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ABSTRACT: Denervation of skeletal muscles results in loss of muscle mass and contractile force. Recent evidence suggests that local immune system activation plays a key role in these processes, but the mechanisms underlying muscle–immune system cross-talk are not understood. The purpose of this study was to address the mechanisms by which muscle responds to denervation and to elucidate the specific role played by FYN in local immune system activation. We studied initial events taking place in the gastrocnemius of wild-type and *Fyn*$^{-/-}$ mice following sciatic nerve transection. Discontinuous sucrose gradient centrifugation was used to prepare lipid rafts at different time-points (1, 7, and 14 days) after surgery. Activation of FYN, cytokine expression (IL-1β and TNF-α), and T-cell activation (CD3 and IL-15) were followed by in vitro kinase assays, enzyme-linked immunoassay (ELISA), Western blotting, and immunoprecipitation. Sciatic nerve injury resulted in increased SRC kinase activity in gastrocnemius lipid rafts. Production of both IL-1β and TNF-α was increased, peaking after 1 day, followed after 7 and 14 days by upregulation of IL-15 and CD3 expression and the development of caveolin-3 and CD3 complexes. The integrity of lipid rafts and the upregulation of SRC kinase activity, cytokine expression, and T-cell activation and cross-talk with muscle cells following denervation were abolished in *Fyn*$^{-/-}$ mice. The integrity of FYN-dependent lipid rafts is required for local immune system activation within denervated muscle, and lipid rafts are implicated in orchestrating muscle–immune-cell cross-talk. These results are likely to provide new insights into the therapy of neuromuscular injury.

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FYN-DEPENDENT MUSCLE–IMMUNE INTERACTION AFTER SCIATIC NERVE INJURY

HUANG HANWEI, PhD[1] and ZHAO HUI, PhD[2]

[1] Department of Orthopaedics, Fifth Hospital of Shanghai, Fudan University, Shanghai, China

[2] Department of Integrative Medicine and Neurobiology, National Key Lab of Medical Neurobiology, Institute of Brain Sciences, Fudan University, 138 Yixueyuan Road, Shanghai 200032, China

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Disruption of nerve supply to skeletal muscles results in a rapid loss of muscle mass and contractile force. Following short-term denervation these losses are largely reversible by grafting and nerve implantation. However, restoration is significantly poorer following long-term denervation,[1] and understanding the early changes taking place in the muscle is of crucial importance for the development of treatments for neuromuscular injury.

Sciatic nerve injury leads to a variety of changes in muscle morphology and biochemistry, including inflammation.[2,3] The immune system is known to play a specific role in certain muscle disorders,[4,5] as well as after denervation. Following sciatic nerve injury, immunological changes in the gastrocnemius muscle include invasion by T cells and increased expression of immune regulators such as major histocompatibility complex (MHC) and B7 molecules.[6,7] The acute response is also associated with increased interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) production.[8,9] A potential role for the immune system in the adverse changes following denervation is supported by clinical

Abbreviations: ANOVA, analysis of variance; APC, antigen-presenting cell; ATP, adenosine triphosphate; BSA, bovine serum albumin; CD3, cluster of differentiation 3; ELISA, enzyme-linked immunoassay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRP, horseradish peroxidase; IL-1β, interleukin-1β; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PBST, PBS plus Tween 20; TCA, trichloroacetic acid; TCR, T-cell receptor; TNF-α, tumor necrosis factor-α

Key words: caveolin-3; FYN; gastrocnemius; muscle–immune interactions; AQ3 sciatic nerve injury

AQ4 **Correspondence to:** Z. Hui; e-mail: mayzhao5375@yahoo.com

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evidence showing that approaches that target immune and/or cytokine responses can shorten recovery time.[1] Although local changes in muscle are therefore likely to contribute to the adverse effects of denervation, little is known of the mechanisms by which denervation can produce immune and inflammatory changes.

Caveolae or lipid rafts, membrane microdomains rich in cholesterol and sphingolipids, are important for cell signaling, because they facilitate colocalization of transmembrane receptors with adaptor and signaling proteins.[10] Caveolin proteins are prominent components of caveolae and play key roles in signal transduction.[11] Caveolin-3, first identified by Parton et al.,[12] is the predominant caveolin in skeletal muscle. Several functions have been ascribed to caveolin-3, including formation of the transverse tubule system and coordination of signal transduction.[12] FYN, an SRC-family tyrosine kinase, has been shown to associate with caveolins.[13] FYN has been reported to be localized in caveolae and is thought to stabilize the neuromuscular junction.[14] It is therefore possible that muscle denervation might lead to alterations in the properties of FYN-dependent lipid rafts. Importantly, there is evidence for direct communication between muscle and immune cells that potentially could act as a relay of SRC-family signaling. Trogocytosis is a cell–cell contact-dependent process whereby membrane fragments and associated molecules can be transferred rapidly between cells.[15] Newly acquired molecules are subsequently found on the recipient cell surface in the correct orientation and functional confirmation. Trogocytosis taking place between antigen-presenting cells and T cells has been reported to stimulate T-cell proliferation and the priming immune responses.[16,17] It was recently reported that muscle cells are capable of exchanging membrane components with T cells.[15] Because FYN activation is central to the initiation of T-cell receptor signaling pathways,[18] it is possible that alterations of lipid raft FYN signaling in muscle cells could be exchanged with cells of the immune system, including T cells, wherein they elicit changes in cell activation.

We therefore hypothesized that FYN signaling in muscle cells, and cross-talk with T cells, might play a key role in aberrant immune and inflammatory processes following denervation. To address this possibility we investigated whether local immune activation in the gastrocnemius muscle following sciatic nerve transection is affected in homozygous $Fyn^{-/-}$ knockout mice. We report that loss of FYN abolishes local immune system activation following denervation.

METHODS

Animals and Surgical Denervation. Mice of the $Fyn^{-/-}$ strain B6 129S7-Fyntm1Sor and of the approximate control wild-type strain B6 129SF2/J were kindly provided by Dr. Loh and Dr. Law (Department of Pharmacology, University of Minnesota). All animal procedures were performed in strict accordance with the U.S. National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*. All experiments were designed to minimize the number of animals used and their suffering.

Animals (18–20 g, all male) were divided into four groups ($n = 5$ in each group): unoperated controls, and animals after 1, 7, or 14 days of sciatic nerve transaction. For muscle denervation, after anesthesia with pentobarbital sodium (35 mg/kg, intraperitoneally), the right sciatic nerve was surgically exposed, and a 1-cm segment was removed from the upper thigh. Body temperature was maintained at 37°C throughout surgery, and there was no evidence of postoperative infection. At the specified time-points the animals were euthanized, and the gastrocnemius was cleaned of fat and connective tissues, weighed, and quickly frozen in liquid nitrogen for later analysis. Contralateral muscle was used as a control.

Discontinuous Gradient Centrifugation for Lipid Raft Extraction. Gastrocnemius muscle tissue was washed twice with phosphate-buffered saline (PBS, pH 7.4) at 4°C and then homogenized in 2 ml of 500 mM $NaCO_3$ (pH 11.0) solution with complete protease inhibitor mixture (Roche Applied Science, Indianapolis, Indiana). The tissue suspension was further sonicated with one 30-s burst at setting 4 and one 30-s burst at setting 7 (Model W-220F; Ultrasonics, Inc., Bartlett, Illinois). The homogenate was adjusted to 45% sucrose by adding 2 ml of 90% (w/v) sucrose prepared in MBS buffer [25 mM MES (pH 6.5), 0.15 M NaCl] and placed at the bottom of an ultracentrifuge tube. Four milliliters of 35% sucrose and 4 ml of 5% sucrose (both in MBS buffer containing 250 mM sodium carbonate) were then overlaid upon the sample to form a 5–45% discontinuous sucrose gradient. The sample was centrifuged at 32,000 rpm for 16 h in a Beckman ultracentrifuge in an SW-41Ti rotor. Fractions of 1 ml each were collected from the top, and total

proteins in each fraction were precipitated with 5% trichloroacetic acid (TCA). The resulting pellets were washed with acetone and then resuspended in Laemmli buffer. A light-scattering band at the 5% and 35% sucrose interface (Fraction 4) represented the lipid raft fractions.[19,20]

In Vitro SRC Kinase Assay. Proteins in gastrocnemius Fraction 4 were precipitated with 5% (w/v) TCA. The resulting pellets were washed with acetone and incubated at 30°C with 5 μg of SRC substrate peptide (KVEKIGEGTYGVVYK, corresponding to amino acids 6–20 of p34cdc2; Upstate Biotechnology, Lake Placid, New York) in kinase buffer containing 5 μCi of [γ-^{32}P]-adenosine triphosphate ([γ-^{32}P]-ATP; PerkinElmer Life Sciences, Waltham, Massachusetts), 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 25 μM ATPase, 1 mM dithiothreitol, and 100 μM Na_3VO_4. After 30 min, the reaction was terminated by the addition of 10 μl of 40% (w/v) TCA, and samples were spotted onto P81 cellulose phosphate paper (Upstate Biotech). The paper was washed three times with 1% (w/v) phosphoric acid and once with acetone. Radioactivity retained on the P81 paper was quantified by liquid scintillation counting. Blank counts (without tissue lysate) were subtracted from each result, and radioactivity (cpm) was converted to picomoles per minute (pmol/min).

Enzyme-Linked Immunoassay Analysis of IL-1β and TNF-α Levels. Levels of IL-1β and TNF-α production were measured by sandwich enzyme-linked immunoassay (ELISA) according to the manufacturer's instructions (R&D Systems, Inc., Minneapolis, Minnesota). A 96-well plate was coated with 2 μg/ml monoclonal anti-mouse IL-1β or TNF-α antibody (MAB401) at 4°C overnight and then blocked with 1% bovine serum albumin (BSA) in PBS for 1 h. The plates were washed three times with PBS containing 0.2% Tween 20 (PBST). Aliquots of tissue lysates were diluted to 100 μl with Hanks' balanced salt solution (HBSS) with calcium and magnesium, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 1% fetal bovine serum, added to the plates, and then incubated for 2 h at room temperature. The plates were washed three times with PBS, and 100-μl aliquots of 0.1-μg/ml biotinylated mouse IL-1β or TNF-α affinity-purified polyclonal antibody (BAF401) were added and incubated for 2 h. After a further three washes with PBST, the immune complexes were colorimetrically detected using horseradish peroxidase (HRP)–streptavidin conjugate. The reaction was halted by the addition of 1 M H_2SO_4, and the absorbance at 450 nm was measured using a microplate reader (VERSAmax; Molecular Devices, Sunnyvale, California). Experiments were performed independently three times, and the data presented as mean ± SEM.

Immunoprecipitation and Western Blotting. For immunoprecipitation, gastrocnemius tissue was homogenized in lysis buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1% Triton X-100, and one complete protease inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany). After centrifugation for 5 min at 14,000 rpm, the supernatants were incubated with anti–caveolin-3 antibody (1:200; BD Transduction Laboratories, Lexington, Kentucky) at 4°C overnight with slow rotation. Sixty microliters of protein G–agarose beads (Invitrogen, Carlsbad, California) were added, and the mixture was further incubated at 4°C for 3 h with slow rotation. The protein G–agarose beads were then pelleted by centrifugation at 12,000*g* for 15 min at 4°C and washed five times with wash buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl]. Proteins were resuspended in a 20-μl Laemmli sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecylsulfate, 5% glycerol, 0.03% bromophenol blue, 0.9% (v/v) β-mercaptoethanol] and boiled for 5 min at 65°C. They were then probed in parallel with antibody against CD3.

For Western blot analysis, protein samples were resolved by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Membranes were blocked with 10% non-fat milk and 1% Tween 20 in Tris-buffered saline. The membrane was subsequently probed with primary antibodies anti–IL-15 and anti-CD3 (1:200; Santa Cruz Biotechnologies, Santa Cruz, California), anti–caveolin-3 (1:1000; Transduction Laboratories, Heidelberg, Germany), and anti-FYN (1:200; Cell Signaling Technology, Danvers, Massachusetts). Protein bands were detected using alkaline phosphatase–conjugated secondary antibodies (1:5000) and ECF substrate (Amersham Biosciences, Piscataway, New Jersey), and then scanned using a Storm 860 imaging system (GE Healthcare, Piscataway, New Jersey). Band intensities were quantified and analyzed with ImageQuant software (GE Healthcare).

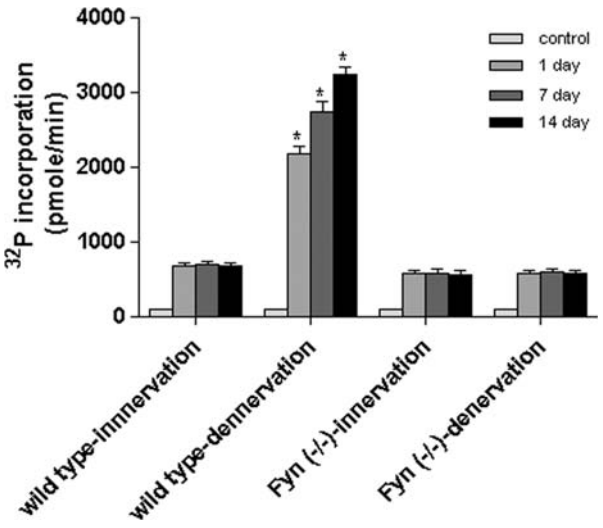

FIGURE 1. Dynamic alteration of SRC kinase activity after sciatic nerve injury. SRC kinase activity was measured in gastrocnemius muscle tissue rafts 1, 7, and 14 days following sciatic nerve transection. Tissues were separated by discontinuous sucrose centrifugation, and SRC kinase activity in fractions enriched in lipid rafts were assayed using an SRC-specific substrate peptide (see Methods). $^*P < 0.05$ ($F = 4.741$) vs. unoperated contralateral muscle.

Statistical Analyses. All experiments were performed with 5 animals per group ($n = 5$). Data are presented as mean ± SEM and analyzed with Prism 5 software. For all data sets, normality and homoscedasticity assumptions were reached, validating the application of the one-way analysis of variance (ANOVA), followed by *t*-test with Bonferroni's correction for multiple comparisons. Differences were considered significant at $P < 0.05$.

RESULTS

Muscle Denervation Increases SRC Kinase Activity within Lipid Rafts. Members of the SRC family or protein kinases are expressed in many cell types, including muscle. It was previously reported that SRC kinase activity is modulated by muscle cell damage,[21] and lipid rafts provide a platform for SRC-mediated signal transduction.[22] We therefore examined SRC kinase activity within gastrocnemius lipid rafts from animals undergoing sciatic nerve resection. Kinase-catalyzed phosphorylation of synthetic target peptides was quantified by the incorporation of radiophosphorus from [γ-^{32}P]-ATP into an SRC-specific substrate peptide (corresponding to p34cdc2 amino acids 6–20). As shown in Figure 1, there was a significant increase in raft-associated SRC kinase activity following sciatic nerve transaction: levels of radiolabel incorporated at 1, 7, and 14 days following surgery were increased by factors of 3.2, 4.0, and 4.7, respectively, relative to controls. These data indicate that denervation of the gastrocnemius significantly increased lipid raft SRC kinase activity.

We then investigated whether similar upregulation takes place following denervation in $Fyn^{-/-}$ mice. In these animals there was no significant increase in SRC kinase activity in gastrocnemius lipid rafts following sciatic nerve injury. There was no detectable increase in SRC activity at any timepoint following surgery. This result indicates that the enhancement of SRC kinase activity following muscle denervation was abolished in *Fyn* knockout mice, demonstrating that FYN kinase is required for upregulation of lipid raft SRC activity.

Local Immune Activation following Muscle Denervation in Wild-Type and $Fyn^{-/-}$ Mice. Peripheral nerve injury is associated with immune reactions in denervated muscle; it has been suggested that this may be of protective value against loss of muscle function.[8,9] Upregulation of proinflammatory cytokines IL-1β and TNF-α is considered to represent an initial event in the induction of a local immune response.[23] We therefore sought to determine whether IL-1β and TNF-α are upregulated following muscle denervation in wild-type and mutant mice. In wild-type mice, only low levels of IL-1β and TNF-α were found in gastrocnemius tissues. However, tissue levels of both cytokines were markedly increased 1 day following sciatic nerve injury: IL-1β levels rose approximately 4-fold, whereas TNF-α levels increased ~3-fold over control values (Fig. 2A and B). The increase was transient and, by 7 and 14 days following surgery, IL-1β levels returned to control values. TNF-α displayed a similar profile with values also falling to control levels at 7 and 14 days following surgery. There were no significant changes in IL-1β and TNF-α content in control contralateral muscle (Fig. 2A and B). These results indicate that denervation of the gastrocnemius led to a robust but transient 3– 4-fold upregulation of muscle levels of both IL-1β and TNF-α.

We then investigated whether a similar upregulation of IL-1β and TNF-α takes place in response to denervation in $Fyn^{-/-}$ mice. In contrast to the changes observed in wild-type animals, there was no significant elevation of either cytokine in animals deficient in FYN kinase. On days 1, 7, and 14 following surgery, IL-1β and TNF-α levels were unchanged versus levels in control animals or in

the contralateral unoperated gastrocnemius (Fig. 2A and B).

The stimulation of memory T cells by IL-15 is thought to reflect antigen-mediated activation,[24] and the expression of IL-15 and the T-cell receptor CD3 complex can afford a measure of T-cell recruitment and activation.[25,26] We therefore measured IL-15 and CD3 expression levels in gastrocnemius muscle following sciatic nerve surgery. As shown in Figure 2C and D, Western blot analysis using specific antibodies against IL-15 and CD3 revealed a progressive increase in expression levels following denervation, and the increase was sustained at 14 days after surgery. Upregulation 1 day following surgery was not significant, but levels of both IL-15 and CD3 rose to between 2- and 3-fold above control levels at days 7 and 14 (Fig. 2C and D). No changes in the levels of either IL-15 or CD3 were observed at any time-point in control contralateral muscle tissue.

We then aimed to determine whether IL-15 and CD3 upregulation takes place in $Fyn^{-/-}$ mice. In knockout animals there was no significant change in the levels of either IL-15 or CD3 at any time-point following surgery (Fig. 2C and D). These data indicate that deficiency in FYN kinase activity abolishes IL-15 and CD3 upregulation, markers of T-cell recruitment and activation, following denervation of the gastrocnemius muscle.

Muscle–T-Cell Interactions after Sciatic Nerve Injury. Plasma membrane caveolae provide a platform for signal transduction. The predominant caveolin in skeletal muscle caveolae is caveolin-3.[12] Because FYN knockout appeared to abolish immune activation produced by denervation, we investigated whether this was associated with disruption of caveolar function. Muscle tissues were separated by discontinuous sucrose density gradient centrifugation, and different fractions were analyzed by Western blotting for the presence of caveolin-3. As shown in Figure 3A, caveolin-3 protein in wild-type muscle was strongly enriched in the lipid raft fractions from the 5–35% sucrose interface (Fraction 4). By contrast, in tissues from $Fyn^{-/-}$ mice, caveolin-3 immunopositive signals were distributed across multiple fractions and were not localized preferentially in Fraction 4. This result suggests that FYN kinase is required for proper localization of caveolin-3 to lipid rafts/caveolae.

We then investigated whether denervation resulted in a change in FYN levels in lipid rafts. Gastrocnemius tissue samples were pooled by anti–caveolin-3 antibody and analyzed by Western blotting using a specific anti-FYN antibody. Signal quantitation revealed that FYN levels were upregulated between ~2- and ~3.4-fold following denervation (Fig. 3B). As shown in Figure 3C, in anti–caveolin-3 antibody immunoprecipitated fraction, sciatic nerve injury enhanced CD3 expression in wild-type mice, but not in $Fyn^{-/-}$ mice. In wild-type mice, the relative intensities of CD3 immunoreactive signals were unchanged at day 1 following surgery, but were increased ~2.5- and ~3.3-fold, respectively, versus controls at 7 and 14 days following surgery. In contrast, there were no significant changes in the expression level of CD3 in $Fyn^{-/-}$ mice at any time-point following surgery. These data suggest that FYN expression is required for upregulation of CD3 in lipid rafts following denervation.

DISCUSSION

Muscle denervation following nerve injury causes profound structural and functional changes within skeletal muscles and can lead to marked impairment in the function of the affected limb.[27] Early changes taking place in denervated muscle include immune activation and inflammation, and infiltration of macrophages and T cells have been suggested to play a role in the recovery process.[28] However, it appears that different target cell populations can play different roles during different phases of the post-injury response. Because immediate treatment is most effective for structural and functional recovery[2,3] we focused on the initial events taking place in the denervated muscle following sciatic injury.

Because lipid rafts are inferred to play a key role in signal transduction pathways activated following denervation, and in communication with cells of the immune system,[29] we prepared lipid rafts from gastrocnemius muscles and studied levels of SRC kinase in the raft fractions. Denervation resulted in a marked increase in raft-associated SRC kinase activity, and this was sustained over the period from 1 to 14 days after surgery. Mice deficient in FYN, a key member of the SRC tyrosine kinase family,[30,31] suffer from muscle wasting and also fail to show increased muscle loss following denervation (our unpublished data), and we therefore addressed whether FYN might play a specific role in the local immunological changes that take place after denervation. We report that there was no increase in raft-associated SRC activity in $Fyn^{-/-}$ mutant mice.

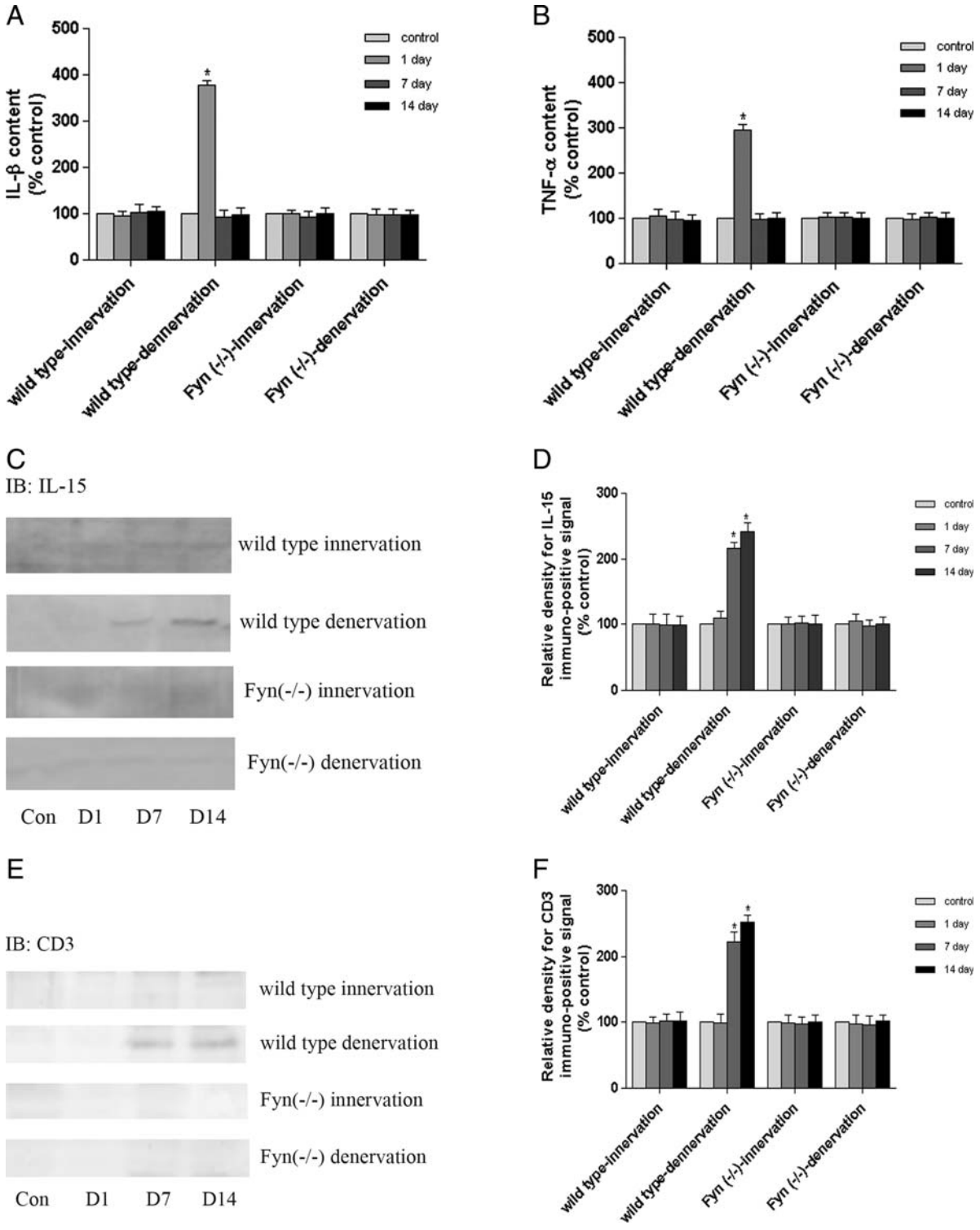

FIGURE 2. Local immune system upregulation following denervation. Cytokine levels and markers of T-cell activation were measured in control and denervated gastrocnemius tissue samples from wild-type and $Fyn^{-/-}$ mice at days 1, 7, and 14 days after sciatic nerve transection. (**A**) IL-1β and (**B**) TNF-α levels measured by ELISA ($F = 3.516$ and 3.625, respectively). (**C**) IL-15 and (**D**) CD3 levels measured by Western blotting ($F = 3.549$ and 3.792, respectively). Experiments were independently performed three times. Data are expressed as mean $\pm$ SEM. $^*P < 0.05$ vs. unoperated contralateral muscle.

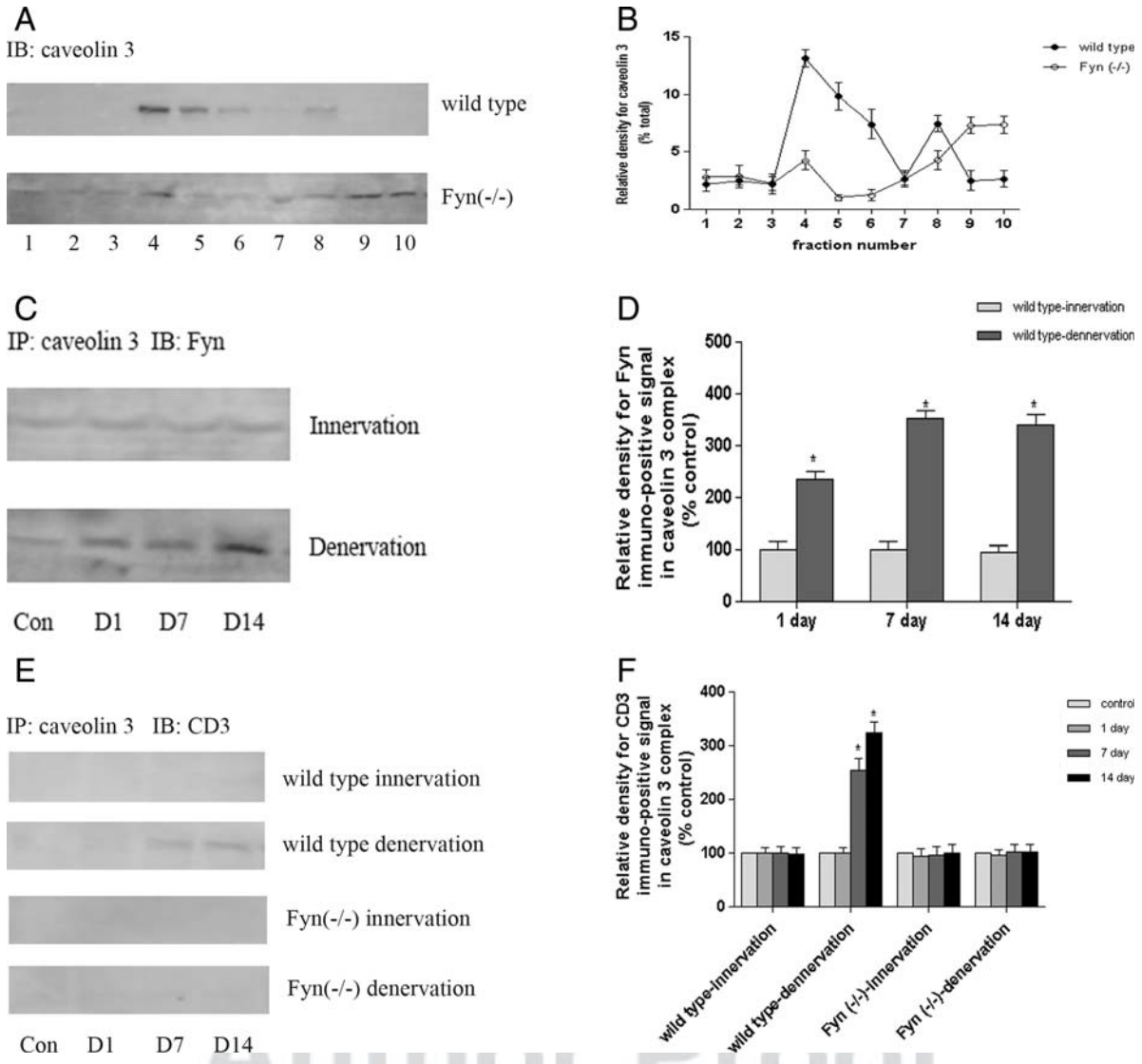

FIGURE 3. Muscle–T-cell interactions following sciatic nerve injury. Lipid rafts from gastrocnemius tissues were separated by discontinuous sucrose centrifugation and immunoprecipitation with anti–caveolin-3, and raft proteins were analyzed by Western blotting. (**A**) The resultant pellets from discontinuous sucrose centrifugation were probed by anti–caveolin-3. When precipitated anti–caveolin-3 by immunoprecipitation assay, (**B**) FYN and (**C**) CD3 expression was examined by Western blotting ($t = 5.698$, $F = 3.513$ respectively). Experiments were independently performed three times. Data are expressed as the relative densities to control. *$P < 0.05$ vs. unoperated control muscle.

Lipid rafts are specialized compartments in the plasma membrane that act as platforms to facilitate intermolecular association of molecules involved in signal transduction.[29] FYN is an important component of lipid rafts and is known to regulate diverse processes including cell proliferation, differentiation, survival, adhesion, migration, and cytoskeletal organization.[30] Importantly, FYN is known to govern the integrity of lipid rafts where it acts to recruit adaptor proteins.[29] The finding that SRC activation was abolished in FYN knockout mice indicates that lipid rafts are likely to play a central role in propagating the specific signaling pathways that are activated following denervation.

We have further reported that levels of proinflammatory cytokines IL-1β and TNF-α are strongly increased in denervated muscle. The increase was most marked 1 day following surgery, but levels of both cytokines decreased to control levels at 7 and 14 days after surgery. This suggests that these cytokines might only play a role in the earliest stages following denervation. We have also provided

evidence for persistent immunological changes in response to denervation. Levels of CD3 and IL-15 were markedly upregulated at 7 and 14 days following surgery, but not at 1 day after denervation, suggesting that these represent a later response phase.

IL-1β and TNF-α are predominantly produced by inflammatory cells and regulate many aspects of the acute immune response.[23] Following muscle denervation it is possible that they might provoke the removal of cellular debris phagocytosis and thus facilitate the switch from a pro- to anti-inflammatory phenotype in regenerating muscle.[32,33] However, cytokines have a wide spectrum of autocrine, paracrine, and endocrine effects, depending on the cell target and the profile of cytokine production.[34] In the experiments reported herein, it was not possible to determine whether IL-1β and TNF-α in denervated muscle derived from intrinsic inflammatory cells or from monocytes or macrophages migrating into denervated muscle. However, it appears likely that the changes taking place in muscle following denervation were associated with T-cell migration and activation. IL-15 is capable of selectively stimulating memory T cells and has been identified as a cue for T cell activation.[35] Furthermore, TCR/CD3 engagement has been reported to induce IL-15 expression.[36] Both IL-15 and CD3 were upregulated in denervated gastrocnemius at 7 days following sciatic nerve injury, and this could indicate influx of T cells into the muscle. It is of note that muscle cells are fully competent for the presentation of both exogenous and endogenous antigens. Indeed, expression of B7, a peripheral membrane protein found on activated antigen-presenting cells, can be detected in muscle under pathologic conditions.[6,7]

Importantly, knockout of FYN prevented induction of IL-1β and TNF-α following denervation, and in the mutant mice we also observed no upregulation of either IL-15 or CD3 in response to surgery. Therefore, it is presumed that loss of FYN activity prevents the proliferation of muscle cells or the recruitment of newly formed myoblasts into damaged muscle fibers, and this is consistent with the muscle wasting seen in $Fyn^{-/-}$ mice (our unpublished observations). Together these results suggest that muscle has important immunoregulatory capacities that include both muscle-derived regulators and the recruitment of immune effector cells.[36] However, local immune activation in response to damage is a complex multistep process,[37] and the mechanisms whereby denervated muscle cells can orchestrate activation of the immune system are not understood.

It was recently suggested that trogocytosis, a process of intercellular transfer of active membrane fragments, might mediate muscle–immune-cell interactions.[15] Trogocytosis is typically considered to take place between antigen-presenting cells and T cells[16,17] and can be triggered by a variety of lymphocyte-specific surface receptors, either individually or in combination. Trogocytosis is considered to be an essential prerequisite for the formation of the immunological "synapse" between different cells of the immune system.[26] A recent report by Waschbisch et al. showed that T cells and muscle cells can exchange cell surface molecules.[15] In addition to muscle to T-cell transfer, it seems likely that fragments of T-cell membranes, including functional proteins, can be incorporated into muscle cells. Caveolae/lipid rafts are membrane microdomains that are associated with a range of signaling components, including ion channels, receptors, and enzymes,[22] and they are likely to contribute to the regulation of muscle–immune-cell interactions. We observed that CD3 levels in lipid fractions enriched in caveolin-3 were increased after 7 days of sciatic nerve injury and this increase was sustained up to 14 days. This corresponds to the timing of the upregulation of IL-15 and CD3 in denervated muscle. The association of caveolin-3, the muscle-specific caveolin isoform,[38,39] with CD3, a component of T-cell receptor complex,[24] suggests that muscle cells might acquire cell-surface components from local T cells.

We have also reported that muscle denervation upregulates FYN levels within caveolin-3–enriched lipid rafts from gastrocnemius. The role of FYN in caveolae-mediated muscle-immune signaling is therefore emphasized in wild-type mice, but not in $Fyn^{-/-}$ mice. These findings are consistent with the interpretation that FYN might be required for caveolin-3 and lipid raft assembly, and therefore provide a substrate for muscle–T-cell trogocytosis. FYN activation is one of the prerequisite events for T-cell receptor (TCR) stimulation, and FYN kinase is known to catalyze specific tyrosine phosphorylation of the TCR and to play a crucial role in TCR-mediated signal transduction.[36]

In conclusion, we have reported that muscle denervation is followed by a rapid increase in the levels of proinflammatory cytokines that is then followed by an increase in markers of T-cell activation as well as muscle–T-cell cross-talk. The induction of a local immunological response following

denervation appears to be pivotally dependent on the integrity of lipid rafts. Our data suggest that the integrity of lipid rafts is markedly impaired in muscles from $Fyn^{-/-}$ mice, and no cytokine production or T-cell activation was observed following denervation in these knockout mice. We conclude that FYN kinase plays a pivotal role in inducing the muscle immunological response that follows denervation. In addition to their potential in the therapy of neuromuscular injury, reagents that target immunomodulation[32,33] and FYN signaling pathways are likely to be of broad clinical application in tissue repair and regeneration.

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REFERENCES

1. Park JW, Kim KM, Oh KJ, Rhyu IJ, Jang HS. Proteasome inhibition promotes functional recovery after peripheral nerve reperfusion injury. J Trauma 2009;66:743–748.
2. Salmons S, Jarvis JC. Functional electrical stimulation of denervated muscles: an experimental evaluation. Artif Organs 2008;32:597–603.
3. Yan M, Cheng C, Ding F, Jiang J, Gao L, Xia C, et al. The expression patterns of beta1,4 galactosyltransferase I and V mRNAs, and Galbeta1–4GlcNAc group in rat gastrocnemius muscles post sciatic nerve injury. Glycoconj J 2008;25: 685–701.
4. Tidball JG, Wehling-Henricks M. Macrophages promote muscle membrane repair and muscle fibre growth and regeneration during modified muscle loading in mice in vivo. J Physiol 2007;578:327–336.
5. Segawa M, Fukada S, Yamamoto Y, Yahagi H, Kanematsu M, Sato M, et al. Suppression of macrophage functions impairs skeletal muscle regeneration with severe fibrosis. Exp Cell Res 2008;314:3232–3244.
6. Wiendl H, Mitsdoerffer M, Schneider D, Melms A, Lochmuller H, Hohlfeld R, et al. Muscle fibres and cultured muscle cells express the B7.1/2-related inducible co-stimulatory molecule, ICOSL: implications for the pathogenesis of inflammatory myopathies. Brain 2003;126:1026–1035.
7. Wiendl H, Mitsdoerffer M, Schneider D, Chen LP, Lochmuller H, Melms A, et al. Human muscle cells express a B7-related molecule, B7-H1, with strong negative immune regulatory potential: a novel mechanism of counterbalancing the immune attack in idiopathic inflammatory myopathies. FASEB J 2003;17:1892–1894.
8. Kiefer R, Kieseierb BC, Stollc G, Hartung HP. The role of macrophages in immune-mediated damage to the peripheral nervous system. Prog Neurobiol 2001;64:109–127.
9. Kielian T, Haney A, Mayes PM, Garg S, Esen N. Toll-like receptor 2 modulates the proinflammatory milieu in *Staphylococcus aureus*-induced brain abscess. Infect Immun 2005;73: 7428–7435.
10. Nabi IR, Le PU. Caveolae/raft-dependent endocytosis. J Cell Biol 2003;161:673–677.
11. Parton RG. Caveolae and caveolins. Curr Opin Cell Biol 1996;8:542–548.
12. Parton RG, Way M, Zorzi N, Stang E. Caveolin-3 associates with developing T-tubules during muscle differentiation. J Cell Biol 1997;136:137–154.
13. Smith CL, Mittaud P, Prescott ED, Fuhrer C, Burden SJ. Src, Fyn, and Yes are not required for neuromuscular synapse formation but are necessary for stabilization of agrin-induced clusters of acetylcholine receptors. J Neurosci 2001; 21:3151–3160.
14. Cheng ZJ, Singh RD, Marks DL, Pagano RE. Membrane microdomains, caveolae, and caveolar endocytosis of sphingolipids. Mol Membr Biol 2006;23:101–110.
15. Waschbischa A, Meutha SG, Herrmanna AM, Wrobela B, Schwaba N, Lochmüllerb H, et al. Intercellular exchanges of membrane fragments (trogocytosis) between human muscle cells and immune cells: a potential mechanism for the modulation of muscular immune responses. J Neuroimmunol 2009;209:131–138.
16. Chen G, Wang X, Yu J, Varambally S, Yu J, Thomas DG, et al. Cutting edge: in vivo trogocytosis as a mechanism of double negative regulatory T cell–mediated antigen-specific suppression. J Immunol 2008;181:2271–2275.
17. Wiendl H. Fast track to becoming a regulatory T cell: "trogocytosis" of immune-tolerogenic HLA-G. Blood 2007;109: 1796–1797.
18. Salmond RJ, Filby A, Qureshi I, Caserta S, Zamoyska R. T-cell receptor proximal signaling via the Src-family kinases, Lck and Fyn, influences T-cell activation, differentiation, and tolerance. Immunol Rev 2009;228:9–22.
19. Macdonald JL, Pike LJ. A simplified method for the preparation of detergent-free lipid rafts. J Lipid Res 2005;46: 1061–1067.
20. Song KS, Li S, Okamoto T, Quilliam LA, Sargiacomo M, Lisanti MP. Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains. J Biol Chem 1996;271:9690–9697.
21. Tanowitz M, Sun HW, Mei L. Surgical denervation increases protein tyrosine phosphatase activity in skeletal muscle. Brain Res 1996;712:299–306.
22. Zhu D,XiongWC Mei L. Lipid rafts serve as a signaling platform for nicotinic acetylcholine receptor clustering. J Neurosci 2006;26:4841–4851.
23. Authier FJ, Chazaud B, Mhiri C, Eliezer-Vanerot MC, Poron F, Barlovatz-Meimon G, et al. Interleukin-1 expression in normal motor endplates and muscle fibers showing neurogenic changes. Acta Neuropathol 1997;94:272–279.
24. Liu K, Catalfamo M, Li Y, Henkart PA, Weng NP. IL-15 mimics T cell receptor crosslinking in the induction of cellular proliferation, gene expression, and cytotoxicity in $CD8^+$ memory T cells. Proc Natl Acad USA 2002;99: 6192–6197.
25. Gómez-Nicola D, Valle-Argos B, Suardíaz M, Taylor JS, Nieto-Sampedro M. Role of IL-15 in spinal cord and sciatic nerve after chronic constriction injury: regulation of macrophage and T-cell infiltration. J Neurochem 2008;107: 1741–1752.
26. Riond J, Elhmouzi J, Hudrisier D, Gairin JE. Capture of membrane components via trogocytosis occurs in vivo during both dendritic cells and target cells encounter by CD8+ T cells. Scand J Immunol 2007;66:441–450.
27. Liu M, Zhang D, Shao C, Liu J, Ding F, Gu X. Expression pattern of myostatin in gastrocnemius muscle of rats after sciatic nerve crush injury. Muscle Nerve 2007;35:649–656.
28. Nielsena S, Pedersena BK. Skeletal muscle as an immunogenic organ. Curr Opin Pharmacol 2008;8:346–351.
29. Calder PC, Yaqoob P. Lipid Rafts—composition, characterization, and controversies. J Nutr 2007;137:548–553.
30. Filipp D, Julius M. Lipid rafts: resolution of the "fyn problem"? Mol Immunol 2004;41:645–656.
31. Calautti E, Grossi M, Mammucari C, Aoyama Y, Pirro M, Ono Y, et al. Fyn tyrosine kinase is a downstream mediator of Rho/PRK2 function in keratinocyte cell–cell adhesion. J Cell Biol 2002;156:137–148.
32. Lockwood DNJ. Leprosy. Medicine 2005;7:26–29.

33. Vilquin JT, Guerette B, Kinoshita I, Roy B, Goulet M, Gravel C, et al. FK506 immunosuppression to control the immune reactions triggered by first-generation adenovirus-mediated gene transfer. Hum Gene Ther 1995;6:1391–1401.
34. Cuneo AA, Autieri MV. Expression and function of anti–inflammatory interleukins: the other side of the vascular response to injury. Curr Vasc Pharmacol 2009;7:267–276.
35. Carson DA. Unconventional T-cell activation by IL-15 in rheumatoid arthritis. Nature Medicine 1997;3:148–149.
36. Tang Q, Subudhi SK, Henriksen KJ, Long CG, Vives F, Bluestone JA. The Src family kinase Fyn mediates signals induced by TCR antagonists. J Immunol 2002;168:4480–4487.
37. Löwenberg M, Tuynman J, Bilderbeek J, Gaber T, Buttgereit F, van Deventer S, et al. Rapid immunosuppressive effects of glucocorticoids mediated through Lck and Fyn. Blood 2005;106:1703–1710.
38. Krajewska WM, Masowska I. Caveolins: structure and function in signal transduction. Cell Mol Biol Lett 2004;9:195–220.
39. Mermelstein CS, Martins ER, Portilho DM, Costa ML. Association between the muscle-specific proteins desmin and caveolin-3 in muscle cells. Cell Tissue Res 2007;327:343–351.

AQ1: Spell out "MBS" and add to "Abbreviations" footnote?

AQ2: Spell out "MES" and add to "Abbreviations"?

AQ3: Please supply two or three more "key words for indexing purposes."

AQ4: Is it "Zhao Hiu" or "Hiu Zhao"? That is, "Z. Hiu" or "H. Zhao"?

AQ5: Clarify wording in the sentence. Not clear as is.