ACTIVATED SATELLITE CELLS ARE PRESENT IN UNINJURED EXTRAOCULAR MUSCLES OF MATURE MICE

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ABSTRACT

Purpose: We recently demonstrated that there is a continuous process of myonuclear addition into normal, uninjured adult myofibers in rabbit extraocular muscles (EOM). This phenomenon is not seen in skeletal muscles from normal, adult limbs. These features may explain the selective involvement of the EOM in progressive external ophthalmoplegia and oculopharyngeal muscular dystrophy due to an accumulation of damaged DNA in mitochondria and nuclei within the EOM as a result of repeated cycling of the muscle satellite cells. Many testable hypotheses flow from these observations. We investigated whether continuous myonuclear addition is present in normal mouse EOM so that mouse models of genetic disorders can be used to study the pathogenic mechanisms and to test potential therapies for human muscle disorders.

Methods: Bromodeoxyuridine (brdU) was injected intraperitoneally into C57 adult mice every 2 hours for 12 hours. Twenty-four hours later the animals were sacrificed, and the globes with the muscles attached were prepared for immunohistochemical localization of brdU-positive nuclei within the EOM. All cross sections were immunostained for both brdU and either dystrophin or laminin.

Results: All the rectus muscles from the mouse EOM examined contained both satellite cells and myonuclei that were positive for brdU. This demonstrates the division of satellite cells and the fusion of their daughter cells with existing adult EOM myofibers in mice.

Conclusions: These data indicate that the process of continuous myonuclear addition is also active in mouse EOM. These findings will allow various mutant mouse models to be used to study the pathogenesis and treatment of various muscle disorders. The existence of continuous myonuclear addition in adult, uninjured EOM fundamentally changes the accepted notion that EOM myofibers are postmitotic.

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INTRODUCTION

Skeletal muscles at all locations are increasingly regarded as heterogeneous at the single myofiber level. At the molecular level, it is becoming clear that even the myosin proteins are expressed in subsets that are much more heterogeneous than was originally thought.[1] In particular, it appears that the extraocular muscles (EOM) represent a unique allotype.[2] Extraocular muscles possess anatomical and physiologic features, as well as disease propensities, that distinguish them from other skeletal muscles. The EOM are distinct from limb muscle in that they have a different developmental origin as well as an unusual set of contractile properties. The EOM express a subset of molecules that are normally down-regulated in adult skeletal muscle, such as the immature form of the acetylcholine receptor[3] and N-CAM,[4] as well as a set of molecules that are uniquely expressed in the EOM, such as the EOM-specific myosin heavy chain isoform.[5] These properties set them apart from other skeletal muscle groups.

Normal skeletal muscle fibers of adult mammals are not replaced or remodeled unless injured. However, skeletal muscles contain a quiescent population of progenitor cells know as satellite cells. When these cells become activated after injury, they divide and result in regeneration of the injured muscle.[6] We have recently shown that uninjured EOM of mature rabbits undergo a process of continuous myonuclear addition.[7] Using a series of bromodeoxyuridine (brdU) labeling strategies, we demonstrated brdU-positive myonuclei that became stably integrated into normal, adult rabbit EOM myofibers. The limb skeletal muscles of the same rabbits did not contain brdU-positive myonuclei. These findings suggest that the EOM of

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adult mammals maintain a population of activated satellite cells that divide in the normal adult muscle, and their progeny slowly fuse into existing myofibers.

The existence of continuous myonuclear addition in the EOM could be of pathogenic significance in those skeletal muscle diseases that either spare the EOM or preferentially involve them.[8,9] For example, the EOM are spared in Duchenne's muscular dystrophy[10-12] and preferentially involved in other conditions, such as progressive external ophthalmoplegia and myasthenia gravis.[13] The phenomenon of continuous myonuclear addition and remodeling of the EOM may have implications for understanding the etiology of strabismus, the late changes following strabismus surgery, and the responses of the EOM to denervation.

It is important to demonstrate that continuous myonuclear addition into single myofibers of adult, uninjured rabbit EOM occurs not only in rabbits but also in mammalian EOM in general. We examined whether this process of continuous myonuclear addition also occurs in the EOM of adult mice. Adult mice were injected with brdU in order to label any dividing satellite cells in the EOM. BrdU is a thymidine analogue, which is incorporated into the DNA during its synthesis in the dividing cell. Following intraperitoneal injection, brdU is rapidly absorbed into the blood and incorporated into dividing cells. Nuclei that are brdU-positive are assumed to have divided shortly after brdU injection. A series of repeated brdU injections increases the window of time for identification of dividing satellite cells. The presence of brdU-positive myonuclei within existing myofibers was demonstrated by using double-labeling techniques in order to identify and quantify the number of brdU-positive myonuclei within the dystrophin-positive sarcolemma of single EOM myofibers in cross section.

METHODS

Adult C57 mice were obtained from Jackson Laboratories (Bar Harbor, Maine) and were housed in AALAC-approved animal quarters at the University of Minnesota. All studies were approved by the Institutional Animal Care and Use Committee at the University of Minnesota and adhered to standards for animal care as defined by the National Institutes of Health.

Mice were injected intraperitoneally with 50 mg brdU/g of body weight every 2 hours for a total of 12 hours. The animals were allowed to survive for 24 hours, after which they were euthanized with an overdose of carbon dioxide. The globes with the EOM attached were removed, embedded in tragacanth gum, and frozen in methylbutane chilled to a slurry on liquid nitrogen. Tibialis anterior muscles were also removed to serve as a control. Serial sections were prepared at 12 µm using a cryostat. The sections were immunostained for the presence of dystrophin and brdU using a procedure previously described.[7] Briefly, the sections were incubated in an antibody against dystrophin (Novocastra Labs, Vector Laboratories, Burlingame, California) at a concentration of 1:20 and reacted with the Vectastain peroxidase ABC kit. The peroxidase was developed using diaminobenzidine. For the brdU localization, the sections were incubated in 2N HCl for 1 hour at 37ºC, followed by neutralization in borate buffer and a PBS rinse. The sections were incubated in the primary antibody to brdU (Boehringer Mannheim) at a concentration of 1:1,000. The sections were rinsed in PBS, incubated using reagents from the alkaline phosphatase ABC kit (Vector Laboratories), and reacted with the alkaline phosphatase purple substrate kit. The staining for dystrophin was brown, and the brdU-positive myonuclei were purple. A second set of sections was prepared and immunostained for brdU and for laminin (1:100, Sigma Chemical Co, St Louis, Missouri) in order to identify brdU-positive satellite cells. BrdU-myonuclei were identified by their position within the dystrophin-positive sarcolemma. BrdU-positive satellite cells were identified by their position with the laminin-positive basal lamina.

Cross sections through the superior and inferior rectus muscles and the tibialis anterior muscle were analyzed to determine the number of myofibers that contained a brdU-positive myonucleus or a brdU-positive satellite cell. A minimum of three cross sections was counted for each of the four sets of globes and EOM analyzed. Counts were performed with aid of the Bioquant Nova morphometry program (R & M Biometrics, Nashville, Tennessee). All data are presented as means ± SEM. All statistical analyses were performed using the Prism and Statmate statistical software (Graphpad Software, Inc, San Diego, California). Statistical significance was defined as $P<.05$. The percent positive of both brdU-positive satellite cells and brdU-positive myonuclei for the global and orbital layers was compared, and there was no significant difference between the two groups using an unpaired, two-tailed *t* test. An F test indicated that the variances were not significantly different.

RESULTS

Multiple brdU injections were administered in order to maximize the chance of having brdU available to all the satellite cells undergoing DNA replication during the 12-hour period that was studied. By waiting 24 hours after the final brdU injection, it was more likely that the labeled progeny of the satellite cells would have time to fuse into a myofiber. This injection schedule was based on empirical observations. By 24 hours after the last brdU injection, brdU-positive myonuclei were present and allowed definitive identification of the fate of single dividing satellite

cells. Cross sections of the EOM from the adult, uninjured mice orbits contained myofibers with brdU-positive extraocular muscle myonuclei (Figure 1A). While these brdU-labeled myonuclei were usually peripherally located, in contrast to the rabbit EOM myofibers previously studied,[7] myofibers were found with brdU-positive myonuclei that were centrally located within single myofiber cross sections (Figure 1B).

The percent of myofibers in cross section containing a brdU-positive myonucleus was 0.68% in the orbital layer and 0.59% in the global layer (Figure 2). Extrapolated out to a 24-hour labeling period, this would mean approximately one myofiber would have a brdU-positive nucleus out of every 80 to 90 myofibers in cross section. Interestingly, approximately 1% of the myofibers in cross section had brdU-labeled satellite cells associated with them after the 24-hour survival period. There were essentially no brdU-positive myonuclei in the cross sections of tibialis anterior muscle.

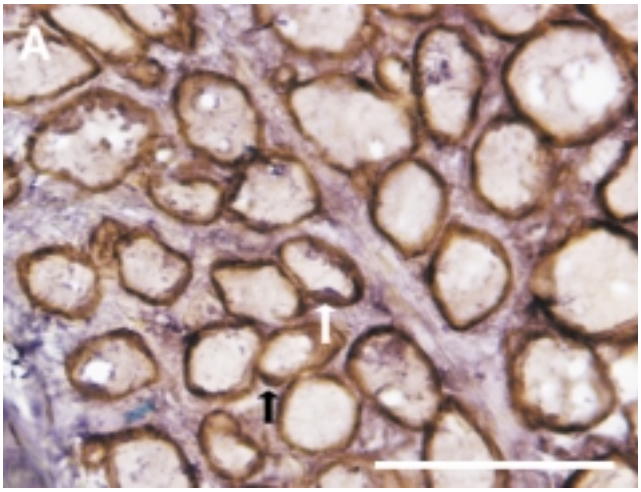

FIGURE 1A
Cross sections through inferior rectus from a mouse immunostained for both brdU (purple) and dystrophin (brown). White arrow indicates a brdU-positive myonucleus in a peripheral location within the cross section. Black arrow indicates a brdU-positive satellite cell. Bar is 50 µm.

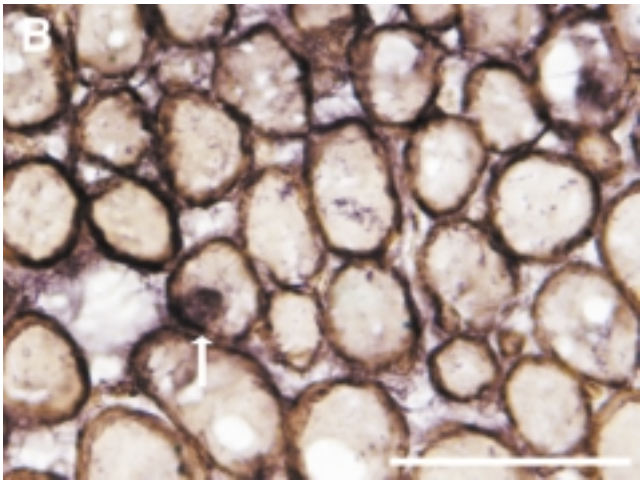

FIGURE 1B
White arrow indicates a brdU-positive myonucleus in a central location within the myofiber cross section. Bar is 50 µm.

DISCUSSION

These studies demonstrate that myonuclei are added in a continuous manner to existing myofibers in adult mouse EOM. It has been well established that adult myonuclei are postmitotic in normal, uninjured limb musculature. We conclude that in all likelihood, the brdU-labeled myonuclei found within the uninjured EOM myofiber cross sections must have resulted from fusion of progeny from muscle satellite cells that divided during the period of brdU exposure. This is supported by evidence that activated muscle satellite cells reside within the uninjured adult EOM.[7] However, it is also possible that an occasional muscle progenitor cell from the blood stream may have entered the extraocular muscles. In both skeletal and cardiac muscle, a number of studies demonstrated the presence of muscle nuclei derived from blood-borne stem cells that ultimately came to reside within existing myofibers.[14,15]

The presence of activated and dividing satellite cells within adult, uninjured EOM is in contrast to the current view that satellite cells are quiescent in adult muscle unless injured.[6] Activated satellite cells can be identified using either cell cycle markers such as Ki-67[16,17] or myogenic lineage markers, including Pax-7[18] and MyoD.[19] Using a number of these markers, we have demonstrated that there is a population of activated satellite cells in mammalian EOM, including rabbit,[7] monkey, and human EOM.[20] It is inter-

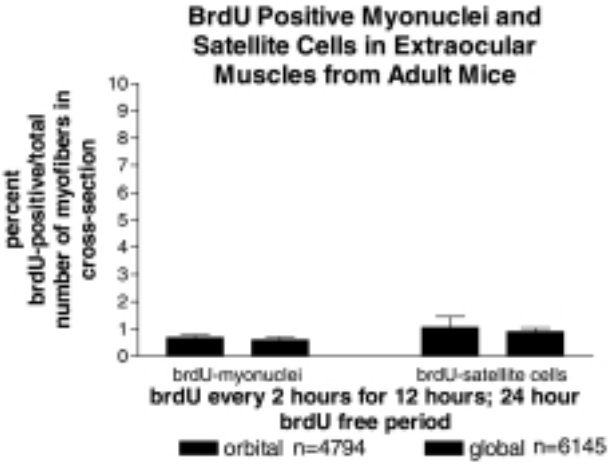

FIGURE 2
Quantification of brdU-labeling of both satellite cells and myonuclei in cross sections through adult mouse extraocular muscles. Mice received brdU injections every 2 hours for 12 hours, followed by a 24-hour brdU-free period before they were euthanized. N is the total number of myofibers counted.

esting that many of the molecules known to be involved in the control of myogenic cell division and differentiation remain up-regulated in normal adult EOM, including insulin growth factor and its receptor.[21] A recent study of the EOM expression profile confirmed the presence of a number of genes for muscle growth, development, and regeneration.[22]

We are beginning to examine possible candidates for control and maintenance of this process of myonuclear addition in mature EOM. Previous work demonstrated that the EOM are dependent for survival in vitro on innervation specifically from their cranial motor neurons; spinal motor neurons could not substitute for them.[23] We are currently assessing the role of innervation on this process of myonuclear addition in adult EOM. Denervation of the EOM occurs as a result of various neuropathic disorders and trauma. Temporary denervation of portions of an EOM may occur iatrogenically as a result of strabismus surgery or chemodenervation. The concept that even normal, uninjured EOM are always engaged in myofiber remodeling means that the EOM presumably respond to local changes in their environment over time. Thus the phenomenon of continuous myonuclear addition could be relevant to our understanding of the causes of strabismus, the late changes (including loss of initial success) following strabismus surgery, and the response to denervation.

There are many important implications of continuous myonuclear addition that may help explain the propensity for, or sparing of, the EOM in specific myopathic conditions.[9] Certain myopathies occur preferentially in extraocular muscle, such as progressive external ophthalmoplegias and oculopharyngeal muscular dystrophy. It is presumed that continuous myonuclear addition is the result of continuous myocyte addition, resulting from fusion of entire satellite cells with existing myofibers.[8] In progressive external ophthalmoplegia, continuous myocyte addition would mean addition of mitochondria from the replicating satellite cells, which in turn could result in increased accumulation of random mitochondrial DNA mutations. These mutated mitochondria could then become clonally amplified within individual postmitotic myofibers.[24] In oculopharyngeal muscular dystrophy, myonuclear addition from replicating satellite cells could result in the abnormal trinucleotide repeats seen in individual myonuclei that comprise the affected myofibers.[25] In oculopharyngeal muscular dystrophy, a progressive nuclear accumulation of abnormal poly(A) binding protein 2[26] results in loss of the normal function of this molecule. Myofiber loss occurs, followed by muscle regeneration. The myopathology of oculopharyngeal muscular dystrophy may be accelerated by the rate of satellite cell replication and addition into existing myofibers within the EOM. Uninjured nonocular skeletal muscle in adult mammals does not have ongoing satellite cell division, and this may result in the protection of the body muscles from the accumulation of mutant DNA seen in the EOM.

The demonstration that the process of myonuclear addition occurs in mouse EOM opens the door for a number of studies looking at transgenic mice with muscle-specific genetic defects. This will allow us to test various hypotheses relating to possible mechanisms that control this process. Hopefully, the result will be the development of new therapeutic treatments for skeletal muscle myopathies.

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DISCUSSION

DR IRENE H LUDWIG. The authors present microscopic evidence of the presence of cells, known to be involved in muscle fiber regeneration, in the extraocular muscles of mice. Other skeletal muscles do not show these cells. The authors also reference other work of theirs indicating similar findings in rabbits, monkeys, and humans. At the recent ARVO meeting, Dr McLoon presented evidence of markers, which indicated activation of satellite cells in normal adult human and monkey extraocular muscles.

As a clinician, I may be underqualified to evaluate the methods used in this study, but I am assured by my research colleagues at the LSU Eye Center, Drs Jacob and Gebhardt, that the methods were sound. From the clinician's perspective, there would be less potential for bias if the observer were masked as to the origin of each tissue cross section before counting satellite cells. Although required in clinical trials, masking is not generally done in laboratory research.

The implications of the authors' findings are intriguing. They provide plausible explanations for the predilection of the extraocular muscles to be affected by conditions such as myasthenia and chronic progressive external ophthalmoplegia. They may also explain why the eye muscles are spared in Duchenne's muscular dystrophy. Perhaps they will also add to our understanding of strabismus mechanisms, leading to improved strabismus management.

For a strabismologist, it is heartening to witness the expansion of basic research in the study of extraocular muscles. At the recent ARVO meeting, numerous presentations were devoted to this subject. For too long, we have utilized diagnosis and treatment strategies based upon empirical evidence and observation. Recently, I read the correspondence surrounding a rejected strabismus paper. The reviewers had objected to findings that contradicted traditional views. One reviewer wrote, "150 years ago, the great physiologists of the 19th century...conclusively demonstrated," and used those early studies to dismiss the writer's observations.

Vision science research has lead to remarkable advances in most branches of ophthalmology in recent decades. Hopefully, today's presentation, and others like it, will pave the way for meaningful discoveries in the strabismus field, which has existed in a state of "pre-science" for too long.

DR JOHN T. FLYNN. It was the great German microscopists of the late 19th and early 20th century who developed the dogma that muscles were postmitotic. They never saw a mitotic figure. What does a satellite cell look like in the resting state and when it's doing its work in making the myoblasts and putting them back to repair the muscle cell? As strabismologists, we're constantly taking pieces of extraocular muscle; should we be doing something with these specimens?

DR ALLAN J. FLACH. I wonder whether the muscles involved in presbyopia, the smooth muscles, and perhaps even cardiac muscles underlying congestive heart failure may be affected by these satellite cells?

DR JONATHAN D. WIRTSCHAFTER. Dr Ludwig, you are correct: masked counting was not used. Since there was no brdU uptake to see in one set of specimens and it was readily seen in the other, I doubt that masking would have made any difference. For me, this has been an interesting journey of discovery too, and we, in fact, did not expect to

see the satellite cells turning over.

Dr Flynn questions why both apoptosis and mitosis had not been detected previously using standard histological methods. Apoptosis of the myonucleus and mitosis of the muscle satellite cell are both processes that occur very quickly. Probably a nucleus is lost in less than an hour, so if one leaf falls from a tree, you just wouldn't see it among all the other activity going on in the forest. But if the leaves all fall at once, taking advantage of the Latin root for the word "apoptosis," you would see a lot of leaves on the ground. That's why we can see myogenic activity in skeletal muscle after a widespread injury, while we can't easily detect a very low basal turnover rate. Also, we don't see the mitosis in normal extraocular muscles because there aren't enough going on at one time. We see mitosis in tumor tissue or in skin cells, where the cells are, in fact, turning over rapidly and they have some place to grow or shed.

The extraocular muscles have to be stochastically in balance, that is, you have the same amount of muscle mass, more or less, at the end of each day, as you had before. This concept opens a whole opportunity to rethink what we know about the extraocular muscles. They're not just meat; probably each fiber is a story of it's own. I think of an extraocular muscle as a big condominium. Each myofiber is a single unit within the condominium, and the family is made up of many myonuclei that represent each of the family members. And, as each grandfather myonucleus dies, it's replaced by a baby myonucleus that's ready to be made in a little room (the muscle satellite cell) next door. That's how the myofiber keeps its number of myonuclei in balance. Still, there are many things we don't yet know. For example, is each muscle satellite cell pluripotent or is it specific to the muscle fiber type that's within its myocyte?

Dr Flach, we have not studied the smooth or cardiac muscles.