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Original Article

Myonuclear domain size varies along the lengths of maturing skeletal muscle fibers

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ABSTRACT In a skeletal muscle fiber, each myonucleus is responsible for gene expression in its surrounding cytoplasm. The region of cytoplasm associated with an individual myonucleus is termed myonuclear domain. However, little is known about domain size variation within individual muscle fibers. This study tests the hypothesis that myonuclear domains expressing neonatal myosin within end regions of maturing fibers will be smaller than domains elsewhere in the fibers. The model used is chicken pectoralis, where we have previously shown that during development repression of neonatal myosin radiates from the central region towards the fiber ends. Samples excised from birds aged nine through to 115 days after hatching were sectioned transversely. Using computer image analysis and immunocytochemistry, fiber profiles were classified as neonatal, transforming or adult. Each profile was also located in an adjacent dystrophin-labelled section, where myonuclei were visualized using haematoxylin and bisbenzamide. Variation in myonuclear length with age was not found to be significant (p = 0.925) . Myonuclei were counted, and formulae used to calculate mean myonuclear domain size for each profile type. Myonuclear number/mm fiber was calculated to be adult (mean = 108.57 myonuclei/mm), transforming (65.82) and neonatal (25.23). Transforming profiles had significantly (p=0.027) more myonuclei/mm than neonatal, as did adult (p=0.005). Volume of cytoplasm/myonucleus was adult (mean = 16,132 μm^3/myonucleus), transforming (12,899) and neonatal (8,130). Transforming and adult profiles had significantly ($p \leq 0.001$) larger myonuclear domains than did neonatal profiles. Transforming and adult profiles did not differ in either myonuclei/mm (p=0.302) or volume of cytoplasm/myonucleus (p=0.413). This study demonstrates smaller domains at the terminal tips of maturing muscle fibers.

KEY WORDS: *muscle, muscle fiber, myosin, nucleus, myonuclear domain*

Introduction

Vertebrate skeletal muscle fibers are, after their embryonic stages, generally elongate, fusiform-shaped, multinucleate cells (Trotter, 1991; McComas, 1996). A typical muscle fiber may contain hundreds or thousand of myonuclei (Cullen and Landon, 1994; Tseng *et al.*, 1994). It has been shown that each myonucleus regulates gene expression within the surrounding portion of the fiber (Hall and Ralston, 1989; Pavlath *et al.*, 1989; Ono *et al.*, 1994; Newlands *et al.*, 1998). These and other observations support the theory of the myonuclear domain (Landing *et al.*, 1974; Hikida *et al.*, 1997; Allen *et al.*, 1999) or DNA unit (Cheek, *et al.*, 1971; Cheek, 1985; Mozdziak *et al.*, 1997). Myonuclear domain has been defined as the theoretical volume of cytoplasm associated with a single myonucleus (Allen *et al.*, 1995 and 1999).

Myonuclear domain size is correlated with muscle fiber type and myosin heavy chain (MyHC) expression. Cytoplasmic volume per myonucleus is smaller in fibers expressing slow as compared to fast MyHC (Tseng *et al.*, 1994; Allen *et al.*, 1999; Schmalbruch and Lewis, 2000). In chickens 66 days old, the anterior latissimus dorsi (slow) and posterior latissimus dorsi (fast) muscles had, respectively, means of 6,849 and 13,158 μm^3 cytoplasm/myonucleus (Matthew and Moore, 1987).

It has been hypothesized that fibers highly active in protein synthesis have smaller domains (Edgerton and Roy, 1991). The greater concentration of myonuclei in slow fibers may be related

Abbreviations used in this paper: ANOVA, analysis of variance; DNA, deoxyribonucleic acid; LSD, least significant difference; MyHC, myosin heavy chain; PB, phosphate buffer; PBS, phosphate buffered saline.

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to a higher rate of protein turnover (Tseng *et al.*, 1994). In chicken anterior latissimus dorsi muscle, myonuclear domains are smaller in developing and regenerating fibers than in mature fibers (Winchester and Gonyea, 1992). Moss (1968) demonstrated that there was a constant ratio of cytoplasm per nucleus throughout development in growing chicken pectoralis and gastrocnemius muscles. However, subsequent studies of chicken pectoralis muscle showed an inverse correlation between myonuclear domain size and the rate of muscle growth (Knizetova *et al.*, 1972). In developing turkey pectoralis, younger smaller fibers were shown to have smaller myonuclear domains (Mozdziak *et al.*, 1994). Similar results have been reported from a recent study of rat muscle (Ohira *et al.*, 2001).

The ends or terminal tips of skeletal muscle fibers have been shown to be the site of longitudinal growth (Swatland, 1994; Zhang and McLennan, 1995). We have recently established that within developing chicken pectoralis the switch from the neonatal to the adult MyHC isoform is initiated near the centrally located motor endplate of each muscle fiber thereafter progressing toward the fiber ends (Rosser *et al,* 2000). The ends of mature fibers retain neonatal isoform (Rosser *et al*, 1995, 2000).

The purpose of this study is to test the hypothesis that myonuclear domains expressing neonatal MyHC within the end regions of maturing muscle fibers will be smaller than domains in other regions of the fibers. The experimental model used is the pectoralis muscle of the chicken. This muscle consists almost exclusively of fast-twitch glycolytic fibers (George and Berger 1966; Rosser *et al.*, 1996). As these fibers are arranged in series overlapping one another from origin to insertion of the muscle (Gaunt and Gans, 1993), a transverse section through the muscle will intersect many fiber ends (Rosser *et al.*, 2000). Also, myosin transformations within this muscle have been well documented (Bandman and Rosser, 2000). Embryonic myosin isoforms are rapidly replaced after hatching by a neonatal isoform which is, in turn, gradually supplanted by an adult isoform (Tidyman *et al.*, 1997). We demonstrate that during maturation, neonatal myosin heavy chain expression is correlated with smaller myonuclear domains.

Results

Identification of Myonuclei

The anti-dystrophin antibody allowed for the identification and exclusion from this study of all nuclei other than myonuclei, which would include those of satellite cells, blood cells, connective tissues proper and neuroglia. Figure 1 compares the location of myonuclei deep to the plasmalemma to those nuclei external to it.

We were also aided in discerning myonuclei by their more central location within the muscle fibers. In embryonic mammalian muscles, myonuclei are typically located centrally within the fiber (Gould, 1972). During later stages of development, they are located peripherally just within the plasmalemma (Gould, 1972; Landing *et al.*, 1974). Central nuclei within mature mammalian fibers are regarded as a sign of muscle pathology (McComas, 1996). Myonuclei in mature healthy chicken pectoralis muscle, however, are typically found deep within the fiber sarcoplasm as well as immediately within the plasmalemma (George and Berger, 1966).

Expression of Neonatal MyHC

Using the same birds and muscles as in the present study, we have previously shown that the smaller fiber profiles containing neonatal myosin are in fact the tapered ends of much larger adult fiber profiles (Rosser *et al.*, 2000). Furthermore, during development, repression of neonatal MyHC isoform raditates from central regions of the fibers towards the fiber ends (Rosser *et al.*, 2000). In the present study, the expression of neonatal myosin heavy chain isoform was similarly repressed during development.

General fiber composition during development is illustrated in Fig. 2. At 16 days after hatching there was uniform labelling by the 2E9 antibody for neonatal myosin among the fiber profiles (Fig. 2A), demonstrating that during this early stage all fibers express neonatal MyHC along their entire length. The larger more lightly labelled transforming profiles were first apparent at 23 days after hatching (not shown). By 41 days posthatch, neonatal and transforming profiles were present in roughly equal numbers (Fig. 2D). Adult fiber profiles, bereft of neonatal MyHC, were first observed 63 days after hatching (not shown). Subsequently, adult profiles increased in number at the expense of both transforming and neonatal profile numbers (Fig. 2G). By 115 days neonatal myosin was restricted to all but the smallest fiber profiles (Fig. 2J) which we have previously shown to consist of neonatal and transforming fiber profiles comprising, respectively, one and nine percent of the fiber population (Rosser *et al.*, 2000).

The location of nuclei relative to plasmalemmae in the preceding representative sections, Fig. 2 A,D,G and J, are shown, respectively, by bright-field in Fig. 2 B,E,H and K, and by epifluorescence in Fig. 2 C,F,I and L. All myonuclei were marked by both hematoxylin (Fig. 2 B,E,H and K) and bisbenzamide (Fig. 2 C,F,I and L).

Lengths of Myonuclei during Development

As evident in Fig. 3, there was no significant ($p = 0.925$, $r = 0.050$) variation in mean myonuclear length throughout development and maturity. The mean lengths of myonuclei within tissue 9, 30, 41, 115, and ~365 days were, respectively, 10.64 ± 1.34 (n=90), 10.58

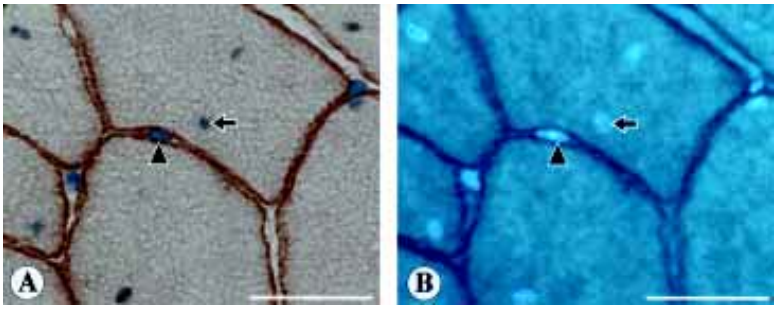


Fig. 1. Myonuclei and other nuclei, within transverse sections of muscle fibers. *A transverse section from chicken pectoralis aged 115 days after hatching. The same section is viewed in (A) and (B). The section is labelled by the anti-dystrophin antibody, and nuclei indicated by both Hoescht 33258 and Haematoxylin.* **(A)** *is viewed by bright-field. All nuclei are dark blue and the plasmalemma, as indicated by dystrophin labeling, appears as a golden-brown line around the circumference of each muscle fiber profile.* **(B)** *is viewed by epi-fluorescence. All nuclei fluoresce bright blue and plasmalemmae are a blackish-brown. Several myonuclei are visible within the cytoplasm of the muscle fibers, deep to the dystrophin ringing each fiber profile. The arrow shows one such nucleus. Other types of nuclei, including satellite cells, are seen superficial to the dystrophin. The arrowhead indicates one of these nuclei. Bar, 20 μm.*

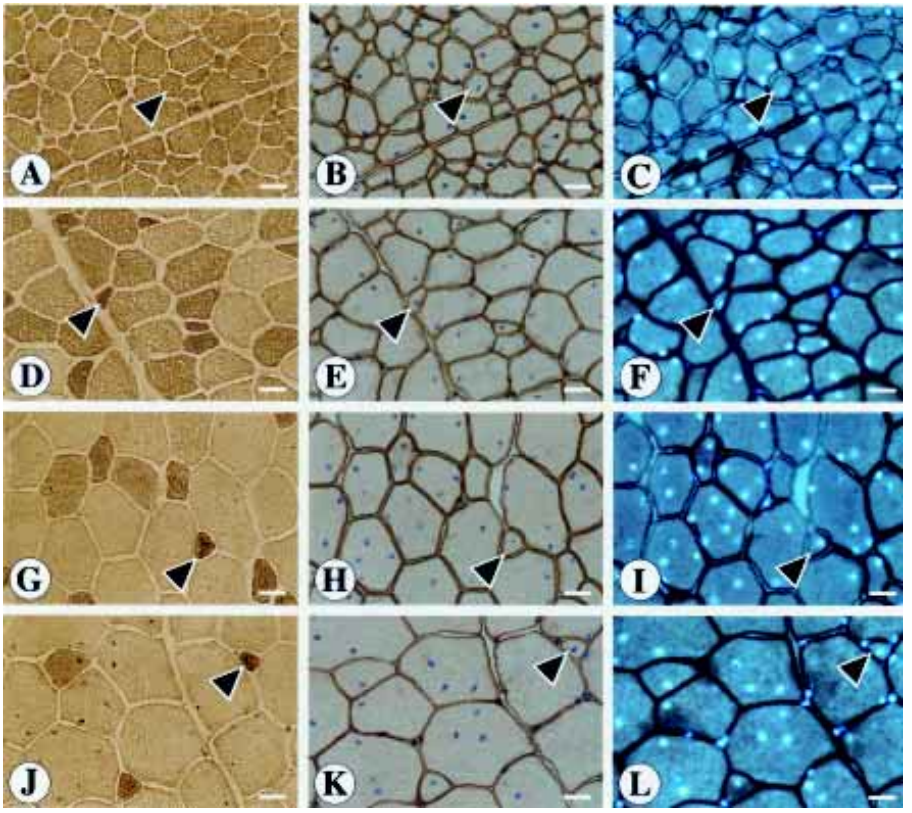


Fig. 2. Representative transverse serial sections of chicken pectoralis. *Chicken pectoralis aged 16 (A,B,C), 41 (D,E,F), 79 (G,H,I) and 115 (J,K,L) days posthatch.* **(A,D,G,J)** *are viewed by bright-field and labeled by 2E9 for neonatal myosin heavy chain (MyHC). Neonatal MyHC is coloured a darker brown.* **(B,E,H,K)** *are, respectively, sections cut serial to A, D, G and J, labelled by the anti-dystrophin antibody and stained by haematoxylin. Under bright-field, nuclei are coloured a deep blue, and dystrophin dark brown. The same sections as B,E,H and K are shown under epi-fluorescence as C,F,I and L. Due to the presence of Hoescht 33258 in the mounting media, all nuclei fluoresce a bright blue. Within each age shown, an arrowhead identifies the same fiber profile in the sections. Bar, 20 μm.*

± 1.60 (n=67), 10.68 ± 1.76 (n=79), 10.55 ± 2.26 (n=77), and 10.47 ± 1.59 (n=52) μm (± standard deviation; n= number of nuclei). The overall mean length was 10.58 μm ± 0.08 (± standard deviation).

Number of Myonuclei per Milimeter of Muscle Fiber

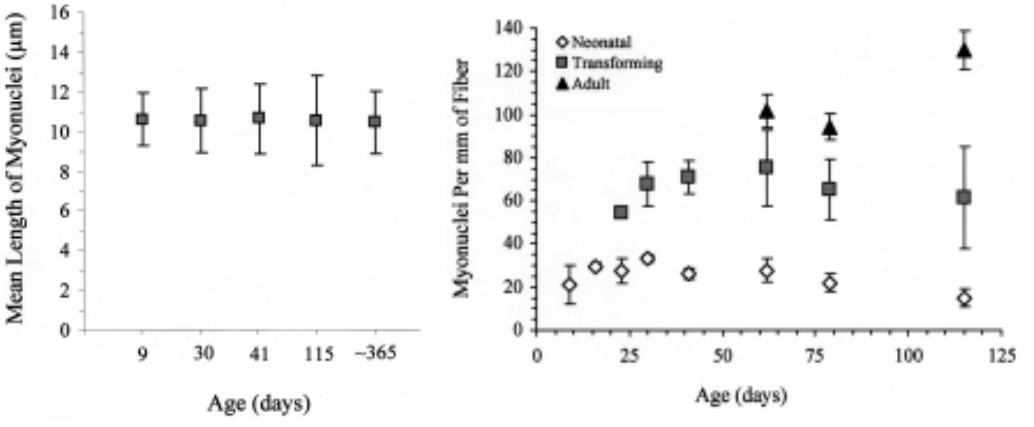
The overall pattern of myonuclear number per mm of fiber for all ages was adult or transforming > neonatal (Fig. 4). In neonatal fibers and neonatal fiber profiles, the number of myonuclei along a mm of fiber decreased significantly (p=0.009; r=0.521) with age as shown by linear regression. Neonatal fibers and neonatal fiber profiles had mean myonuclear numbers ranging from 10.63 to 34.84 myonuclei/mm/bird, with an overall mean of 25.23 myonuclei/mm. In transforming fiber profiles, the number of myonuclei per mm of fiber did not vary with age (p=0.975, r =0.008). Transforming profiles ranged from 44.29 to 89.85 myonuclei/mm/bird, with a mean of 65.82 myonuclei/mm. In adult fiber profiles the number of myonuclei per mm of fiber increased (p=0.009, r=0.806) with age. Adult fiber profiles ranged from 87.28 to 139.67 myonuclei/mm/bird, with a mean of 108.57 myonuclei/mm. ANOVA shows that number of myonuclei per mm varies significantly (p=0.014) with developmental phase. A Least Significant Difference (LSD) test demonstrates that adult fiber profiles have significantly (p=0.005) more myonuclei per unit length than neonatal profiles. Similarly, transforming profiles have more (p=0.027) myonuclei per mm than neonatal profiles. Adult and transforming profiles, however, are not significantly different (p=0.302).

Volume of Cytoplasm per Myonucleus (Myonuclear Domain)

Among the various fiber profiles, myonuclear domain size was adult or transforming > neonatal (Fig. 5). Linear regression shows that within neonatal muscle and neonatal fiber profiles myonuclear domain size did not vary (p=0.542, r=0.131) with age. Neonatal fiber profiles had mean cytoplasmic volumes ranging from 6,080 to 9,324 μm^3/myonucleus/bird, with an overall mean of 8,130 μm^3/myonucleus. Domain size within transforming fiber profiles increased significantly ($p \leq 0.001$, r=0.778) with age. Transforming profiles ranged from 9,231 to 15,523 μm^3/myonucleus/bird, with a mean of 12,899 μm^3/myonucleus. Domain size within adult fiber profiles also increased significantly (p=0.004, r=0.847) with age. Adult fiber profiles ranged from 14,630 to 17,844 μm^3/myonucleus/

Fig. 3. (Left) Mean length of myonuclei at representative ages. *Data are expressed as mean ± SD. An Analysis of Variance (ANOVA) shows no significant difference (p=0.925) in mean myonuclear length with age. The overall mean myonuclear length is 10.58 μm.*

Fig. 4. (Right) Mean number of myonuclei along a millimeter of fiber in maturing chicken pectoralis. *Data are expressed as mean ± SD. The overall pattern of myonuclear number per mm of fiber is adult or transforming > neonatal.*



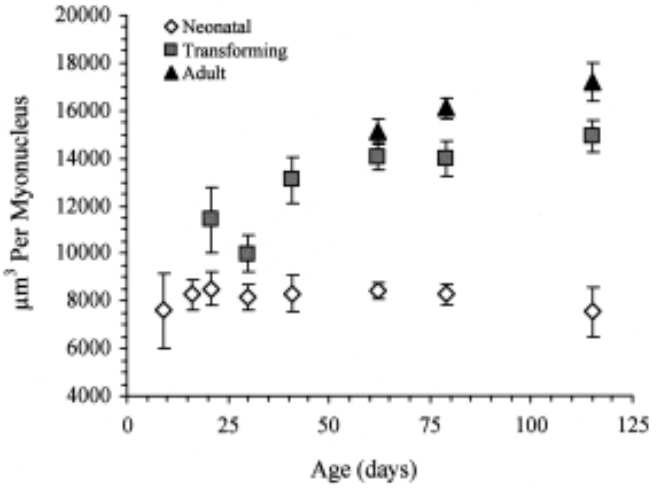


Fig. 5. Myonuclear domain (volume of cytoplasm per myonucleus) in maturing chicken pectoralis. *Values are expressed as mean ± SD. The overall pattern of myonuclear domain size is adult or transforming > neonatal.*

bird, with a mean of 16,132 μm^3/myonucleus. ANOVA showed that myonuclear domain size varied significantly ($p \leq 0.001$) with developmental phase. A LSD test demonstrated no significant difference ($p=0.413$) between myonuclear domain size of transforming and adult fiber profiles. Adult profiles were significantly ($p \leq 0.001$) larger than those of neonatal profiles, however, as were transforming profiles ($p \leq 0.001$).

Discussion

This study verifies, with a caveat, the hypothesis that myonuclear domains expressing neonatal MyHC within the tapered end regions of maturing muscle fibers are smaller than domains in other regions of the fibers. The caveat is that this hypothesis holds true only for those fiber regions that we classified as neonatal; the optical density for labelled neonatal MyHC was as high as that obtained for our 16 day (neonatal) control pectoralis. We have shown that these regions of intense labelling for neonatal MyHC are located i) throughout fibers of young birds and ii) within the tapered ends of maturing and mature fibers (Rosser *et al.,* 2000). In those regions that we classified as transforming, in which the expression of neonatal MyHC was less than that found in neonate control muscle but more than that observed in control adult muscle, domain size was not significantly different from that found in those fiber regions not expressing neonatal MyHC. We have shown that these transforming regions are located i) within the ends of some mature fibers, ii) in maturing fibers between tapered ends more heavily expressing neonatal MyHC and iii) in those regions of maturing and mature fibers located between the small tapered ends heavily expressing neonatal MyHC and the larger central regions not expressing neonatal MyHC (Rosser *et al.*, 2000).

Myonuclear domains in the present study ranged from an average of 6,080 μm^3 cytoplasm/myonucleus/bird in muscle expressing neonatal myosin to 17,844 μm^3 cytoplasm/myonucleus/bird in fibers where neonatal myosin was absent. Overall, there was an increase in the size of myonuclear domains as the muscle developed. Our values and trends are comparable to those in the literature. The pectoralis of Rose Comb Bantam chickens showed an increase in the size of myonuclear domain from 5,662 μm^3/myonucleus at 14 days after hatching to 16,233 μm^3/myonucleus at 84 days (calculated from Knizetova *et al.*, 1972). The pectoralis of Large White Tom turkeys (*Melleagris gallopavo*) showed an increase from approximately 3,000 μm^3/myonucleus at 21 days after hatching to approximately 13,000 μm^3/myonucleus at 189 days (from Mozdziak *et al.*, 1994). While White Leghorn chicken anterior latissimus dorsi (slow myosin) muscle at three days posthatch averaged 2,639 μm^3/myonucleus, the same muscle at 66 days averaged 6,849 μm^3/myonucleus (calculated from Matthew and Moore, 1987). Similarly, the posterior latissimus dorsi (fast myosin) was 1,203 μm^3/myonucleus at three days and 13,158 μm^3/myonucleus at 66 days (Matthew and Moore, 1987). Myonuclear domains within predominantly fast contracting mature mammalian muscles and fibers tend to be larger than those in chicken and their allies. Rat gastrocnemius and tibialis anterior muscles were, respectively, on average 18,100 and 20,800 μm^3 cytoplasm/myonucleus (Kasper and Xun, 1996). In fast fibers of cat plantaris muscle, Allen *et al.* (1995) determined the cytoplasmic volume per myonucleus to be 25,952 μm^3. Slow fibers in the same muscle averaged 14,103 μm^3 cytoplasm/nucleus. Adult rat soleus, consisting mainly of slow fibers, had a nuclear domain of approximately 13,000 μm^3/nucleus (Allen *et al.*, 1997). Mature human soleus fibers had nuclear domains averaging approximately 10,000 μm^3/nucleus (Ohira *et al.*, 1999). Rat domains, like myonuclear domains of the chicken and their allies, also increase in size during development (Ohira *et al.,* 2001).

Myonuclear length averaged 10.58 μm and did not vary significantly among the development stages that we studied. These findings are similar to those in the literature. In White Leghorn chicken, throughout the developmental stages studied myonuclear length did not vary significantly in anterior (average 8.37 μm) or posterior (average 9.37 μm) latissimus dorsi muscles (derived from Matthew and Moore, 1987). Comparing mature rat soleus and extensor digitorum longus muscles, myonuclear length did not vary and averaged 14.3 um in each muscle (Schmalbruch and Lewis, 2000).

Our work differs from previous studies, in that we demonstrate significant variation in the size of myonuclear domains along the lengths of muscle fibers. Specifically, we show greater myonuclear density at the ends or terminal tips of maturing and mature muscle fibers. While it has been demonstrated that nuclear density is higher in the immediate vicinity of the centrally located motor endplate, as compared to adjacent regions along the length of a muscle fiber (Tseng *et al.,* 1994), it is widely held that elsewhere skeletal muscle fibers are covered by nuclear territories of uniform size and shape (Landing *et al.*, 1974; Tseng *et al.,* 1994).

Traditionally, most muscle biologists have believed that biochemical and physiological properties were homogeneous along the length of individual muscle fibers. However, this paradigm continues to be challenged. For example, myosin heavy chain expression can certainly vary along the length of a fiber (Peuker and Pette, 1997; Rosser *et al.*, 2000). In addition, the expression of certain myogenic regulatory factors has been reported to be greater at the ends of the fibers in mature rat soleus (Zador *et al.*, 1999). In mature human muscle, contractile properties have recently been shown to vary in adjacent segments of individual fibers. An explanation offered for this observation was variability

of protein expression among nuclear domains (Wilkins *et al*., 2001).

The ends or terminal tips of skeletal muscle fibers have been shown to be the site of longitudinal growth (Swatland, 1994; Zhang and McLennan, 1995). Presumably the smaller nuclear domains that we observe at the ends of maturing and mature muscle fibers are correlated with a need for greater protein synthesis. Winchester and Gonyea (1992) in studying quail (*Coturnix*, species not given) anterior latissimus dorsi muscle concluded that nuclear domains might need to be smaller to govern protein synthesis during hypertrophy. In comparing pectoralis muscle growth between three strains of chickens, Knizetova *et al.* (1972) found an inverse correlation between the intensity of growth and the cytoplasmic volume per nucleus. Edgerton and Roy (1991) state that fibers highly active in protein synthesis will have smaller domains.

Our results provide an important insight into how muscle fibers are adapted to meet the demands of protein synthesis. In most eukaryotic cells, a nucleus affects the level of protein expression by modulating the rates of protein synthesis and/or protein degradation. Muscle fibers also employ these strategies (Booth and Criswell, 1997). However, unlike the overwhelming majority of normal animal cell types, skeletal muscle fibers are highly unusual in that they are multinucleate. Therefore, skeletal muscle has an additional avenue available with which to effect protein expression (Allen *et al*., 1999). Myonuclei can apparently be more concentrated where there is a greater demand for protein synthesis in a muscle fiber. Certainly, this deduction is corroborated by studies of muscle growth (Knizetova, 1972; Mozdziak *et al*., 1994; Ohira *et al*., 2001). Conversely, presumably through incorporation of satellite cells into muscle fibers, the size of the myonuclear domain appears to be maintained during hypertrophy of mature muscle (Winchester and Gonyea, 1992; Allen *et al*., 1999).

Materials and Methods

Animals

The same White Leghorn chickens (*Gallus gallus*; Highline W-36, Clark Highline, Brandon, Manitoba) utilized in our earlier work (Rosser *et al*., 2000) were used for the present study. All birds were hatched at the same time and raised under identical conditions at the University of Saskatchewan, Department of Animal and Poultry Science. The birds were put on a 23 hours light, one-hour dark cycle at an initial environment temperature of 35°C. Subsequently, temperature was decreased 3.5°C at weekly intervals until 21°C was reached when the birds were five weeks old. Feeding was done *ad libitum* and initially consisted of commercial chick starter that was replaced with a commercial chick grower (Federated Co-operatives Ltd., Saskatoon, Saskatchewan) when the birds were 42 days old. Following the Canadian Council on Animal Care Guidelines, three birds were killed by inhalation of Carbon Dioxide at each of the following ages: 9,16, 23, 30, 41, 62, 79, and 115 days, post-hatch. Unrelated White Leghorns aged ~365 days were used as a control.

Tissue Preparation and Sectioning

Three muscle samples were excised from the superficial regions of the proximal one-half of the left pectoralis muscle of each bird. Each sample was approximately $0.5 \times 0.5 \times$ 2-4cm. The long axis of each sample was parallel to the direction of the muscle fibers. Immediately after excision, each sample was coated with Tissue-Tek O.C.T. Compound (Miles Inc., Elkhart, Indiana) and then frozen in 2-methylbutane cooled via liquid nitrogen to -160°C. Samples were then stored at -80°C.

Serial cross-sections of alternating thickness of 8 and 12 μm were cut, in a cryostat at -20°C, from samples representative of each bird studied. After two sections of the same thickness were cut, the microtome within the cryostat was adjusted and two sections of the other thickness were cut. Each two adjacent sections of the same thickness were picked up on a ProbeOn Plus microscope slide (Fisher Scientific Canada, Ottawa, Ontario), such that any given slide would bear only two 8 μm or two 12 μm sections. We have found that 8 μm sections work well for density measurements (Rosser *et al*., 2000; see Image Analysis and Data Acquisition in following). The 12 μm sections were employed to assist in locating more myonuclei than would be encountered using 8 μm sections, and are the same thickness as earlier work that used the same techniques to determine myonuclear domain (Hikida *et al*., 1997; see Differentiation of Myonuclei from Satellite Cells and Other Nuclei in following). The rationale for collecting two sections on each slide was that the second section could be used to both confirm that which was observed on the first section, as well assist in the identification of those artifacts that can occur when using histochemical and immunocytochemical procedures on muscle. In addition, a second section provides the option of choosing a better section. All slides were stored at -20°C.

Immunocytochemical and Histological Techniques

The two primary monoclonal antibodies used were 2E9 and anti-dystrophin. 2E9 is produced in our laboratory and is specific for chicken neonatal MyHC (Bandman, 1985; Moore *et al*., 1992). The anti-dystrophin antibody is commercially available and strongly labels dystrophin of chicken, as well as that of several mammals (Sigma Chemical Co., Missouri; catalogue number D8168; clone number MANDYS8).

Slides bearing sections were allowed to air dry at room temperature for approximately one-half hour. Sections were treated for 15 minutes with at least 200μl of blocking solution, which consisted of 5mM ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS; 0.02 M sodium phosphate buffer, 0.15 M sodium chloride, pH 7.2), 5% horse serum and 1% percent bovine serum albumin. Blocking solution was then drained from each slide onto a paper towel, taking care not to damage the sections. Before the sections could begin to dry, 200μl of a primary antibody (either 2E9 or anti-dystrophin) was applied. The 2E9, at a dilution of 1:500 in blocking solution, was pipetted onto the 8 μm thick sections. The anti-dystrophin at a dilution of 1:400 in blocking solution was applied to the 12μm thick sections. Slides were then placed into a Plexiglas box containing strips of filter paper that had been soaked in H_20 to provide humidification. The lid of the box was then sealed by strips of Parafilm (VWR Canlab, Ontario), and the entire box was placed into an oven maintained at 27°C. After 75 minutes the slides were removed from the box, returned to room temperature, and twice washed in fresh PBS for five minutes per wash.

A secondary antibody, biotinylated goat anti-mouse IgG (Sigma Chemical Co., Missouri) diluted to 1:500 in PBS, was applied to the sections on each slide for 30 minutes. The slides were again washed twice in fresh PBS for five minutes per wash. The slides were then placed in 3% hydrogen peroxide in methanol for five minutes, after which they received two washes in fresh PBS for five minutes each.

Next 200μl of Avidin DH-biotinylated horseradish peroxidase in 10mM pH 7.5 PBS solution (ABC Elite Kit, Vector Laboratories Inc., California) was applied for 60 minutes to the sections on each slide. Slides were then washed twice in phosphate buffer (PB; 0.02 M sodium phosphate buffer, pH 7.2) for five minutes per wash. To bring about a specific colour reaction visible using bright-field microscopy, slides were immersed in 0.05% diaminebenzidine in PB for five minutes. This was followed by a five minute wash in fresh PB, and then a five minute fresh PBS wash. A solution of 4% formaldehyde in PBS was applied for three minutes, after which slides were washed in fresh PBS for five minutes and then in running tap water for five minutes.

Slides treated with 2E9 were dehydrated in an ascending ethanol series, placed in xylene substitute (BDH Chemicals, Ontario), and then mounted with Cytoseal (Stephens Scientific, Michigan). Anti-dystrophin slides were treated with Mayer's Haematoxylin (Presnell and Screibman, 1997) for 15 minutes, followed by a five minute tap water wash, and then mounted with

Citifluor (MARIVAC, Nova Scotia) containing 0.001% Hoechst No. 33258 (bisbenzamide; Sigma Chemical Co., Missouri).

Image Analysis and Data Acquisition

Images were obtained from a Leitz Diaplan microscope using a Panasonic Colour CCTV Camera (Model WV-CP410) and a Quadra 800 Macintosh computer equipped with a RasterOps Framegrabber card (RasterOps Corp., Indiana) and IP Lab Spectrum software (Scanalytica Inc., Virginia). NIH Image, a public domain program developed at the US National Institutes of Health (available on the Internet by anonymous FTP from Zippy.nimh.nih.gov) was used with a Macintosh Performa 5200 computer to digitize and analyze results. Images were converted from colour to grey scale. Using "Uncalibrated" Optical Density white and black were, respectively, assigned densities of 0 and 255 at the ends of a perfect arithmetic linear regression.

Densities of individual fiber profiles were computed. As 16-day-old pectoralis exhibits neonatal MyHC almost exclusively (Bandman, 1985), this tissue was used as a neonatal density control throughout all experiments. Similarly, the large fiber profiles of the unrelated control white leghorns aged ~365 days were used as a control for the absence of neonatal myosin which was indicative of adult profiles (Rosser *et al.*, 2000). Control neonatal fiber profiles always had densities ≥120, while control adult profiles always had values of ≤ 90. Thus, we classify fiber profiles from all animals as neonatal (optical density ≥120), transforming (optical density <120 and >90) or adult (optical density ≤90). Approximately 200 fiber profiles per bird were examined and categorized as neonatal, transforming or adult based on their respective densities. Those fibers chosen for study were contiguous to one another within randomly selected fascicles.

In this publication, the term neonatal muscle refers to chicken pectoralis muscle when it consists primarily of neonatal fibers. Here we define a neonatal fiber as one that contains almost exclusively the neonatal myosin heavy chain isoform. In our work, neonatal muscle was found in chickens aged 9 and 16 days after hatching (see Bandman and Rosser, 2000). For the purposes of this study a neonatal fiber profile, or neonatal profile, is a muscle fiber cross-section that contains densities of neonatal myosin heavy chain comparable to those found in a neonatal fiber. In animals older than 16 days, fiber profiles from different regions of the same fiber could contain varying amounts of neonatal myosin. In addition, we define maturing fibers as those older than 16 days posthatch in which there is evidence of the repression neonatal myosin heavy chain.

Using the NIH program, fiber profile cross-sectional area was measured in an 8 μm 2E9 section from each animal. Within an adjacent 12 μm serial section, the same fiber was found and the number of myonuclei per cross-section counted. Using a Zeiss Axioskop 20 Microscope, myonuclei were identified by both bright-field demonstration of haematoxylin staining and their affinity for the Hoechst 33258 dye (bisbenzamide) as revealed by epi-fluorescence.

Calculation of Myonuclear Length

Longitudinal sections were cut from the pectoralis muscle of chickens aged 9, 30, 41, 115 or ~365 days. Sections were obtained at a thickness of 8 μm in the same manner outlined in the preceding. Slides were stained with Mayer's Haematoxylin for 15 minutes, followed by a 5-minute wash with tap water, and then mounted with Citifluor containing Hoechst 33258. Using NIH Image, the lengths of 52-90 myonuclei for each age were quantified. As no significant differences (see "Results") were observed in mean myonuclear length among the muscles of the five ages in which this parameter was measured, an overall average of the means (10.58 μm) was used as myonuclear length in all calculations of myonuclear numbers and myonuclear domain.

Differentiation of Myonuclei from Satellite Cells and Other Nuclei

Haematoxylin stains all nuclei a deep blue colour that is visible with conventional bright-field microscopy (Presnell and Screibman, 1997). Hoechst 33258 labels DNA, and this labelling can be viewed by epi-fluorescence microscopy (Araki *et al.,* 1987; Pavlath *et al.*, 1989). When applied to the same sections, as we did, double labelling by haematoxylin and Hoechst 33258 provide convincing evidence that structures are nuclei.

Satellite cells are myogenic stem cells situated between the basal lamina and plasmalemma of the sarcolemma (Moss and Leblond, 1971; McComas, 1996). It has been estimated that approximately one to five percent of all nuclei associated with a normal muscle fiber are satellite cells (Edgerton and Roy, 1991; Cullen and Landon, 1994; McComas, 1996). However, as satellite cells are not within the sarcoplasm, they need be excluded from counts of nuclei when determining myonuclear domain (Hikida *et al.*, 1997).

Dystrophin is a structural protein located on the cytoplasmic face of the plasmalemma (Matsumura and Campbell, 1994). Therefore, an anti-dystrophin antibody can be used to identify the plasmalemma. Nuclei deep to the plasmalemma are myonuclei situated within the sacroplasm, and nuclei external to the plasmalemma either belong to satellite cells or are other non-myonuclei (Winchester and Gonyea, 1992; McComas, 1996; Hikida *et al.*, 1997).

To count myonuclei, we used sections (12 μm thick) labelled by the anti-dystrophin antibody. To avoid counting a myonucleus twice, only one of the two 12 μm sections from each animal was used. These sections were viewed by both bright-field and epi-fluorescence to locate and confirm the presence of nuclei. All nuclei observed were labelled by both haematoxylin and Hoechst 33258. For each fiber profile assessed, nuclei (myonuclei) situated deep to the darkly labelled plasmalemma were included in the count. Those nuclei external to the plasmalemma, satellite cell nuclei and other non-myonuclei, were not counted. To assign each of these fiber profiles to a developmental stage (neonatal, transforming or adult), the same fiber was located in an adjacent 2E9 labelled section (8μm thick) and its optical density measured (see "Image Analysis and Data Acquisition").

Calculation of Myonuclear Domain

Schmalbruch and Hellhammer (1977) developed a formula that has been routinely employed (Hikida *et al.*, 1997; Schmalbruch and Lewis, 2000) to calculate the number of nuclei in a muscle fiber segment (X)

$$X = (NL) / (d + l) \qquad (1)$$

This formula utilizes the number of nuclei counted in a cross-section of a particular fiber profile (N), the desired length of the fiber segment (L), the thickness of the cryostat cut cross-section (d), and the average length of a muscle nucleus (l). Using earlier studies as our guideline (Schmalbruch and Hellhammer, 1977; Hikida *et al.*, 1997), "L" was set at 1 mm and "d" at 12 μm. The "l" was determined to be 10.58 μm (see "Calculation of Myonuclear Length").

We used a second formula to compute the volume of cytoplasm per myonucleus (Y)

$$Y = (C\ L) / X \qquad (2)$$

This formula uses the quantified cross-sectional area of a muscle fiber profile (C), the length of the segment (L) and the number of myonuclei per fiber segment (X) of the same fiber profile. "L" was again set at 1mm. "X" was determined using the Schmalbruch and Hellhammer formula (1).

Statistical Analyses

A one-way analysis of variance was used to examine differences in myonuclear length with age of birds. To discern the relationship of number of myonuclei per mm of fiber or volume of cytoplasm per myonucleus versus age of birds for each developmental phase (adult, transforming or neonatal), simple linear regressions were used. Statistical differences in number of myonuclei per mm of fiber or volume of cytoplasm per myonucleus among developmental phases were each determined by a nested one-way analysis of variance (ANOVA) on a subset of the data (birds aged 62-115 days) in which all phases were present in each bird. When the ANOVA was significant, a Fisher's Least Significant Difference (LSD) test was used for pairwise comparisons between different developmental phases. A significance level of $p < 0.05$ was used in all tests. All statistical tests were performed using SYSTAT (1997, Standard Version 7.0.1 for Windows, SPSS, Inc.).

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References

ALLEN, D.L., MONKE, S.R., TALMADGE, R.J., ROY, R.R. and EDGERTON, V.R. (1995). Plasticity of myonuclear number in hypertrophied and atrophied mammalian skeletal muscle fibers. *J. Appl. Physiol.* 78:1969-1976.

ALLEN, D.L., LINDERMAN, J.K, ROY, R.R., GRINDELAND, R.E., MUKKU, V. and EDGERTON, V.R. (1997). Growth hormone/IGF-I and/or resistive exercise maintains myonuclear number in hindlimb unweighted muscles. *J. Appl. Physiol.* 83:1857-1861.

ALLEN, D.L., ROY, R.R. and EDGERTON, V.R. (1999). Myonuclear domains in muscle adaptation and disease. *Muscle and Nerve* 22:1350-1360.

ARAKI, T., YAMAMOTO, A. and YAMADA, M. (1987). Accurate determination of DNA content in single cell nuclei stained with Hoechst 33258 fluorochrome at high salt concentration. *Histochemistry* 87: 331-338.

BANDMAN, E. (1985). Continued expression of neonatal myosin heavy chain in adult dystrophic skeletal muscle. *Science* 227:780-782.

BANDMAN, E. and ROSSER, B.W.C. (2000). Evolutionary significance of myosin heavy chain heterogeneity in birds. *Microsc. Res. Tech.* 50:473-491

BOOTH, F.W. and CRISWELL, D.S. (1997). Molecular events underlying skeletal muscle atrophy and the development of effective countermeasures. *Inter. J. Sports Med.* 18: S265-S269, Suppl. 4.

CHEEK, D.B. (1985). The control of cell mass and replication. The DNA unit – a personal 20-year study. *Early Human Dev.* 12:211-239.

CHEEK, D.B., HOLT, A.B., HILL, D.E. and TALBERT, J.L. (1971). Skeletal muscle cell mass and growth: The concept of the deoxyribonucleic acid unit. *Pediatr. Res.* 5:312-328.

CULLEN, MJ and LANDON, DN (1994). The normal ultrastructure of skeletal muscle. In: Walton J, Karpati G, Hilton-Jones D (editors). *Disorders of Voluntary Muscle*. New York, Churchill Livingstone, 87-137.

EDGERTON, V.R. and ROY, R.R. (1991). Regulation of skeletal muscle fiber size, shape and function. *J. Biomechanics* 24:123-133.

GAUNT, A.S. and GANS, C. (1993). Variations in the distribution of motor end-plates in the avian pectoralis. *J. Morphol.*, 215: 65-88.

GEORGE, J.C. and BERGER, A.J. (1966). *Avian Myology*. Academic Press, New York.

GOULD, R.P. (1972). The microanatomy of muscle. In: *The structure and function of muscle*. G.H. Bourne, editor 2 ed. Academic Press, New York, pp. 2: 186-243.

HALL, Z.W. and RALSTON, E. (1989). Nuclear domains in muscle cells. *Cell* 59:771-772.

HIKIDA, R.S., VAN NOSTRAN, S., MURRAY, J.D., STARON, R.S., GORDON, S.E. and KRAEMER, W.J. (1997). Myonuclear loss in atrophied soleus muscle fibers. *Anat. Rec.* 247:350-354.

KASPER, C.E. and XUN, L. (1996). Cytoplasm-to-myonucleus ratios following microgravity. *J. Musc. Res. Cell Mot.* 17:595-602.

KNIZETOVA, H., KNIZE, B., KOPECNY, V. and FULKA, J. (1972). Concentration of nuclei in chicken muscle fibre in relation to the intensity of growth. *Ann. Biol. Anim. Bioch. Biophys.,* 12:321-328.

LANDING, B.H., DIXON, L.G. and WELLS, T.R. (1974). Studies on isolated human skeletal muscle fibers. *Hum. Pathol.* 5:441-461.

MATSUMURA, K. and CAMPBELL, K.P. (1994). Dystrophin-glycoprotein complex: Its role in the molecular pathogenesis of muscular dystrophies. *Muscle and Nerve* 17:2-15.

MATTHEW, C.A. and MOORE, M.J. (1987). Numbers of myonuclei and satellite cell nuclei in latissimus dorsi muscles of the chicken. *Cell Tissue Res.* 248:235-238.

McCOMAS, A.J. (1996). *Skeletal Muscle Form and Function.* Human Kinetics, Champaign.

MOORE, L.A., ARRIZUBIETA, M.J., TIDYMAN, W.E., HERMAN, L.A. and BANDMAN, E. (1992). Analysis of the chicken myosin fast heavy chain family: Localization of isoform-specific antibody epitopes and regions of divergence. *J. Mol. Biol.* 225:1143-1151.

MOSS, F.P. (1968). The relationship between the dimensions of the fibres and the number of nuclei during normal growth of skeletal muscle in the domestic fowl. *Am. J. Anat.* 122:555-564.

MOSS, F.P. and LEBLOND, C.P. (1971). Satellite cells as the source of nuclei in muscles of growing rats. *Anat. Rec.* 170:421-436.

MOZDZIAK, P.E., SCHULTZ, E. and CASSENS, R.G. (1994). Satellite cell mitotic activity in posthatch turkey skeletal muscle growth. *Poultry Science* 73:547-555.

MOZDZIAK, P.E., SCHULTZ, E. and CASSENS, R.G. (1997). Myonuclear accretion is a major determinant of avian skeletal muscle growth. *Am. J. Physiol.* 272:C565-C571.

NEWLANDS, S., LEVITT, L.K., ROBINSON, C.S., CARMEN-KARPF, A.B., HODGSON, V.M.R., WADE, R.P. and HARDEMAN, E.C. (1998). Transcription occurs in pulses in muscle fibers. *Genes Dev.* 12:2748-2758.

OHIRA, Y., TANAKA, T., YOSHINAGA, T., KAWANO, F., NOMURA, T., NONAKA, I., ALLEN, D.L., ROY, R.R. and EDGERTON, V.R. (2001). Ontogenetic, gravity dependent development of rat soleus muscle. *Am.J. Physiol. Cell Physiol.* 280: C1008-C1016.

OHIRA, Y., YOSHINAGA, T. OHARA, M., NONAKA, I., YOSHIOKA, T., YAMASHITA-GOTO, K., SHENKMAN, B.S., KOZLOVSKAYA, I.B., ROY, R.R. and EDGERTON, V.R. (1999). Myonuclear domain and myosin phenotype in human soleus after bed rest with or without loading. *J. Appl. Physiol.* 87: 1776-1785.

ONO, T., ONO, K., MIZUKAWA, K., OHTA, T., TSUCHIYA, T. and TSUDA, M. (1994). Limited diffusibility of gene products directed by a single nucleus in the cytoplasm of multinucleated myofibers. *FEBS Lett.* 337:570-573.

PAVLATH, G.K., RICH, K., WEBSTER, S.G. and. BLAU, H.M (1989). Localization of muscle gene products in nuclear domains. *Nature* 337:570-573.

PEUKER, H. and PETTE, D. (1997). Quantitative analysis of myosin heavy chain mRNA and protein isoforms in single fibers reveal a pronounced fiber heterogeneity in normal rabbit muscles. *Eur. J. Biochem.* 247: 30-36.

PRESNELL, J.K. and SCREIBMAN, M.P. (1997). *Humason's Animal Tissue Techniques*. John Hopkins Press, Baltimore.

ROSSER, B.W.C., WALDBILLIG, D.M., LOVO, S.D., ARMSTRONG, J.D. and BANDMAN, E. (1995). Myosin heavy chain expression within the tapered ends of skeletal muscle fibers. *Anat. Rec.* 242:462-470.

ROSSER, B.W.C., WICK, M., WALDBILLIG, D.M. and BANDMAN, E. (1996). Heterogeneity of myosin heavy chain expression in fast-twitch fiber types of mature avian pectoralis muscle. *Biochem. Cell Biol.* 74:715-728.

ROSSER, B.W.C., FARRAR, C.M., CRELLIN, N.K., ANDERSEN, L.B. and BANDMAN, E. (2000). Repression of myosin isoforms in developing denervated skeletal muscle fibers originates near motor endplates. *Dev. Dyn.* 217:50-61.

SCHMALBRUCH, H. and HELLHAMMER, U. (1977). The number of nuclei in adult rat muscles with special reference to satellite cells. *Anat. Rec.* 189:169-176.

SCHMALBRUCH, H. and LEWIS, D.M. (2000). Dynamics of nuclei of muscle fibers and connective tissue cells in normal and denervated rat muscles. Muscle and Nerve 23:617-626.

SWATLAND, H.J. (1994). *Structure and Development of Meat Animals and Poultry*. Technomic, Lancaster, Pennsylvania.

SYSTAT. (1997). Standard Version 7.0.1 for Windows. SPSS, Inc.

TIDYMAN, W.E., MOORE, L.A. and BANDMAN, E. (1997). Expression of fast myosin heavy chain transcripts in developing and dystrophic chicken skeletal muscles. *Dev. Dyn.* 208:491-504.

TROTTER, J.A. (1991). Dynamic shape of tapered skeletal muscle fibers. *J. Morphol.* 207:211-223.

TSENG, B.S, KASPER, C.E. and EDGERTON, V.R. (1994). Cytoplasm-to-myonucleus ratio and succinate dehydrogenase activities in adult rat slow and fast muscle fibers. *Cell Tissue Res.* 275:39-49.

WILKINS, J.T., KRIVICKAS, L.S., GOLDSTEIN, R., SUH, D. and FRONTER, A.R. (2001). Contractile properties of adjacent segments of single human muscle fibers. *Muscle and Nerve* 24:1319-1326.

WINCHESTER, P.K. and GONYEA, W.J. (1992). A quantitative study of satellite cells and myonuclei in stretched avian slow tonic muscle. *Anat. Rec.* 232:369-377.

ZADOR, E., DUX, L. and WUYTACK, F. (1999). Prolonged passive stretch of rat soleus provokes an increase in the mRNA levels of the muscle regulatory factors distributed along the entire length of the fibers. *J. Musc. Res. Cell Mot.* 20:395-402.

ZHANG, M. and McLennan, I.S. (1995). During secondary myotube formation, primary myotubes preferentially absorb new nuclei at their ends. *Dev. Dyn.* 204:168-177.

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