



## Aging-associated exacerbation in fatty degeneration and infiltration after rotator cuff tear

Jonathan P. Gumucio, BS<sup>a,b</sup>, Michael A. Korn<sup>a,b</sup>, Anjali L. Saripalli<sup>a</sup>,  
Michael D. Flood, BS<sup>a</sup>, Anthony C. Phan, BS<sup>a</sup>, Stuart M. Roche, BS<sup>a</sup>, Evan B. Lynch, BS<sup>a,b</sup>,  
Dennis R. Claflin, PhD<sup>c,d</sup>, Asheesh Bedi, MD<sup>a</sup>, Christopher L. Mendias, PhD, ATC<sup>a,b,\*</sup>

<sup>a</sup>Department Orthopaedic Surgery, University of Michigan Medical School, Ann Arbor, MI, USA

<sup>b</sup>Department of Molecular & Integrative Physiology, University of Michigan Medical School, Ann Arbor, MI, USA

<sup>c</sup>Department of Biomedical Engineering, University of Michigan Medical School, Ann Arbor, MI, USA

<sup>d</sup>Section of Plastic Surgery, Department of Surgery, University of Michigan Medical School, Ann Arbor, MI, USA

**Background:** Rotator cuff tears are one of the most common musculoskeletal complaints and a substantial source of morbidity in elderly patients. Chronic cuff tears are associated with muscle atrophy and an infiltration of fat to the area, a condition known as “fatty degeneration.” To improve the treatment of cuff tears in elderly patients, a greater understanding of the changes in the contractile properties of muscle fibers and the molecular regulation of fatty degeneration is essential.

**Methods:** Using a full-thickness, massive supraspinatus and infraspinatus tear model in elderly rats, we measured fiber contractility and determined changes in fiber type distribution that develop 30 days after tear. We also measured the expression of messenger RNA and micro-RNA transcripts involved in muscle atrophy, lipid accumulation, and matrix synthesis. We hypothesized that a decrease in specific force of muscle fibers, an accumulation of type IIb fibers, and an upregulation in atrophic, fibrogenic, and inflammatory gene expression would occur in torn cuff muscles.

**Results:** Thirty days after the tear, we observed a reduction in muscle fiber force and an induction of RNA molecules that regulate atrophy, fibrosis, lipid accumulation, inflammation, and macrophage recruitment. A marked accumulation of advanced glycation end products and a significant accretion of macrophages in areas of fat accumulation were observed.

**Conclusions:** The extent of degenerative changes in old rats was greater than that observed in adults. In addition, we identified that the ectopic fat accumulation that occurs in chronic cuff tears does not occur by activation of canonical intramyocellular lipid storage and synthesis pathways.

**Level of evidence:** Basic Science Study, Molecular and Cell Biology, Animal Model.

© 2014 Journal of Shoulder and Elbow Surgery Board of Trustees.

**Keywords:** Fatty degeneration; rotator cuff; sarcopenia; atrophy; lipid droplets; macrophages

This study was approved by the University of Michigan Institutional Animal Care and Use Committee (approval number 10500).

\*Reprint requests: Christopher L. Mendias, PhD, ATC, Department of Orthopaedic Surgery, University of Michigan Medical School, 109 Zina Pitcher Pl, BSRB 2017, Ann Arbor, MI 48109-2200, USA.

E-mail address: [cmendias@umich.edu](mailto:cmendias@umich.edu) (C.L. Mendias).

Shoulder pain is a common complaint among the elderly population in the United States, with 25% of elderly individuals experiencing moderate to severe shoulder pain.<sup>25</sup> The most common cause of shoulder pain in elderly

patients is rotator cuff disease.<sup>25,56</sup> Elderly patients with rotator cuff disease often present with complaints of pain and a loss of strength and functional capacity that result in significant morbidity and disability.<sup>25,35</sup> Because full-thickness rotator cuff tears do not spontaneously heal, nonoperative treatments, such as physical therapy, anti-inflammatory medication, and activity modification, are directed at improving compensatory mechanisms to manage the functional deficits, but up to 74% of patients report persistent shoulder pain and dysfunction that continue to worsen with time.<sup>25</sup> Even with rotator cuff tears that are successfully repaired arthroscopically, elderly patients are more likely to have reduced shoulder function than younger patients.<sup>16</sup> The reasons behind the aging-associated decrease in rotator cuff regeneration are not well understood, and further insights in this area are critical to improve the treatment of elderly patients with rotator cuff tears.

After a rotator cuff tear develops, there is often an atrophy of muscle fibers, fibrosis, and an accumulation of fat within and around muscle fibers.<sup>13,15,21,42</sup> This set of pathophysiologic changes is often referred to as "fatty degeneration" and is frequently exacerbated in elderly patients with rotator cuff tears.<sup>25</sup> Compounding the decline in the regenerative capacity of muscles in elderly patients is sarcopenia, which is broadly defined as the loss of skeletal muscle mass and strength that occurs with aging.<sup>39</sup> There is an elevation in the proteolysis of muscle fiber contractile proteins, a reduction in contractile protein synthesis, and an increase in connective tissue accumulation that impairs the normal function of skeletal muscle in individuals with sarcopenia.<sup>18</sup> We previously showed that rotator cuff tears in adult rats result in reduced muscle fiber force production, conversion of type I and IIA fibers to type IIB, increased total intramyocellular and intermyocellular lipid content, and an accumulation of lipid-laden macrophages.<sup>17</sup> However, the effect of rotator cuff tear on muscle fiber force production and on lipid and macrophage accumulation in elderly rats was not known.

To gain greater insight into aging-associated changes in fatty degeneration, we used a well-established chronic experimental model of full-thickness rotator cuff tear<sup>17,27,45</sup> in elderly male rats. We evaluated changes in muscle fiber contractility, fiber type distribution and size, and the expression of messenger RNAs (mRNAs) and micro-RNAs (miRNAs) involved in lipid synthesis, lipid storage, extracellular matrix production, inflammation, and autophagy in old rats that underwent a full thickness supraspinatus and infraspinatus tear. We hypothesized that 30 days after a rotator cuff tear in old rats, there would be a reduction in muscle-specific fiber force production and an induction in the expression of mRNA and miRNA transcripts that regulate atrophy, inflammation, fibrosis, lipid accumulation, and autophagy.

## Materials and methods

### Animals

The study used 24-month-old male Sprague-Dawley rats ( $n = 6$ ) obtained from the National Institutes on Aging (NIA) Aged Rodent Colony (National Institutes of Health, Bethesda, MD, USA). This age was chosen because the maximum lifespan of rats is 32 to 36 months, and the aging-related decline in force generating and regenerative capacities of rats of this age closely reflect what is often observed in elderly humans.<sup>5,6</sup>

Full-thickness tears of the supraspinatus and infraspinatus tendons were created as previously described.<sup>17</sup> Briefly, a full-thickness tenectomy of the right supraspinatus and infraspinatus was performed using a deltoid-splitting transacromial approach to simulate a massive rotator cuff tear and to prevent scarring and healing of the detached tendons in the rodent model. The left shoulder served as a sham-operated control in which a deltoid splitting procedure was performed, but the supraspinatus and infraspinatus tendons were left intact. After induction of tear, rats were given postoperative buprenorphine (0.05 mg/kg) for analgesia.

After 30 days, rats were anesthetized with sodium pentobarbital (50 mg/kg), and the supraspinatus and infraspinatus muscles were harvested. The distal end of the muscle was free and showed no signs of lateral adhesion formation. Rats were then humanely euthanized with pentobarbital overdose. Supraspinatus muscles were weighed, finely minced, and prepared for biochemical measures. Infraspinatus muscles were weighed and separated at the midbelly for histologic analysis and single fiber contractility.

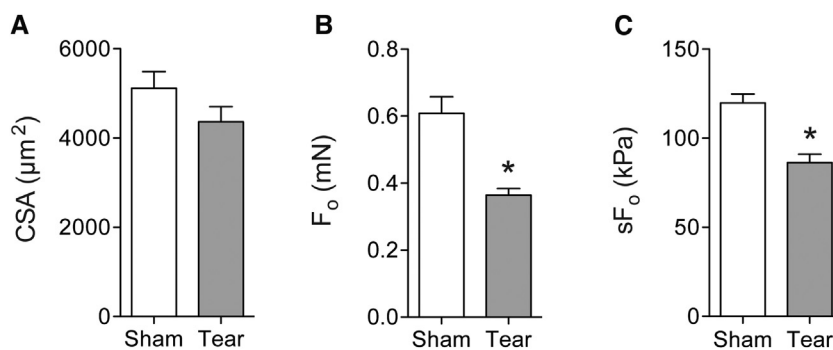
### Muscle fiber contractility

Contractile measurements of permeabilized muscle fibers of the proximal infraspinatus were performed as previously described.<sup>17,32</sup> Fibers were placed in a temperature-controlled chamber containing relaxing solution, and one end was secured to a servomotor (Model 322C, Aurora Scientific, Aurora, ON, Canada) and the other end to a force transducer (Model 403A, Aurora Scientific). Fiber length was adjusted to obtain a sarcomere length of 2.5  $\mu\text{m}$ . Fiber cross-sectional area (CSA) was calculated by analyzing 5 different diameters along the fiber from high-magnification images of the top and side views. Maximum isometric force ( $F_0$ ) was elicited by immersing the fiber in a high calcium solution, and specific force ( $sF_0$ ) was calculated by dividing  $F_0$  by CSA. Ten to 20 fast fibers were tested from each infraspinatus muscle.

### Histologic analysis

Distal infraspinatus segments were frozen in Tissue-Tek (Sakura, AJ Alphen aan den Rijn, The Netherlands) using isopentane cooled liquid nitrogen, and stored at  $-80^\circ\text{C}$  until needed. Muscles were cryosectioned at a thickness of 10  $\mu\text{m}$  and stained with Oil red O and hematoxylin or prepared for immunohistochemistry. Histologic analysis was conducted as previously described.<sup>17</sup>

The distribution of fiber types was determined by labeling sections with antibodies against type I, type IIA, and type IIB myosin heavy chain (all from Developmental Studies Hybridoma



**Figure 1** Permeabilized fiber contractility size and force. (A) Permeabilized fiber cross-sectional area (CSA), (B) maximum isometric force ( $F_0$ ), and (C) specific force ( $sF_0$ ) of sham and torn rotator cuff muscles. Values are mean  $\pm$  standard error SE for  $n = 6$  muscles from each group. \* $P < .05$  indicating significant difference from sham group.

Bank, University of Iowa, Iowa City, IA USA). Extracellular matrix (ECM) was identified using wheat germ agglutinin (WGA) lectin conjugated to AlexaFluor 488 (Invitrogen, Grand Island, NY, USA). Type IIX fibers were detected by the absence of signal.

To identify macrophages and areas of lipid accumulation, slides were incubated with antibodies against the macrophage-specific antigen F4/80 Abcam (Cambridge, MA, USA) and the lipid stain boron-dipyrromethene (BODIPY) 493/503 (Invitrogen). Primary antibodies were detected using highly cross-adsorbed secondary antibodies conjugated to AlexaFluor probes (Invitrogen, Carlsbad, CA, USA). Images were obtained using a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) with AxioCam cameras (Zeiss). Quantitative measurements were performed using ImageJ software (National Institutes of Health).

### Gene expression

Total RNA was isolated from supraspinatus muscles using a miRNeasy kit (Qiagen, Valencia, CA, USA), treated with DNase I (Qiagen), and the concentration of isolated RNA was measured on a NanoDrop (Thermo Fisher Scientific Inc, Waltham, MA, USA). RNA was reverse transcribed to complementary DNA using the RT<sup>2</sup> First strand kit (Qiagen) and amplified in a CFX96 real time thermal cycler (Bio-Rad, Hercules, CA, USA) using a custom array of primers for specific mRNA and miRNA species and an RT<sup>2</sup> SYBR Green qPCR mix (Qiagen). Expression of mRNA transcripts was normalized to the stable housekeeping gene  $\beta$ -actin, and miRNA transcripts to the stable housekeeping gene *Rnu6*.<sup>41</sup> A list of transcripts and RefSeq and miRBase information is presented in [Supplementary Tables I and II](#).

### Measurement of advanced glycation end product and N<sup>ε</sup>-(carboxymethyl) lysine adducts

Finely minced supraspinatus muscle (25 mg) was digested into peptides by placing samples in a 20 mg/mL solution of proteinase K (Invitrogen) for 24 hours at 55°C. After digestion, the proteinase was inactivated by heating samples to 95°C for 10 minutes. Enzyme-linked immunosorbent assays (Cell Biolabs, Inc, San Diego, CA, USA) for advanced glycation end product (AGEs) and N<sup>ε</sup>-(carboxymethyl) lysine (CML) protein adducts were performed according to manufacturer's directions. Enzyme-linked

immunosorbent assay plates were read in a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Measured AGE and CML adduct content was normalized to input wet muscle mass.

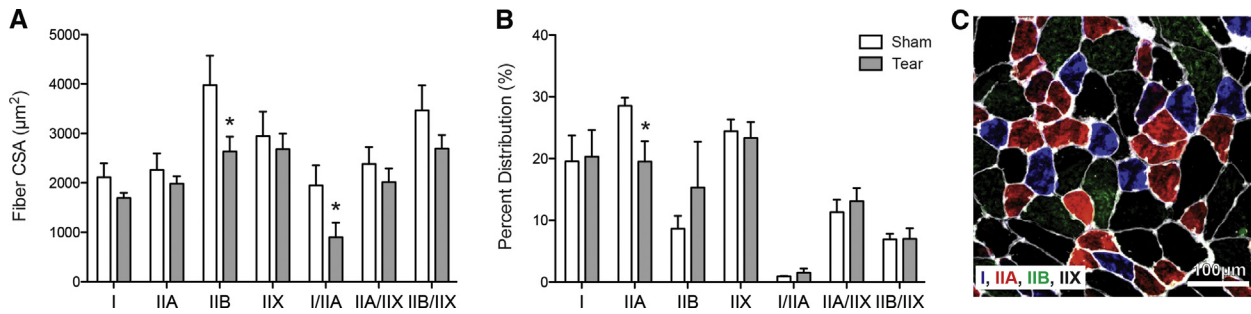
### Statistical analysis

Data are presented as mean  $\pm$  standard error. Differences between the sham and tear side were tested using *t* tests ( $\alpha = 0.05$ ). Prism 5.0b software (GraphPad, La Jolla, CA, USA) was used for calculations.

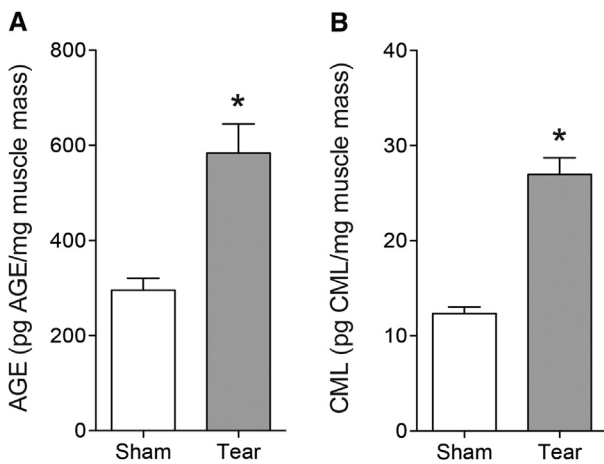
### Results

At 30 days after creation of the rotator cuff tear, there was a 40% decrease in the mass of supraspinatus muscles (sham: 386  $\pm$  37.6 mg; tear: 230  $\pm$  22.7 mg;  $P < .05$ ) and a 50% decrease in the mass of infraspinatus muscles (sham: 394  $\pm$  27.1 mg; tear: 198  $\pm$  12.7 mg,  $P < .05$ ). For single muscle fibers, rotator cuff tear had no effect on CSA ([Fig. 1, A](#)), but there was a 40% reduction in  $F_0$  and a 30% decrease in  $sF_0$  ([Fig. 1, B and C](#)). Infraspinatus muscles were then analyzed for myosin heavy-chain isoforms to determine fiber type size and distribution. The size of type IIB and hybrid type I/IIA muscle fibers ([Fig. 2, A](#)) and the amount of type IIA fibers ([Fig. 2, B](#)) was reduced in torn rotator cuff muscles. A representative image of muscle fiber typing is shown in [Figure 2, C](#). We next measured the levels of the oxidative stress markers AGE and CML and observed an increase in AGE and CML adducts in torn rotator cuff muscles ([Fig. 3](#)).

A marked increase in lipid accumulation within and around muscle fibers was observed ([Fig. 4, A and B](#)), and fatty macrophage deposition was also frequently noted in torn rotator cuff muscles ([Fig. 4, C and D](#)). Gene expression of torn muscles showed increases in the expression of peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) and CCAAT-enhancer-binding proteins (C/EBP)- $\alpha$  and a decrease in the expression of glycerol-3-phosphate acyltransferase (GPAT4, [Fig. 5, A](#)). There was no change in diglyceride acyltransferase



**Figure 2** Fiber type size and percent composition. (A) Cross-sectional area (CSA) and (B) percentage distribution of myosin heavy-chain (MHC) isoform of muscle fibers from sham and torn rotator cuff muscles. (C) Representative immunohistochemistry image demonstrates different fiber types: *white*, extracellular matrix (wheat germ agglutinin-lectin); *blue*, MHC I; *red*, MHC IIA; *green*, MHC IIB; *black*, MHC IIX; *blue-red*, hybrid MHC I/IIA; *red-black*, hybrid MHC IIA/IIX; *green-black*, hybrid MHC IIB/IIX. Values are mean  $\pm$  standard error for  $n = 6$  muscles from each group. \* $P < .05$  indicating significant difference from sham group.



**Figure 3** Oxidative stress markers. (A) Advanced glycation end product (AGE) protein adducts and (B) N<sup>ε</sup>-(carboxymethyl) lysine (CML) protein adducts in sham and torn rotator cuff muscles, normalized to wet muscle mass. Values are mean  $\pm$  standard error for  $n = 6$  muscles from each group. \* $P < .05$  indicating significant difference from sham group.

(DGAT1), the fatty acid transporter, fatty acid translocase (FAT)/cluster of differentiation 36 (CD36), or adipose triglyceride lipase (ATGL), a lipolytic enzyme (Fig. 5, A). For lipid storage transcripts, there was a dramatic increase in the expression of perilipin-1 (PLIN1), fat-specific protein of 27 kDa (FSP27), and phospholipase D1 (PLD1, Fig. 5, B), with no change in PLIN2 (ADRP), PLIN5 (OXPAT), the perilipin binding partner, CGI58, or the endoplasmic reticulum-associated lipid droplet binding proteins, fat storage-inducing transmembrane proteins 1 and 2 (FIT1 and FIT2; Fig. 5, B).

For transcripts involved in fibrosis and ECM maintenance, there were increases in collagen type I- $\alpha$ 2, FSP-1, matrix metalloproteinase (MMP)-14, MMP-2, platelet-derived growth factor receptor (PDGFR) $\alpha$ , tissue inhibitor of matrix metalloproteinase (TIMP)-1, TIMP-2, and tenomodulin (Fig. 5, C). There was no change in MMP-9 and a decrease in scleraxis expression (Fig. 5, C). Transcripts

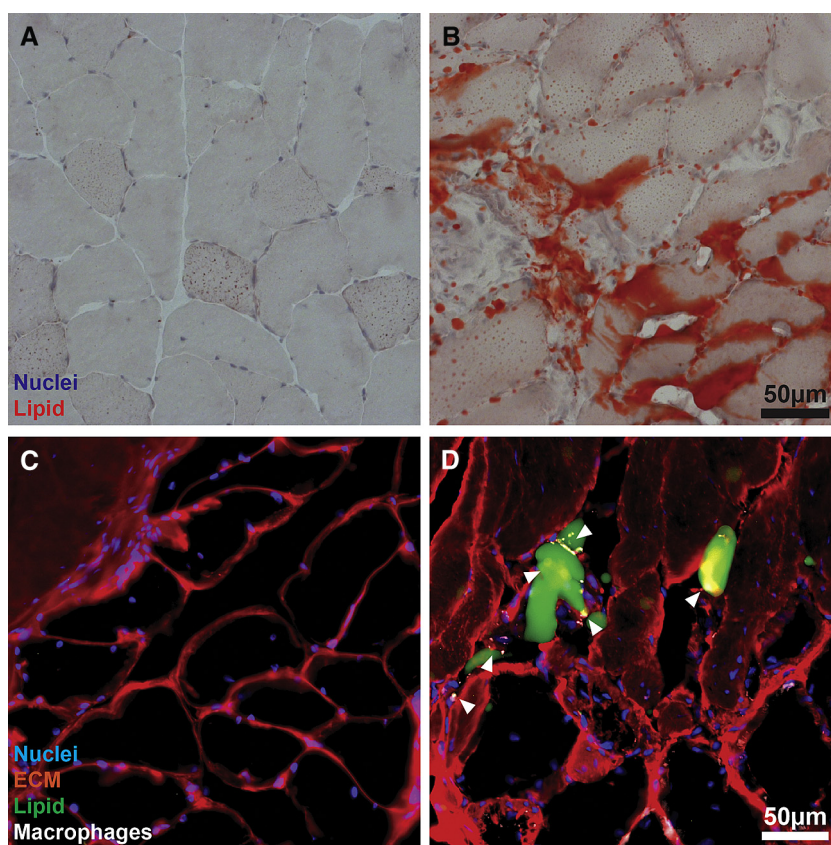
involved in autophagy, autophagy-related protein (Atg) 16L1, Atg5, Beclin-1, and Vps34 were unchanged after rotator cuff tear (Fig. 5, D). For atrophy and inflammation gene expression, there was a decrease in the E3 ubiquitin ligase, muscle RING-finger protein-1 (MuRF-1), but no change in Atrogin-1 (Fig. 5, E). Additionally, there were increases in 5-lipoxygenase (5-LOX), cyclooxygenase (COX)-2, interleukin (IL)-10, and IL-1 $\beta$ , but no change in COX-1 or IL-6 (Fig. 5, E).

For transcripts associated with macrophage accumulation, an increase in the pan macrophage marker F4/80 (Fig. 5, F) was observed. No change was detected for the expression of the proinflammatory M1 macrophage marker, C-C chemokine receptor type 7 (CCR7), but an increase was observed in 2 other M1 macrophage markers, CD11b and CD68 (Fig. 5, F). The anti-inflammatory M2 macrophage marker, CD168, was unchanged after tear (Fig. 5, F). Apolipoprotein E (ApoE), a transcript related to fatty macrophage formation, was increased after the tear, but there was no change in the primary fatty macrophage cholesterol storage transcript acetyl-co-A cholesterol acyl-transferase (ACAT-1) (Fig. 5, F).

We also evaluated the expression of several miRNAs. For miRNAs associated with muscle adaptation, miR-499, -133a, -29b, and -143 were downregulated, but miR-23a was upregulated in torn muscles (Fig. 6, A). There was no change in miR-133b, -126, -23b, -1, or -206 (Fig. 6, A). Lipid metabolism and adipogenesis-related transcript miR-130a was upregulated after tear, but no changes in miR-27a, -138, -103, -196a, and -107 were detected (Fig. 6, B). miRNAs associated with ECM synthesis, miR-21, -381, -221, -214, and -31, were upregulated after tear, but miR-338 and -222 (Fig. 6, C) did not change. All transcripts measured from the let-7 cluster were upregulated (Fig. 6, D).

## Discussion

Understanding the effect of full-thickness, massive rotator cuff tear on muscle fiber force production and lipid and

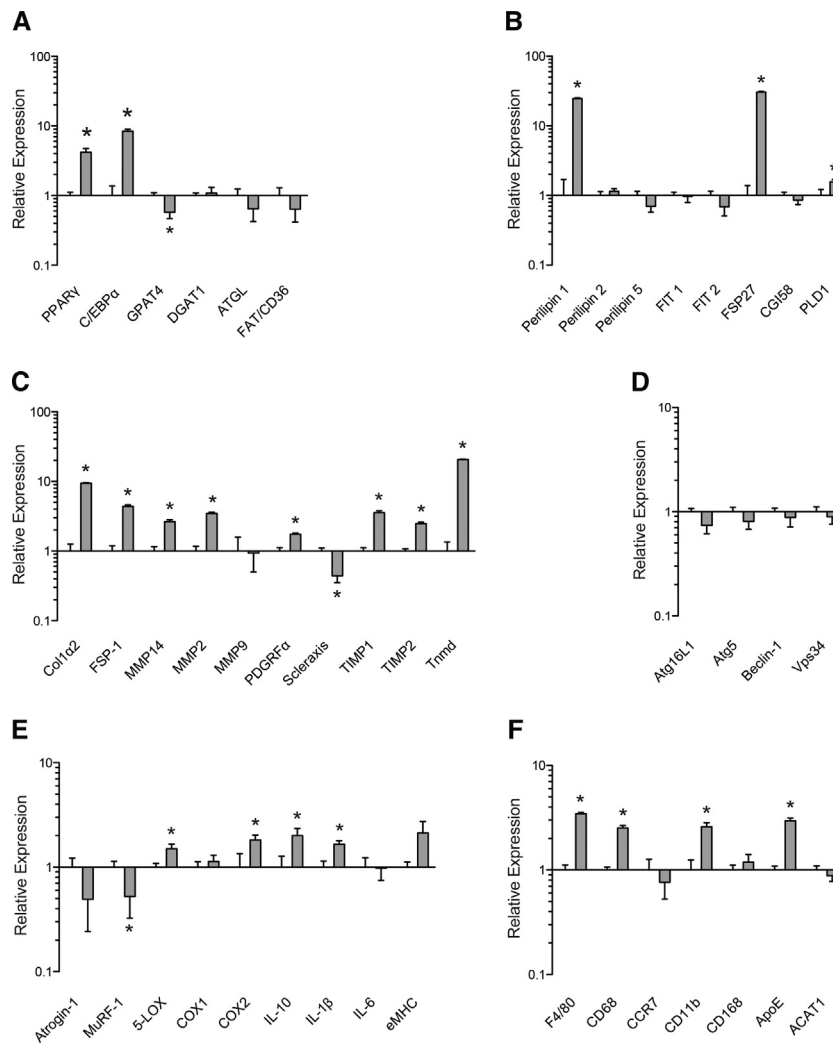


**Figure 4** Lipid and macrophage immunohistochemistry. Representative Oil red O sections from (A) sham and (B) torn rotator cuff muscles: *blue*, hematoxylin; *red*, Oil red O. Representative immunohistochemistry from (C) sham and (D) torn rotator cuff muscles: *blue*, nuclei (4',6-diamidino-2-phenylindole); *red*, extracellular matrix (wheat germ agglutinin lectin); *green*, lipid (boron-dipyrromethene); *white*, macrophages (F4/80). The *arrowheads* indicate areas of macrophage accumulation concomitant with lipid deposition.

macrophage accumulation is of critical importance to improve outcomes after rotator cuff repair surgery in elderly patients. This was the first study, to our knowledge, that measured changes in contractility, fiber type distribution, gene expression of various mRNAs and miRNAs, and markers of oxidative stress after a rotator cuff tear in an elderly rodent model. Rotator cuff tear reduced  $F_o$  and  $sF_o$ , indicating there was a functional decrease in the number of force-generating myofibrils within muscle fibers. The relative decrease in force production was more pronounced in elderly rats than in adult rats from our previous study.<sup>17</sup> Adult rats showed decreases in the CSA of type I, IIX, and IIA/IIX fibers after rotator cuff tear, but the old rats in the current study had decreases in the size of type IIB and I/IIA fibers. We also expected an increase in the number of type IIB fibers but did not see this in the elderly rats. Combined, these results suggest that elderly rats experience greater decreases in force production after tear and that the aging-related effect of rotator cuff tear is fiber-type specific.

Interleukins, prostaglandins, and leukotrienes have diverse roles in promoting inflammation, fibrosis, and atrophy after muscle injury.<sup>23,33,43,46</sup> IL-1 $\beta$ , a proinflammatory cytokine that generally promotes muscle atrophy,<sup>44</sup> was upregulated after tear. Also upregulated were COX-2,

which catalyzes the formation of proinflammatory prostaglandins that inhibit muscle stem cell proliferation and muscle regeneration,<sup>33,43</sup> and 5-LOX, which converts arachidonic acid into proinflammatory leukotrienes.<sup>46</sup> AGEs and CML are markers of chronic oxidative stress in muscle that promote local inflammation,<sup>1,8</sup> and elevations in AGE and CML protein adducts were observed in torn rotator cuff muscles of the old rats. For miRNAs associated with myogenesis and muscle regeneration,<sup>11</sup> miR-1, -133b, and -206 were unchanged after tear. However, miR-133a and miR-499, which promote differentiation of myoblasts,<sup>11</sup> were downregulated after tear. The let-7 cluster of miRNAs are important in regulating myogenesis,<sup>9</sup> and all let-7 transcripts were elevated in the torn rotator cuff muscles. Although induction in autophagy-related transcripts was observed in torn rotator cuff muscles from adult rats, no changes in the autophagy-related transcripts Atg16L1, Atg5, Beclin-1, or Vps34 were observed in the current study. MuRF-1 and atrogin-1 are the major E3 ubiquitin ligases in skeletal muscle and serve as important rate-limiting enzymes in protein degradation.<sup>40</sup> miR-23a is a miRNA that binds to the 3' untranslated region of MuRF-1 and atrogin-1 transcripts and is important in inhibiting MuRF-1 and atrogin-1 translation.<sup>49</sup> Unlike adult

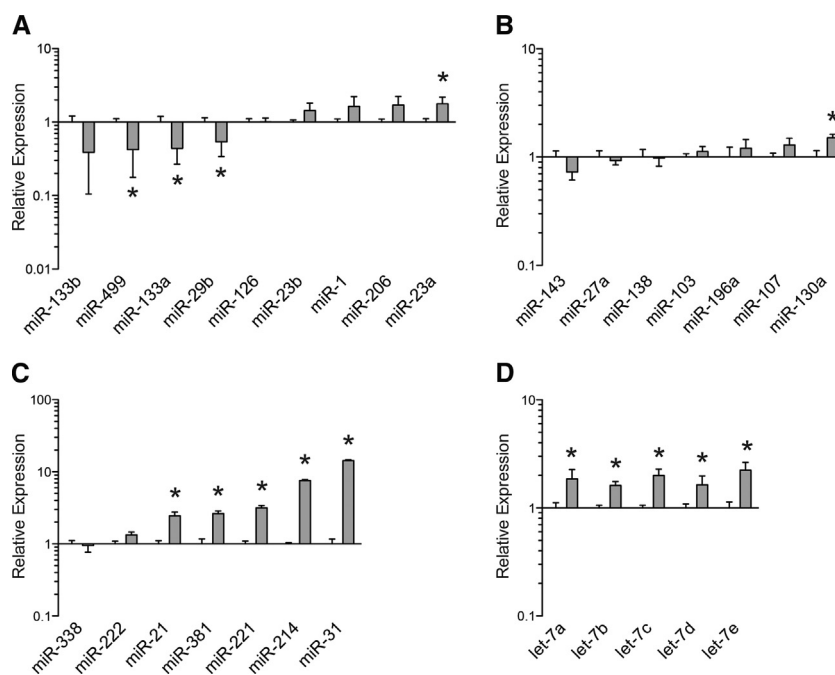


**Figure 5** Expression of genes (see Supplemental Table I) associated with (A) adipogenesis, (B) lipid storage, (C) extracellular matrix synthesis and fibrosis, (D) autophagy, (E) inflammation and atrophy, and (F) macrophage and fatty macrophage accumulation. Target gene expression was normalized to  $\beta$ -actin. Values are mean  $\pm$  standard error for  $n = 6$  muscles from each group. \* $P < .05$  indicating significant difference from sham group.

rats, which showed no change in MuRF-1, atrogin-1, or miR-23a after rotator cuff tear,<sup>17</sup> old rats had an upregulation in miR-23a expression and a corresponding downregulation of MuRF-1 expression. Although MuRF-1 and atrogin-1 have a well-described role in acute muscle atrophy after injury or immobilization, there are conflicting data regarding the role of ubiquitin-mediated proteolysis in chronic muscle atrophy.<sup>18,42</sup> The results from the current study suggest that although several proinflammatory markers are upregulated after prolonged rotator cuff tear, activation of autophagy pathways and sustained induction of MuRF-1 and atrogin-1 are not required for chronic atrophy in torn rotator cuff muscles of old rats.

Chronic rotator cuff tears often have substantial fibrosis,<sup>27,29</sup> and immunohistochemistry of torn muscles in the current study showed apparent gross accumulation of connective tissue. For gene expression, type 1 collagen expression was elevated in the torn rotator cuff muscles.

Elevations of miR-214, -21, -221, and -222 are associated with fibrosis in other tissues,<sup>3,19,36</sup> and although miR-222 expression was not different, miR-214, -21, and -221 expression was increased in torn rotator cuff muscles. The ECM degradation transcripts MMP-2 and MMP-14 were elevated, but increases in the MMP inhibitors TIMP-1 and TIMP-2 were observed. The fibroblast proliferation markers FSP-1, PDGFR- $\alpha$ , and tenomodulin were also elevated. Scleraxis, a bHLH transcription factor that promotes tendon fibroblast proliferation,<sup>28</sup> was downregulated, whereas miR-381, which is predicted to inhibit scleraxis expression,<sup>31</sup> was upregulated. The downregulation in scleraxis is likely due to the lack of passive tension in torn muscles, because loss of tensile load reduces scleraxis expression.<sup>28</sup> Overall, the general pattern of changes in mRNA and miRNA molecules that regulate ECM production was similar to what we observed in our previous study in adult rats,<sup>17</sup> although the tear-induced changes were



**Figure 6** Expression of micro-RNAs (*miR*) associated with (A) muscle adaptation, (B) lipid storage and synthesis, (C) extracellular matrix synthesis and fibrosis, and (D) the *let7* cluster. Target *miRNA* expression was normalized to *Rnu6*. Values are mean  $\pm$  standard error for  $n = 6$  muscles from each group. \*  $P < .05$  indicating significant difference from sham group.

generally of greater magnitude for old muscles than for adult muscles.

Macrophages are important in regulating muscle degeneration and regeneration after injury, and at least two well-studied populations of macrophages are thought to contribute to each of these processes. The classically activated M1 macrophages promote atrophy and inflammation, whereas the alternately activated M2 macrophages help to resolve inflammation and promote regeneration.<sup>37,47</sup> We previously demonstrated a marked accumulation of macrophages in torn rotator cuff muscles of adult rats,<sup>17</sup> although we did not evaluate markers of specific M1 and M2 subpopulations of macrophages. For old rats, the expression of the M1 transcripts CD11b and CD68 was increased, but no change in the M2 marker CD168 was observed. CD11b and CD68 levels increase immediately after most muscle injury and return to baseline levels at 4 to 6 days after injury.<sup>47</sup> The persistent elevation of M1 markers in a chronic rotator cuff tear in old rats suggests a prolonged activation of inflammatory and degenerative processes.

In atherosclerosis, a population of fatty macrophages, referred to as foam cells, uptake high levels of lipid and promote local tissue inflammation.<sup>7</sup> Activation of the transcription factor PPAR- $\gamma$  is important in the differentiation of fatty macrophages, which upon differentiation express high levels of ApoE and FSP27 at high levels.<sup>24,48</sup> In the current study, substantial elevations in PPAR- $\gamma$ , ApoE, and FSP27 were observed. In atherosclerosis, foam cells can exacerbate fatty lesions that worsen the severity of

the disorder<sup>7</sup> and may also contribute to chronic inflammation and degeneration in rotator cuff tears.

Although the deposition of large amounts of fat in and around muscle fibers is commonly observed after tear and a negative correlation exists between fat accumulation and clinical outcomes after rotator cuff tear,<sup>14</sup> the molecular etiology of fatty infiltration after tear is not well understood. PPAR- $\gamma$  and C/EBP $\alpha$  have diverse roles in lipid synthesis and storage and are commonly used as general markers of adipogenesis after rotator cuff tear. In agreement with previous studies,<sup>13,17,20,22</sup> both transcripts were upregulated in old rats after rotator cuff tear. The ontogeny of lipid accumulation and the specific cells and signaling pathways, which drive this process in rotator cuff tears, remain largely unknown. A slight aging-associated increase in adipocytes has been reported in a rat model of full-thickness rotator cuff tears.<sup>12</sup>

Marked increases in lipid accumulation have been observed in response to chronic endurance training and in patients with type 2 diabetes mellitus, a phenomenon referred to as the "athlete's paradox."<sup>51</sup> The accumulation of fat in these states can have both adaptive and maladaptive effects on muscle function.<sup>51</sup> To gain a greater understanding of the mechanisms behind the development of fatty degeneration in the rotator cuff, we evaluated the expression of several genes with well-known roles in canonical lipid synthesis, storage, and metabolism.

FAT/CD36 is the primary protein used to transport fatty acids into cells.<sup>51</sup> Once fatty acids have been taken up into the cytosol, they are stored in lipid droplets as triglycerides,

a process catalyzed by the enzymes GPAT4 and DGAT1.<sup>50</sup> Small lipid droplets, such as those typically found in endurance-trained or diabetic muscles, are coated by perilipin 2.<sup>50</sup> FSP27 promotes the formation of large lipid droplets,<sup>38</sup> and perilipin 1 preferentially coats droplets of this size,<sup>10</sup> like those observed in adipocytes and foam cells. When fatty acids are needed for energy metabolism, perilipin-5 coats lipid droplets and targets them to mitochondria.<sup>54</sup> On the lipid droplet surface, CGI-58 activates ATGL to directly liberate fatty acids from stored triglycerides,<sup>57</sup> which can then be put through the  $\beta$ -oxidation pathway for energy production. Although ectopic fat notably increased, there was an upregulation of miR-130a, which is downregulated during adipogenesis,<sup>55</sup> and no change in FAT/CD36 expression after tear. There was a downregulation of GPAT4 and unchanged DGAT1 expression after tear. Consistent with the large intramyocellular lipid droplets observed in torn rotator cuff muscles, there was a marked increase of perilipin 1 expression after rotator cuff tear but no change in perilipin 2 or perilipin 5. The expression of ATGL and its activator, CGI58, was not different after tear. No changes were noted in miR-103 and miR-107, which increase lipid metabolism and mitochondrial function.<sup>52</sup> In Oil red O images, the size of intramyocellular lipid droplets is substantially larger than what is typically observed for endurance athletes or type 2 diabetic individuals,<sup>2,34</sup> and for intermyocellular lipid, many of the fat deposits contained high levels of macrophages. Together, these results suggest the dramatic increase in fat that occurs with fatty degeneration is not being actively used for metabolic purposes and may be a pathologic response to rotator cuff tear.

This study has several limitations. Although the rat is an accepted model of rotator cuff tear because of its morphologic similarities to humans,<sup>45</sup> rats do not develop the severity of fatty degeneration seen in human patients. We chose a single time of 30 days to study an early chronic tear condition to allow for comparisons with our previous study but did not evaluate changes over multiple time points. A tenectomy model was also required to prevent spontaneous healing, which can occur in rodents but does not occur in humans. Although we made comparisons between observations in the current manuscript and our previous work,<sup>17</sup> we did not perform direct statistical analyses to evaluate the effect of age and tear on rotator cuff function and morphology.

For single-fiber measurements, we used type II fibers only and did not measure by specific myosin isoform. We did this due to the greater amount of type II fibers in the cuff, and  $sF_o$  does not change between fibers with different type II myosin isoforms.<sup>4</sup> We used the contralateral side as the control muscle, and although we did not notice any overt signs of muscle injury, the control muscle could possibly have experienced a small amount of muscle hypertrophy. We only measured changes in gene expression as opposed to protein level changes, and did

not measure changes specific to myosin isoform or cell type. Despite these limitations, this study provided insight into the cellular and molecular changes in rotator cuff muscles of old rats and identified many transcripts and cytokines that may be important for causing many of these changes.

An estimated 50% or greater of elderly patients have a full-thickness rotator cuff tear, and many of these patients are symptomatic with pain, weakness, and functional limitations with activities of daily living.<sup>25</sup> The prevalence and size of tears increase with age,<sup>25</sup> and treatment is limited. Nonsteroidal anti-inflammatory drugs (NSAIDs) are used to decrease pain and inflammation; however, use of NSAIDs can inhibit muscle regeneration<sup>33,43</sup> and may not be appropriate for elderly individuals with certain cardiovascular conditions.<sup>30</sup> Physical rehabilitation for elderly patients with rotator cuff tears follows a 3-phase system with coordinated pain control and exercises to improve strength and proprioception,<sup>53</sup> but despite these strategies, the incidence of fatty degeneration does not decrease.

The ectopic fat seen in rotator cuff tears is an attractive therapeutic target because the pathologic lipid that accumulates in muscle has been shown to increase inflammation and inhibit activation of the mammalian target of rapamycin (mTOR)/Akt pathway.<sup>37</sup> Lipid-mediated inhibition of mTOR/Akt signaling has been studied in the context of glucose transport and insulin sensitivity, but many of the mTOR/Akt signaling pathway components also regulate protein synthesis in muscle.<sup>40</sup> Liu et al<sup>26</sup> observed a decreased activation of Akt and mTOR in torn rotator cuff muscles, and the accumulation of lipid in torn rotator cuff muscle might inhibit protein synthesis pathways and reduce the recovery of muscle fiber force production. Further studies exploring the specific nature and regulation of lipid deposition in rotator cuff tears will be critical in development of treatment strategies to combat fatty degeneration and return patients to full function.

## Conclusions

Rotator cuff tears are common upper extremity injuries in the elderly, and treatment options to reverse fatty degeneration are limited. Although the elderly have high rates of rotator cuff tears, most of the animal studies that have evaluated the biology of rotator cuff tears have used juvenile or adult animals. By using an elderly rat model, the present study evaluated the changes in muscle fiber contractility, fiber type distribution, gene expression, and AGE accumulation that occurred after inducing a rotator cuff tear. We observed a reduction in muscle fiber force production, an induction of fibrogenic, mRNA, and miRNA molecules, and a significant accumulation of macrophages in areas of fat accumulation.



In general, the magnitude of degenerative changes in old rats was greater than that observed in adult animals. We also identified that the ectopic fat accumulation that occurs in chronic rotator cuff tears in elderly rats does not occur by activation of canonical intramyocellular lipid storage and synthesis pathways.

## Disclaimer

This work was supported by National Institutes of Health grants AG-024824 and GM-008322.

The authors, their immediate families, and any research foundations with which they are affiliated have not received any financial payments or other benefits from any commercial entity related to the subject of this article.

## Supplementary data

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.jse.2013.04.011>.

## References

- Ahmed KA, Muniandy S, Ismail IS. N(epsilon)-(carboxymethyl)lysine and coronary atherosclerosis-associated low density lipoprotein abnormalities in type 2 diabetes: current status. *J Clin Biochem Nutr* 2009;44:14-27. <http://dx.doi.org/10.3164/jcbn.08-190>
- Amati F, Dubé JJ, Alvarez-Carnero E, Edreira MM, Chomentowski P, Coen PM, et al. Skeletal muscle triglycerides, diacylglycerols, and ceramides in insulin resistance: another paradox in endurance-trained athletes? *Diabetes* 2011;60:2588-97. <http://dx.doi.org/10.2337/db10-1221>
- Bauersachs J. Regulation of myocardial fibrosis by microRNAs. *J Cardiovasc Pharmacol* 2010;56:454-9. <http://dx.doi.org/10.1097/FJC.0b013e3181ee81df>
- Bottinelli R, Canepari M, Pellegrino MA, Reggiani C. Force-velocity properties of human skeletal muscle fibres: myosin heavy chain isoform and temperature dependence. *J Physiol (Lond)* 1996;495:573-86.
- Carlson BM, Dedkov EI, Borisov AB, Faulkner JA. Skeletal muscle regeneration in very old rats. *J Gerontol A Biol Sci Med Sci* 2001;56: B224-33.
- Carlson BM, Faulkner JA. Muscle transplantation between young and old rats: age of host determines recovery. *Am J Physiol* 1989;256: C1262-6.
- Cascieri MA. The potential for novel anti-inflammatory therapies for coronary artery disease. *Nat Rev Drug Discov* 2002;1:122-30. <http://dx.doi.org/10.1038/nrd723>
- Cassese A, Esposito I, Fiory F, Barbagallo APM, Paturzo F, Mirra P, et al. In skeletal muscle advanced glycation end products (AGEs) inhibit insulin action and induce the formation of multimolecular complexes including the receptor for AGEs. *J Biol Chem* 2008;283: 36088-99. <http://dx.doi.org/10.1074/jbc.M801698200>
- Crippa S, Cassano M, Sampaolesi M. Role of miRNAs in muscle stem cell biology: proliferation, differentiation and death. *Curr Pharm Des* 2012;18:1718-29. <http://dx.doi.org/10.2174/138161212799859620>
- Ducharme NA, Bickel PE. Lipid droplets in lipogenesis and lipolysis. *Endocrinology* 2008;149:942-9. <http://dx.doi.org/10.1210/en.2007-1713>
- Eisenberg I, Alexander MS, Kunkel LM. miRNAs in normal and diseased skeletal muscle. *J Cell Molec Med* 2009;13:2-11. <http://dx.doi.org/10.1111/j.1582-4934.2008.00524.x>
- Farshad M, Würzler-Hauri C, Kohler T. Effect of age on fatty infiltration of supraspinatus muscle after experimental tendon release in rats. *BMC Res Notes* 2011;12:530. <http://dx.doi.org/10.1186/1756-0500-4-530>
- Frey E, Regenfelder F, Sussmann P, Zumstein M, Gerber C, Born W, et al. Adipogenic and myogenic gene expression in rotator cuff muscle of the sheep after tendon tear. *J Orthop Res* 2009;27:504-9. <http://dx.doi.org/10.1002/jor.20695>
- Gladstone JN, Bishop JY, Lo IKY, Flatow EL. Fatty infiltration and atrophy of the rotator cuff do not improve after rotator cuff repair and correlate with poor functional outcome. *Am J Sports Med* 2007;35: 719-28. <http://dx.doi.org/10.1177/0363546506297539>
- Goutallier D, Postel JM, Bernageau J, Lavau L, Voisin MC. Fatty muscle degeneration in cuff ruptures. Pre- and postoperative evaluation by CT scan. *Clin Orthop Relat Res* 1994;304:78-83.
- Gulotta LV, Nho SJ, Dodson CC, Adler RS, Altchek DW, Macgillivray JD, et al. Prospective evaluation of arthroscopic rotator cuff repairs at 5 years: part II—prognostic factors for clinical and radiographic outcomes. *J Shoulder Elbow Surg* 2011;20:941-6. <http://dx.doi.org/10.1016/j.jse.2011.03.028>
- Gumucio JP, Davis ME, Bradley JR, Stafford PL, Schiffman CJ, Lynch EB, et al. Rotator cuff tear reduces muscle fiber specific force production and induces macrophage accumulation and autophagy. *J Orthop Res* 2012;30:1963-70. <http://dx.doi.org/10.1002/jor.22168>
- Gumucio JP, Mendias CL. Atrogin-1, MuRF-1, and sarcopenia. *Endocrine* 2013;43:12-21. <http://dx.doi.org/10.1007/s12020-012-9751-7>
- Iizuka M, Ogawa T, Enomoto M, Motoyama H, Yoshizato K, Ikeda K, et al. Induction of microRNA-214-5p in human and rodent liver fibrosis. *Fibrogenesis Tissue Repair* 2012;5:12. <http://dx.doi.org/10.1186/1755-1536-5-12>
- Itoigawa Y, Kishimoto KN, Sano H, Kaneko K, Itoi E. Molecular mechanism of fatty degeneration in rotator cuff muscle with tendon rupture. *J Orthop Res* 2011;29:861-6. <http://dx.doi.org/10.1002/jor.21317>
- Jost B, Pfirrmann CW, Gerber C, Switzerland Z. Clinical outcome after structural failure of rotator cuff repairs. *J Bone Joint Surg Am* 2000;82:304-14.
- Kim HM, Galatz LM, Lim C, Havlioglu N, Thomopoulos S. The effect of tear size and nerve injury on rotator cuff muscle fatty degeneration in a rodent animal model. *J Shoulder Elbow Surg* 2011;. <http://dx.doi.org/10.1016/j.jse.2011.05.004>
- Koh TJ, Pizza FX. Do inflammatory cells influence skeletal muscle hypertrophy? *Front Biosci (Elite Ed)* 2009;1:60-71. doi:<http://dx.doi.org/10.2741/e7>
- Li H, Song Y, Li F, Zhang L, Gu Y, Zhang L, et al. Identification of lipid droplet-associated proteins in the formation of macrophage-derived foam cells using microarrays. *Int J Mol Med* 2010;26:231-9. <http://dx.doi.org/10.3892/ijmm.00000457>
- Lin JC, Weintraub N, Aragaki DR. Nonsurgical treatment for rotator cuff injury in the elderly. *J Am Med Dir Assoc* 2008;9:626-32. <http://dx.doi.org/10.1016/j.jamda.2008.05.003>
- Liu X, Joshi SK, Samagh SP, Dang Y-X, Laron D, Lovett DH, et al. Evaluation of Akt/mTOR activity in muscle atrophy after rotator cuff tears in a rat model. *J Orthop Res* 2012;30:1440-6. <http://dx.doi.org/10.1002/jor.22096>

27. Liu X, Manzano G, Kim HT, Feeley BT. A rat model of massive rotator cuff tears. *J Orthop Res* 2011;29:588-95. <http://dx.doi.org/10.1002/jor.21266>
28. Maeda T, Sakabe T, Sunaga A, Sakai K, Rivera AL, Keene DR, et al. Conversion of mechanical force into TGF- $\beta$ -mediated biochemical signals. *Curr Biol* 2011;21:933-41. <http://dx.doi.org/10.1016/j.cub.2011.04.007>
29. Mannava S, Plate JF, Whitlock PW, Callahan MF, Seyler TM, Koman LA, et al. Evaluation of in vivo rotator cuff muscle function after acute and chronic detachment of the supraspinatus tendon: an experimental study in an animal model. *J Bone Joint Surg Am* 2011; 93:1702-11. <http://dx.doi.org/10.2106/JBJS.J.00184>
30. McGettigan P, Henry D. Cardiovascular risk and inhibition of cyclooxygenase: a systematic review of the observational studies of selective and nonselective inhibitors of cyclooxygenase 2. *JAMA* 2006;296: 1633-44. <http://dx.doi.org/10.1001/jama.296.13.jrv60011>
31. Mendias CL, Gumucio JP, Bakhrin KI, Lynch EB, Brooks SV. Physiological loading of tendons induces scleraxis expression in epitenon fibroblasts. *J Orthop Res* 2012;30:606-12. <http://dx.doi.org/10.1002/jor.21550>
32. Mendias CL, Kayupov E, Bradley JR, Brooks SV, Claffin DR. Decreased specific force and power production of muscle fibers from myostatin-deficient mice are associated with a suppression of protein degradation. *J Appl Physiol* 2011;111:185-91. <http://dx.doi.org/10.1152/jappphysiol.00126.2011>
33. Mendias CL, Tatsumi R, Allen RE. Role of cyclooxygenase-1 and -2 in satellite cell proliferation, differentiation, and fusion. *Muscle Nerve* 2004;30:497-500. <http://dx.doi.org/10.1002/mus.20102>
34. Minnaard R, Schrauwen P, Schaart G, Jorgensen JA, Lenaers E, Mensink M, et al. Adipocyte differentiation-related protein and OXPAT in rat and human skeletal muscle: involvement in lipid accumulation and type 2 diabetes mellitus. *J Clin Endocrinol Metab* 2009;94:4077-85. <http://dx.doi.org/10.1210/jc.2009-0352>
35. Nho SJ, Yadav H, Shindle MK, Macgillivray JD. Rotator cuff degeneration: etiology and pathogenesis. *Am J Sports Med* 2008;36: 987-93. <http://dx.doi.org/10.1177/0363546508317344>
36. Ogawa T, Enomoto M, Fujii H, Sekiya Y, Yoshizato K, Ikeda K, et al. MicroRNA-221/222 upregulation indicates the activation of stellate cells and the progression of liver fibrosis. *Gut* 2012;61:1600-9. <http://dx.doi.org/10.1136/gutjnl-2011-300717>
37. Olefsky JM, Glass CK. Macrophages, inflammation, and insulin resistance. *Annu Rev Physiol* 2010;72:219-46. <http://dx.doi.org/10.1146/annurev-physiol-021909-135846>
38. Puri V, Ranjit S, Konda S, Nicoloso SMC, Straubhaar J, Chawla A, et al. Cidea is associated with lipid droplets and insulin sensitivity in humans. *Proc Natl Acad Sci U S A* 2008;105:7833-8. <http://dx.doi.org/10.1073/pnas.0802063105>
39. Rosenberg IH. Summary comments. *Am. J. Clin. Nutr* 1989;50:1231-3.
40. Sandri M. Signaling in muscle atrophy and hypertrophy. *Physiology (Bethesda, Md)* 2008;23:160-70. <http://dx.doi.org/10.1152/physiol.00041.2007>
41. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 2008;3:1101-8. <http://dx.doi.org/10.1038/nprot.2008.73>
42. Schmutz S, Fuchs T, Regenfelder F, Steinmann P, Zumstein M, Fuchs B. Expression of atrophy mRNA relates to tendon tear size in supraspinatus muscle. *Clin Orthop Relat Res* 2009;467:457-64. <http://dx.doi.org/10.1007/s11999-008-0565-0>
43. Shen W, Li Y, Tang Y, Cummins J, Huard J. NS-398, a cyclooxygenase-2-specific inhibitor, delays skeletal muscle healing by decreasing regeneration and promoting fibrosis. *Am J Pathol* 2005; 167:1105-17. [http://dx.doi.org/10.1016/S0002-9440\(10\)61199-6](http://dx.doi.org/10.1016/S0002-9440(10)61199-6)
44. Smith C, Kruger MJ, Smith RM, Myburgh KH. The inflammatory response to skeletal muscle injury: illuminating complexities. *Sports Med* 2008;38:947-69. <http://dx.doi.org/10.2165/00007256-200838110-00005>
45. Soslowky LJ, Carpenter JE, DeBano CM, Banerji I, Moalli MR. Development and use of an animal model for investigations on rotator cuff disease. *J Shoulder Elbow Surg* 1996;5:383-92.
46. Stables MJ, Gilroy DW. Old and new generation lipid mediators in acute inflammation and resolution. *Prog Lipid Res* 2011;50:35-51. <http://dx.doi.org/10.1016/j.plipres.2010.07.005>
47. Tidball JG, Villalta SA. Regulatory interactions between muscle and the immune system during muscle regeneration. *Am J Physiol Regul Integr Comp Physiol* 2010;298:R1173-87. <http://dx.doi.org/10.1152/ajpregu.00735.2009>
48. Tontonoz P, Nagy L, Alvarez JG, Thomazy VA, Evans RM. PPAR- $\gamma$  promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell* 1998;93:241-52.
49. Wada S, Kato Y, Okutsu M, Miyaki S, Suzuki K, Yan Z, et al. Translational suppression of atrophic regulators by miR-23a integrates resistance to skeletal muscle atrophy. *J Biol Chem* 2011;286:38456-65. <http://dx.doi.org/10.1074/jbc.M111.271270>
50. Watt MJ. Storing up trouble: does accumulation of intramyocellular triglyceride protect skeletal muscle from insulin resistance? *Clin Exp Pharmacol Physiol* 2009;36:5-11. <http://dx.doi.org/10.1111/j.1440-1681.2008.05075.x>
51. Watt MJ, Hoy AJ. Lipid metabolism in skeletal muscle: generation of adaptive and maladaptive intracellular signals for cellular function. *Am J Physiol Endocrinol Metab* 2012;302:E1315-28. <http://dx.doi.org/10.1152/ajpendo.00561.2011>
52. Wilfred BR, Wang WX, Nelson PT. Energizing miRNA research: a review of the role of miRNAs in lipid metabolism, with a prediction that miR-103/107 regulates human metabolic pathways. *Mol Genet Metab* 2007;91:209-17. <http://dx.doi.org/10.1016/j.ymgme.2007.03.011>
53. Wirth MA, Basamania C, Rockwood CA Jr. Nonoperative management of full-thickness tears of the rotator cuff. *Orthop Clin North Am* 1997;28:59-67.
54. Wolins NE, Quaynor BK, Skinner JR, Tzekov A, Croce MA, Gropler MC, et al. OXPAT/PAT-1 is a PPAR-induced lipid droplet protein that promotes fatty acid utilization. *Diabetes* 2006;55:3418-28. <http://dx.doi.org/10.2337/db06-0399>
55. Xie H, Lim B, Lodish HF. MicroRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity. *Diabetes* 2009;58:1050-7. <http://dx.doi.org/10.2337/db08-1299>
56. Yamaguchi K, Ditsios K, Middleton WD, Hildebolt CF, Galatz LM, Teefey SA. The demographic and morphological features of rotator cuff disease. A comparison of asymptomatic and symptomatic shoulders. *J Bone Joint Surg Am* 2006;88:1699-704. <http://dx.doi.org/10.2106/JBJS.E.00835>
57. Zechner R, Kienesberger PC, Haemmerle G, Zimmermann R, Lass A. Adipose triglyceride lipase and the lipolytic catabolism of cellular fat stores. *J Lipid Res* 2009;50:3-21. <http://dx.doi.org/10.1194/jlr.R800031-JLR200>