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Heterogeneous activation of a slow myosin gene in proliferating myoblasts and differentiated single myofibers

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ABSTRACT

Each skeletal muscle contains a fixed ratio of fast and slow myofibers that are distributed in a stereotyped pattern to achieve a specific motor function. How myofibers are specified during development and regeneration is poorly understood. Here we address this question using transgenic reporter mice that indelibly mark the myofiber lineages based on activation of fast or slow myosin. Lineage tracing indicates that during development all muscles have activated the fast myosin gene *Myl1*, but not the slow myosin gene *Myh7*, which is activated in all slow but a subset of fast myofibers. Similarly, most nascent myofibers do not activate *Myh7* during fast muscle regeneration, but the ratio and pattern of fast and slow myofibers are restored at the completion of regeneration. At the single myofiber level, most mature fast myofibers are heterogeneous in nuclear composition, manifested by mosaic activation of *Myh7*. Strikingly, *Myh7* is activated in a subpopulation of proliferating myoblasts that co-express the myogenic progenitor marker Pax7. When induced to differentiate, the Myh7-activated myoblasts differentiate more readily than the non-activated myoblasts, and have a higher tendency, but not restricted, to become slow myotubes. Together, our data reveal significant nuclear heterogeneity within a single myofiber, and challenge the conventional view that myosin genes are only expressed after myogenic differentiation. These results provide novel insights into the regulation of muscle fiber type specification.

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Introduction

The skeletal muscle comprises ~40% of the body mass and functions to empower body movements and regulate systemic energy metabolism (Karagounis and Hawley, 2010; Zierath and Hawley, 2004). The skeletal muscle is mainly composed of mature muscle cells called myofibers, and myofiber composition in each muscle is heterogeneous, containing a mixture of slow and fast type myofibers (Wang et al., 2004). Based on the relative abundance of slow and fast myofibers, a muscle can either be classified as a slow or a fast muscle. For example, the soleus (SOL) muscle is a slow muscle as it contains mostly slow (or type I) myofibers; and the extensor digitorum longus (EDL) muscle is a fast muscle because its myofibers are predominantly fast (type II) (Waddell et al., 2010). The slow and fast myofibers are different in biochemical, structural and physiological properties (Bassel-Duby and Olson, 2006). One main criterion to classify myofiber types is based on the myosin heavy chain (Myh) isoform expression. In this scenario, fast myofibers can be further divided into type IIA, IIX and IIB based on the expression of *Myh2*, *Myh1* and *Myh4* genes, respectively (Chakkalakal et al., 2012). By contrast, type I slow myofibers uniquely express the *Myh7* gene, encoding the β-myosin heavy chain protein that is also expressed in cardiac muscles (Chen and Wang, 2012). The contractile speed of these myofibers ranks in the order of IIB > IIX > IIA > I. Based on energy utilization, myofibers can also be classified as oxidative (I, IIA) and glycolytic (IIX, IIB) myofibers.

When skeletal muscles are damaged, they regenerate to reestablish the preexisting myofiber types (Feldman and Stockdale, 1991). Satellite cells, a population of muscle resident stem cells,

Abbreviations: BSA, bovine serum albumin; CTX, cardiotoxin; DMEM, defined minimal essential medium; EDL, extensor digitorum longus (muscle); EYFP, enhanced yellow fluorescent protein; FACS, fluorescent activated cell sorting; FBS, fetal bovine serum; MYH, myosin heavy chain; MYL, myosin light chain; Myod, myogenic differentiation 1; Pax7, paired box 7; RFP, red fluorescent protein; SOL, soleus (muscle); TA, tibialis anterior (muscle); TdTomato, tandem dimer Tomato.

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are responsible for the regeneration of injured muscles (Relaix and Zammit, 2012). Satellite cells are quiescent in non-injured adult muscles. In response to muscle injury, they are activated and reenter the cell cycle to proliferate, then differentiate and fuse with the damaged myofibers to repair the damages (Kuang and Rudnicki, 2008). Meanwhile, a subpopulation of the proliferating myoblasts undergoes self-renewal to replenish the quiescent satellite cell pool. The self-renewal, proliferation and differentiation status of satellite cells can be distinguished by the expression of Pax7 and MyoD, manifested as $Pax7^+/MyoD^-$, $Pax7^+/MyoD^+$ and $Pax7^-/MyoD^+$, respectively (Olguin and Olwin, 2004; Zammit et al., 2004).

A long-standing unresolved question is whether myofiber specification occurs at the progenitor cell level (i.e. subpopulations of progenitor cells give rise to fast and slow myofibers, respectively) or at the post-differentiation level (i.e. fast and slow myofibers originate from a common progenitor population). Nevertheless, it has been widely accepted that *Myh* genes are only expressed in post-differentiation muscle cells. In the present study, we conducted genetic lineage analyses to determine the specification of fast and slow myofibers during development and regeneration. As *Myh7* and *Myl1* genes are unique markers of mature slow (type I) and fast (type II) myofibers, respectively, we used Myh7-Cre and Myl1-Cre mice, in combination of fluorescent reporter mice, to delineate if mature fast and slow myofibers originate from progenitors expressing the corresponding myosin genes. Using this strategy, we show that *Myl1* gene is activated in all fast and slow myofibers, but *Myh7* is only activated in a subset of fast myofibers in addition to slow myofibers. Surprisingly, we found that *Myh7* is activated in a subset of actively proliferating myoblasts, thus challenging the previous view that *Myh* genes are only activated in differentiated cells. When induced to differentiate, the Myh7-activated myoblasts were not limited to become slow myotubes, though they had a higher tendency to do so. These results provide mechanistic insights into muscle fiber type specification.

Materials and methods

Animals

All procedures involving the use of animals were performed under the guideline of Purdue University's Animal Care and Use Committee. Mice were housed in animal facility with free access to water and standard rodent chow. The reporter mice were purchased from Jackson Laboratory (Bar Harbor, ME) under that stock numbers: Rosa26-EYFP (Madisen et al., 2010), 007903; Rosa26-TdTomato (Madisen et al., 2010), 007905; and Rosa26-mTmG (Muzumdar et al., 2007), 007576. The Myh7-Cre transgenic mouse (Parsons et al., 2004) was provided by Katherine Yutzey (Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio). The Myl1-Cre knockin mouse (Bothe et al., 2000) was provided by Steven Burden (New York University Medical Center, NY). The PCR genotyping was done using protocols described by the supplier.

Cardiotoxin (CTX) injection

CTX (Sigma) was used to induce muscle regeneration. Mice were anesthetized by IP injection of 0.1 ml ketamine cocktail per 10 g body weight and TA/EDL muscles were injected with 50 µl of 10 µM CTX. Muscles were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek) and fresh-frozen in isopentane chilled on dry ice. Frozen samples were cut into 10 µm thickness sections on a CM1850 cryostat (Leica). Muscle sections were collected on Superfrost Plus glass slides (Electron Microscopy Sciences).

Immunostaining and imaging

Cryosections were thawed at room temperature and washed with PBS to wash out the OCT. Samples were then blocked in PBS containing 5% goat serum, 2% BSA, 0.2% triton X-100 and 0.1% sodium azide. Primary antibodies used were anti-Pax7, anti-MYHC I, anti-MYHC IIA, anti-MYHC IIB (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA), anti-MyoD (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-Laminin (Sigma-Aldrich, St. Louis, MO, USA). Samples were incubated in primary antibodies diluted in blocking buffer for overnight at 4 °C, followed by incubating with secondary antibodies and 4′, 6-diamidino-2-phenylindole (DAPI) diluted in PBS for 30 min at room temperature. Cultured cells were fixed by 4% PFA, and then quenched by 100 nM Glycine, followed by similar staining steps. Stained samples were mounted with Dako fluorescent mounting media (DAKO corp., Carpinteria, CA, USA). Fluorescent images were captured with a Coolsnap HQ CCD camera (Photometrics, USA) driven by IP Lab software (Scanalytics Inc, USA) using Leica DMI6000B fluorescent microscope (Leica Microsystems, Mannheim, Germany) with 1.25 ×, 10 ×, or 20 × objectives.

Primary myoblast culture

Hindlimb muscles were collected and trimmed free of connective tissues and tendons, minced with scissors and digested in collagenase/dispase in 6-cm petri dishes at 37 °C for 24 min. The digestion solution contained 1% collagenase B (Roche, Indianapolis, IN, USA) and 2.4 U/ml dispase (Roche). The digestion was stopped by adding 3 × volume of Ham's F-10 medium (Multicell) with 5% FBS and triturated, then centrifuged at 250*g* for 5 min using an IEC CENTRA CL2 centrifuge (Thermo Electron Corporation). After centrifugation, the pellet was resuspended and filtered through 70 µm cell strainer to remove debris, and cells were pelleted and grown in 10 cm collagen-coated dishes with 10 ml of Ham's F-10 medium containing 20% FBS, 1% penicillin/streptomycin (P/S) and 4 ng/ml bFGF at 37 °C and 5% CO_2. The initial culture was refreshed with 5 ml growth medium every 24 h and passaged to a new collagen-coated dish after 72 h. During passages, cells were treated with 0.25% trypsin and closely monitored. To remove fibroblasts, the trypsinization was stopped immediately when most myoblasts are detached from the plate but before fibroblasts detach. Cells were fed with fresh medium every two days. Upon confluence, cells were induced to differentiate in DMEM (Sigma) containing 2% horse serum and 1% P/S.

Isolation and culture of single myofibers

Intact EDL and SOL muscles were dissected out by cutting both tendons and digested in 0.2% type I collagenase (Sigma) in DMEM at 37 °C with agitation for 30 min (EDL muscle) or 70 min (SOL muscle) until fibers began to detach from the muscle. After digestion, muscles were triturated with polished glass Pasteur pipettes to liberate myofibers from the collagenase loosened EDL and SOL muscles. All pipettes and petri dishes were pre-rinsed with horse serum to prevent myofiber attachment. Single myofibers were either directly fixed or cultured in DMEM containing 20% FBS, 1% P/S, 0.5% Chicken Embryo Extract (CEE).

Statistical analysis

All data are presented as mean ± standard deviation (SD). The "*n*" in the results represents biological replicates unless specified. At least three technical replicates were usually conducted within each biological sample and the averaged value was used. For the significance analysis, two-tailed student's *t*-test was used to

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