Steep Increase in Myonuclear Domain Size During Infancy

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ABSTRACT

We investigated whether myonuclear number increases in proportion to the increase in fiber size during maturational growth of skeletal muscle. Thoraco-abdominal muscle tissue was obtained from twenty 6-day to 15-year-old boys and girls during cardiothoracic surgery. Cross-sections were stained by anti-laminin for the basal lamina and by DAPI to identify nuclei. Basal lamina was traced on digital images to measure the fiber cross-sectional area (FCSA). Nuclei located within the basal lamina were considered myonuclei if pax7-negative and satellite cell nuclei if pax7-positive. Samples of two children were excluded from analysis because of clear signs of hypoxia as shown by positive carbonic anhydrase IX staining. Linear regression showed that FCSA increased with age by 187 μm^2·per annum ($R^2 = 0.90$; $P < 0.001$). Satellite cell density showed a dramatic decrease in the first months of life, but this was not accompanied by an increase in myonuclei per muscle fiber cross-section. Till four years of age the number of myonuclei per muscle fiber cross-section remained relatively constant but increased thereafter. Myonuclear domain size showed a steep increase during infancy and reached adult values in the young adolescent phase. Anat Rec, 296:192–197, 2013. ©2012 Wiley Periodicals, Inc.

Key words: skeletal muscle; myonuclei; satellite cell; myonuclear domain; muscle fiber size; maturation

It is generally assumed that postnatal muscle growth due to fiber hypertrophy is of much importance whereas the contribution of hyperplasia is negligible, both in humans (Malina, 1978) and in rodents (Tamaki and Uchiyama, 1995; Tamaki et al., 1997; White et al., 2010). It should be noted that muscle fibers not only increase in cross-sectional area, but also in length. In the medial gastrocnemius muscle, for instance, fiber length in children increased by 5% per year between 5 and 12 years of age (Benard et al., 2011). The observation that each myonucleus is usually associated with a more or less constant cytoplasmic volume has led to the concept of the myonuclear domain (Hall and Ralston, 1989). According to this concept, muscle fiber atrophy is associated with a commensurate loss of myonuclei, whereas hypertrophy is accompanied by a proportional increase in myonuclear number (Moss and Leblond, 1970; Allen et al., 1999; Van der Meer et al., 2011a,b). However, exercise induced hypertrophy in rats can occur to some extent without any change in number of

Abbreviations used: DAPI = 4',6-Diamidino-2-phenylindole; FCSA = fiber cross-sectional area; RT = room temperature; SEM = standard error of the mean.

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myonuclei (Petrella et al., 2006; van der Meer et al., 2011b). The myonuclear domain size also increases during maturational growth. In rodents it increases its size 4-fold between 4 days and 3 months after birth (Kelly, 1978; Kawano et al., 2008; White et al., 2010). Though in humans the myonuclear domain has also been found to increase 2.5-fold from toddler to adolescent (Vassilopoulos et al., 1977), a detailed time course of this increase is yet lacking, especially during infancy.

Muscular diseases like Duchenne Muscular Dystrophy and Pompe disease become manifest at young age. In these diseases loss of muscle mass and function result from the ultimate failure of satellite cell proliferation and differentiation to repair the ongoing muscle damage during these conditions (Hawke and Garry, 2001). Reference values for satellite cell densities in healthy children are, however, still missing. The observation that in rodent skeletal muscle the number of satellite cells per myonucleus decreases rapidly in the first weeks after birth (Moss and Leblond, 1970; White et al., 2010) suggests that the accretion of myonuclei occurs at its highest rate early after birth. If a similar situation applies to human muscle, this would be revealed as a rapid increase in myonuclear number in the first months after birth followed by a more gradual increase thereafter.

The objective of this study was therefore to determine the time course of changes in myonuclear domain area and satellite cell density during human muscle growth from one week after birth till adolescence.

MATERIALS AND METHODS

Thoraco-abdominal muscle tissue was obtained from twenty 6-day to 15-year-old boys and girls during cardiothoracic surgery. The study was approved by the medical ethics committee of the University of Leuven, Belgium and written informed consent was given by the parents or guardians of the children prior to the surgery. Any visible nonmuscle tissue was removed from the biopsy samples, which were then embedded in Tissue-Tek (Sakura Finetek, Zoeterwoude, The Netherlands), immediately frozen in liquid nitrogen-cooled isopentane and stored at −80°C until further analyses. Cross-sections (5 μm) were cut in a cryostat at −20°C, air-dried, and stored at −80°C. Sections were fixed for 5 minutes in acetone, air-dried, and then incubated with mouse monoclonal pax7 antibody (1:20 dilution in PBS-0.05%Tween20 (PBS-T), developed by A. Kawakami and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA, USA) and rabbit polyclonal anti-laminin antibody (1:50 dilution in PBS-T; Sigma, Zwijndrecht, The Netherlands) for 45 minutes at room temperature (RT). After washing in PBS-T (1x) and PBS (2x), sections were incubated with the appropriate secondary antibodies: AlexaFluor 555 goat anti-mouse (1:500, Molecular Probes, Invitrogen, Breda, The Netherlands) and AlexaFluor 488 goat anti-rabbit (1:200, Molecular Probes) for 45 minutes at RT. Sections were again washed with PBS-T and PBS before incubated with 300nM 4′,6-Diamidino-2-Phenylindole (DAPI; Molecular Probes) to visualize the nuclei. After a final wash with PBS sections were mounted with Mowiol (Calbiochem, Amsterdam, The Netherlands). Hypoxia affected cells were detected as follows: sections were fixated for 60 minutes with 3.7% formaldehyde (Merck; VWR; Amsterdam, The Netherlands) in PBS at RT. After a washing step with PBS, sections were incubated for 60 min at RT with the carbonic anhydrase IX antibody (1:100 dilution in PBS-T; Novus Biologicals; Cambridge, United Kingdom) (Garcia-Parra et al., 2011). After washing in PBS-T (1x) and PBS (2x), sections were incubated with the appropriate secondary antibody, AlexaFluor 488 goat anti-rabbit (1:200, Molecular Probes) for 45 minutes at RT. Also these sections were incubated with 300 nM DAPI to visualize the nuclei and after a final wash with PBS sections were mounted with Mowiol.

From each section, four to seven randomly selected images were captured using a Nikon ER800 fluorescence microscope (Nikon Instruments Europe, Badhoevedorp, The Netherlands) coupled to a Basler A113 C progressive scan color CCD camera with a Bayer color filter. Epifluorescence signal was recorded using a Texas red excitation filter (540–580 nm) for the detection of pax7, an FITC (fluorescein isothiocynate) excitation filter (465–495 nm) for the detection of laminin or carbonic anhydrase IX, and a UV excitation filter (340–380 nm) to detect the DAPI stained nuclei. To enable valid computer-assisted quantitative analysis, images for pax7, laminin and DAPI were captured with identical camera settings (i.e., the same exposure time, gain and offset settings) and magnification (40×). Images for CaIX and DAPI were captured with 20× magnification. Image processing was done using Lucia 4.81 software package (Nikon). For the analysis of myonuclear domain size, 50 muscle fibers of each subject were randomly selected from the captured digital images. Nuclei located within the basal lamina were considered myonuclei if pax7-negative and satellite cell nuclei if pax-7 positive (Fig. 1). The public domain software package *ImageJ* (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2007) was used to trace the laminin-stained basal lamina of the muscle fibers to compute the fiber cross-sectional area (FCSA). Image size was calibrated using a slide micrometer. Myonuclear domain area was calculated by dividing the mean FCSA by the mean number of myonuclei per muscle fiber cross-section. The satellite cell density was expressed as the number of satellite cells per mm^2 muscle fiber cross-sectional area. Satellite cells located in partly visible fibers were only counted from the bottom and right hand sides of the images.

Statistics

Due to the limited number of biopsies, we decided to pool data for boys and girls. The effect of age on FCSA and number of myonuclei per fiber was assessed using linear regression analysis. The effect of age on myonuclear domain area and density of satellite cells was assessed by negative exponential regression analysis (SPSS 16.0, Chicago, IL, USA). Data are presented as mean ± standard error of the mean (SEM). Effects were considered significant at $p < 0.05$.

RESULTS

Muscles and Age of the Children

The muscles from which the biopsies were taken and the ages of the children are shown in Table 1. Two children showed clear signs of hypoxia in their biopsies as

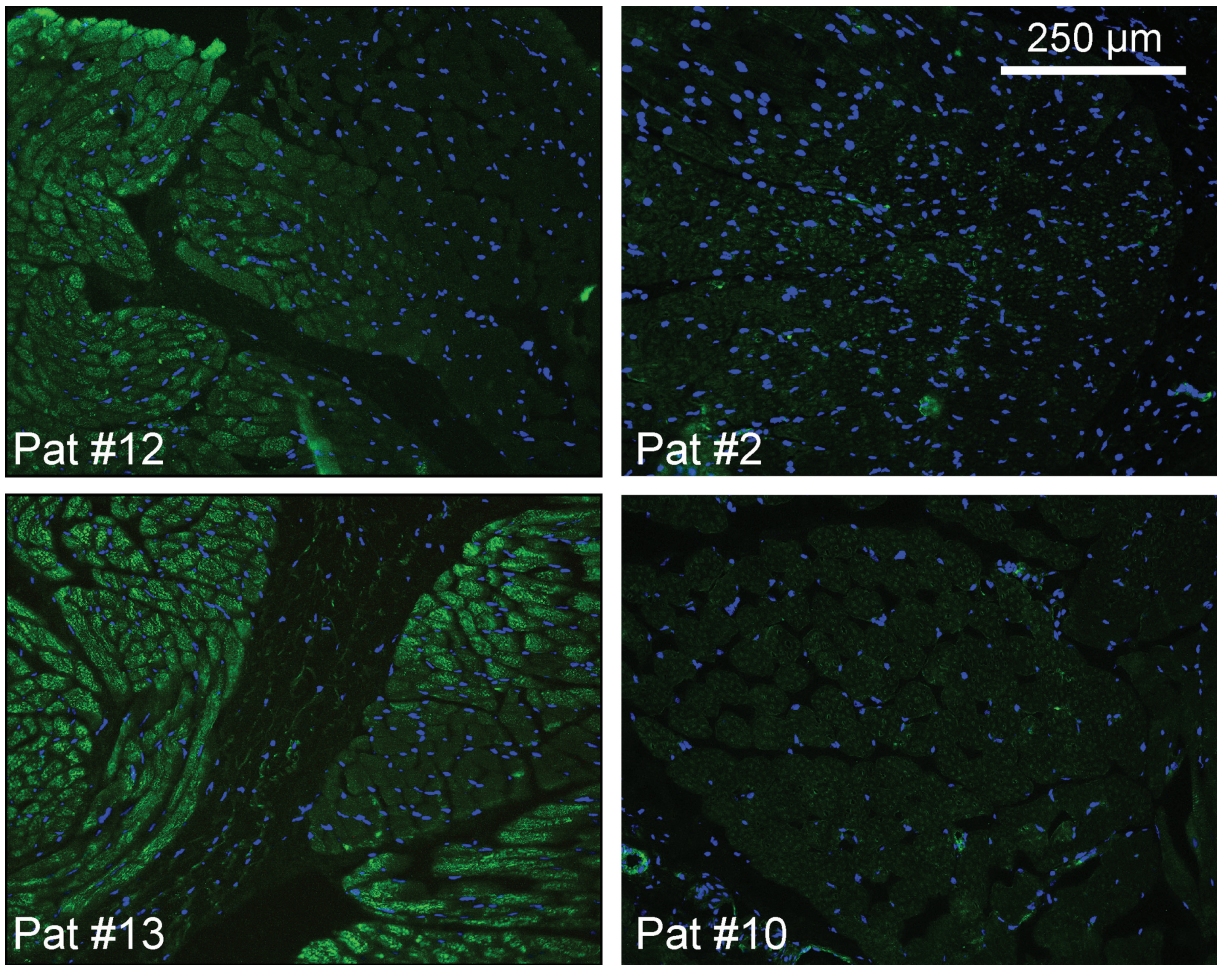

Fig. 1. Carbonic anhydrase IX immuno-stained cross-sections showing clear signs of hypoxia (green cells) in outlier children #12 and #13 (left panel). Sections of example patients #2 and #10 are negative for carbonic anhydrase IX. Nuclei are stained blue.

TABLE 1. Age, gender, and type of muscle

Subject	Age	Gender	Muscle
1	0.2 m	Boy	m. rectus abdominis
2	0.2 m	Boy	m. pectoralis major
3	1.3 m	Girl	m. pectoralis major
4	1.5 m	Girl	Abdominal muscle wall
5	3.9 m	Girl	m. latissimus dorsi
6	5.7 m	Boy	m. rectus abdominis
7	6.2 m	Girl	m. pectoralis major
8	8.3 m	Boy	m. rectus abdominis
9	1 y 1.0 m	Boy	m. pectoralis major
10	2 y 1.0 m	Girl	m. rectus abdominis
11	2 y 6.0 m	Girl	m. pectoralis major
12	3 y 1.0 m	Boy	m. pectoralis major
13	3 y 1.2 m	Boy	m. pectoralis major
14	3 y 2.5 m	Boy	m. rectus abdominis
15	3 y 3.0 m	Boy	m. pectoralis major
16	3 y 9.0 m	Boy	m. gluteus maximus
17	5 y 2.8 m	Girl	m. rectus abdominis
18	7 y 3.6 m	Boy	m. rectus abdominis
19	10 y 5.6 m	Boy	unknown
20	15 y 1.0 m	Girl	m. rectus abdominis

Y, indicates years; m, indicates months.

shown by positive carbonic anhydrase IX staining (Fig. 1). Because the results of these two boys, both aged 3-year-and-1-month, were clear outliers (very low FCSA, high myonuclei density, very small myonuclear domain area), we decided to exclude these results from further analysis.

Muscle Characteristics

Typical examples of immuno-stained cross-sections are shown in Fig. 2. From these images it is evident that FCSA increases with age. Figure 3 shows plots of FSCA, satellite cell density, myonuclei per fiber, and myonuclear domain area as a function of age. Because the boys ($n = 10$) and girls ($n = 8$) as a group did not differ significantly with respect to age, FCSA, satellite cell density, myonuclei per muscle fiber cross-section, and myonuclear domain area, we pooled the data of boys and girls for further analysis. Linear regression analysis revealed an increase in FCSA of 187 μm^2·per annum ($R^2 = 0.90$, $P < 0.001$). Satellite cell density showed a dramatic decrease in the first months of life, but this was not accompanied by an increase in myonuclei per muscle

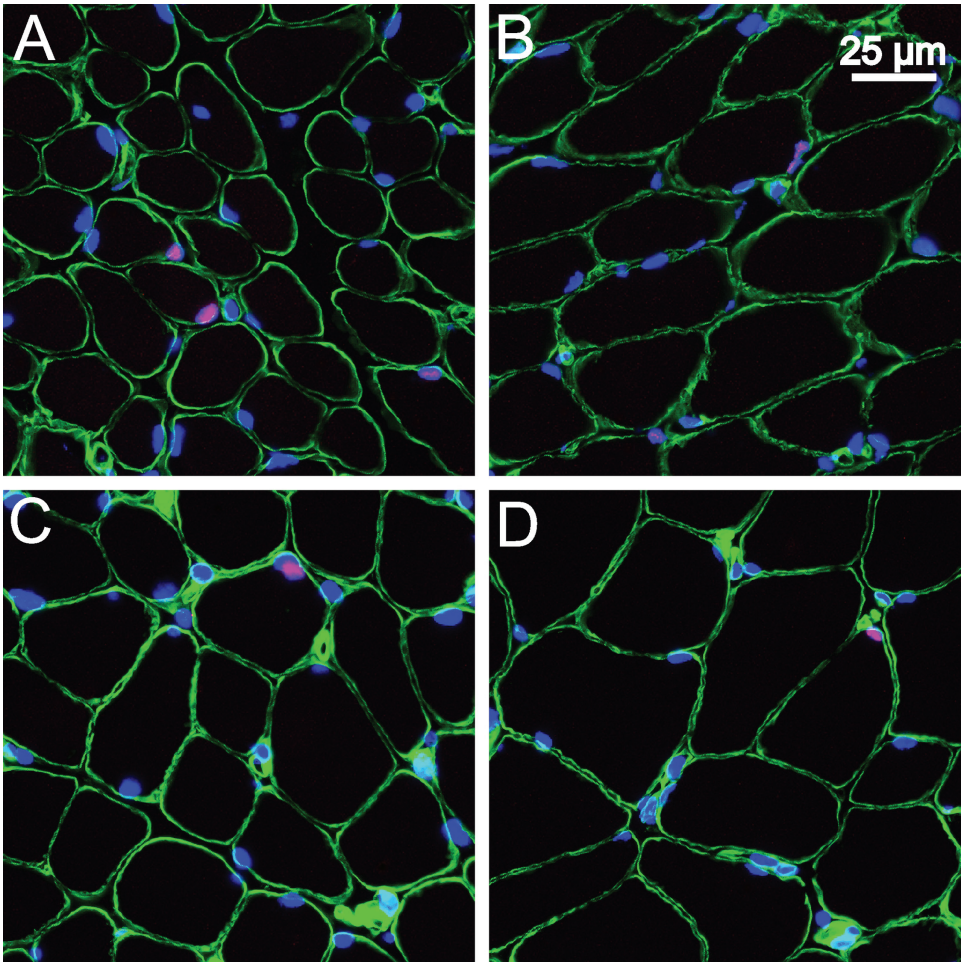

Fig. 2. Typical example of immuno-stained cross-sections of muscles from (**A**) 6-day-old, (**B**) 8-month-old, (**C**) 2-years-and-1-month-old and (**D**) 7-year-and-4-month-old children. Cell membranes are stained green, myonuclei blue, and satellite cells red.

fiber cross-section. Till four years of age the number of myonuclei per muscle fiber cross-section remained relatively constant but increased thereafter. Myonuclear domain area showed a steep increase during infancy and reached adult values in the young adolescent phase.

DISCUSSION

In corroboration with the general understanding of human muscle growth (Brooke and Engel, 1969; Malina, 1978), we observed that the FCSA increased with age. The principle finding of this preliminary study is, however, the steep decline in satellite cell density in early infancy that was not accompanied by a commensurate increase in the number of myonuclei per muscle fiber cross-section. Consequently, myonuclear domain size increased rapidly in the first months after birth followed by an attenuated rate of increase.

The only other study on human myonuclear domain size development that we are aware of reported that the nucleo-cytoplasmic ratio remained constant between 1 and 2.5 years to 12 and 30 years (Vassilopoulos et al., 1977). At first glance this appears at odds with our data, but it should be noted that they used fiber diameter as a measure of fiber size. Calculating the FCSA from the reported diameters shows that in their study myonuclear domain size did in fact also increase during maturation.

The increase in FCSA in the absence of a proportional increase in myonuclear number per fiber cross-section might indicate that during normal muscle growth the transcriptional activity of the individual myonuclei and/or the rate of protein translation increases to accommodate this growth and that protein synthesis is not limited by the transcriptional capacity of muscle fibers. Another possibility is that adults use muscle protein synthesis almost exclusively for turnover of protein, while in particular during early maturation it is used for both protein turnover and the accretion of new proteins for growth. If so, myonuclear transcriptional activity is expected to be higher postnatally than at later stages of maturation and, hence, requires a smaller myonuclear area.

In this study biopsies were obtained from different thoraco-abdominal muscles, which may have introduced some variance. It has been reported that myonuclear number per fiber cross-section does indeed vary between muscles within animals (Schmalbruch and Hellhammer,

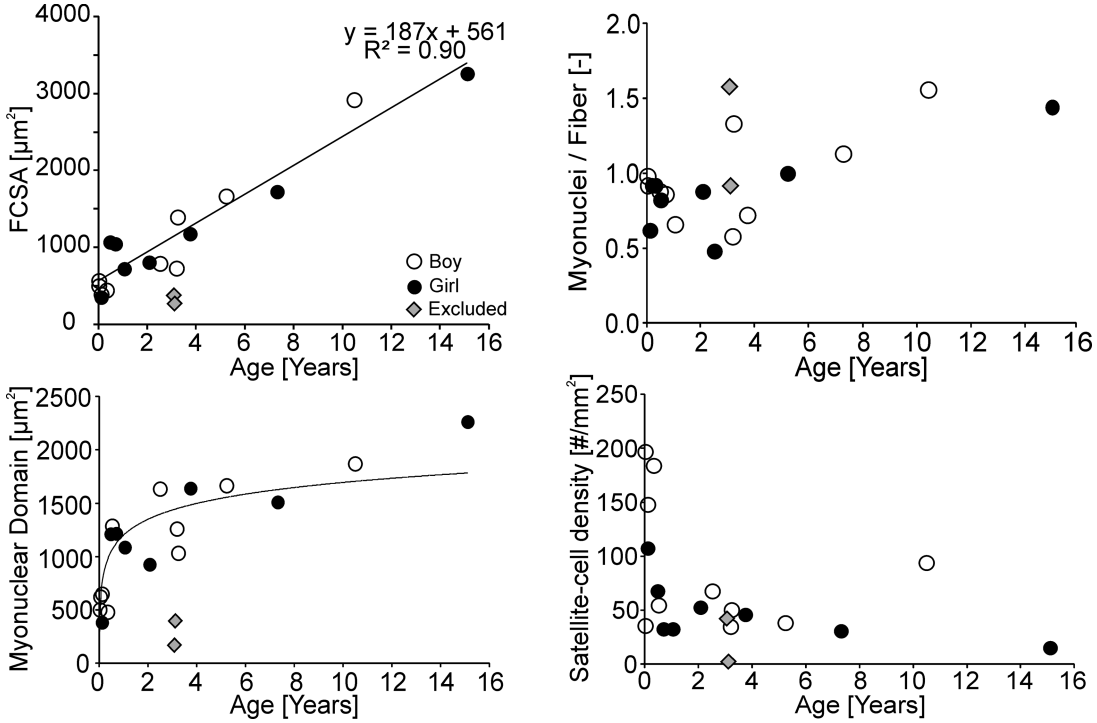

Fig. 3. Plots of muscle fiber cross-sectional area (FCSA), satellite cell density (#/mm^2), myonuclei per fiber cross-section, and myonuclear domain area as a function of age in years.

1977), and between fiber types in human muscle, e.g. type I fibers have more total nuclei and satellite cells (Verdijk et al., 2007). Though type I and II fibers from arm and leg muscles have a similar size and grow similarly in both boys and girls up to 15 years of age (Brooke and Engel, 1969; Oertel, 1988), it remains to be established to what extent changes in myonuclear domain size during maturation differ between fiber types and to what extend the loss of satellite cells in infancy is affected by the fiber type of the muscle studied. To test the possibility that changes in fiber type distribution as a function of age interfered with the increase of myonuclear domain, we performed additional stainings and measurements on non-serial sections using the following antibodies: A4.840 (Developmental Studies Hybridoma Bank [DSHB], Department of Biological Sciences, University of Iowa, 028 BBE, Iowa City, USA) a mouse IgM monoclonal antibody raised against myosin heavy chain to visualize human slow (type I) fibers; N2.261, a mouse IgG1 monoclonal antibody raised against myosin heavy chain, to visualize human fast (type IIa) fibers. We found no significant relationship between percentage type I fibers and age ($I\% = 0.82^*$ age $[y] + 61$, $r^2 = 0.08$, $P = 0.16$).

In this study muscle growth was estimated by changes in FCSA. It should be noted however, that not only the girth, but also the length of the muscle changes during growth. Benard et al. showed with a three-dimensional ultrasound analysis that fiber length increases proportionally to tibia length in human medial gastrocnemius medial muscle between 5 and 12 years of age (Benard et al., 2011). Furthermore, the consensus is that the number of muscle fibers is set at birth (Gollnick et al., 1981), but some investigators have reported that postnatal muscle growth may be realized to some extent by an increase in number of fibers (Sjostrom et al., 1991; Antonio and Gonyea, 1993; Tamaki and Uchiyama, 1995). These changes however do not affect the parameters used in our study, as they are normalized to the muscle volume contained within a 5-μm cross-section.

In human studies the number of myonuclei per fiber CSA is a widely used parameter (Kadi et al., 2004; Verdijk et al., 2007). To facilitate comparison with rodent data, myonuclear domain size was also calculated in terms of volume. Thereto, our data was corrected for slice thickness (β; 5 μm) and nucleus length (λ) (Jaspers et al., 2006). As we did not find any data on nucleus length as a function of age, we corrected our data using an adult myonucleus (~12 μm) and satellite cell nucleus length (~8 μm) (Watkins and Cullen, 1988). Under the assumption that nuclei are perpendicular oriented with respect to the plane of the slice and with an estimated minimal visible length γ of 1 μm, the correction factors, calculated as $CF = ((\lambda - 2\gamma)/\beta) + 1)$, are 3 for myonuclear domain size and 2.2 for satellite cell density. Though these factors will influence the absolute size of the myonuclear domain or satellite cell density, it will not affect its course as a function of age.

In conclusion, our study shows that satellite cell density steeply declines in early infancy. The skeletal muscle growth between birth and the age of 15 years is due to an increase in the size of the fibers without a proportional increase in myonuclear number per fiber. As a result, the myonuclear domain area [μm^2] increases rapidly in the first months after birth and reaches adult values in the young adolescent phase. Our data on the time courses of changes in satellite cell densities and

myonuclear domain area during maturational muscle growth provide some reference values for normally growing muscle and may be used for evaluation of therapies for muscle diseases in children.

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