HHS Public Access

Author manuscript

*Curr Ophthalmol Rep*. Author manuscript; available in PMC 2018 September 01.

Published in final edited form as:

*Curr Ophthalmol Rep*. 2017 September ; 5(3): 207–215. doi:10.1007/s40135-017-0141-4.

# Extraocular Muscle Repair and Regeneration

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## Abstract

**Purpose of Review—**The goal of this review is to summarize the unique regenerative milieu within mature mammalian extraocular muscles (EOMs). This will aid in understanding disease propensity for and sparing of EOMs in skeletal muscle diseases as well as the recalcitrance of the EOM to injury.

**Recent Findings—**The EOMs continually remodel throughout life and contain an extremely enriched number of myogenic precursor cells that differ in number and functional characteristics from those in limb skeletal muscle. The EOMs also contain a large population of Pitx2-positive myogenic precursor cells that provide the EOMs with many of their unusual biological characteristics, such as myofiber remodeling and skeletal muscle disease sparing. This environment provides for rapid and efficient remodeling and regeneration after various types of injury. In addition, the EOMs show a remarkable ability to respond to perturbations of single muscles with coordinated changes in the other EOMs that move in the same plane.

**Summary—**These data will inform Ophthalmologists as they work toward developing new treatments for eye movement disorders, new approaches for repair after nerve or direct EOMs injury, as well as suggest potential explanations for the unusual disease propensity and disease sparing characteristics of human EOM.

### Keywords

extraocular muscle; satellite cells; Pax7; Pitx2; strabismus surgery

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**Compliance with Ethical Guidelines**

**Human and Animal Rights and Informed Consent**

Animal studies described in this review were performed with approval by the University of Minnesota Animal Care and Use Committee and followed the NIH Animal Care and Use Guidelines.

**Conflict of Interest**

Mayank Verma, Krysta Fitzpatrick, and Linda McLoon declare no conflict of interest.

## Introduction

### EOM Characteristics Compared to Limb Skeletal Muscles

Skeletal muscles are composed of long multinucleated myofibers that are responsible for the control of body movement. Different skeletal muscles display distinctly different microscopic anatomy as well as different contractile properties and form a continuum based on the complexity of their molecular and anatomical organization. For example, myofibers in the soleus muscle in the leg run the full length of the muscle resulting in a defined endplate zone [1], and they have a relatively uniform and simple internal fiber architecture [2]. Soleus muscle fibers are almost completely positive for the slow twitch myosin heavy chain isoform (*MYH7*) [3] with a small number of myofibers expressing fast twitch myosins (*MYH1, MYH2, MYH4*). This makes them apt for low intensity, long term contraction required for standing or postural control. At the far end of the skeletal muscle "continuum" are the extraocular muscles (EOMs) [4], whose complexity is significantly greater than limb skeletal muscles. The six EOMs in each orbit are able to produce the wide range of eye movements that are finely controlled. The EOM diverge from limb and body skeletal muscles in a number of fundamental ways. In contrast to a single endplate zone, neuromuscular junctions in EOMs are dispersed throughout the length of the muscles. The EOMs also contain multiply innervated myofibers, with specialized *en grappe* endings with multiple small synapses along a single muscle fiber, along with the traditional *en plaque* endings found in other skeletal muscles [5, 6]. Additionally, the myofibers in EOMs are short and overlapping, ending and beginning throughout the muscle length [7, 8]. While body and limb skeletal muscles contain varying proportions of the same 4 myosin heavy chain isoforms as soleus, the EOMs contain 9 different isoforms, including an EOM specific MyHC isoform (*MYH13*), and express multiple isoforms within single myofibers [9, 10]. These combined traits result in EOMs being densely innervated, with the fastest contraction speeds of mammalian muscles [11]. In contrast to limb muscles, the EOMs are also fatigue-resistant [12]. These differences extend to their gene and protein expression profiles, which are considerably different from that of non-cranial skeletal muscles [13, 14].

The EOMs also differ from limb skeletal muscle in their developmental origin and the genetic control over their embryonic development. While limb skeletal muscles are derived from the somite, the craniofacial muscles, including the EOMs, are derived from prechordal and paraxial head mesoderm. While much of the transcriptional myogenic differentiation program remains the same, the EOMs have the distinct feature of not being derived from a Pax3-positive lineage. In fact, when the transcription factor Pax3 is knocked out in the embryo, no limb or body muscles develop, but the EOMs are completely normal [15]. Conversely, mice lacking the transcription factor Pitx2 do not develop EOMs while the rest of the skeletal muscles develop normally [16]. In a series of experiments using a transgenic mouse where Pitx2 is conditionally knocked out when creatine kinase is expressed, in the absence of Pitx2 expression in these mice the EOMs lose many of their specific characteristics including the expression of the ultrafast EOM-specific MyHC isoform (*MYH13*) and multiply innervated myofibers [17, 18]. In summary, the EOMs are strikingly different in their embryonic origin, normal anatomy, physiology, and protein expression profiles when compared to non-cranial skeletal muscles.

## EOM Regenerative Cell Populations

### EOM Myogenic Progenitor Cells

Skeletal muscles have the ability to regenerate in disease and after injury in part due to myogenic precursor cells that reside around the individual muscle fibers. In adult skeletal muscles, these cells have been classically defined as satellite cells, which were shown to reside outside of the sarcolemma of the myofiber but inside the basal lamina [19]. Satellite cells are defined by their expression of Pax7, and were considered to be largely quiescent in the absence of disease or injury [20]. However, recent lineage tracing experiments show that these Pax7-expressing cells continuously fuse into myofibers during normal homeostasis in both developing and adult mice [21, 22]. The rate of fusion of these cells is significantly greater in the EOMs than in many of the skeletal muscles examined in these studies.

As might be expected from the differences in genetic control of their early embryological origin combined with their unique array of adult muscle characteristics, the myogenic precursor cells in the EOMs also differ from those in limb skeletal muscles in a number of substantive ways. Similar to limb muscles, the EOMs contain Pax7-positive satellite cells, but morphometric analysis of histological sections show there are significantly more Pax7-positive cells relative to myofiber number than seen in limb skeletal muscle [23, 24]. This was confirmed with the use of flow cytometry, where again the EOMs contain significantly more Pax7-positive cells than limb skeletal muscle [25]. It should be noted that two recent studies suggested that there were equal numbers of Pax7-positive cells in EOMs and limb muscle; these studies were largely based on immunostaining with antibodies to Pax7 [26, 27]. Recent reports have described reliable Pax7 lineage reporter mice ($Pax7^{CreER};Rosa26R^{Stop\text{-}Flox\text{-}Stop\text{-}tdTomato}$) that allow for accurate quantification of Pax7-expressing myogenic precursor cells using both FACS and microscopy [21, 22]. Microscopic examination of histological sections from the tibialis anterior and EOMs from these mice show that not only are there more Pax7-positive cells in EOMs, but these Pax7-positive cells are larger in the EOMs, with more extensive filopodia-like processes (Figure 1). Using flow cytometry, we examined the number of Pax7-positive satellite cells in tibialis anterior (TA), extensor digitorum longus (EDL), soleus, diaphragm, and EOMs in the Pax7 lineage reporter mice (Figure 2). When examined as percent of live mononuclear cells, the soleus is significantly greater than all the other muscles (Figure 2A); however, when this is compared to the total number of live cells isolated, EOMs have over 3 times the number of live mononuclear cells compared to soleus. Interestingly, the diaphragm has10 times the number of live mononuclear cells compared to soleus. The data were reanalyzed as the number of Pax7 cells relative to muscle mass, and in this case the EOMs have significantly more Pax7-positive cells than TA, EDL, and soleus (Figure 2B). It should be noted that unlike somite-derived muscle stem cells, the stem cells from head muscles do not have a developmental history that includes Pax7 expression, but rather it emerges *de novo* [28]. This provides further evidence of the unique properties of the cranial mesoderm-derived skeletal muscles.

Recent data suggest that skeletal muscles contain Pax3-positive myogenic precursor cells, which would normally co-express Pax7, and these appear to be responsible for the muscle regeneration seen in the absence of Pax7-positive satellite cells [29]. Using a Pax3 lineage

reporter mouse (*Pax3*$^{CreER}$;*Rosa26R*$^{Stop\text{-}Flox\text{-}Stop\text{-}tdTomato}$), Pax3-positive cells are easily found in limb muscle cross-sections (Figure 3A); however, the EOMs are completely devoid of Pax3 expression (Figure 3B).

All skeletal muscles have far greater numbers of live mononuclear cells than the number of cells that are positive for Pax7. These consist of hematopoietic cells, endothelial cells, pericytes, fibroblasts, and other non-muscle specific cell types. We have used flow cytometric studies to identify a myogenic precursor cell population that expresses the stem cell marker CD34 but is negative for Sca1, CD31, and CD45, which we now call the EECD34 cells [25]. These are significantly enriched in the EOMs compared to limb skeletal muscle, and *in vitro* the EECD34 cells isolated from EOMs are significantly more proliferative and have a higher fusion index than those isolated from limb skeletal muscle [25, 30].

We demonstrated that there is a myogenic precursor cell population enriched in mammalian EOMs that expresses the transcription factor Pitx2 [30]. Pitx2 is a homeobox transcription factor that plays a critical role in development of the eye and myogenesis in the head region [31, 32], and its expression is essential for EOM formation in development [16]. Using flow cytometry, we examined EECD34 cells for Pitx2 expression, and showed that 80% of these cells are positive for Pitx2 [30]. In addition, when the EECD34 cells are placed *in vitro*, immunostaining of the cultured cells showed that 100% of these cells were Pitx2-positive. Another source of endogenous myogenic progenitors in skeletal muscles can be obtained using a Hoechst dye exclusion method for flow cytometry, and these are termed side population (SP) cells [33]. Interestingly, the EOMs contain 15 times higher numbers of SP cells compared to the limb skeletal muscle. Microarray studies reveal that the EOM SP cells also express higher levels of Pitx2 [34]. Pitx2-positive myonuclei are also abundant in mammalian EOM [17]. Pitx2-positive mononuclear cells reside both in the traditional satellite cell location as well as in the interstitial connective tissue (Figure 4). The Pitx2 myogenic precursor cells do not co-express Pax7, and thus represent a second large population that is involved in remodeling, repair, and regeneration in the EOMs [30]. High dose gamma irradiation (18Gy) injury to limb muscle in the *mdx:utrophin*$^{+/-}$ mouse model of muscular dystrophy results in a loss of Pax7-positive cells that do not recover and a permanent loss of muscle regenerative capacity over time [24]. In contrast, gamma irradiation of the EOMs in the same mouse model results in a short term increase in both the Pax7 and Pitx2 populations of myogenic precursor cells, a short-term dystrophic appearance, and ultimately a return of the EOMs to normalcy. The potential differential roles each of these regenerative cell populations plays in maintaining the EOMs is an area of active study.

Other transcription factors have been implicated in regulating cranial mesoderm development. These include Twist1, whose absence results in compromised development of the EOMs [35]. Absence of Twist1 causes abnormalities in neural crest functional development [36]. This may be the precipitating alteration that impacts normal EOM formation, as neural crest cells are crucial for normal EOM development through their expression of retinoic acid [37, 38]. Interestingly, a recent report has shown a Twist1-positive mesenchymal cell population in skeletal muscle which can contribute to the regeneration and maintenance of type II fibers [39]. The potential role of these cells in the

EOMs has yet to be determined. PW1 expression also has been implicated in maintaining EOM resistance to ageing and disease, and these PW1-positive interstitial cells are retained at normal numbers throughout life [40]. The relatedness of these Twist1 and PW1 cells is unclear at the present time.

Unique differences are seen in zebrafish EOMs, which do not express Pax7 or Pitx2 myogenic precursor cells [41]. Instead it appears that when there is a large injury to the EOMs in the zebrafish, the remaining cells are able to de-differentiate into myogenic precursor cells that express MyoD and result in completely normal regeneration. It will be interesting to see if other non-mammalian species known to have robust capacity for regeneration of a myriad of tissues and organs will also have the same absence of traditional myogenic precursor cells.

## EOM after Surgery and Drug Treatments

### EOM Surgery

The EOMs are one of the few muscles whose lengths are routinely altered by surgical manipulation. Unlike the movements that result from most other skeletal muscles, eye movements are conjugate. This means that the EOMs are functionally organized in dynamic pairs where each eye moves the same amount in the same direction to ensure that the identical part of the visual world falls on the fovea of each eye. Strabismus is a common eye misalignment disorder, found in 3–5% of children, where this conjugacy is lost [42, 43]. After eye patching, the next most common treatment method is the surgical alteration of EOM tension. Very few surgery-related problems are associated with this common procedure, with the exception of stretched scars – essentially a tendon/connective tissue problem [44, 45]. This is true even in surgical management of head posture in nystagmus, where 5–13 mm of individual EOMs are removed [46]. However, the long-term success rate for producing binocularity in the children who receive strabismus surgery averages around 50% [47–49].

In a series of experiments examining changes in EOMs after strabismus surgeries, single muscle surgery resulted in coordinated changes in the yoked muscles, i.e. the right medial rectus muscle and the left lateral rectus muscle that move the eye in the same direction at the same time [50] and to coordinated but often reciprocal changes in agonist/antagonist pairs, i.e. the right medial rectus and the right lateral rectus muscle [51]. For example, after a lateral rectus muscle resection, which increases the tension on the shortened muscle, both the shortened muscle and the antagonist medial rectus muscle exhibit similar increases in myofiber size [51]. Similar coordinated changes in muscle tension are seen after adductor weakening, where a similar decrease in tension is seen in the untreated antagonist muscle [50]. All changes in both these studies show a return to normal, pre-surgical values by 6 weeks after surgery. The potential mechanisms for these coordinated EOM changes in the unoperated EOM were examined in a series of studies. Increased satellite cell proliferation as well as rapid integration of these cells into myofibers are seen after similar surgeries in rabbits, suggesting a vigorous remodeling 10 response as a result of either lengthening or shortening a single EOM [52, 53]. In addition, these surgeries result in similar and coordinated activation of myogenic precursor cells in the untreated contralateral muscles and

reciprocal changes in the antagonist muscles on the same globe [53]. These changes are associated with altered expression of neurotrophic factors, including insulin growth factor-I and -II and transforming growth factor β-1 [52, 54]. Similar types of coordinated responses are also seen in myosin heavy chain isoform expression, a property that controls shortening velocity. The ability of the unoperated EOMs to adapt after surgery of a single EOM suggests that single muscle surgery could be sufficient, and this approach has significant proponents in the clinic. It appears to be sufficient to improve eye alignment in many cases, and leaves other EOMs untouched if future surgery is needed [reviewed in 55]. We hypothesize that the ability of the ocular motor system to modify the yoked and agonist/antagonist pairs may be sufficiently strong to ensure coordinated improvements in eye alignment in unilateral surgical approaches to strabismus treatment, at least under a subset of conditions.

## Botulinum Toxin Injections

The EOMs often respond in a manner quite different from limb skeletal muscles after direct intramuscular administration of drug treatments such as botulinum toxin. Botulinum toxin A is widely used for the treatment of focal dystonia disorders, and acts by paralyzing the neuromuscular junction [56]. Botulinum toxin injections directly into most other skeletal muscles result in significant myofiber atrophy [57–59]. In contrast, animal studies show that myofiber atrophy does not develop after botulinum toxin injections into the EOMs [60, 61], even when it is injected into developing EOMs [62]. In fact, hypertrophy of orbital singly innervated myofibers is described as a result of botulinum toxin muscle paralysis in an EOM of an adult monkey [60]. One potential explanation is that botulinum toxin injection into the EOMs causes a large increase in satellite cell activation, division, and myonuclear addition into existing myofibers [63]. We hypothesize that the EOMs, even after neuromuscular junction paralysis, are able to maintain relatively normal morphology by active myofiber remodeling [63]. This hypothesis is supported by studies that show the importance of satellite cells in the maintenance of the neuromuscular junction [64].

## Local Anesthetic Injections into the EOM

Another common procedure routinely performed prior to a variety of intraorbital procedures is the retrobulbar injection of local anesthetics, such as bupivacaine and lidocaine, exposing the EOMs to the known myotoxins [65]. One common morbidity associated with these injections is the subsequent development of diplopia [66]. Orbital injection of local anesthetics during strabismus surgery can cause complications from this myotoxicity, which are exacerbated when the injections are inadvertently made intramuscularly [67]. Direct injection of bupivacaine into the EOMs, particularly exacerbated by the presence of epinephrine, causes significant myonecrosis [68, 69]. This is followed by relatively rapid regeneration, but with some scar formation due to increased interstitial connective tissue [68, 69]. However, these studies show that over time essentially all of the myofibers repair and regenerate and return to normal size [68, 70] and normal function [71]. Studies in the non-human primate suggest that retrobulbar injections, in the absence of epinephrine, actually cause little damage to the EOMs, with only the global singly-innervated myofibers affected, and even these fibers return to normal size within one month [70]. These varied results are interesting in light of the proposed use of bupivacaine to treat strabismus [72]. Based on the

extensive literature on postoperative diplopia after use of 12 local anesthetics in the orbit and accidently directly into an EOM, changes which largely resolve, it may be that the addition of epinephrine to the bupivacaine injections would predict the formation of increased connective tissue scarring within thusly treated EOMs. This would then be expected to produce a “tighter” muscle due to the resultant fibrosis within the treated EOM. Further studies will need to resolve these conflicting data concerning what is happening at the muscle level.

## EOM and Sparing in Muscle Disease

While beyond the scope of this review, the EOMs have a distinct disease propensity and disease sparing profile. The cause of the morphological and functional sparing of the EOMs in Duchenne and related muscular dystrophies is a long standing question [73, 74]. Our recent studies support the hypothesis that it is the incredible regenerative capacity within the EOMs that allows them to remain both morphologically and functionally spared in many forms of muscular dystrophy [25]. As discussed in an earlier section, the EOMs not only contain a large population of Pax7-positive myogenic precursor cells, but also express an abundant Pitx2-positive myogenic precursor cell population that is sparse in other skeletal muscles [30]. Reduction in the numbers of these cells by high dose gamma irradiation of the EOMs in the *mdx* mouse model of muscular dystrophy results in the transient appearance of dystrophic muscle changes, such as central nucleation [24]. Interestingly, these irradiated EOMs return to normal morphology within one month after these high irradiation doses [24]. At least one of these populations of myogenic precursor cells is radiation-resistant, as we showed using bromodeoxyuridine labeling of dividing cells, that the irradiated EOMs still contain cells capable of replicating their DNA and dividing, 13 as evidenced by the restoration of normal population numbers within one month after radiation injury.

Our preliminary data demonstrate that in the absence of Pitx2 expression on the *mdx* mouse background, the EOMs succumb to dystrophic changes that are even more severe than those seen in the limb muscles of the same mice (Figure 5). A recent study suggests that the milieu in which these cells reside also plays a role in long-term maintenance of the EOMs in disease and aging. It was demonstrated that the stem cell niche within the EOMs of the *mdx* mouse is maintained throughout life and provides a supportive location for maintaining stable populations of both myogenic stem cells and PW1-positive interstitial cells [FAPS] [40]. Thus, the continued presence of large numbers of highly regenerative myogenic precursor cells is able to maintain both EOM structure and function throughout life. What allows the maintenance of these cells and their environment in which they reside over a lifetime is the subject of intense investigation.

## Summary

In summary, EOMs are dynamic skeletal muscles with the lifelong capacity to remodel existing myofibers through the presence of at least 3 or 4 different and partially overlapping myogenic precursor cell populations: Pax7-positive satellite cells [21–25; Figure 1, 2], CD34-positive cells (EECD34 cells) of which 80% are Pitx2-positive (but Pax7-negative) [25, 30], and PW1-positive cells, half of which express Pax7 [40] (Figure 6). This myogenic

precursor cell-rich environment may be supported by the maintained expression in adult EOMs of a number of neurotrophic factors that are normally down-regulated in skeletal muscles [75], including insulin-like growth factor-1 and -2 [76, 77], brain derived neurotrophic factor [78, 79], glial derived neurotrophic factor [80, 81], and neurotrophin-3 [78]. All of these neurotrophic factors are critical for maintenance of the ocular motor neurons in development [82, 83]. This specialized communication between the EOMs and their innervating cranial motor neurons is critical for their development [84]. The EOMs provide a unique tissue in which to study robust regenerative capacity and the cells and trophic factors responsible for retention of this large and active myogenic precursor cell population throughout life [85]. The unique embryology, complex fiber types and contractile properties, adaptability to various types of external perturbations, and differential disease sparing capacity all demonstrate that the behavior of the EOMs cannot be predicted by studying limb skeletal muscles. The study of these specialized muscles provides an opportunity to ask critical questions about how the EOMs retain their preferential sparing characteristics, and provides a rich area for developing strategies for the potential treatment of muscle pathology associated with disease, injury, and aging.

## Acknowledgments

This work was supported by NIH EY55137 (LKM), NIH RA066454 (MV), NIH P30 EY11375, NIH P30AR0507220, Minnesota Lions Foundation, and an unrestricted grant to the Department of Ophthalmology and Visual Sciences from Research to Prevent Blindness, Inc. In addition, this work was supported in part by NIH P30 CA77598 supporting the University Flow Cytometry Resource.

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••Of major importance

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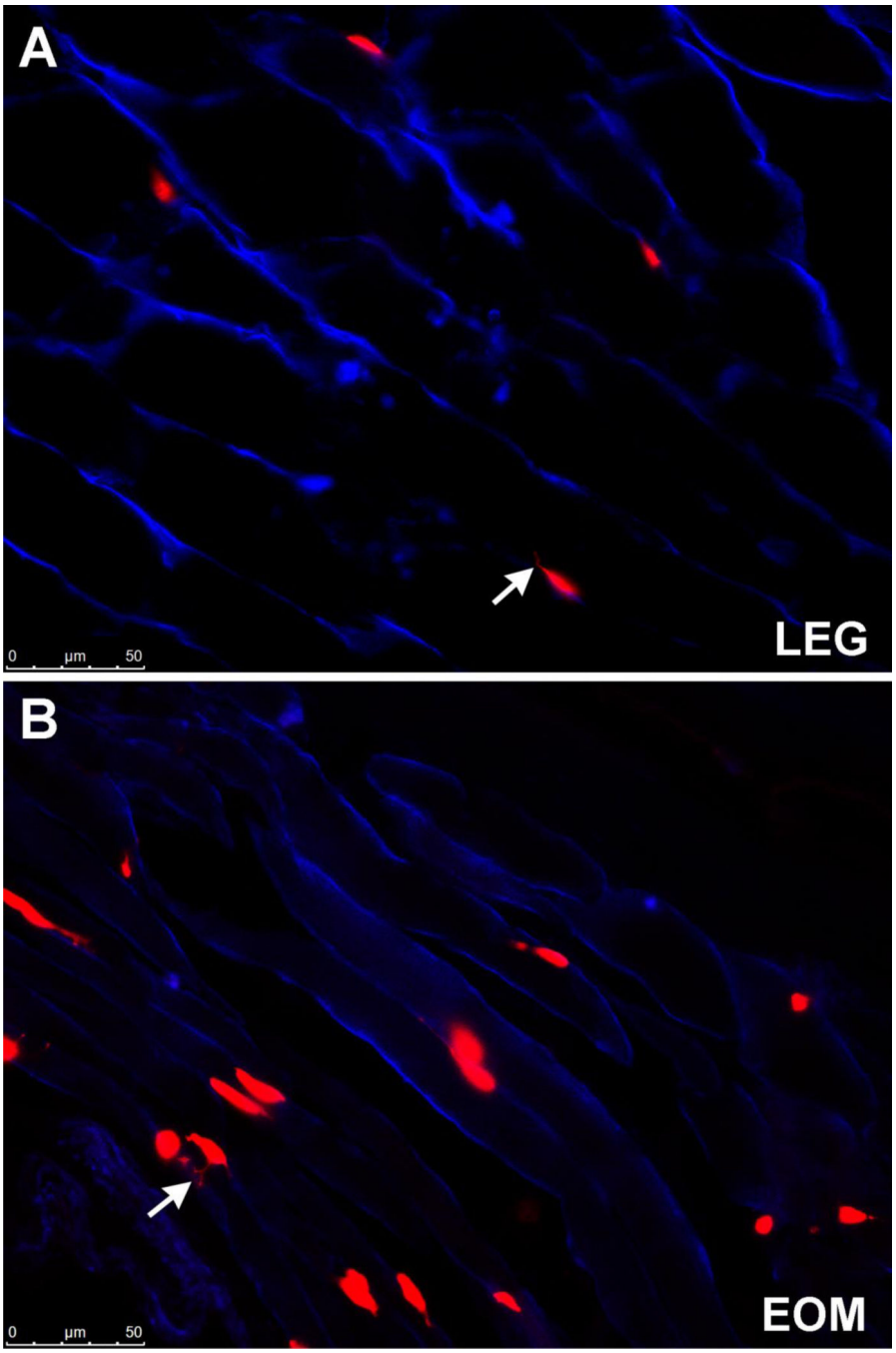


**Figure 1.**
Photomicrograph of (A) tibialis anterior and (B) extraocular muscle from the Pax7-lineage reporter mouse (red) immunostained for dystrophin (blue). Arrows indicate cell filopodia

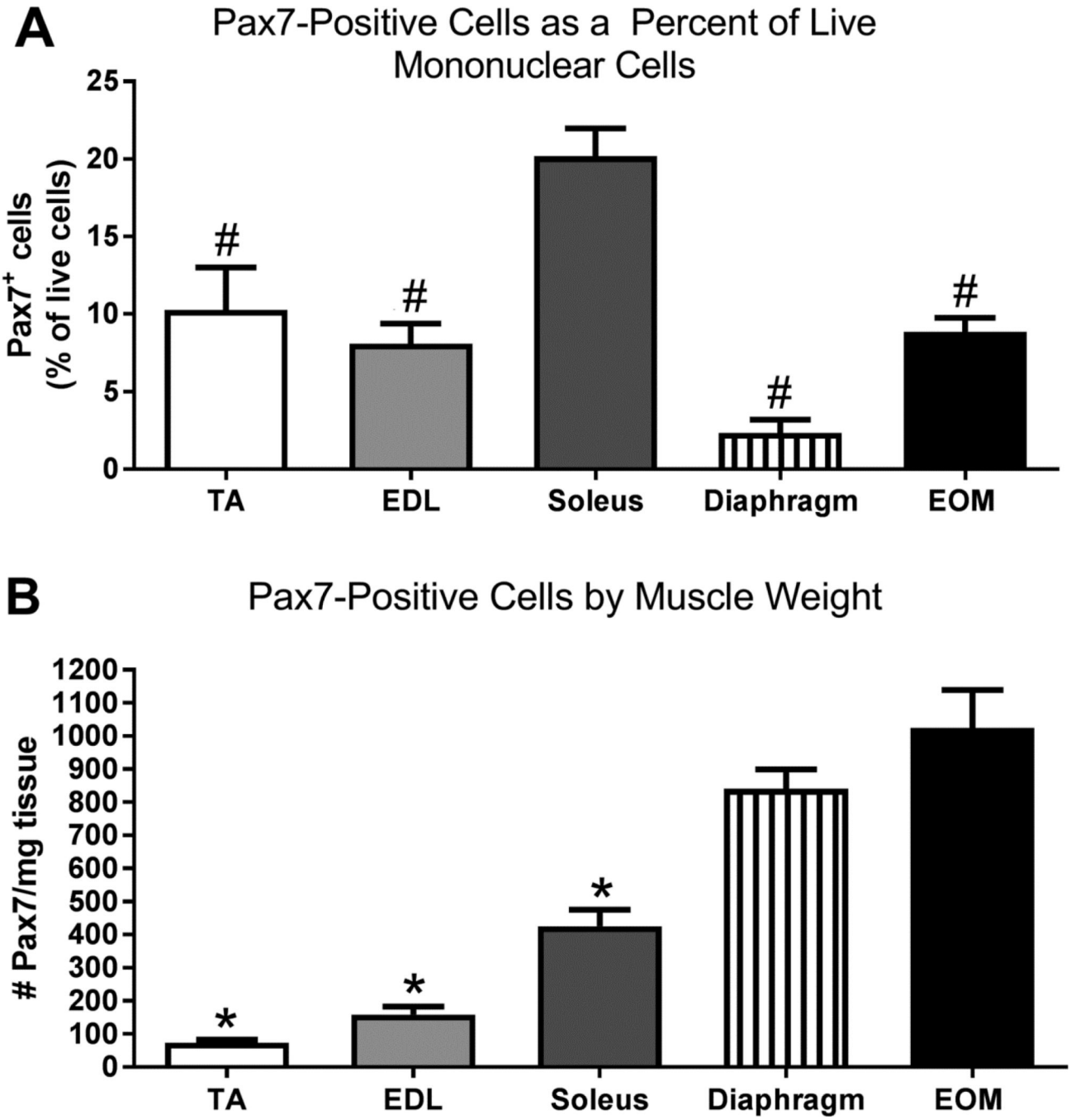


**Figure 2.**

Pax7 cells from a Pax7-tdTomato mouse isolated using flow cytometry analyzed (A) as a percent of all live mononuclear cells and (B) as number per milligram (mg) muscle weight. # indicates significant difference from soleus. * indicates significant difference from both diaphragm and EOM. Data analyzed with an ANOVA followed by a Tukey's multiple comparisons test. Significance is $p<0.05$.

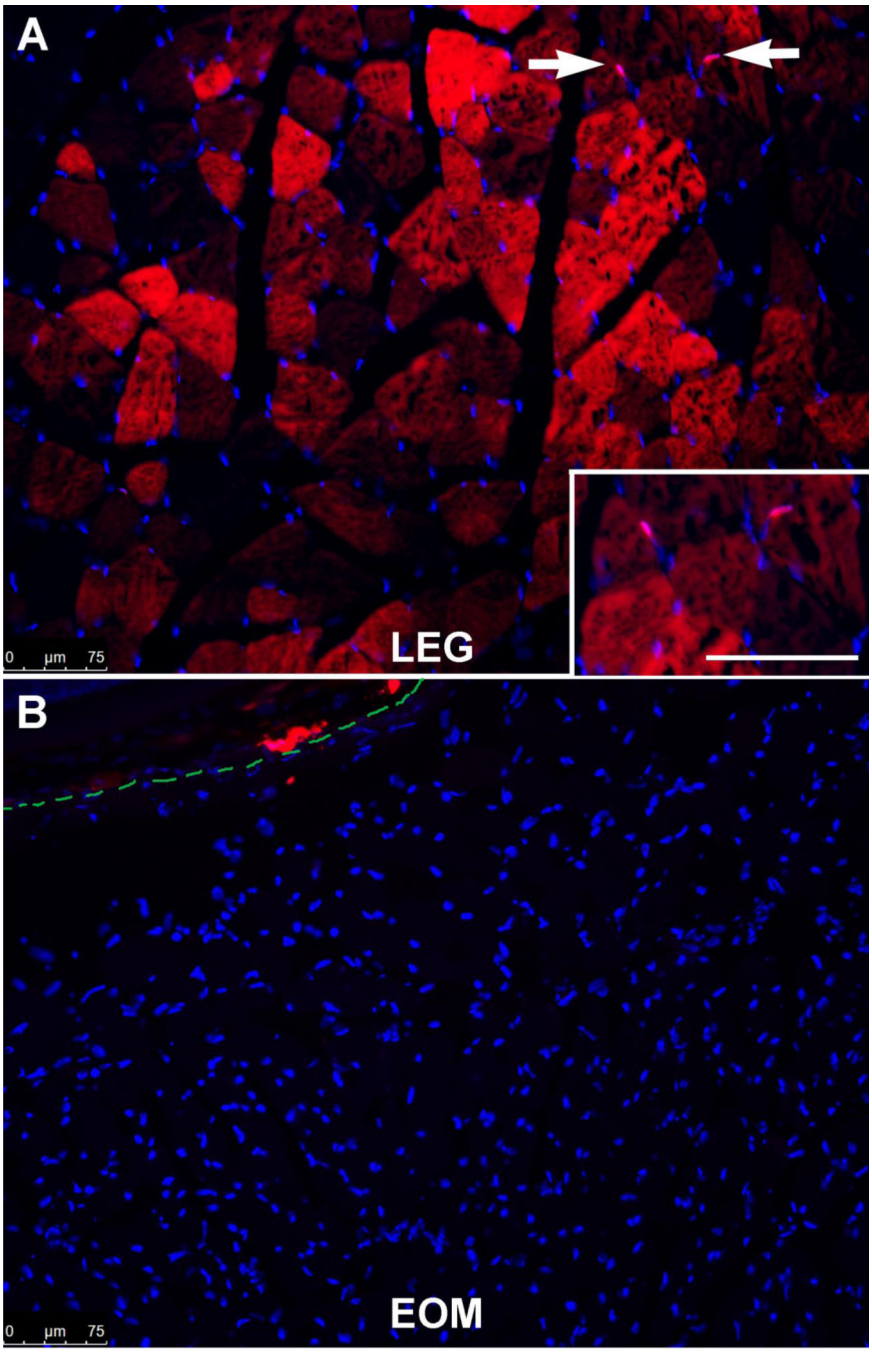


**Figure 3.**

Photomicrograph of (A) triceps and (B) extraocular muscle from a Pax3 lineage reporter mouse (red) stained with dapi (blue). (A) Arrows indicate Pax3-positive nuclei (pink). Inset shows the Pax3-positive cells (pink). Bar is 75µm. Red myofibers indicate a previous contribution of Pax3-positive cells. (B) The dotted green line represents the edge of the sclera of the globe.

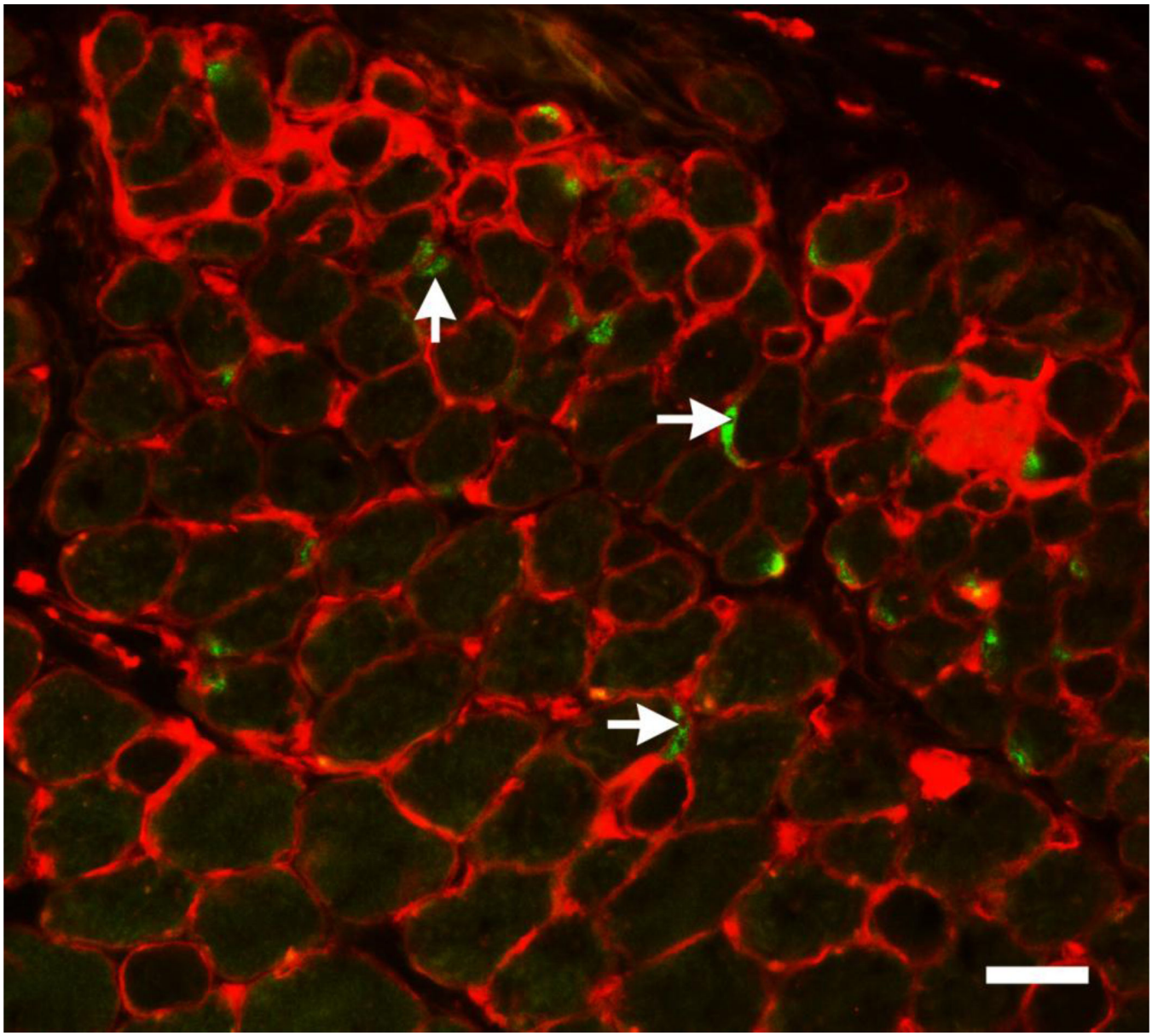

**Figure 4.**

Photomicrograph of mouse EOM immunostained for pax7 (green) and wheat germ agglutinin (red). Pax7-positive cells can be seen both inside the sarcolemma (vertical arrow) and outside the sarcolemma (horizontal arrows). Bar is 20μm.

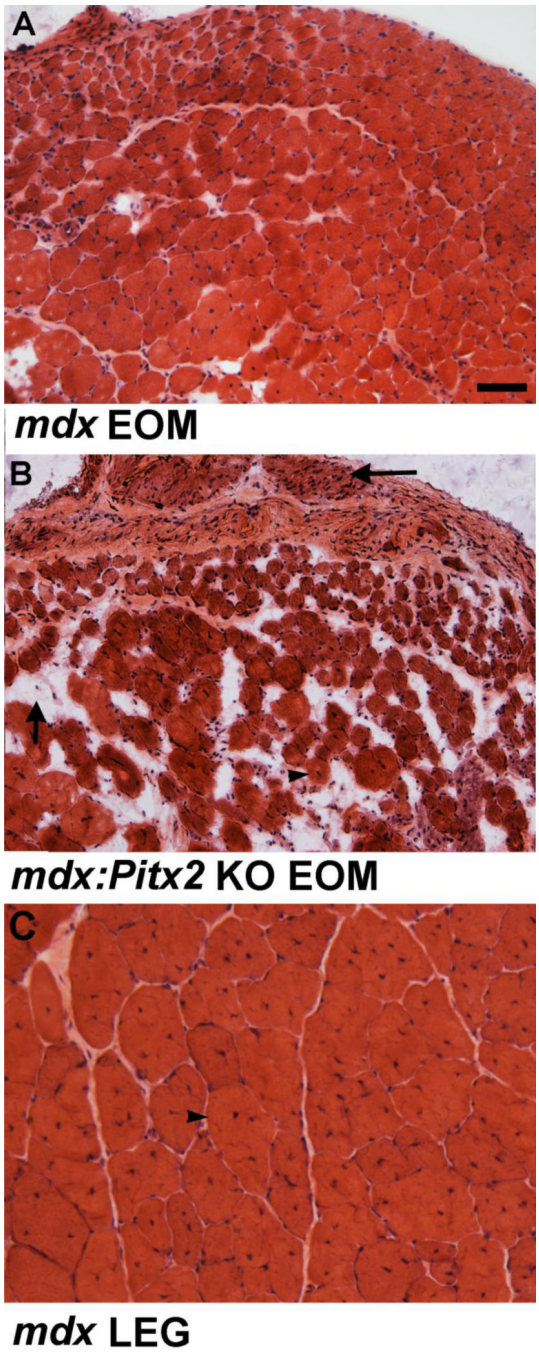


**Figure 5.**

Photomicrograph of cross-sections stained for hematoxylin and eosin of mouse EOM at 18 months of age from an (A) *mdx4cv* mouse model of Duchenne muscular dystrophy and (B) an *mdx4cv;mck-cre+/−;Pitx2fl/fl* mouse. Note the relatively normal morphology in the mdx mouse EOM and the severe pathology in the *mdx4cv;mck-cre+/−;Pitx2fl/fl* EOM. Vertical arrow shows fatty infiltration, horizontal arrow denotes what is left of the levator palpebrae superioris muscle, which normally is affected in the *mdx* mouse. Arrow head indicates a centrally nucleated myofiber, which is a hallmark of the process of degeneration/

regeneration that occurs in diseased or injured skeletal muscles. (C) Cross-section through the tibialis anterior of an *mdx4cv* mouse at 18 months of age. Bar is 100μm.

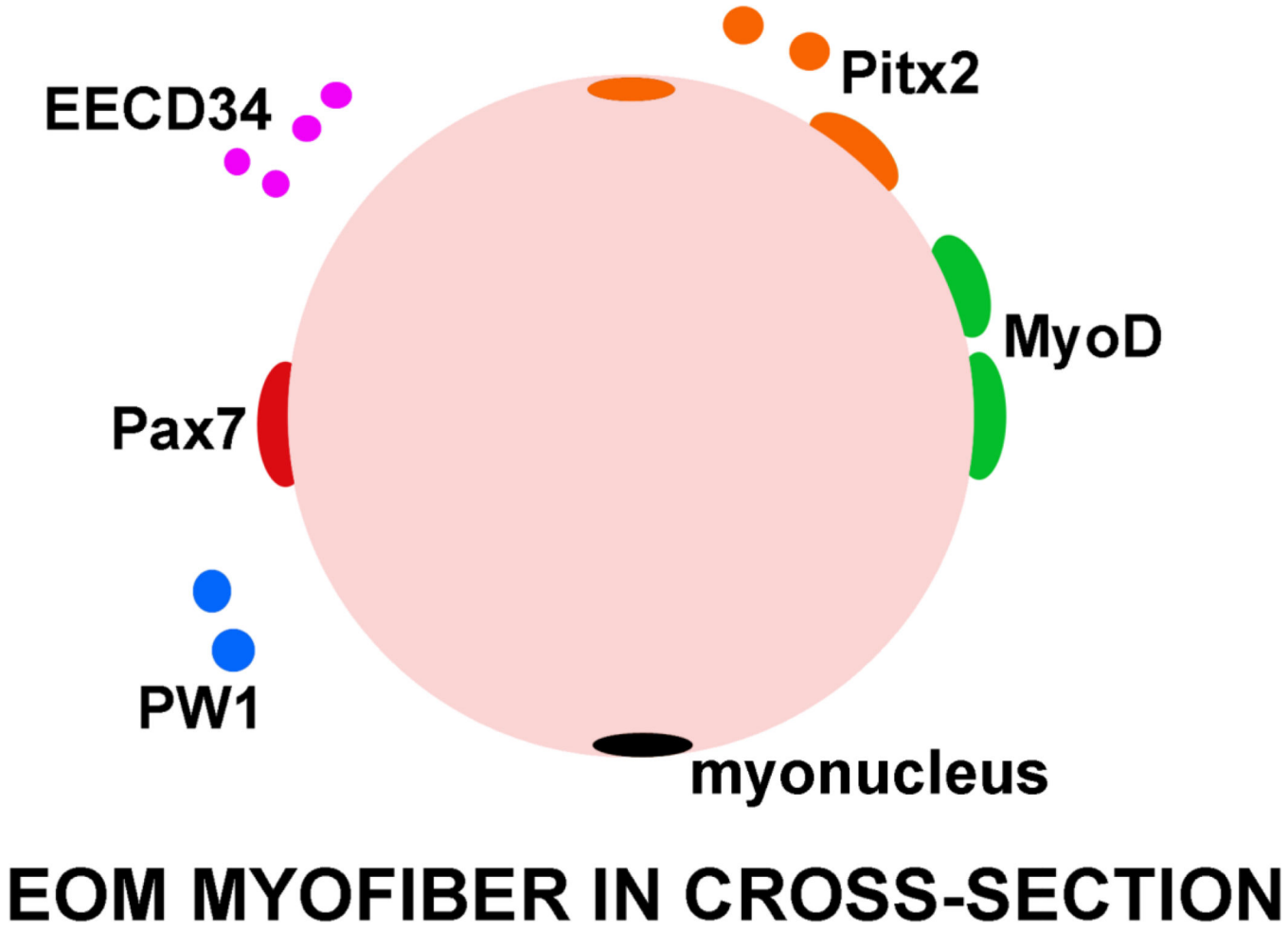


**Figure 6.**

Cartoon of a single myofiber from the EOM in cross-section with the myogenic precursor cells that have been identified thus far indicated. Red: Pax7; Green: MyoD; Purple: EECD34; Orange: Pitx2; Blue: PW1; Black: myonucleus.