Developmental effects on myonuclear domain size of rat diaphragm fibers

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Mantilla CB, Sill RV, Aravamudan B, Zhan WZ, Sieck GC. Developmental effects on myonuclear domain size of rat diaphragm fibers. J Appl Physiol 104: 787–794, 2008. First published January 10, 2008; doi:10.1152/japplphysiol.00347.2007.—During early postnatal development in rat diaphragm muscle (Dia_m), significant fiber growth and transitions in myosin heavy chain (MHC) isoform expression occur. Similar to other skeletal muscles, Dia_m fibers are multinucleated, and each myonucleus regulates the gene products within a finite volume: the myonuclear domain (MND). We hypothesized that postnatal changes in fiber cross-sectional area (CSA) are associated with increased number of myonuclei so that the MND size is maintained. The Dia_m was removed at postnatal days 14 (P-14) and 28 (P-28). MHC isoform expression was determined by SDS-PAGE. Fiber CSA, myonuclear number, and MND size were measured using confocal microscopy. By P-14, significant coexpression of MHC isoforms was present with no fiber displaying singular expression of MHCNeo. By P-28, singular expression was predominant. MND size was not different across fiber types at P-14. Significant fiber growth was evident by P-28 at all fiber types (fiber CSA increased by 61, 93, and 147% at fibers expressing MHC_Slow, MHC2A, and MHC2X, respectively). The number of myonuclei per unit of fiber length was similar across fibers at P-14, but it was greater at fibers expressing MHC2X at P-28. The total number of myonuclei per fiber also increased between P-14 and P-28 at all fiber types. Accordingly, MND size increased significantly by P-28 at all fiber types, and it became larger at fibers expressing MHC2X compared with fibers expressing MHC_Slow or MHC2A. These results suggest that MND size is not maintained during the considerable fiber growth associated with postnatal development of the Dia_m.

respiratory muscles; postnatal development; skeletal muscle; fiber type; myosin heavy chain

SIMILAR TO OTHER SKELETAL muscles, diaphragm muscle (Dia_m) fibers are multinucleated. Each myonucleus regulates gene products within a finite volume, the myonuclear domain (MND) (4, 18, 32). It has been suggested that MND size is maintained under different circumstances that stimulate muscle fiber hypertrophy through activation of satellite cells (28, 35, 36). However, in type-identified rat Dia_m fibers, our laboratory found that CSA of fibers expressing MHC_Slow or MHC2A increases to a lesser extent (e.g., ~50% from P-14 to P-28 and 90% from P-14 to P-84). Importantly, the Dia_m must be functional at birth, is highly active from birth onward, and is not as influenced by changes in gravitational load. Whereas previous studies have focused primarily on postnatal changes in Dia_m fiber CSA or the expression of MHC protein and/or mRNA (13, 24, 27, 41, 42, 49), little is known about changes in MND size. The Dia_m has a mixed fiber-type composition (24, 26, 27) and thus is ideally suited to examine fiber type-specific changes in MND size. We hypothesized that postnatal changes in fiber CSA are associated with increased number of myonuclei so that the MND size is maintained. The results of this study can then elucidate whether MND size is maintained during postnatal fiber growth across different fiber types, and thus whether myonuclear incorporation into growing fibers is proportional to the change in fiber volume, which is the key underlying question.

METHODS

Animals. To evaluate the effects of age on MND size of Dia_m fibers, Sprague-Dawley rat pups were studied at P-14 and P-28. Pregnant mothers were obtained, and pups were randomly assigned to one of these developmental time points after birth. Litter sizes were culled at birth to eight pups per litter. Pups stayed with their lactating mothers until postnatal day 21. Thereafter, Purina rat chow and water were provided ad libitum to weaned rats. All animals were housed in separate cages under a 12:12-h light-dark cycle. The Institutional

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Animal Care and Use Committee at Mayo Clinic approved all experimental procedures.

**Tissue preparation.** Animals were anesthetized with xylazine (10 mg/kg) and ketamine (90 mg/kg), and the costal Diam was removed. Midcostal Diaₘₘ samples were cut into rectangular strips; stretched to ~1.5 times their resting length, which is the optimal fiber length; and pinned to a piece of cork. After muscle strips were attached to the cork, they were placed in a relaxing solution consisting of (in mM) 59 potassium acetate, 6.7 magnesium acetate, 5.6 NaATP, 10 EGTA, 2 dithiothreitol, and 50 imidazole (all reagents were acquired from Sigma-Aldrich, St. Louis, MO). The total ionic strength of this solution was 200 mM (pH 7.0 at 4°C). After 24 h, the muscle strips were stored in 50% glycerol-50% relaxing solution at −20°C until single-fiber dissection occurred.

**Single-fiber dissection.** Similar to previous studies (14, 16, 44), Diaₘₘ strips were pinned on a Sylgard-coated (Dow Corning, Midland, MI) culture dish containing cooled 50% glycerol-50% relaxing solution. From each strip, 20–30 fibers were dissected by using a dissecting microscope (SZ40 Zoom Stereo Microscope, Olympus America, Melville, NY). Single fibers were cut into two segments. Aluminum clips were attached to the ends of one fiber segment, which was then placed in a 0.1% Triton X-100 relaxing solution for 20 min.

**Single-fiber electrophoresis.** MHC isoform expression was determined in the remaining fiber segment. This segment was placed and dissolved in 25 μl of sodium dodecyl sulfate (SDS) sample buffer consisting of 62.5 mM Tris-HCl, 2% (wt/vol) SDS, 10% (vol/vol) glycerol, and 0.001% (wt/vol) bromophenol blue at pH 6.8. As previously described (13, 15, 24), the samples were boiled for 2 min and run on SDS-polyacrylamide gel electrophoresis. The separating gel contained 5–8% acrylamide (pH 8.8) with 25% glycerol, and the stacking gel contained 3.5% acrylamide (pH 6.8). Gels (8 × 10 cm, 0.75 mm thick; Hoefer SE250) were run overnight at a constant current of 20 mA. Thereafter, the gels were silver stained (31). Myosin standards were used as a reference to determine the migration pattern of MHC isoform(s) expressed in each single-fiber segment.

Electrophoretic separation of MHC isoforms at the different postnatal ages was confirmed by immunoblotting of whole Diaₘₘ samples, as previously described (24, 26, 27). Embryonic and/or postnatal rat Diaₘₘ were quickly removed and snap frozen. The tissues were then homogenized in a modified RIPA lysis buffer (1% Igepal CA-630, 1% sodium deoxycholate, 1% Triton-X 100, 0.1% SDS, 10 mM EDTA, and Complete Mini protease inhibitors; Roche, Indianapolis, IN) in Tris-buffered saline (TBS). Homogenates were clarified by centrifugation and 50 μg of total protein was electrophoretically separated on a 7.5% SDS-polyacrylamide gel. After transfer to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA), the membrane was blocked in TBS containing 5% milk and 0.1% Tween 20 (Sigma-Aldrich) before an overnight incubation in primary antibodies derived from the following clones: F1.652 for embryonic MHC (DSHB, Iowa City, IA); NCL (Novocastra) for MHCslow; SC.71 (ATTCC, Manassas, VA) for MHC2A; BF-F3 (DSMZ, Braunschweig, Germany) for MHC2B; and BF-35 (DSMZ) for all but the MHC2X isoform. The specificity of all of these primary antibodies has been validated previously (22, 37). The membrane was then incubated with horse-radish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) of the appropriate species and isotype. Immunodetection was performed using enhanced chemiluminescence according to the manufacturer’s protocol (Pierce Biotechnology, Rockford, IL).

**Fluorescent labeling.** The fiber segment with aluminum clips was carefully placed on a glass slide held slightly in place with the utilization of a boundary created by a PAP pen (Super HT, Research Products International, Mt. Prospect, IL), and then it was fixed in 2% paraformaldehyde for 3 min followed by a wash in 0.1 M phosphate buffer (PB; pH 7.4). Next, fibers were treated for 3 min with 1 mg/ml of the membrane-specific dye N-(3-triethylammoniumpropyl)-4-(4-(4-(diethylamino)phenyl)butadienyl)pyridinium dibromide (RH 414; Molecular Probes, Eugene, OR), washed in 0.1 M PB and treated for 5 min with 0.01 mM propidium iodide (Molecular Probes), which stained the myonuclei. After the final wash in 0.1 M PB, the fibers were mounted in 100% glycerol (Sigma-Aldrich) and covered with a coverslip. The aluminum clips at each end of the fiber served as struts, so compression of the fiber by the coverslip was minimized.

**Single-fiber imaging and MND size determination.** An Olympus Fluoview confocal microscope mounted on a BX50W1 microscope (Olympus America, Melville, NY) was utilized to image the fluorescently stained fibers. Using a krypton laser, fibers were illuminated and imaged with an Olympus DApO ×40/1.3-numerical aperture oil-immersion objective. A representative single Diaₘₘ fiber is shown in Fig. 1. Serial confocal optical sections (step size = 0.5 μm) were imaged by moving the stage in only one direction from the top to the bottom of the fiber. This eliminated any backlash error in the stepper motor. Optical sections were digitized and stored individually in arrays of 800 × 600 pixels. Pixel dimensions were calibrated using a stage micrometer and were found to be 0.5 × 0.5 μm for the xy-plane (by convention, parallel to the microscope stage). The calculated thickness of optical sections was matched to this dimension, such that each voxel was 0.125 μm³. Using this technique, we previously...
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Determined (4, 44) that the average distortion in the z-plane is ~9%; and in the y-axis, distortion was <1%. Stacks of confocal optical slices were analyzed using Metamorph software (Molecular Devices, Sunnyvale, CA). Volume and muscle fiber CSA were estimated on the basis of optical sections obtained at three different randomly selected positions along the length of the fiber. The number of myonuclei in each fiber segment was counted along with segment length using the image obtained with the ×20 objective. With the ×40 objective, sarcomeres were counted and the length those sarcomeres spanned was measured. Fiber length measurements were adjusted to a sarcomere length of 2.5 μm.

In a subset of single fibers at each postnatal age, our laboratory confirmed that the fiber dissection technique results in inclusion of few satellite cells in single fibers (5, 44). The staining technique was further confirmed in Diaₘ fibers obtained from 3-day old pups (P-3) in which similar fixation and immunohistochemical procedures were used. In 30-μm-thick Diaₘ sections, incubation in propidium iodoide, anti-m-cadherin antibody (Santa Cruz), and anti-dystrophin antibody (Millipore, Billerica, MA) was followed by incubation with fluorescence-tagged secondary antibodies raised to the appropriate species. Sections were visualized using confocal microscopy as described. In these sections, 42 ± 2% of nuclei located within or in the immediate vicinity of Diaₘ fibers displayed m-cadherin immunoreactivity at P-3. Few satellite cells were identified in single fibers (<2%) at P-14 or P-28. Thus all propidium iodoide labeled nuclei in single fibers were considered to be myonuclei.

The total number of myonuclei per fiber was calculated from the average number of myonuclei per micrometer taking into account the adjustment for a 2.5-μm sarcomeric length and normalized for the average developmental age-specific fiber length. The number of myonuclei per fiber segment and fiber volume were then used to calculate average MND size for individual fibers (MND units of μm²/myonucleus).

Statistical analysis. One-way ANOVA was used to analyze the differences in MND size, number of myonuclei per fiber length, total number of myonuclei per fiber and fiber CSA across fibers containing MHC isoforms, and experimental groups based on age and fiber type. Tukey-Kramer honestly significant different test was used to analyze differences post hoc when appropriate. All statistical evaluations were performed with standard statistical software (JMP 6.0.0, SAS Institute, Cary, NC). Statistical significance was established at the 0.05 level. Values are means ± SE, unless otherwise specified.

RESULTS

Body weight. During postnatal development, rats rapidly increased in body weight (P-14: 29.6 ± 1.3 g, n = 6 pups; P-28: 64.9 ± 4.2 g, n = 8 pups). Pups from at least four different litters were used at each age group.

Developmental transitions in MHC isoform expression. A total of 208 single Diaₘ fibers were dissected and analyzed. In agreement with previous studies (15, 40), considerable developmental transitions in MHC isoform expression were observed in the rat Diaₘ between P-14 and P-28. MHC isoform expression was identified electrophoretically and confirmed using Western blot analyses of the entire Diaₘ as in previous studies (24, 26, 27). Expression of MHCEmb was not present at either P-14 or P-28 (but detectable embryonically; data not shown). At P-14, expression of MHCNeo, MHCSlow, MHC2A, and MHC2X was detected (but not of MHC2B). At P-28, MHCNeo was no longer present and MHC2B expression became evident. It is important to note that estimates of the relative distribution of Dia fiber types across developmental ages are limited by the single-fiber dissection technique and may not be reliable. At P-14, many fibers expressed MHCNeo (41 out of 69 fibers), but no singular expression of MHCNeo was detected. Coexpression of MHCNeo with MHCSlow, MHC2A, or MHC2X was common. Among fibers coexpressing MHC isoforms, 32 fibers coexpressed MHC2A and MHCNeo, 6 fibers coexpressed MHC2X and MHCNeo, and 3 fibers coexpressed MHC2A, MHC2X, and MHCNeo. Among fibers displaying singular expression of MHC isoforms, 8 expressed MHCSlow; 15 expressed MHC2A; and 5 fibers expressed MHC2X. At P-28, MHCNeo was no longer detected. Most fibers singularly expressed adult MHC isoforms (137 out of 139 fibers) at P-28: 41 fibers expressed MHCslow; 54 fibers expressed MHC2A; and 42 fibers expressed MHC2X. Two fibers showed coexpression of MHC2X and MHC2B which is frequently detected in the adult rat Diaₘ as well (15, 40).

Single Diaₘ fibers and MHC expression. Single Diaₘ fibers were grouped based on their MHC isoform expression for subsequent measurements. Three groups were considered: 1) fibers expressing MHCslow (singularly or in coexpression with MHCNeo), 2) fibers expressing MHC2A (singularly or in coexpression with MHCNeo or MHCslow), and 3) fibers expressing MHC2X (singularly or in coexpression with MHCNeo, MHCslow, MHC2A, or MHC2B). There were at least seven fibers from each group obtained per age. No differences in fiber CSA, myonuclear number or MND size were noted within these fiber groupings.

Diaₘ fiber dimensions. As expected, Diaₘ fibers increased in length from 8.6 ± 0.1 mm at P-14 to 11.4 ± 0.4 mm at P-28 (a 33% increase). The CSA of Diaₘ fibers varied with fiber type at both developmental ages (Fig. 2). At P-14, CSA for Diaₘ fibers expressing MHC2X was larger than for those expressing MHC2A. No significant difference in CSA was observed between fibers expressing MHCslow or MHC2A. At P-28, CSA was larger for fibers expressing MHC2X than for those expressing MHCslow or MHC2A. There was significant fiber growth from P-14 to P-28 for all fiber types. Fibers expressing MHCslow exhibited a 61% increase in mean CSA from P-14 to P-28. The CSA of fibers expressing MHC2A increased by 93% and that of fibers expressing MHC2X in-

Fig. 2. Fiber cross-sectional area of type-identified single Diaₘ fibers. The number of fibers in each postnatal group and type are shown below the corresponding bar and are the same for all figures. Values are means ± SE. *Significantly different from P-14 fibers of the same type, P < 0.05. †Significantly different from fibers expressing MHCslow in the same postnatal group, P < 0.05.
creased by 147% from P-14 to P-28. Consequently, mean volume of fibers expressing MHCSlow increased from $2.1 \pm 0.2 \times 10^6 \mu m^3$ at P-14 to $4.6 \pm 0.2 \times 10^6 \mu m^3$ at P-28 (114%); volume of fibers expressing MHC2A increased from $1.7 \pm 0.1 \times 10^6 \mu m^3$ at P-14 to $4.3 \pm 0.3 \times 10^6 \mu m^3$ at P-28 (156%); and volume of fibers expressing MHC2X increased from $2.2 \pm 0.2 \times 10^6 \mu m^3$ at P-14 to $7.0 \pm 0.4 \times 10^6 \mu m^3$ at P-28 (227%).

**Number of myonuclei.** The number of myonuclei per unit of fiber length (mm) did not vary between fiber types at P-14 (Fig. 3). At P-28, the number of myonuclei per millimeter of fiber length was greater at fibers expressing MHC2X than at fibers expressing MHCSlow or MHC2A. The number of myonuclei per millimeter of fiber length did not change from P-14 to P-28 in fibers expressing MHCSlow, but it increased at fibers expressing MHC2A or MHC2X. Irrespective of fiber type, there is poor correlation between fiber CSA and the number of myonuclei per fiber at either P-14 or P-28 (Fig. 4).

The total number of myonuclei per fiber were also estimated assuming an even distribution of myonuclei across the fiber and thus considering the increase in fiber length associated with greater postnatal age. In agreement with the findings on number of myonuclei per fiber length, the total number of myonuclei per fiber did not vary between fiber types at P-14, whereas at P-28 there was a significant difference in the total number of myonuclei per fiber across fiber types, being greater at fibers expressing MHC2X than at fibers expressing MHCSlow or MHC2A. Similarly, there was a significant increase in the total number of myonuclei per fiber from P-14 to P-28 at all fiber types. The total number of myonuclei increased by 52, 57, and 63% at fibers expressing MHCSlow, MHC2A, and MHC2X, respectively. Irrespective of fiber type, there is poor correlation between fiber CSA and the number of myonuclei per fiber at either P-14 ($r^2 = 0.11$) or P-28 ($r^2 = 0.38$). Thus the number of myonuclei (whether normalized to a unit of fiber length or estimated to the entire fiber) does not depend on fiber CSA (Fig. 4).

**MND size.** Consistent with the developmental differences in CSA and total number of myonuclei per fiber, MND size was not different across fiber types at P-14, whereas at P-28, fibers expressing MHC2X had larger MND size compared with fibers expressing MHCSlow or MHC2A (Fig. 5). From P-14 to P-28, MND size increased significantly at all fiber types (by 49% at fibers expressing MHCSlow or MHC2A and 105% at fibers expressing MHC2X). In general, MND size was larger in fibers with greater CSA at both P-14 and P-28. As shown in Fig. 6, MND sizes display only moderate correlation with fiber CSA, independent of fiber type or developmental age ($r^2$ for fibers expressing MHCSlow = 0.74 vs. 0.40 for P-14 and P-28 groups, respectively).
expressing MHC2A grew, and the size of fibers expressing MHC2B increased by ~150% while MND size increased by ~100%. Thus, although the increase in MND size is not exactly proportionate to the increase in fiber CSA, the volume of a muscle under control of a single myonucleus does expand during this period of rapid fiber growth across all fiber types.

Myonuclei may critically determine transcriptional rates, and thus they indirectly affect translational rates and fiber hypertrophy (1, 33, 34). If the mRNA transcription rate per myonucleus were to remain constant (11, 18, 28, 36), the larger MND size would suggest that there is a decrease in the local concentration of mRNA available for protein synthesis. In a previous study (13), the amount of MHCSlow and MHC2X mRNA (expressed as a fraction of total RNA) increased slightly, whereas the amount of MHC2A mRNA remained unchanged in the postnatal rat Diam between P-14 and P-28. Importantly, the amount of total RNA (expressed per tissue mass) was unchanged between these time points. Thus transcription of gene products necessary for fiber growth is controlled independently of myonuclear number. Rapid postnatal growth of the Diam likely results from increased protein accumulation within fibers independent of addition of new myonuclei to individual muscle fibers or a proportionate increase in MHC mRNA.

Changes in the number of myonuclei during postnatal development. The number of myonuclei may increase postnatally as a result of satellite cell activation (20). Accordingly, in the present study, there was a significant increase in the number of myonuclei per fiber length and total number of myonuclei per fiber across fiber types (Fig. 3). Although apoptosis occurs during early postnatal development of hindlimb muscles (12), it is most prominent between 5 and 9 days postnatally, and it is minimal beyond P-14. Thus our findings are consistent with a net gain of myonuclei resulting predominantly from satellite cell activation (29).

Although satellite cell activation and incorporation into myofibers is crucial for postnatal fiber growth (6, 29), the number of myonuclei does not seem to determine the extent of growth in fiber CSA. There is a weak correlation between myonuclear number and fiber CSA at both P-14 and P-28 (Fig. 4), which is modest at best (r² = 0.5) across developmental stages. As a general trend, as CSA increases from P-14 to P-28, so does myonuclear number; and it does so similarly across fiber types (~60%) despite considerable differences in fiber cross-sectional growth. Satellite cells are abundant postnatally, comprising 11–25% of all nuclei in myofibers at P-14 and P-28 (10). Consistent with the abundance of satellite cells in postnatal muscles, we found that ~40% of nuclei surrounding Diam fibers were positive for the satellite cell attachment protein m-cadherin at P-3. Satellite cells show high levels of mitotic activity between P-14 and P-28 and thus most certainly participate in fiber growth (20, 38). Satellite cell proliferation and incorporation into muscle fibers may be particularly important at fiber ends (near the myotendinous junction). In the present study, the maintenance of MND size during a period of fiber rapid increase in fiber CSA was explored, and thus only missections of costal Diam were examined. Whether different mechanisms are at play in the longitudinal vs. cross-sectional growth of fibers during postnatal development cannot be ascertained from this study, but this possibility is certainly intriguing.

Fig. 6. Scatterplot of the MND size vs. fiber CSA at single type-identified Diam fibers. A: fibers expressing MHCslow. B: fibers expressing MHC2A. C: fibers expressing MHC2X. Values for each fiber in the P-28 group; r, values for each fiber in the P-14 group. Regression lines fitted for each fiber type independent of postnatal group are shown (MHCslow: r² = 0.47; MHC2A: r² = 0.58, and MHC2X: r² = 0.54). All regression lines showed a statistically significant correlation (P < 0.0001).
Changes in MND with developmental growth. The original concept of MND implied that with conditions that bring about muscle adaptation and remodeling, MND is a strictly regulated, controlled variable whose size is maintained (18, 32). However, there is conflicting evidence regarding the maintenance of MND size across conditions associated with fiber growth. Indeed, MND size may be differentially regulated across fibers of different size or MHC isoform expression. In the present study, MND size increased at all Dia<sub>an</sub> fibers from P-14 to P-28, and, although proportionate, MND size is poorly correlated with fiber CSA regardless of postnatal age or fiber-type composition ($r^2 < 0.5$; Fig. 6). In agreement with previous studies in the adult rat Dia<sub>an</sub>, MND size varies across fiber types, with MND size in fibers expressing MHC<sub>2X</sub> being ~80\% larger than fibers expressing MHC<sub>Slow</sub> or MHC<sub>2A</sub> (5, 44).

During postnatal growth of mouse hindlimb muscles, MND size increased in the tibialis anterior muscle (0 days to 5 wk) because of a greater increase in fiber volume compared with the change in myonuclear number (45). The mouse tibialis anterior muscle is fairly homogeneous in composition, comprising primarily myofibers expressing MHC<sub>2X</sub> and/or MHC<sub>2B</sub>. In contrast, MND size remained constant in the soleus muscle (a mixed muscle in the mouse comprising fibers expressing MHC<sub>Slow</sub> and MHC<sub>2A</sub>), but it increased in the extensor digitorum longus (composed predominantly of fibers expressing MHC<sub>2B</sub>) between 2 and 23 mo of age (8).

Previous studies observed that MND size was maintained in hindlimb muscles under other conditions that lead to muscle fiber hypertrophy. MND size remained unchanged in the rat soleus (28), rat (36) and cat (3) plantaris, and rat extensor digitorum longus (35) muscles after functional overload or removal of synergistic muscles. In accordance, after treadmill exercise (36) or muscle fiber hypertrophy induced by IGF-1 treatment (28), MND size did not change. However, during normal growth of adult rat diaphragm muscle (~11 days; mean initial body weight: 267 g, final: 320 g), MND size increased significantly at fibers expressing MHC<sub>Slow</sub> (~56\%), MHC<sub>2A</sub> (~67\%), and MHC<sub>2X</sub> (~33\%), but it did not increase at fibers expressing MHC<sub>2B</sub> (44). Fourteen days after phrenic nerve section (5), fibers expressing MHC<sub>Slow</sub> hypertrophy (~15\% increase in CSA), and MND size increases (~26\%). It is possible that the type of stimulus for fiber growth determines the maintenance (or not) of MND size and/or that fiber-type differences exist in the mechanisms underlying cross-sectional growth of muscle fibers. Regardless of fiber type, MND size shows a poor correlation to fiber CSA at either P-14 or P-28 (Fig. 6), indicating that the postnatal increase in fiber CSA is not associated with maintenance of MND size independent of postnatal age, fiber type, or fiber CSA.

**MHC isoform expression during development.** During the first 4 wk postnatal development, the rat diaphragm muscle shows significant changes in MHC isoform expression, muscle contractile properties, and fiber dimensions (13, 24, 25, 27, 41, 42, 49). Whereas the majority of fibers express protein for the MHC<sub>Neo</sub> isoform at birth, by P-28, the expression of this isoform has disappeared. Fibers express protein for the MHC<sub>2X</sub> and MHC<sub>2B</sub> isoforms only beyond P-14. Importantly, the growth of muscle fibers is most notable for those expressing MHC<sub>2X</sub> and MHC<sub>2B</sub> (15, 24, 41, 47–50). The transition in MHC isoform phenotype during postnatal development results in a larger proportion of fast-type isoforms being expressed in the adult rat Dia<sub>an</sub>. Such a transition has important physiological and functional relevance as the repertoire of respiratory behaviors increases.

In agreement with a previous study from our laboratory (40), we found a predominance of fibers coexpressing MHC isoforms at P-14, whereas most fibers had singular MHC isoform expression at P-28. The distribution of fibers expressing MHC<sub>Neo</sub>/2A, MHC<sub>Neo</sub>/2A/Slow, MHC<sub>2A</sub>, and MHC<sub>2X</sub> in the present study is similar to that previously reported for P-14 fibers, although single-fiber studies have significant limitations in estimating the distribution of MHC isoform expression. In general, fibers expressing MHC<sub>Neo</sub>/2A, MHC<sub>Neo</sub>/2A/Slow, and MHC<sub>Slow</sub> isoforms predominate in the P-14 Dia<sub>an</sub>. Also in agreement with our laboratory’s previous report, at P-28 there is a predominance of MHC<sub>Slow</sub>, MHC<sub>2A</sub>, and MHC<sub>2X</sub> expressing Dia<sub>an</sub> fibers. These findings also confirm previous findings using histochemical methods for fiber-type classification (43). Caiozzo et al. (9) examined MHC polymorphisms in a large sample of single muscle fibers from various muscles, including the adult rat Dia<sub>an</sub>. Up to 55\% of fibers expressed more than one MHC isoform, and nearly all possible 15 combinations for coexpression of adult MHC isoforms were found. It is possible that the differences in MHC expression may relate to the use of female (9) vs. male rats (17, 40, 43) or even differences in suppliers of Sprague-Dawley rats. These possibilities remain to be explored.

In present study, three fiber type groups were considered based on the expression of MHC isoforms for the purpose of examining fiber-type-dependent effects on MND size across developmental ages. Although differences in MND size were evident across fiber types, MND was not maintained at any fiber type during this period of rapid increase in fiber CSA. Single fibers were identified based on their MHC isoform expression. The identity of MHC isoforms expressed was determined electrophoretically. MHC isoform determination by this method was confirmed by immunoblotting of whole Dia<sub>an</sub> samples, as in previous studies (24, 26, 27, 43). Importantly, electrophoretic separation permits clear identification of MHC isoform expression in the adult Dia<sub>an</sub>. By electrophoretic migration pattern alone, MHC<sub>2B</sub> and MHC<sub>Neo</sub> as well as MHC<sub>2A</sub> and MHC<sub>Emb</sub> might not be as readily identified. However, we confirmed by immunoblotting that MHC<sub>Emb</sub> was no longer present at P-14 (data not shown). In addition, our laboratory has previously determined that MHC<sub>2B</sub> was not present in the rat Dia<sub>an</sub> at P-14 (when MHC<sub>Neo</sub> is commonly expressed); and, MHC<sub>Neo</sub> is no longer expressed at P-28 (24, 40, 43). Thus we are confident that MHC isoform expression in single fibers was correctly identified in this study. However, studies at other time points might require alternative methods for fiber-type identification.

**Mechanisms underlying Dia<sub>an</sub> fiber growth.** During early postnatal development, transcriptional control and other regulatory processes likely play a significant role in the overall growth of rat Dia<sub>an</sub> fibers, especially for those expressing adult MHC isoforms. Based on the results of the present study, addition of myonuclei [likely through fusion of activated satellite cells (29)] is not proportionate to the increase in fiber volume, resulting in increased MND size. However, the present study examined midsections of Dia<sub>an</sub> fibers rather than their ends. It is possible that the mechanisms underlying the longitudinal growth of fibers differ from those underlying...
cross-sectional growth of fibers. Clearly, postnatal growth of muscle fibers, including those in the Dia\textsubscript{an}, occurs in both dimensions. Analyses of MND size in fiber midsections rather than their ends likely is more relevant to the postnatal increase in fiber CSA than to longitudinal growth, if the concept of MND size as an overall determinant of local transcriptional activity holds (4, 18, 32). Thus our findings that MND size is not maintained provide important novel information indicating that the postnatal growth in Dia\textsubscript{an} fiber CSA does not involve a proportionate increase in myonuclear number. It is possible that fiber growth may result from the transcriptional activity level in myonuclei rather than the total myonuclear number. Indeed, the level of transcriptional activity may differ across myonuclei within the same myofiber (30). The expression of muscle-specific factors or other proteins (including contractile proteins) may also differ across myonuclei within a single fiber (7, 19). However, these possibilities were not examined in the present study. This notwithstanding, mRNA amounts in the rat Dia\textsubscript{an} do not increase in proportion to fiber growth (13), suggesting that changes in transcriptional activity (although present) do not exclusively determine the postnatal growth of the Dia\textsubscript{an}.

Transitions in MHC isoform expression and fiber cross-sectional growth may depend on shared, preprogrammed fiber-type differences. However, several factors are known to only modify the timing of postnatal MHC isoform expression. For example, low thyroid levels, altered patterns of muscle innervation, and phrenic denervation all temporally alter postnatal MHC isoform transitions and blunt fiber cross-sectional growth (2, 23, 41). However, the adult MHC phenotype is eventually expressed. Taken together, these results suggest that during postnatal development in the rat Dia\textsubscript{an} complex differential regulatory mechanisms control MHC isoform expression.

In conclusion, the present study demonstrates that during postnatal development, there are significant increases in fiber CSA and MND size while still increasing the number of myonuclei in Dia\textsubscript{an} fibers (i.e., postnatal fiber cross-sectional growth takes place without maintenance of MND size). Although addition of myonuclei play a critical role in fiber growth, the number of myonuclei that are incorporated during a period of rapid increase in Dia\textsubscript{an} fiber CSA is not regulated to maintain MND size.

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GRANTS

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