In human muscle fibres (III), MND size was not affected by ageing in type I fibres while type IIa fibres had significantly ($p < 0.001$) smaller MNDs in elderly men and women (III, Tab. 2). The MND size plotted against muscle fibre CSA showed a significant linear relationship in both genders irrespective age and MyHC isoform type ($r^2 = 0.20-0.62$, $p < 0.05-0.001$, III, Fig. 5). Thus the smaller MNDs in type IIa fibres represent an ageing-related preferential atrophy of these fibre types. In the twin study (IV), type I fibres showed a 27% smaller ($p < 0.05$) MND size in HRT users than their non-user counterparts (IV, Fig. 2A). This was due to the combined effects of trends, albeit not statistically significant, of both smaller fibres and a higher myonuclei number in HRT users. In muscle fibres expressing the type IIa MyHC isoform, MND size did not differ significantly between HRT users and non-user twins.

Type I fibres had smaller MNDs than type IIa fibres within the same species except the two smallest mammals, the rat and mouse where MND size did not differ between type I and type IIa fibres (I, Tab. 2). In contrast to larger mammals, rodents have higher mitochondrial activity in type IIa than type I fibres (Nemeth & Pette 1981), suggesting that mitochondrial proteins beside MyHC isoform type may be important determinants of MND size in smaller mammals. Thus, serial cross-sections from control, $Mstn^{-/-}$ and $mlgf1^{+/+}$ mice were assessed for SDH activity of type I and IIa fibres identified by mATPase staining. All three groups showed deeper staining of type IIa than type I fibres despite the fact that MND size was bigger in type IIa fibres from $Mstn^{-/-}$ mice, suggesting that mitochondrial content may not
be the primary factor determining MND size, at least not in *Mstn*−/− mice muscle fibres.

**Comments:** All MND size measurements were performed at a fixed sarcomere length optimal for force generation (Burkholder & Lieber 2001). This has advantages over measurements taken from cross-sections where sarcomere length and fibre CSA can be highly variable. Further, altered shape and aggregates of myonuclei (III, IV) may lead to an over-estimation of nuclei from cross-sections and both these factors may lead to erroneous measurements of MND size and nuclei count. Myonuclei close to terminal part of the muscle fibre segment were either omitted (I, II, III) or half of them randomly selected for analysis (IV) since their domains may extend outside the fibre segment and give rise to errors in MND size measurements.
Discussion

What determines myonuclear domain size? (I, II, III, IV)

MND size appears to differ between different fibre types and in accordance with previous studies, a smaller MND size was observed in muscle fibres expressing the slow MyHC isoform (I, II, III). Slow-twitch fibres have a higher protein turnover rate, with a half life of 7-8 days, almost half that of fibres expressing fast-twitch MyHC isoforms (Kelly et al. 1984), hence a demand for higher mRNA transcription and a greater concentration of myonuclei. Furthermore, MND size is inversely correlated with muscle fibre oxidative capacity and mitochondrial contents (Tseng et al. 1994). In human muscle, mitochondrial density is higher in fibres expressing type I MyHC isoform followed by type IIa and type IIx fibres. However, this notion is not observed in smaller mammals such as rodents where muscle fibres expressing type IIa MyHC isoforms have higher mitochondrial enzyme activity than type I fibres (Zot & Potter 1982). This was associated with slightly smaller or equal MND size in type IIa fibres of rat and mouse compared to type I fibres (I, II), suggesting that mitochondrial proteins beside MyHC isoform type may play a role in determining MND size. However, Mstn\(^-\) mice (II) had significantly larger MND size in type IIa than type I fibres, despite the fact that SDH staining was stronger for type IIa fibres in serial cross-sections. Thus, mitochondrial proteins may not be the major determinant of MND size, at least not in Mstn\(^-\) mice.

It should be noted that MND size is not ‘fixed’ in a given fibre type in a certain species and varies with changes in cell size, for instance during altered functional demand (II), ageing (III), or under the influence of hormone therapy (IV).

Myonuclear domain size increases with increasing body mass (I)

A strong linear relationship was found between MND size and body mass for the two muscle fibre types observed in all six species, i.e., muscle fibres expressing type I and type IIa MyHC isoforms. This is explained by increased metabolic and functional demands and higher protein turnover rates in small compared to large mammals (With 1960; Weibel & Hoppeler
The higher metabolic rate is the result of greater mitochondrial volume in muscle fibres of small mammals. For instance, mitochondrial volume can be up to 35% of fibre volume in smaller mammals (Mathieu-Costello et al. 2002) compared with 2-5% of fibre volume in human muscle fibres (Howald et al. 1985). Although body size and mitochondrial volume had a strong scaling effect on MND size in type I and type IIa fibres, some deviations from the scaling effect were also observed. For instance, muscle fibres expressing the type IIa MyHC isoform from pigs had larger MND sizes than in horse and rhinoceros despite the fact that the body size was smaller in the pig. However, MND size is also dependent on fibre size and pigs had the largest muscle fibres expressing type IIa MyHC isoform among the six species (I, Tab. 2). On the other hand, in muscle fibres expressing the type I MyHC isoform, MND size scaled with body mass despite the fact that the largest fibres were observed in the pig among the six species. Thus, the impact of fibre size and body mass on MND size seems to be fibre type dependent. However, it is also important to remember that the pig has been bred over many years for meat production with consequences for muscle fibre size and MND. In addition, it was recently shown that fibres from two different regions of the same muscle (van der Meer et al. 2011b) or different muscles from the same species (Mantilla et al. 2008) expressing similar MyHC isoform type may have significantly different MND size, but these differences most probably have a minor impact on deviations from scaling effects compared with the large body size range among the six species studied.

Thus, it is concluded that MND size scales with body size, but MyHC isoform expression and mitochondrial contents are also playing an important role in modulating MND size.

Functional hypertrophy requires extra myonuclei in fast- but not slow-twitch muscle fibres (II)

Specific force and MND size in hypertrophic EDL muscle fibres

MND size was larger in muscle fibres expressing fast- compared to slow-twitch MyHC isoforms confirming earlier observations (Bruusgaard et al. 2003; Bruusgaard et al. 2006). We report that these MNDs are optimally tuned for force generation and any additional enlargement of these large MNDs will result in compromised function as observed in Mstn−/− mice. In contrast, the hypertrophy in mIgf1+/− mice is accompanied by myonuclear accretion resulting in maintained specific force and MND size.

The fibre hypertrophy in Mstn−/− mice occurred without myonuclear incorporation resulting in enlarged MNDs. This is probably due to a lack of satellite cell activation in Mstn−/− mice as reported recently (Amthor et al. 2005).
The loss in specific force in skinned fibres is due to a decreased fraction of cross-bridges in strong bound state according to stiffness measurements and is supported by the proportional loss of the myosin content (II, Fig. 5). Skinned fibre preparations rely on the final functional contractile unit, i.e., the sarcomere for force generation and it is possible that other mechanisms such as a decreased mitochondrial density may also contribute to an in-vivo loss in specific force in myostatin deficient mice (Amthor et al. 2007).

In contrast, the hypertrophic mlgf1+/+ mice show a proportional increase in size and force resulting in maintained specific force, as reported in the transgenic mice model over-expressing IGF-1 (Gonzalez et al. 2003; Colombini et al. 2009). There was an increase in myonuclei count in these mice, but the rate of myonuclear incorporation lags behind the increase in muscle size, resulting in 10% larger MNDs compared to control. We suggest that the resulting MND size of 31,700 µm³ is probably close to the maximum transcriptional capacity of the myonucleus in order to maintain an optimum of functioning myofibrillar proteins in its cytoplasmic territory.

Specific force and MND size in hypertrophic soleus muscle fibres
The slow-twitch soleus has a higher density of myonuclei and smaller MND size than the fast-twitch EDL muscle fibres. The mouse soleus is primarily composed of fibres expressing type I and type IIa MyHC isoforms. Despite the more prominent hypertrophy in type IIa fibres from Mstn-/- and mlgf1+/+ mice, the nuclei count per unit fibre length remained unchanged in both fibre types, resulting in a strong linear relationship between MND size and fibre CSA in both experimental groups (II, Fig. 4).

Specific force was unchanged in hypertrophic soleus muscle fibres which confirms previous observations in myostatin-null (Mendias et al. 2006) and mlgf1+/+ mice (Del Prete et al. 2008). Thus, an increase in MND size to ~24,000um³ does not compromise the force-generating capacity of muscle fibre nor the myonuclear transcriptional capacity and transport distances are still sufficient to maintain specific force and myosin content.

In accordance with observations in myostatin-null mice, lack of myonuclear incorporation in both experimental groups indicates a hypertrophy without satellite cells activation (Zhu et al. 2000; Amthor et al. 2009). The hypertrophy of soleus muscle fibres in mlgf1+/+ mice is surprising since IGF-1 transgene expression has been reported to be restricted to fast-twitch muscle (Musaro et al. 2001). We cannot rule out compensatory hypertrophy of the soleus muscle due to hypertrophy of the fast-twitch antagonists, however this is a less likely mechanism since satellite cell activation is typically associated with compensatory hypertrophy (Snow 1990; Rosenblatt et al. 1994). Nevertheless, satellite cell-independent hypertrophy has been reported in IGF-1 over-expression mice in 2009; Matsakas et al. 2009).
differentiated muscles (Barton-Davis et al. 1999) as well as in cell culture (Quinn et al. 2007).

Thus, myostatin deficiency and IGF-1 over-expression have different and muscle specific effects on the force generating capacity and myonuclear organization at the single muscle fibre level. MNDs in the fast-twitch EDL muscle fibres are optimally tuned for force generation and hypertrophy must be accompanied by myonuclear incorporation to maintain myosin content and specific force. In contrast, MNDs from slow-twitch soleus muscle fibres have a broad dynamic range for hypertrophy with a proportional increase in force, without a need for extra myonuclei. These changes should be considered when developing therapeutic strategies to boost human muscle mass and strength.

Ageing is associated with an altered spatial organization and aggregation of myonuclei (III)

Myonuclei were arranged in long chains forming aggregates in muscle fibres from old men and women (III, Fig. 2). This is consistent with the observations in old mice (Bruusgaard et al. 2006) suggesting that the positioning of myonuclei in adult muscle fibre is not fixed (Ralston et al. 2006). The exact mechanism by which myonuclei are positioned in the muscle fibre is unknown; however many factors may contribute to these observed changes, such as microtubuli, desmin and blood vessels (Bruusgaard et al. 2006; Ralston et al. 2006). The ageing-related denervation-reinnervation process (Larsson & Ansved 1995) may play a role in formation of nuclear aggregates since long-term denervation can result in aggregation of myonuclei (Viguie et al. 1997). However, the ageing-related denervation-reinnervation process is reported in both slow- and fast-twitch motor units (Larsson & Ansved 1995) while an increased variability in nuclei nearest-neighbour distance was only observed in muscle fibres expressing the type I MyHC isoform (III, Fig. 7).

Ageing was associated with an increased number of myonuclei per unit length in muscle fibres expressing type I and type IIa MyHC isoforms irrespective gender (III, Fig. 3). However, an increased CSA of type I fibres was reported in old age and a correlation has been suggested between the nuclei count and age in young and old mice (Bruusgaard et al. 2006), but this correlation was weak in human muscle fibres and limited to women (III, Fig. 4).

In muscle fibres expressing the type I MyHC isoform, the average MND size was not affected by ageing despite an increased variability in myonuclei organization. This finding of an increased myonuclei variability and altered spatial organization probably represents a more important biological finding...
than the unchanged MND size. Increased MND size variability may be a contributing factor to an altered myofibriller protein synthesis in old age, since not all the nuclei are transcriptionally active in a muscle fibre at a given time (Newlands et al. 1998). Interestingly, localized mitochondrial abnormalities have been reported adjacent to a phenotypically normal portion of the same fibre segment (Cao et al. 2001; Oldfors et al. 2006). On the other hand, in muscle fibres expressing the type IIa MyHC isoform, a significant decrease in MND size was reported in old age which confirms earlier observations in fast-twitch mouse muscle (Brack et al. 2005). Thus, the smaller MNDs in type IIa fibres may represent a physiological adaptation of myonuclei to an ageing-related preferential fibre atrophy.

Consequently, an ageing-related, more heterogeneous distribution of myonuclei may decrease efficiency of the transcriptional machinery and inter-nuclear corporation leading to an impaired local protein turnover, hence directly influencing the amount and quality of contractile proteins.

**Hormone replacement therapy preserves the strength without affecting myonuclei count (IV)**

HRT has beneficial effects on specific force which is fibre type specific in terms of stiffness and myonuclear organization. In muscle fibres expressing the type I MyHC isoform, the increased fraction of strongly attached cross-bridges contribute to about 50% of the increase in specific force in HRT users (IV, Fig. 3A), suggesting that the force per cross-bridge accounts for the remaining increase in specific force. On the other hand, in fibres expressing the type IIa MyHC isoform, specific force and stiffness values are in good accord (IV, Fig. 3B), suggesting that an increased fraction of strongly attached cross-bridges primarily contribute to the increased specific force in HRT users. These findings are in accordance with Phillips’s hypothesis (Phillips et al. 1993) and EPR spectroscopy studies (Moran et al. 2007; Lowe et al. 2010) suggesting that the primary site of action of HRT is at the cross-bridge level. While myonuclei count and fibre CSA did not differ significantly between hormone user and non-user, a significant decrease in MND size was reported in fibres expressing the type I MyHC isoforms in the HRT user group.

The fibre type specific effects of HRT may be explained by the higher concentration of estrogen receptors in slow- than in fast-twitch fibres (Meeuwsen et al. 2000; Lemoine et al. 2002) and anti-oxidant properties of estrogen (Persky et al. 2000). The increased post-translational modification of myosin by free radicals is a major mechanism contributing to the ageing-related contractile dysfunction (Lowe et al. 2001; Lowe et al. 2004). Thus,
HRT may accordingly reduce impaired myosin function in post-menopausal women more efficiently in type I fibres by protecting against PTMs.

Further, ageing-related oxidative damage has more profound effects in fibres expressing the slow MyHC isoform (McArdle et al. 2002). Oxidative damage reduces transcriptional capacity leading to a reduced specific force and a need for smaller myonuclear domains. It is possible that due to higher concentration of its receptors in slow-twitch fibres, estrogen not only arrests ageing-related oxidative damage but also reduces myonuclear domain size to restore force generating capacity in ageing fibres. This also optimizes transport distances and transcriptional capacity of myonuclei to maintain functional capacity.

HRT has qualitative effects on muscle and single muscle fibres resulting in improved force generating capacity without affecting size. This effect is fibre-type specific and is obtained by modulating number and quality of actin-myosin interactions, as well as myonuclear domain size. These findings have an important prospect for developing future pharmacological interventions in countering the ageing-related decrease in muscle mass and strength.
Conclusions

This study provides new information related to myonuclear organization and myonuclear domain size during the adaptation process of skeletal muscle to different triggering events. A successful new method was elaborated to study myonuclear distribution and phenotypical observations along the length of single skeletal muscle fibres. Several conclusions can be drawn from our data. First, myonuclear domain size increases with increasing body mass, hence the critical cytoplasmic volume that individual nuclei can support optimally, varies from species to species. Second, a MND size ‘ceiling’ is proposed during muscle fibre hypertrophy beyond which myonuclear accretion is necessary to avoid functional compromise. Third, an ageing-related change in the spatial organization of myonuclei may impair local protein turnover leading to a decreased force-generating capacity at the single fibre level. Fourth, hormone replacement therapy arrests the ageing-related loss in specific force and has a fibre-type specific effect on MND size in post-menopausal MZ-twins. Lastly, MND size is not fixed and does vary between the fibre type and with the oxidative capacity of fibre.

1. A comparative study of the MND size was undertaken in mammalian species representing a 100 00 fold difference in body mass ranging from 25g to 2500 kg. Larger MNDs were observed in muscle fibres expressing fast than slow MyHC isoforms in all mammalian species except the two smallest mammals (rat and mouse) where muscle fibres expressing the type I MyHC isoform did not have a MND size smaller than fibres expressing the type IIa MyHC isoform. MND size increased with body mass in both the type I and type IIa fibres. Thus, independent of species, MND size is highly dependent on MyHC isoform type and there is a strong scaling effect of body size. However, MyHC isoform is not the sole factor determining MND size and other protein systems such as mitochondrial proteins co-varying with MyHC isoform expression may be an equally or more important determinant of MND size.

2. In an attempt to explore its role on functional capacity of muscle, MND size was studied together with specific force and the content of the molecular motor protein, myosin, at the single fibre level from myostatin knock-out (Mstn−/−) and IGF-1 over expressing (mIgf1+/+) mice. A significant effect of size of MNDs on specific force and myosin
content was observed. This effect was muscle cell type specific and showed that the bigger MNDs in fast-twitch muscles are optimally tuned for force production while the smaller MNDs in the slow-twitch soleus had a much larger dynamic range for hypertrophy without a need for adding more myonuclei. This indicates a critical volume individual myonuclei can support efficiently for a proportional gain in muscle force and size.

3. In human muscle fibres, effects of ageing and gender were investigated on MND size showing a strong linear relationship with fibre size irrespective age, gender and MyHC isoform expression. The spatial organization of myonuclei was influenced by both ageing and MyHC isoform expression. In fibres expressing the type I MyHC isoform, an increased MND size variability was observed in old age reflecting aggregates of myonuclei although average MND size did not change. In fibres expressing the type IIa MyHC isoform, the average MND size was smaller in old age reflecting the smaller size of muscle fibres. This may influence the transcriptional activity per myonucleus and/or local cooperativity of myonuclei in a gender and muscle fibre type-specific manner.

4. Finally the effects of hormone replacement therapy (HRT) were studied on MND size together with specific force at the single fibre level in post-menopausal HRT discordant monozygous twin pairs. A smaller MND size was recorded in slow-twitch fibres while an increase in specific force was observed in both fibre types expressing type I and type IIa MyHC isoforms. The positive effect on force was mainly due to the quantitative effect in fibres expressing the fast myosin isoform while the effect was both quantitative and qualitative in fibres expressing the slow myosin isoform. Thus, the response difference in MNDs should be considered when developing therapeutic strategies for combating menopause related muscle weakness.
Kortfattad svensk sammanfattning

Skelettmuskelceller är multinukleär a celler med upp till 1000 kärnor per muskelcell. Varje kärna försörjer en specifik cytoplasmatisk volym vilken benämns myonukleär domän (MND). Vi har utvecklat och implementerat en unik imagingteknik tillsammans med Imaging Center, UU, där vi kan analysera enskilda kärnors myonukleära domän i enskilda muskelceller från människa och försöksdjur. Effekterna av åldrande, kön, hormon och kroppstorlek på MND har studerats och den långsiktiga målsättningen är att använda denna kunskap vid framtida interventionsstudier vilka syftar till att motverka muskelförtvining vid olika patologiska och fysiologiska tillstånd. Resultaten från våra studier visar att MND storlek är påverkbar, dvs att såväl den spatiala organisationen som antalet muskecellkärnor kan påverkas vid ändrade fysiologiska och patofysiologiska förhållanden.

I jämförande studier i delarbete 1 har vi studerat MND storlek hos däggdjur vilka har en 100 000-faldig skillnad i kroppsmassa, dvs från mus (25 g) till noshörning (2500 kg). I detta delarbete är det visat att MND storleken är beroende av muskelfibertyp, dvs myosin isoform uttryck. Denna skillnad var tydligast bland stora men ej små däggdjur (mus, råtta). Myosin isoform uttryck är dock ej den enda faktorn som bestämmer MND storleken och andra faktorer såsom oxidativ kapacitet är sannolikt också av stor betydelse. MND storleken visade även ett starkt beroende av kroppsmassa oavsett muskelfibertyp (type I och IIa)

Inom delarbete 2 har MND storleken studerats för muskeltillväxt och muskelfunktion studerats i extremt hypertrofierad muskulatur från möss vilka saknar den negativa regulatorn för muskeltillväxt myostatin (myostatin knock out möss) och transgena möss vilka överuttrycker tillväxthormonet IGF-1. Resultaten från dessa studier visar på ett komplext mönster tydande på att det finns en kritisk volym enskilda muskelkärnor kan effektivt försörja för produktion av motorproteinet myosin och detta är sannolikt även förklaringen till muskelfibertyp specifika skillnader.

Effekten av åldrande och kön på MND organistraion studerades i enskilda muskelceller från människa i delarbete 3. Inom ramen för detta delarbete är det visat att MND organisationen påverkades av såväl åldrande som muskelfiber typ. Det mest framträdande fyndet var dock den ökande storleksvariabiliteten av MND med stigande ålder i muskelfibrer vilka uttrycker myosin isoform av långsam typ.
Inom delarbete 4 har vi studerat effekterna av hormonsubstitutionsterapi hos postmenopausala kvinnor. Muskelfiberfunktion, proteinkoncentration av myosin och MND har studerats i enskilda muskelceller från monozygota tvillingpar där endast den ene av tvillingen använder substitutionsterapi. Denna typ av hormonsubstitutionsterapi hade signifikanta såväl kvantitativa som kvalitativa effekter på muskelcellfunktion vilken var relaterad till uttryck av specifika myosinisoformer.

Sammantaget visar resultaten från detta doktorandprojekt att reglering/modulering av muskelcellkärnors antal och cytoplasmatiska försörjningsområde utgör en viktig process i skelettmuskels adaptation. Detta utgör sannolikt en mycket viktig mekanism vilken bör beaktas vid olika terapeutiska behandlingsförsök vilka ämnar förbättra muskelmassa och funktion vid olika sjukdomstillstånd.
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