Rotator Cuff Tear Reduces Muscle Fiber Specific Force Production and Induces Macrophage Accumulation and Autophagy

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ABSTRACT: Full-thickness tears to the rotator cuff can cause severe pain and disability. Untreated tears progress in size and are associated with muscle atrophy and an infiltration of fat to the area, a condition known as "fatty degeneration." To improve the treatment of rotator cuff tears, a greater understanding of the changes in the contractile properties of muscle fibers and the molecular regulation of fatty degeneration is essential. Using a rat model of rotator cuff injury, we measured the force generating capacity of individual muscle fibers and determined changes in muscle fiber type distribution that develop after a full thickness rotator cuff tear. We also measured the expression of mRNA and miRNA transcripts involved in muscle atrophy, lipid accumulation, and matrix synthesis. We hypothesized that a decrease in specific force of rotator cuff muscle fibers, an accumulation of type IIb fibers, and an upregulation in fibrogenic, adipogenic, and inflammatory gene expression occur in torn rotator cuff muscles. Thirty days following rotator cuff tear, we observed a reduction in muscle fiber force production, an induction of fibrogenic, adipogenic, and autophagocytic mRNA and miRNA molecules, and a dramatic accumulation of macrophages in areas of fat accumulation. J Orthop Res 30:1963–1970, 2012

Keywords: rotator cuff; muscle fiber contractility; fatty degeneration; autophagy

Large or massive tears of the rotator cuff can cause severe pain, substantially limit mobility, and negatively impact quality of life.[1] An estimated 50% of patients still have symptoms at 6 months, and 40% continue to have symptoms 1 year after surgery,[2] demonstrating that surgical repair is often unable to fully restore the normal function and strength of the involved muscles. Common pathophysiological changes that occur in torn rotator cuff muscles are atrophy of muscle fibers, fibrosis, and an accumulation of fat in the muscle extracellular matrix (ECM),[3] changes collectively referred to as "fatty degeneration." Despite improvements in surgical technique that result in biomechanically strong repairs, studies of rotator cuff muscles using MRI or CT scans often fail to demonstrate a reduction in fatty degeneration following repair.[4] The molecular etiology of fatty degeneration following rotator cuff tears is not fully understood, and gaining greater insight into the mechanisms that lead to the development of atrophy, fibrosis, and fat accumulation will likely improve patient recovery.

The adaptation of muscle to chronic injuries is a multifactorial process involving many cell types and several signaling pathways. A loss of muscle strength frequently occurs after rotator cuff tear, and even after a successful and structurally intact repair of the tear, strength often times does not fully return to pre-injury levels.[5] Satellite cells are a resident population of muscle stem cells that help regenerate muscle after injury.[6] At several time points after a tear, an increase occurs in the expression of several myogenic transcription factors that regulate satellite cell activity,[7–9] suggesting that satellite cells are actively involved in remodeling injured muscles. Adipocytes, identified by their signet ring appearance using H&E staining, accumulate in torn rotator cuff muscles[39] and are thought to play a role in structural reorganization of torn muscles.[10] While satellite cells have a well established role in muscle remodeling, the full extent of the physiological function of adipocytes in regeneration and remodeling remains unknown.

Macrophages are important regulators of muscle degeneration and regeneration,[11] and accumulate substantial amounts of intracellular lipid in atherosclerotic plaques.[12] Macrophages also play an important role in regulating autophagy in fatty atherosclerotic plaques. Autophagy is the process whereby cells break down organelles and other components of the cytosol in order to adapt to new environmental stressors, and an emerging body of literature has indicated that autophagy plays an important role in the remodeling of muscle cells to injury or disease.[13] However, the roles that macrophages and autophagocytic pathways play in the regeneration and remodeling of rotator cuff tears are undefined.

MicroRNA (miRNA) molecules are small non-coding RNAs that participate in post-transcriptional regulation of many protein-coding mRNA molecules, often by binding to the 3′ UTR of a mRNA and targeting the mRNA for rapid degradation.[14] Several miRNA

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molecules play a role in the mechanical adaptation of skeletal muscle,[15] adipogenesis,[16] and fibrosis.[17] However, the expression of miRNA molecules that regulate muscle atrophy, fibrosis, and lipid accumulation have not been previously investigated in the context of rotator cuff tears.

As the mechanisms that regulate muscle strength and fatty degeneration in the rotator cuff have yet to be fully elucidated, we utilized a well-established rat model of a full-thickness rotator cuff tear[18,19] to investigate the effects of fatty degeneration on the function and molecular adaptations in skeletal muscle fibers. We evaluated changes in canonical lipogenesis pathways and the role of macrophages and autophagy using immunohistochemistry and gene and miRNA expression analysis. We hypothesized that, 30 days following a rotator cuff tear, there would be a reduction in muscle specific fiber force production, an accumulation of macrophages in areas of fat deposition, and an induction in the expression of mRNA and miRNA transcripts that regulate atrophy, inflammation, fibrosis, lipid accumulation, and autophagy.

METHODS

Animals and Surgical Procedure

This study was approved by the University of Michigan IACUC. Six-month-old male Sprague Dawley rats ($N = 6$) were used. Rats were anesthetized with 2% isoflurane, and the skin above the shoulder was shaved and scrubbed with povidone iodine. To create a full thickness supraspinatus and infraspinatus tear and prevent tendon reattachment, the right shoulders underwent a supraspinatus and infraspinatus tenectomy[19] after visualizing the tendons through a field established by a deltoid splitting incision and transacromial approach.[20] The left shoulder underwent a sham surgical operation in which a transacromial approach was performed, but the tendons were left intact. The deltoid was closed using absorbable 3-0 chromic gut suture (J&J, New Brunswick, NJ), and the skin and fascia closed using 5-0 nylon (J&J) and GLUture (Abbott, Abbott Park, IL). Subcutaneous buprenorphine (0.05 mg/kg) was administered for analgesia during post-operative recovery. Ad libitum weightbearing and cage activity were allowed, and rats were monitored for signs of distress or infection. Thirty days after later, rats were anesthetized with pentobarbital (50 mg/kg), the supraspinatus and infraspinatus muscles were removed and their masses were recorded. The rats were euthanized by anesthetic overdose and induction of bilateral pneumothorax. Supraspinatus muscles were finely minced and prepared for RNA isolation. The distal halves of the infraspinatus muscles were used for histology, and the proximal halves were used for muscle fiber contractility.

Muscle Fiber Contractility

Single fiber contractility experiments were performed as previously described[2122]. Briefly, fiber bundles ~5 mm in length and 0.5 mm in diameter were dissected from the proximal half of infraspinatus muscles, placed in skinning solution for 30 min and then in storage solution for 16 h at 4°C, followed by storage at −80°C. On the day of an experiment, bundles were thawed on ice, and single fibers were pulled from bundles using fine mirror-finished forceps. Fibers were then placed in a chamber containing relaxing solution and secured at one end to a servomotor (Aurora Scientific, Aurora, ON, Canada, Model 322C) and the other end to a force transducer (Aurora Model 403A) using two ties of 10-0 monofilament nylon suture at each fiber end. Fiber length was adjusted to obtain a sarcomere length of 2.5 μm using a laser diffraction measurement system; fiber length (L_f) was then measured. The average fiber cross-sectional area (CSA) was calculated assuming an elliptical cross-section, with diameters obtained at five positions along the fiber from high-magnification images of the top and the side views. Maximum isometric force (F_o) was elicited by immersing the fiber in a high-calcium concentration solution. Specific force (sF_o) was calculated by dividing F_o by CSA. Ten to 20 fast fibers were tested from each infraspinatus muscle. Fibers were categorized as fast or slow by examining their force response to rapid, constant-velocity shortening.[23]

Histology

The distal half of the infraspinatus was snap frozen in Tissue-Tek (Sakura) using isopentane cooled in liquid nitrogen, and stored at −80°C until use. Muscles were sectioned at a thickness of 10 μm, and stained with Oil Red O and hematoxylin or prepared for immunohistochemistry (IHC). To determine fiber type, sections were permeabilized in 0.2% Triton X-100 in PBS, blocked in 5% goat serum, and incubated with monoclonal antibodies (Developmental Studies Hybridoma Bank) against type I myosin heavy chain (BA-D5, mouse IgG2b), type IIA myosin heavy chain (SC-71, mouse IgG1) and type IIB myosin heavy chain (BF-F3, mouse IgM). Primary antibodies were detected with highly cross-adsorbed secondary antibodies from goat that were specific to each mouse Ig isoform and conjugated to AlexaFluor probes (Invitrogen, Grand Island, NY). Type IIX muscle fibers were detected by the absence of signal. Wheat germ agglutin (WGA) lectin conjugated to AlexaFluor 488 (Invitrogen) was used to identify extracellular matrix. To identify lipid deposition and macrophages, sections were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 in PBS, blocked in 5% goat serum, and incubated with biotinylated antibodies against the macrophage-specific antigen F4/80 (AbCam, Cambridge, MA) and BODIPY 493/503 to identify lipid (Invitrogen), DAPI (Sigma, St. Louis, MO) to identify nuclei, and WGA-Texas Red (Invitrogen) to identify extracellular matrix. Streptavidin conjugated to AlexaFluor 647 (Invitrogen) was used to detect F4/80 antibodies. Sections were visualized using a Zeiss Axioplan 2 microscope equipped with AxioCam cameras. For quantitative histomorphometry, measurements were performed on four fields/section taken from the superficial, deep, cephalad, and caudal muscle regions, and quantified using ImageJ (NIH). We pooled all four regions as no regional differences in fiber type distribution were noted.

Gene Expression

RNA was isolated from supraspinatus muscles using an miRNeasy kit (Qiagen, Valencia, CA) and treated with DNase I (Qiagen). RNA integrity was verified using a Bioanalyzer RNA system (Agilent, Santa Clara, CA). RNA was reverse transcribed using an RT^2 First Strand kit (Qiagen) and amplified in a CFX96 real time thermal cycler (BioRad, Hercules, CA) using an RT^2 SYBR Green qPCR system (Qiagen) using primers for specific mRNA or miRNA transcripts

(Qiagen). Expression of mRNA transcripts was normalized to β-actin and miRNA transcripts were normalized to Rnu6 using the methods of Schmittgen and Livak.[24] A list of transcripts and corresponding RefSeq and miRBase information is listed in Supplementary Tables 1 and 2.

Statistical Analyses

Data are presented as mean ± SD. Differences between the control and tear side were tested using paired *t*-tests ($\alpha = 0.05$) in GraphPad Prism 5.0.

RESULTS

Thirty days after inducing a tear, there was a 53% reduction in supraspinatus muscle mass and a 45% reduction in infraspinatus muscle mass compared with control muscle groups (Table 1). At the single fiber level, for fast muscle fibers rotator cuff tear reduced infraspinatus fiber CSA by 32%, maximum isometric force (Fo) by 43% and specific force (sFo, Fo normalized to CSA) by 18% (Table 1). Rotator cuff tear reduced the size of type I, type IIX, and type IIA/IIX muscle fibers (Fig. 1A), decreased the frequency of type I and IIA muscle fibers, and increased the frequency of type IIB muscle fibers (Fig. 1B). A representative example of muscle fiber types is shown in Figure 1C.

For atrophy and inflammation gene expression, supraspinatus muscles that suffered a tear had no change in the expression of the E3 ubiquitin ligases that regulate skeletal muscle atrophy, atrogin-1 and MuRF-1, but did have a decrease in the anti-inflammatory gene IL-10, with no change in the pro-inflammatory genes IL-6 and IL-1β (Fig. 2A). For genes that regulate fibroblast proliferation, an upregulation of PDGFRα and tenomodulin and a downregulation in scleraxis expression occurred, while the expression of the major components of the muscle ECM, type I collagen and type III collagen, were upregulated (Fig. 2B). The lipid metabolism genes PPARγ and C/EBPα were upregulated in torn rotator cuff muscles, as was the lipid droplet gene perilipin-1; however, the expression of intramuscular lipid droplet formation gene ADRP was not different from controls (Fig. 2C). There was an increase in the expression of the macrophage specific marker F4/80, and ApoE and CIDEC that regulate lipid accumulation, and the autophagy related genes Vps34 and Beclin-1 (Fig. 2D). miR-130a, -138, -221, and -29b were upregulated in torn muscles, but no change in expression was detected between control and torn rotator cuff muscles for miR-1, -103, -107, -133b, -143, -206, -23a, -23b, and -27a (Fig. 3).

A dramatic increase in lipid accumulation within and around infraspinatus fibers and the aponeurosis was observed (Fig. 4). As the expression of F4/80 was upregulated, we then used immunohistochemistry to identify the location of macrophages. A marked accumulation in macrophages was present in areas of fatty degeneration in torn rotator cuff muscles (Fig. 5).

DISCUSSION

Previous studies reported a reduction in whole muscle force production and changes in muscle architecture and ECM accumulation after rotator cuff tear.[9,19,25–27] By analyzing contractility at the single fiber level, we could specifically measure force production without the confounding effects that fibrosis and chronic detachment could have on whole muscle force generation. While the reduction in F_o was expected in a muscle that has undergone atrophy, the decrease in sF_o in torn rotator cuff muscle fibers suggests that the tear leads to pathological changes that reduce the inherent force generating capacity of the muscle. The number of type I and type IIA fibers markedly decreased, and the number of type IIB fibers markedly increased following rotator cuff tear. The accumulation of type IIB fibers is often correlated with chronic diseases or injuries of skeletal muscle,[28] and combined with the reduction in sF_o, suggests that 30 days after tear bona fide pathological changes are present in rotator cuff muscle fibers.

We also measured the expression of several mRNA and miRNA molecules that regulate muscle atrophy, inflammation, and regeneration. miR-1, miR-133b, and miR-206, which play important roles in regulating multiple steps of myogenesis,[29] were not different between control and torn rotator cuff muscles. Atrogin-1 and MuRF-1 are muscle-specific E3 ubiquitin ligases that direct the polyubiqutination of proteins and are important rate limiting enzymes in skeletal muscle protein degradation.[30] While atrogin-1 and MuRF-1 are elevated following acute muscle injuries,[31] despite substantial atrophy we observed no change in atrogin-1 or MuRF-1

Table 1. Muscle Mass and Infraspinatus Single Fiber Contractility Data

	Control	Tear
Supraspinatus mass (mg)	740 ± 30	349 ± 22[a]
Infraspinatus mass (mg)	726 ± 32	396 ± 24[a]
Fiber CSA (μm^2)	8,270 ± 1,570	5,610 ± 390[a]
Fiber F_o (mN)	1.05 ± 0.21	0.60 ± 0.12[a]
Fiber sF_o (kPa)	130 ± 10	107 ± 17[a]

Data are shown as mean ± SD.
[a]Significantly different from control muscle ($p < 0.05$). $N = 6$ muscles from each group.

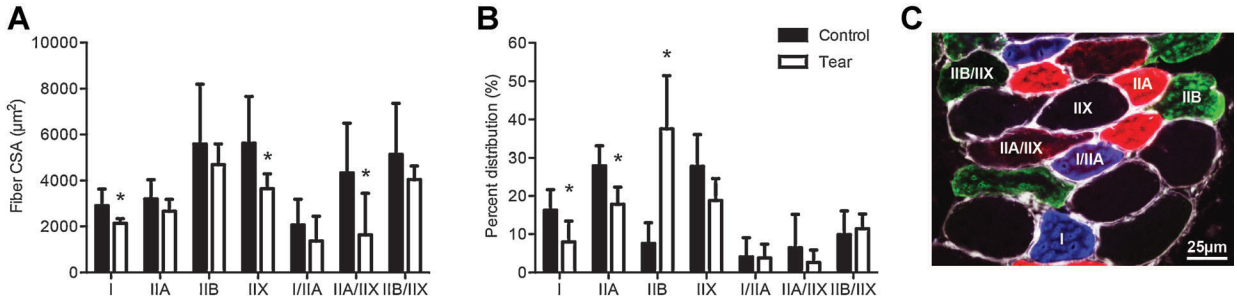

Figure 1. Rotator cuff tear changed the (A) size and (B) myosin heavy chain isoform distribution of muscle fibers. (C) A representative section demonstrating the different muscle fiber types. White, extracellular matrix (WGA-lectin); blue, MHC I; red, MHC IIA; green, MHC IIB; black MHC IIX; blue-red, hybrid MHC I/IIA; red-black, hybrid MHC IIA/IIX; green-black, hybrid MHC IIB/IIX. Values are mean ± SD. $N = 6$ infraspinatus muscles from each group. *, significantly different from control ($p < 0.05$). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jor]

expression between intact or torn rotator cuff muscles. Consistent with this finding, compared to patients with intact rotator cuff muscles there was no change in atrogin-1 and MuRF-1 expression from the muscles of patients with torn rotator cuff tendons.[32] In support of the mRNA expression data, miR-23a, which binds to the 3′ UTR of atrogin-1 and MuRF-1 transcripts to block translation,[33] was not different between control and torn rotator cuff muscles. IL-6 and IL-1β, cytokines that generally promote inflammation and protein degradation after a muscle injury,[11] were not different between intact and torn muscles. IL-10, generally considered to be an anti-inflammatory cytokine that promotes muscle regeneration,[34] was downregulated in torn rotator cuff muscles. The combined muscle fiber contractility and gene expression data suggest that by 30 days the muscle has reached an early chronic, weakened condition.

The accumulation of fibrotic ECM is a common feature in cross-sections of muscle following rotator cuff tear.[19,25,35] We observed an increase in the expression of type I and type III collagen. Fibroblasts are thought to be important regulators of ECM formation in muscle, and we observed a dramatic increase in the expression of the fibroblast proliferation gene tenomodulin[36] in torn muscles. Scleraxis is a bHLH transcription factor that also promotes fibroblast proliferation[37–39] and enhances the formation of new entheses following rotator cuff tear,[40] but interestingly we observed a decrease in its expression in torn muscles. The reason for this downregulation in scleraxis is likely due to the lack of passive tension present in the torn muscles, as Maeda and colleagues [37] observed that a loss of tensile load dramatically reduces scleraxis expression in fibroblasts. PDGFRα, a receptor that has been suggested to be a marker of fibroblast and adipocyte precursor cells,[41] was also upregulated in torn rotator cuff muscles. miR-29b, a miRNA molecule that is enriched in fibroblasts and inhibits the translation of type I collagen mRNA,[42–44] was upregulated in torn muscles. miR-23b is also associated with the regulation of type I collagen gene expression,[42] but the expression of miR-23b was not different between torn and intact muscles. Taken together, these results indicate that active ECM remodeling occurs following rotator cuff tear, and that even though there is an increase in the anti-fibrotic miR-29b, it was insufficient to block collagen accumulation in torn rotator cuff muscles.

A common feature in many rotator cuff tears is dramatic accumulation of intramyocellular and extramyocellular fat.[3,9,19,45] The nature of this fat, and the reason that the rotator cuff is particularly prone to fat accumulation after injury, are not well understood. PPAR-γ and C/EBPα are transcription factors that work in a coordinated fashion to promote adipogenesis.[46] Several reports documented an increase in PPAR-γ or C/EBPα after rotator cuff tear,[8,9,45] and our findings are consistent with this. The expression of the lipid-droplet binding protein, perilipin-1, which is highly expressed in adipocytes, was also upregulated in torn rotator cuff muscles. However, while PPAR-γ, C/EBPα and perilipin-1 are expressed in adipocytes, they are also expressed in other types of cells found in

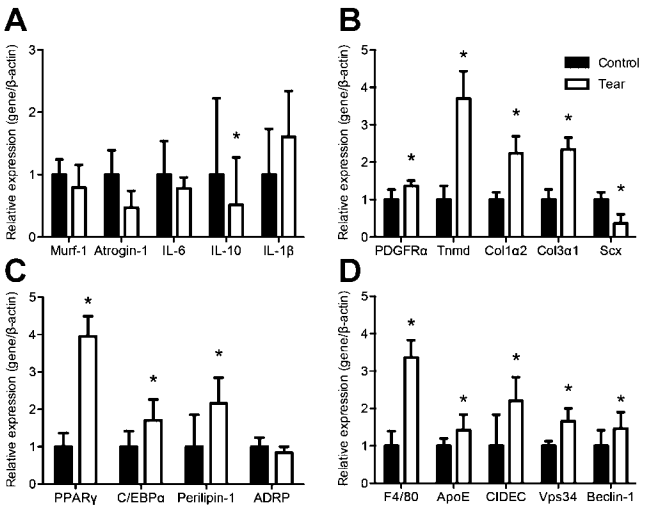

Figure 2. Rotator cuff tear changed the expression of genes related to (A) atrophy and inflammation, (B) fibrosis, (C) adipogenesis, and (D) macrophage accumulation and autophagy. Target gene expression was normalized to that of the stable housekeeping gene β-actin. Values are mean ± SD. $N = 6$ supraspinatus muscles from each group. *, significantly different from control ($p < 0.05$).

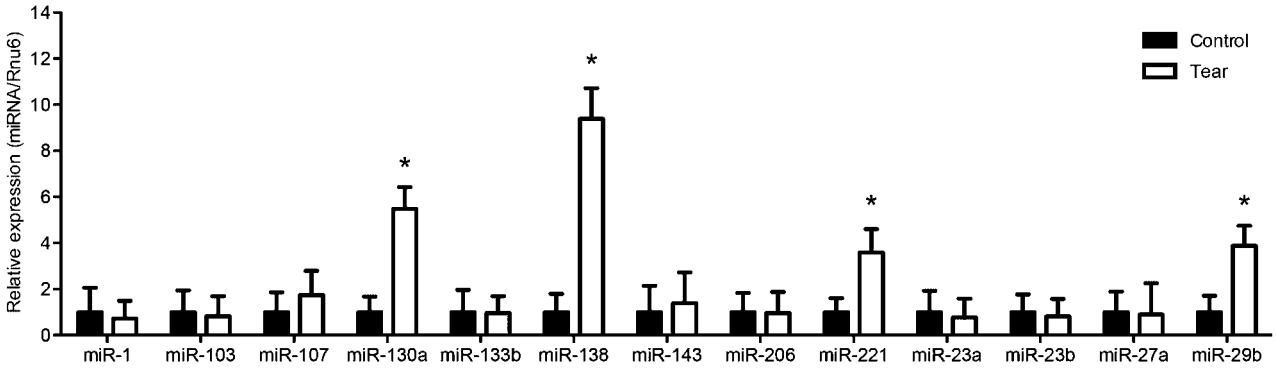

Figure 3. Rotator cuff tear changed the expression of several miRNA transcripts. Target gene expression was normalized to that of the stable housekeeping non-coding RNA Rnu6. Values are mean ± SD. $N = 6$ supraspinatus muscles from each group. *, significantly different from control ($p < 0.05$).

skeletal muscle, including macrophages.[47–49] ADRP is the predominant lipid droplet-associated protein in skeletal muscle[50] and plays a central role in the formation and localization of normal lipid droplets. Even though a dramatic accumulation of intramyocellular lipid was found in torn rotator cuff muscles, there was no difference in ADRP expression. These results suggest that although members of canonical adipogenic pathways may be activated following a tear, the processes that regulate lipid accumulation in injured muscles may be quite different from normal lipid storage physiology.

MicroRNAs also have critical functions in the control of adipogenesis and lipid accumulation. miR-103 and miR-107 play important roles in regulating adipocyte size and lipid storage, but we did not detect a difference in their expression between control and torn rotator cuff muscles.[51] Surprisingly, miR-130a and miR-138, which suppress adipogenesis by inhibiting the translation of PPAR-γ and C/EBPα mRNA, respectively,[52,53] and miR-221, which is downregulated during adipogenesis,[54] were upregulated in torn rotator cuff muscles. The expression of miR-143, which promotes proliferation and differentiation of intramuscular adipocytes,[55] and miR-27a, which inhibits adipocyte differentiation,[56] were not different between control and torn muscles. Taken in consideration with the histology and mRNA expression data, the miRNA data add further support to the notion that the fat accumulation occurring following rotator cuff tears is a different process than metabolic lipid storage.

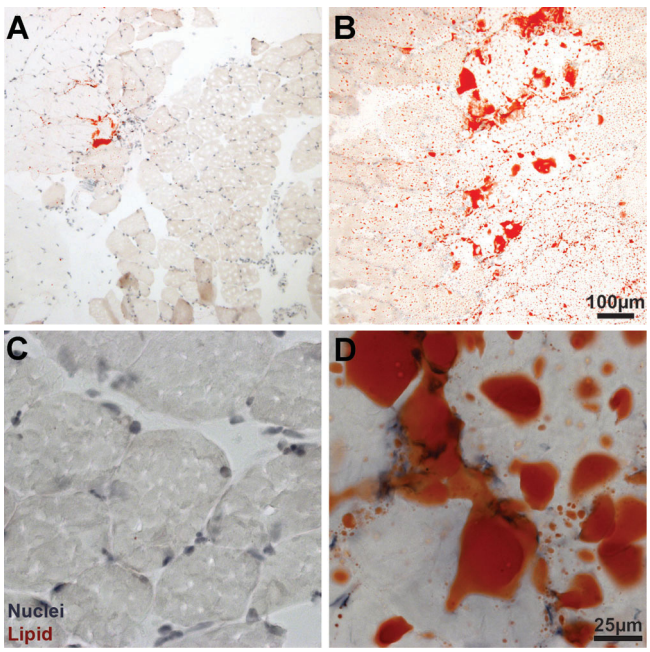

Figure 4. Compared with (A,C) control muscles, (B,D) rotator cuff tear increased intramyocellular and extramyocellular lipid deposition in infraspinatus muscles. Blue, hematoxylin; red, Oil Red O. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jor]

Macrophages play an important role in remodeling and regenerating injured skeletal muscles.[11] In atherosclerosis, another pathophysiological condition characterized by dramatic accumulation of lipid, macrophages are central regulators of fatty plaque formation. We therefore thought it was important to evaluate their role in rotator cuff tears. Using the macrophage-specific marker F4/80,[57] we observed an increase in F4/80 gene expression and a marked accumulation of macrophages in torn muscles. In fatty atherosclerotic plaques, macrophages differentiate into a "foam cell" phenotype and serve as the core cellular component of the plaque.[47] Once they have differentiated into foam cells, they express Apolipoprotein E (ApoE) and CIDEC at high levels.[58] ApoE is a central component of very-low density lipoproteins and chylomicron particles,[58] and CIDEC plays an important role in lipid droplet formation and fat metabolism.[59] Along with an accumulation of macrophages that co-localize with areas of lipid accumulation, we observed an increase in both CIDEC and ApoE expression in torn muscles, consistent with the notion that these macrophages have a similar phenotype to foam cells found in atherosclerotic plaques. While PPAR-γ plays an important role in promoting adipogenesis, it is also central in the differentiation of macrophages into foam cells.[60] In addition to possibly increasing adipogenesis, the increase in whole muscle PPAR-γ expression may

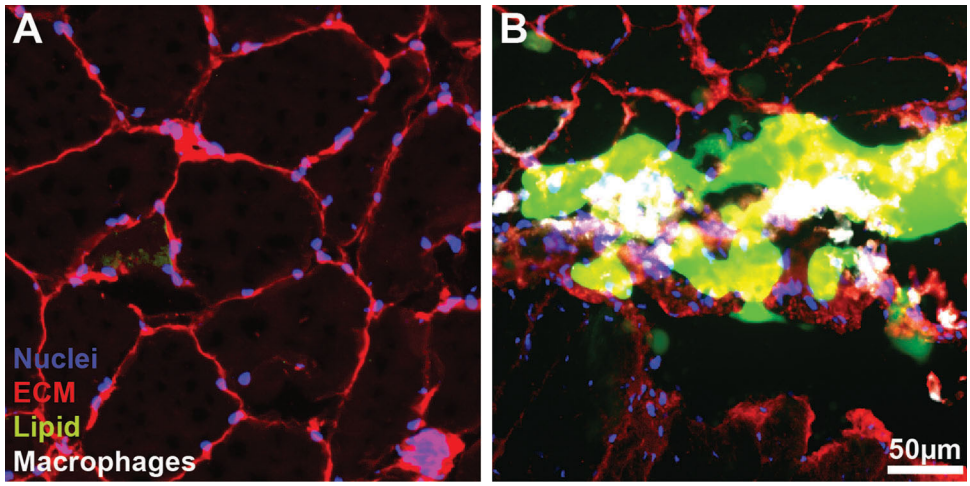

Figure 5. Compared with (A) control infraspinatus muscles, (B) infraspinatus muscles that suffered rotator cuff tears demonstrated areas of lipid deposition with concomitant macrophage accumulation. Blue, nuclei (DAPI); red, extracellular matrix (WGA lectin); green, lipid (BODIPY); white, macrophages (F4/80). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jor]

therefore reflect an increase in foam cell deposition in torn rotator cuff muscles.

Autophagy is an important cellular phenomenon, whereby cells remodel their cytosolic components in response to environmental stressors.[61] Autophagy can be initiated by a number of factors, including oxidized lipid accumulation, endoplasmic reticulum stress, macrophage recruitment, metabolic stress, and inflammation.[62] As we observed substantial increases in macrophage recruitment and lipid accumulation in torn rotator cuff muscles, we looked at the expression of two key components in the initiation of autophagy, Vps34 and Beclin-1.[61] Compared to controls, there was an increase in both Vps34 and Beclin-1 in torn rotator cuff muscles. These results suggest torn rotator cuff muscles utilize autophagocytic pathways to remodel and adapt to their chronically unloaded states.

This study has several limitations. We used a rat model of rotator cuff injury. Although it shares many anatomical features with human cuff muscles, it does not have the same extent of fatty degeneration observed in humans. We only analyzed force production of type II muscle fibers, and did not determine the contractility of fibers by individual myosin heavy chain isoform. We chose this approach due to the substantially greater abundance of type II fibers in the cuff, and because sF_o does not vary significantly across different type II fibers.[63] We also only examined a single time point likely similar to early chronic changes in human tears, and did not look at changes in cellular and molecular function following acute injury. While we measured the expression of several mRNAs, we did not quantify protein levels, and changes in mRNA may not reflect changes in protein content. Finally, although the expression of several mRNA and miRNA transcripts changed as a result of a tear, we examined the expression of transcripts from whole muscle homogenates and did not localize changes to specific cell types. Despite these limitations, this work provided novel insight into changes in muscle fiber contractility, for the first time identified miRNA transcripts that are differentially regulated after rotator cuff tear, and identified macrophages and autophagy as important factors that are likely behind some of the remodeling and regeneration that occur in torn rotator cuff muscles.

For many patients, the fatty degeneration that has set in over time is irreversible.[4,64] Developing strategies that promote the regeneration of torn rotator cuff muscles at the cellular and molecular level will likely be important in restoring full function after surgical repair. The lipid that accumulates in torn rotator cuff muscles is an appealing therapeutic target, as fat promotes local and systemic inflammation,[49] and the accumulation of fat weakens the structural integrity of muscle tissue.[65] Further studies identifying the specific lipid species that accumulate in torn rotator cuff muscles and the molecular pathways that regulate this deposition are necessary to develop an effective strategy to reverse rotator cuff fatty degeneration.

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