Damaged muscle fibers might masquerade as hybrid fibers – a cautionary note on immunophenotyping mouse muscle with mouse monoclonal antibodies

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Abstract

We report that, labeling mouse muscle tissue, with mouse monoclonal antibodies specific to slow or fast myosin heavy chain (sMyHC and fMyHC, respectively), can lead to artefactual labeling of damaged muscle fibers, as hybrid fibers (sMyHC+ and fMyHC+). We demonstrate that, such erroneous immunophenotyping of muscle may be avoided, by performing colabeling or serial-section-labeling, to identify damaged fibers. The *quadriceps femoris* muscle group (QF) in 7-month-old, male, C57BL/6J mice had: 1.21±0.21%, 98.34±1.06%, 0.07±0.01%, and 0.53±0.85% fibers, that were, sMyHC+, fMyHC+, hybrid, and damaged, respectively. All fibers in the tibialis anterior muscle (TA) of 3-month-old, male, C57BL/6J mice were fMyHC+; and at 3 days after injurious eccentric contractions, there was no fiber-type shift, but ~ 18% fibers were damaged.

Introduction

Skeletal muscle tissue possesses the unique property of being able to generate active contractile force for voluntary movement. Muscle contraction is made possible by cyclic interaction of myosin heavy chains (MyHCs) in the thick filaments of sarcomeres with actin in thin filaments, through the hydrolysis of ATP.[1,2] Movement is mediated by the activation of motor units, which are made up of all muscle fibers that receive efferent input from a single motor axon.[3,4] Depending on whether muscle fibers are part of a fast or slow motor unit, they possess slow MyHC (sMyHC, a.k.a. type 1 MyHC) or fast MyHC (fMyHC, a.k.a. type 2 MyHC; subtypes 2A and 2X in humans, along with 2B in other mammalian species).[2,5,6] Immunophenotyping of muscles, based on fiber type, is possible due to the availability of antibodies against specif-

ic MyHC isoforms.[6-8] Either naturally, or due to genetic/epigenetic influences, muscles may have a population of hybrid fibers, which have more than one type of MyHC.[9,10] Based on an incidental finding in our laboratory, we developed a set of experiments, to test the hypothesis that damaged muscle fibers label falsely as hybrid fibers. In order to test our hypothesis, we studied the *quadriceps femoris* (QF) muscle group of healthy middle-aged mice (7 months), since the QF in some of these mice shows small amounts of spontaneous damage. Also, since, intense eccentric muscle contractions are known to cause muscle fiber necrosis by disrupting sarcomeres, the sarcolemma, and the excitation-contraction coupling apparatus; we exposed the *tibialis anterior* (TA) muscle of healthy young mice (3 months) to a bout of medium-strain forced eccentric exercise (MSFEE), to assess if experimentally-injured muscle fibers might also present as hybrid fibers.[11-16]

Materials and Methods

Animal models

Experiments with live animals were performed at Wayne State University (Detroit, MI), according to protocols approved by the Institutional Animal Care and Use Committee. These protocols were in accordance with the Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC). All mice in this study were from the C57BL/6J strain (Stk# 664, The Jackson Laboratory, Bar Harbor, ME), males, 7 month-old, for studies on the QF (n=3 mice), and 3 mo old, for studies on the TA (n=3 mice).

Tissue collection

We removed the QF and TA *en bloc* from euthanized mice, by cutting the distal tendon, reflecting the muscle upward with tweezers, and then releasing the proximal attachments with a scalpel. We quickly dipped the harvested muscles in mineral oil for cryoprotection, blotted of the excess oil with lab wipes, placed the muscles on aluminum foil, and snap froze them by rapidly immersing them in liquid nitrogen.

Histological studies

A brief description of our immunolabeling protocols is provided here; additional details are provided under Supplementary Methods and Supplementary Data.

MyHC labeling on QF muscle sections from 7-month-old mice

We followed the labeled streptavidin biotin (LSAB) method to label MyHC and visualize it under confocal optics.[17] We incubated serial sections overnight, at 4°C, separately, with mouse monoclonal primary antibodies specific to sMyHC or fMyHC (M8421 and M4276, respectively, 1:1000, MilliporeSigma, St. Louis, MO, USA).[18]

After washing off unbound primary antibodies, sections were incubated for 60 min with secondary antibodies conjugated to biotin (goat-anti-mouse IgG, B2763, 1:200, ThermoFisher Scientific, Waltham, MA, USA). After washing off unbound secondary antibodies, we incubated sections for 15 min with streptavidin conjugated to Alexa 568 (S11226, 1:200, ThermoFisher Scientific).

Desmin colabeling on QF muscle sections from 7-month-old mice

Since desmin is highly sensitive to muscle fiber damage, we colabeled QF sections with rabbit polyclonal primary antibodies to desmin (RB-9014-P, 1:200, ThermoFisher Scientific, Waltham, MA), to detect damaged fibers in the QF.[19,20] After overnight incubation and washing, as above; we incubated sections with secondary antibodies conjugated to Alexa 488 for 60 min (goat-
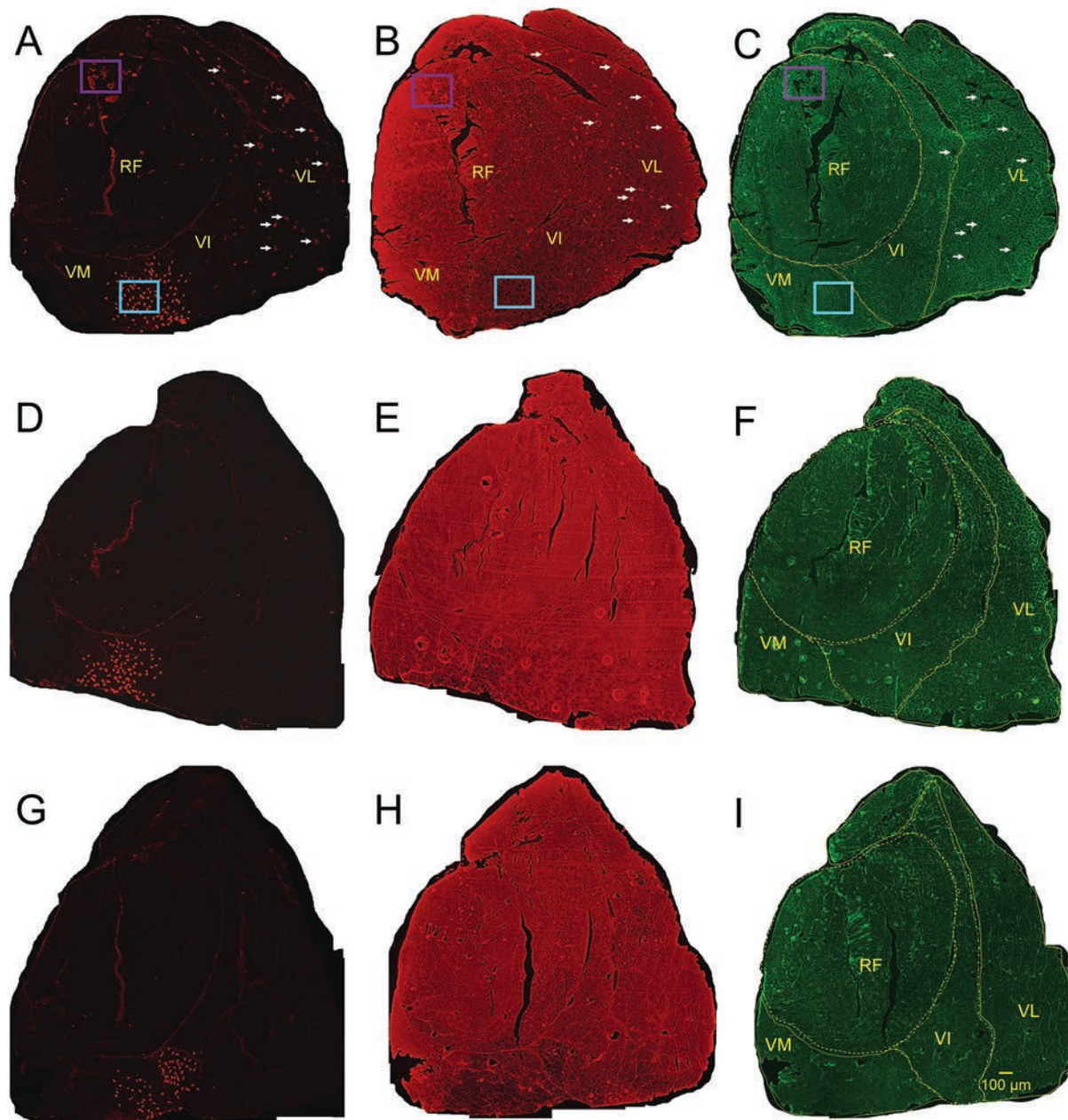
Figure 1. fMyHC+ fibers predominate in the QF of 7-month-old mice with some sMyHC+ fibers in the VM and VI. This figure shows tiled images of serial or colabeled cross sections of the QF muscle group, from 7-mo-old C57BL/6J mice (n = 3; A-C, D-F, and G-I are from animals 1, 2, and 3, respectively). We labeled sections with antibodies to sMyHC (A, D, G) or fMyHC (B, E, H), and colabeled sections with antibodies to desmin to detect muscle fiber damage (C, F, I). In all 3 mice, the QF was mostly composed of fMyHC+ fibers, except for a small number of sMyHC+ fibers that were concentrated in the VM and VI portions of the QF (A-C, region in blue boxes; high magnification images are shown in Figure 2). In 1 of 3 mice (A-C), we found several fibers across the entire QF, which appeared to be sMyHC+ and fMyHC+ (white arrows); however, these fibers were desmin-, and were therefore damaged fibers and not hybrid fibers (region in purple boxes; high magnification images are shown in Figure 2).

Figure 2. Damaged muscle fibers in the QF of 7-month-old mice appear as hybrid fibers. Panels A, B, and C, are images from the QF of animal 1, enclosed within the blue box in Figure 1 A-C,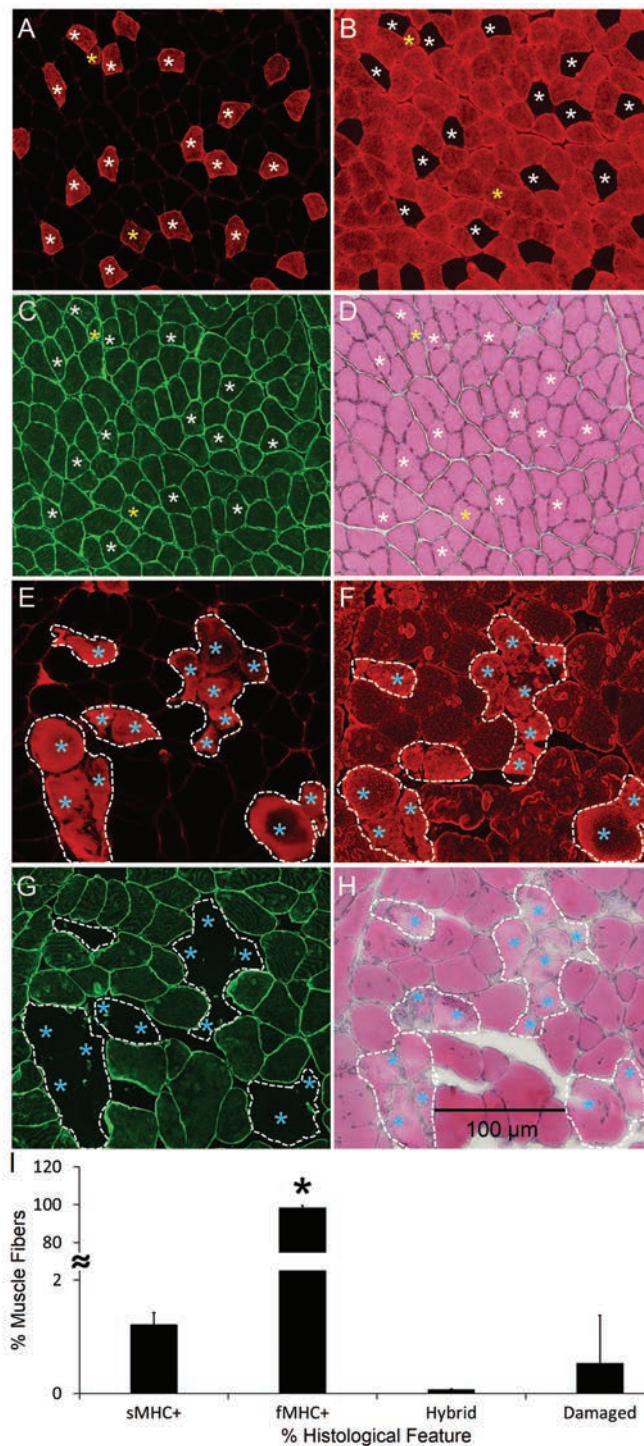 and are results of immunostaining with antibodies to sMyHC, fMyHC, and desmin, respectively. H&E staining of the same region in A-C, is shown in D. The data in A-D suggest that, even though the VM and VI portions of the QF have sMyHC+ fibers, there are very few hybrid fibers. The white asterisks in A-D, denote the same set of sMyHC+ (i.e. fMyHC-) fibers in serial sections. The yellow asterisks in A-D, denote hybrid fibers that are sMyHC+, fMyHC+, desmin+, and show no damage through H&E staining. E, F, and G, are images from the QF of animal 1, enclosed within the purple box in Figure 1 A-C, and are results of immunostaining with antibodies to sMyHC, fMyHC, and desmin, respectively. H&E staining of the same region in E-G, is shown in H. In panels E-H, damaged regions are enclosed by white dashed lines, and damaged fibers are marked with blue asterisks. Damaged fibers are desmin- and show disruption seen through H&E staining (pale and/or fragmented cytoplasm, with or without cellular infiltration). Damaged fibers appear to be positive for both sMyHC and fMyHC, likely due to entry of mouse IgG, which gets labeled by secondary antibodies used for MyHC labeling. Without verifying fiber integrity, damaged fibers, such as those shown in E-H, could be mistaken for hybrid fibers having both sMyHC and fMyHC. Quantitative data from QF muscles of all three 7-month-old mice are summarized in panel I, and confirm that the percentage of fMyHC+ fibers is significantly greater than the percentage of sMyHC+, hybrid and damaged fibers. *P<0.05 for fMyHC+ fibers versus other groups. Mean ± SD.

anti-rabbit F(ab')2 fragments, A-11070, 1:200, ThermoFisher Scientific).

H&E staining on QF muscle sections from 7-month-old mice

On serial sections, we performed hematoxylin and eosin (H&E) staining, as described earlier and in Supplementary Methods.[21,22]

MyHC labeling on TA muscle sections from 3-month-old mice

We labeled TA muscle sections from 3-month-old mice, just as we labeled QF sections from 7- month-old mice, but without colabeling desmin.

Desmin and immunoglobulin G (IgG) labeling on TA muscle sections from 3-month-old mice

On serial sections of the TA muscle, we labeled desmin and IgG, to detect damaged fibers, as described earlier and in Supplementary Methods.[20,22] As above, we incubated sections overnight with primary antibodies to desmin, washed the sections, and then applied goat anti-rabbit secondary antibodies. While applying goat anti-rabbit-secondary antibodies, we simultaneously applied goat anti-mouse IgG antibodies conjugated to biotin, in order to label mouse IgG. After washing off unbound secondary antibodies, we incubated sections for 15 min with streptavidin conjugated to Alexa 568 to visualize mouse IgG under confocal optics. By this method, damaged fibers are detected by their loss of desmin and inclusion of mouse IgG.[19,20,23]

Dystrophin labeling on TA muscle sections from 3-month-old mice

On serial sections of the TA muscle, we labeled the sarcolemma-associated protein dystrophin, as an additional measure of muscle fiber damage.[24] Labeling methods were similar to desmin labeling, above, albeit with rabbit polyclonal antibodies against dystrophin (RB-9024-P, 1:200, ThermoFisher Scientific).

Negative control labeling

For MyHC, desmin and dystrophin labeling experiments, we performed negative control labeling by replacing primary antibodies with equal concentrations of non-specific IgG from the host species. For IgG, we performed negative labeling by omitting goat anti-mouse IgG. Additional details are provided under Supplementary Methods and Supplementary Data.

Muscle injury by eccentric contractions

To demonstrate that damaged fibers label falsely as hybrid fibers, we followed a protocol of injurious eccentric contractions, which has been described in detail.[16] We exposed the left TA to 40 eccentric contractions (under general anesthesia; inhaled, 2-

5% for induction and 1-4% for maintenance), performed in 4 sets of 10 repetitions, with 2 min rest between sets. For each eccentric contraction, the dorsiflexors were tetanically stimulated, and the foot was plantarflexed from 90 to 160°, at 300 /S.[16] The right TA served as an unexercised control.

Statistical analyses

We analyzed quantitative data with SigmaStat 3.5 software (Systat Software, San Jose, CA, USA). We counted all fibers in each QF and TA, and calculated the percentage of fibers in each muscle that were sMyHC+, fMyHC+, hybrid, or damaged. We analyzed fiber counts from the QF and TA, by one-way ANOVA. Where F-ratios were significant, *post-hoc* analyses with Holm-Sidak correction identified significant differences between groups. P-values <0.05 were considered significant. Data are reported as mean ± standard deviation (SD).

Results

Distribution of sMyHC+ and fMyHC+ fibers in the QF of 7-month-old C57BL/6J mice

In the QF, ~98 % fibers were fMyHC+ (Figures 1 and 2; 8783±372 fibers counted per QF; n=3 mice). After identifying the *rectus femoris* (RF), *vastus medialis* (VM), *vastus intermedius* (VI), and *vastus lateralis* (VL) portions of the QF, based on prior data; we evaluated the distribution of sMyHC and fMyHC fibers in these individual portions of the QF.[25,26] We found a concentration of sMyHC+ fibers in the VM and VI (Figures 1 and 2). In the vicinity of the cluster of sMyHC+ fibers in the VM and VI, we found a very small number of hybrid fibers, which were sMyHC+, fMyHC+, desmin+, and showed no disruption through H&E staining.

In one of the QF samples, we observed several fibers that appeared to be sMyHC+ and fMyHC+, and were sporadically distributed across the entire QF (Figure 1 A-C, white arrows); however, these fibers showed a loss of desmin, and disruption through H&E staining, confirming that they were damaged fibers and not hybrid fibers (Figures 1 and 2). As reported earlier, desmin labeling is seen internally in muscle fibers, with enrichment near the sarcolemma.[20,27,28]

Distribution of sMyHC+ and fMyHC+ fibers in the TA of 3-month-old C57BL/6J mice

In the control TA, all fibers assessed were fMyHC+, with no detectable

sMyHC+, or hybrid fibers (Figure 3 A-C, G-I; 2113±80 fibers counted per TA; n = 3). At 3 days after injurious eccentric contractions, we did not detect a change in fiber type, but did see an increase in damaged fibers that were IgG+, desmin-, dystrophin-, and showed disruption through H & E staining (Figure 3 D-F, J-L). As seen in the QF of 7-mo-old mice, damaged fibers appeared to be both sMyHC+ and fMyHC+, based on MyHC staining alone; but, the entry of IgG, the loss of desmin and dystrophin, and fiber disruption seen through H&E staining, confirmed that, these fibers were damaged fibers and not hybrid fibers.

Discussion

Damaged fibers masquerade as hybrid fibers

In 7-month-old mice, fMyHC+ fibers predominated in the QF, with a few sMyHC+ fibers in the VM and VI portions, and a very small number of hybrid fibers in the vicinity of sMyHC+ fibers. However, in 1 of 3 animals studied, we found what looked like hybrid fibers (both sMyHC+ and fMyHC+) scattered across the entire cross sectional area of the QF. By labeling serial sections with antibodies to desmin and through H&E staining, we confirmed that, damaged fibers in the QF, were appearing to be sMyHC+ and fMyHC+. To empirically validate our observation that damaged fibers can present as hybrid fibers, we studied the TA in younger mice (3-month-old), after injurious eccentric contractions. We confirmed that, even in the experimentally-injured TA, MyHC labeling alone, gave the impression that damaged fibers were sMyHC+ and fMyHC+. Since damaged fibers are permeable to native IgG (Figure 3 F,J-L), it is likely that damaged fibers showed artefactual labeling with the anti-mouse secondary antibodies used for MyHC labeling.[20,22]

The topographical distribution of sMyHC+, fMyHC+, and hybrid fibers in the QF - a potential outcome measure

Our data suggest that ~98% of the QF in healthy, 7-month-old, C57BL/6J mice, is made up of fMyHC+ fibers. However, we detected a cluster of sMyHC+ fibers in the VM and VI, and a very small number of hybrid fibers in the vicinity of sMyHC+ fibers. This topographical information, could serve as a potential outcome measure to study muscle pathology or adaptation (example: a shift in fiber type), and to pre-clinically evaluate experimental therapies (example: reversal of a fiber type shift).
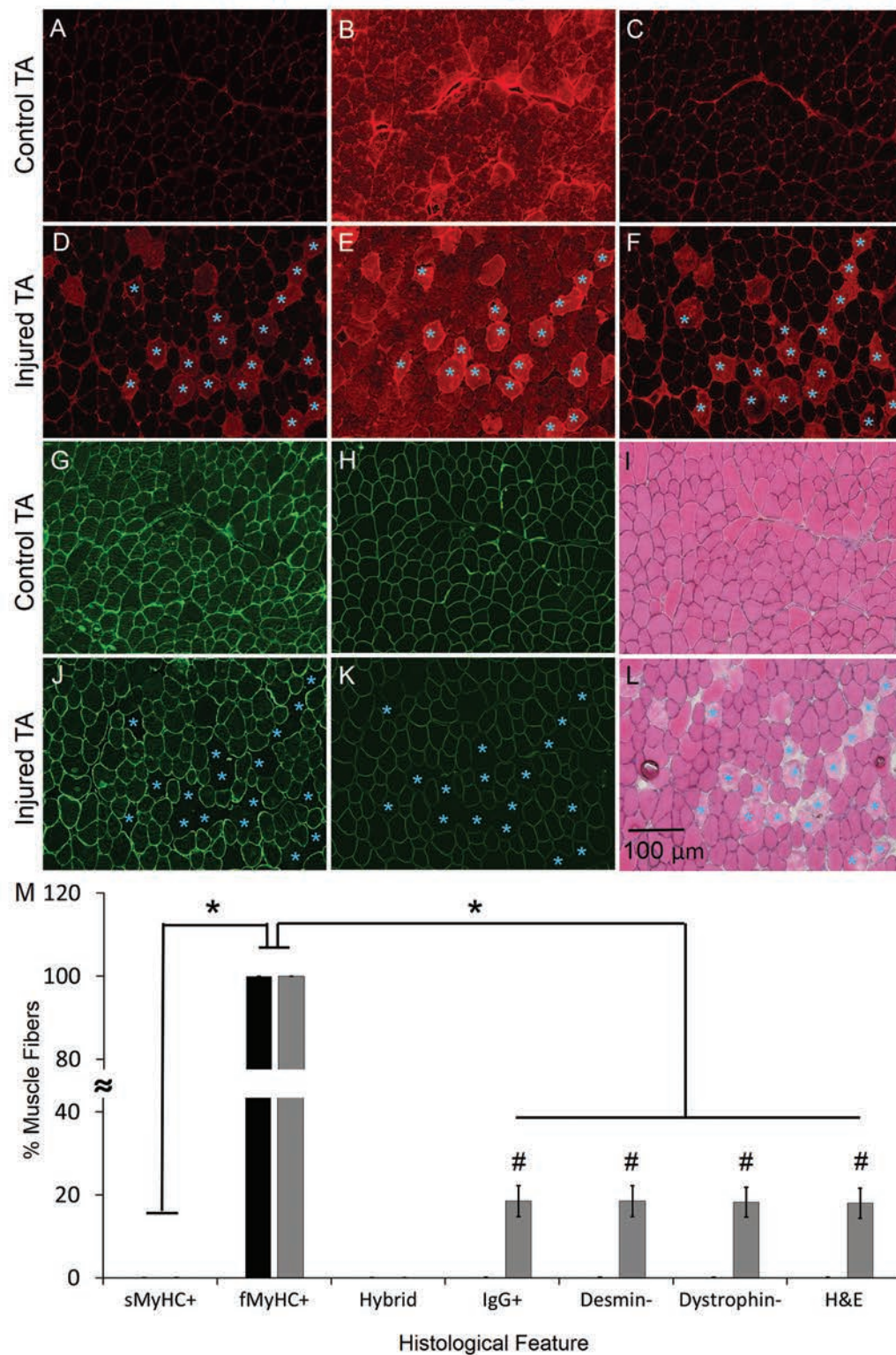
Figure 3. Representative serial sections of control TA muscle (A-C, G-I) and eccentrically-injured TA muscle (D-F, J-L, 3 days post-injury), are shown. Panels A, B, C, G and H, are labeled with antibodies to sMyHC, fMyHC, mouse IgG, desmin, and dystrophin, respectively; and so are D, E, F, J and K. Panels I and L, are serial sections of control and injured TA, respectively, which are stained with H&E. The data suggest that, all fibers in the control TA, are fMyHC+. Damaged fibers in the eccentrically-injured TA, can be identified by their loss of desmin and dystrophin, and inclusion of mouse IgG. Damaged fibers appear as if they are hybrid fibers, which are positive for both sMyHC and fMyHC (marked with blue asterisks); however, this labeling is artefactual. Quantitative data are presented in panel M. The data indicate that, the control TA is composed of fMyHC+ fibers, and that there is no change in fiber type at 3 days after a single bout of injurious eccentric contractions. The black and grey bars in the graph, represent control and injured TA muscle, respectively. *P<0.05 for fMyHC+ fibers versus other groups; #P<0.05 for injured versus control TA. Mean ± SD.

Limitations of current study and alternative approaches

Since muscle fibers can undergo segmental degeneration, studies such as ours, can only provide a snapshot of the status of a particular region, along the length of each muscle fiber observed in a cross section. Furthermore, artefactual MyHC labeling can be reduced by: using mouse-on-mouse labeling kits when using mouse monoclonal antibodies to label mouse tissue; using primary antibodies that have different immunoglobulin isoforms (example: IgG1, IgG2b, IgM); using antibodies raised in species other than the species being studied (example: avoiding mouse-on-mouse labeling); and, pre-conjugating primary antibodies to fluorophores before labeling.[8,29-31] Nonetheless, our data stress the importance of assessing fiber integrity, while immunophenotyping muscle based on MyHC isoforms.

Assessing myofiber integrity is a useful checkpoint, while immunophenotyping muscle for fiber-type distribution, based on MyHC isoforms.

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