Reduced Muscle Degeneration and Decreased Fatty Infiltration after Rotator Cuff Tear in a PARP-1 Knock-Out Mouse Model

PARP-1 Regulates Muscular Deterioration After Rotator Cuff Tear

Michael B. Kuenzler, MD [1, 2], Katja Nuss, DVM [2], Agnieszka Karol, DVM [2], Michael O. Schär, MD [1], Michael Hottiger, DVM, PhD [3,6] Sumit Raniga, MD [1], David Kenkel [4], Brigitte von Rechenberg, DVM [2], ECVS [2,6] Matthias A. Zumstein, MD [1, 5]

[1] *Shoulder, Elbow and Orthopaedic Sports Medicine , Department of Orthopaedic Surgery and Traumatology, Inselspital, Bern University Hospital, Bern, Switzerland*

[2] *Musculoskeletal Research Unit, Equine Department, Vetsuisse Faculty and the Institute of Veterinary Biochemistry and Molecular Biology, University of Zurich, Zurich, Switzerland*

[3] *Institute of Veterinary Biochemistry and Molecular Biology, University of Zurich, Zurich, Switzerland*

[4] *Department of Diagnostic and Interventional Radiology, University Hospital of Zürich*

[5] *Shoulder & Elbow Unit, SportsClinic#1 AG, Papiermuehlestrasse 73, Wankdorf Center, Bern, Switzerland*

[6] *Competence Center for Applied Biotechnology and Molecular Medicine (CABMM), Equine Department, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland*

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Corresponding Author:
Matthias A. Zumstein, MD
Inselspital, Bern University Hospital
Freiburgstrasse 18, Bern 3010, Switzerland
Phone: +41(0)31 632 21 11
Mail: m.zumstein@me.com

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Abstract

Introduction: Disturbed muscular architecture, atrophy and fatty infiltration remain irreversible in chronic rotator cuff tears (RCT) even after repair. Poly (ADP-ribose) polymerase-1 (PARP-1) has shown to be a key regulator of inflammation, apoptosis, muscle atrophy, muscle regeneration and adipocyte development. We hypothesized that the absence of PARP-1 would lead to a reduction in damage to the muscle subsequent to combined tenotomy and neurectomy in a PARP-1 knock-out mouse model**.**

Methods: PARP-1 knock-out (PARP-1 KO group) and wild type C57BL/6 (WT group) mice were analyzed at different time points (1, 6 and 12 weeks, total n=84). In all mice the supraspinatus and infraspinatus muscles of the left shoulder were detached and denervated. Macroscopic analysis, magnetic resonance imaging, gene expression analysis, immunohistochemistry and histology were used to assess the differences in PARP-1 KO and WT mice.

Results: The muscles in the PARP-1 KO group had significantly less retraction, atrophy and fatty infiltration after 12 weeks than in the WT group. Gene expression of inflammatory, apoptotic, adipogenic and muscular atrophy genes was significantly decreased in PARP-1 KO mice in the first 6 weeks.

Discussion: Absence of PARP-1 leads to a reduction in muscular architectural damage, early inflammation, apoptosis, atrophy and fatty infiltration after combined tenotomy and neurectomy of the rotator cuff muscle. Although the macroscopic reaction to injury is similar in the first 6 weeks, the muscles ability to regenerate is much greater in the PARP-1 KO group leading to a near normalization of the muscle after 12 weeks.

Keywords: rotator cuff tear; PARP-1; ARTD1; supraspinatus muscle; mouse model; inflammation; muscle atrophy; fatty infiltration

Introduction

Rotator cuff tears (RCT) cause profound and potentially irreversible structural alterations in the affected muscle. There is significant migration of inflammatory cells within the first few days of a tear and the muscle fibers undergo apoptosis [27; 32]. The infiltrate of inflammatory cells releases Interleukin1-ß (IL1-ß) and Tumor Necrosis Factor α (TNFα), which incites the inflammatory cascade [32]. These factors activate intracellular Nuclear Factor kappa B (NF-κB) which not only induces apoptosis and muscular atrophy, but also inhibits muscle regeneration [23; 32; 35; 42]. Pro-fibrotic factors from the surrounding extracellular matrix (ECM) [24] are released and activated. These factors are members of the Transforming Growth Factor beta (TGFβ) superfamily and are key regulators of gene expression in muscle homeostasis [20]. They lead to the degradation of the injured muscle fibers and the clearance of cellular debris by M1 macrophages. Once the cellular debris have been evacuated, the monocytes transform into anti-inflammatory $M2_{reg}$ macrophages to support myogenesis [1] with the expression of myogenic regulatory factors (MRFs) [45], which in combination with other endocrine growth factors instigate the development mature myocytes from precursor cells [45]. If the tendon remains torn, unloaded and retracted, the macrophages switch to become pro-fibrotic $M2_a$ macrophages and reprogram myogenic precursor cells into the adipogenic pathway, with mature adipocytes infiltrating the free inter- and intramyofibrillar spaces [9]. This phenomenon is termed fatty infiltration [2; 27]. Although reloading the dynamic musculotendinous units leads to partial recovery of atrophy and retraction, fatty infiltration remains irreversible [5; 18]. The degree of fatty infiltration in a chronically torn rotator cuff is a negative predictor for a successful surgical outcome [46].

The complex interplay of molecular and cellular mechanisms, which leads to potentially irreversible structural alterations in skeletal muscle, is well described [22]. However, a single upstream regulator may orchestrate this molecular cascade. The discovery of such a regulator could potentially provide a future target for therapeutic interventions at the molecular level that may enhance the recovery of rotator cuff muscles post surgical repair.

Poly (ADP-ribose) polymerase-1 (PARP-1), also known as ADP-ribosyltransferase (ARTD1), is a key transcription factor involved in the maintenance of cellular homeostasis [37]. It activates NF-κB transcription during the inflammatory response which not only induces apoptosis and muscular atrophy, but also inhibits muscle regeneration [14; 42]; it promotes a caspase independent pathway of apoptosis via the apoptosis inducing factor (AIF) [15]; it regulates the expression of peroxisome proliferator-activated receptor gamma (PPARγ), which has a role in adipogenesis and may induce fatty infiltration of the muscle [7] ; it also induces muscular atrophy and fibrosis whilst depressing regenerative pathways [17; 40]. Hence, PARP-1 may be the upstream regulator that orchestrates the molecular and cellular mechanisms that leads to potentially irreversible structural alterations after RCT.

We therefore hypothesized that the absence of PARP-1 would lead to a reduction in muscular architectural damage, early inflammation, atrophy and fatty infiltration subsequent to combined tenotomy and neurectomy in an established PARP-1 knock-out mouse model [19; 25]. The aim of this study was to investigate the role of PARP-1 in regulating the potentially irreversible structural alterations after RCT utilizing macroscopic, histological, molecular, and radiological techniques.

Methods

Animals

This investigation gained approval from the federal ethics committee (No. 98/2013). PARP-1 knockout mice were originally obtained from Zhao-Qi Wang, PhD (Jena, Germany) and have been crossed back into the C57BL/6 background. These C57BL/6 mice have a PARP-1 gene fragment replaced by the neomycin resistance gene in between the second exon and intron (PARP-1 KO). The wild type (WT) C57BL/6JOlaHsd mice were obtained from Harlan Laboratories (Netherlands). The animals were housed in a specific pathogen free facility under standard enriched housing conditions. Only female mice between the ages of 6-8 weeks at the time of surgery were included in the study.

Study design (Fig. 1)

A total of 42 PARP-1 KO and 42 WT mice were included in the study. These mice underwent combined tenotomy and neurectomy of the supraspinatus (SSP) and infraspinatus (ISP) muscles. In both groups the animals were randomly assigned to three time points. The 1 week and 6 weeks time points included 12 animals each. These mice were then subdivided for either histological (Histology group) or gene expression (PCR group) analysis (n=6 each). The 12 weeks time point contained 18 animals in each of the PARP-1 KO and WT groups. These were then further subdivided for histological (Histology group), gene expression (PCR group) or MRI (MRI group) analysis (n=6 each).

Surgery

Tenotomy and denervation of the SSP and ISP was performed according to published protocols by Liu et al. [25] and Kim et al. [19]. Surgery was carried out on the left shoulder and the contralateral shoulder served as an uninjured control. Anesthesia was induced with

intraperitoneal administration of Ketamine 30mg/kg BW and maintained with inhaled Isoflurane. Intraoperative pain was controlled with subcutaneous injections of Buprenorphine 01.mg/kg BW when indicated. The surgical site underwent sterile preparation and draping with chlorhexidine. All procedures were performed under a surgical microscope using microsurgical instruments. A 2 cm long skin incision was made over the shoulder joint and the deltoid muscle split parallel to its fibers to expose the underlying rotator cuff insertion. The deltoid was retracted with a forceps and the tendons of the SSP and ISP sharply detached from the humeral head. The trapezius was then split along its fibers over the lateral scapular spine. The SSP muscle was bluntly elevated to reach the suprascapular notch. The suprascapular nerve was identified and a 2 mm segment was resected from a point where it enters the notch to a point beyond its division into supraspinatus and infraspinatus branches. The muscular split in the trapezius and deltoid muscles were then repaired with 10-0 Etibond sutures (Ethicon, USA). The skin incision was closed using staples. The animals were allowed free cage activity with food and water ad libidum post surgery. Postoperative pain was controlled with subcutaneous injections of Buprenorphine 01.mg/kg BW in the first day after surgery followed by Buprenorphine 1ml/50ml H_2O in the drinking water for 3 days.

Sacrifice and Sampling

At the specified time points post intervention, the mice in the histology group were euthanized with cervical dislocation under anesthesia followed by harvest of the entire upper extremity of both shoulders with the rotator cuff muscles intact. These samples were immediately fixed in 4% Formalin. The animals in the PCR group underwent further Ketamine 30mg/kg BW induction and anesthesia with Isoflurane. The SSP and ISP muscles from both shoulders were carefully dissected and elevated from the scapula and immediately stored in RNAlater (Quiagen) at -20°C for further analysis. After the muscles were harvested these animals were euthanized with cervical dislocation whilst anaesthetized.

A pilot study with 6 WT mice showed severe retraction of the tendon stump in all 6 animals marked with non-absorbable sutures, macroscopic atrophy and fatty infiltration of the muscles 12 weeks after surgery (data not shown).

Histology

For both immunohistochemistry (IHC) and conventional histological analysis the harvested SSP and ISP muscles were fixed in 4% Formalin overnight, washed with deionised water and stored in 70% Ethanol until parraffin embedding. Once embedded in paraffin, they were sectioned, deparaffinized, rehydrated in xylene and ethanol and then incubated with specific antibodies. For routine histology, H&E and Picrosirius Red staining was performed as per institutional standard operating procedure. The slides were digitalized with a NanoZoomer 2.0-HT Digital slide scanner C9600 (Hamatsu, Japan) in various magnifications to allow further digital processing and analysis.

To visualize intramuscular fat deposition, the midportions of SSP cross-sections were stained with a rabbit anti-mouse antibody against Fabp4 (HPA002188, Sigma-Aldrich, USA). Fatty infiltration, measured by the deposition of adipocytes between the muscle fiber bundles (perimysial) or within the muscle bundles due to replacement of muscle fibers (endomysial), was graded from 0 to 5 (0= no intramuscular fat except around the main vessel; 1= Single intramuscular fat cells or fat cells that penetrate from the vessel into the muscle; 2= Streaks of fat cells into the muscle; 3=Fatty streaks in 2 of 4 quadrants of the muscle; 4=fat cells in all quadrants; 5=severe fatty infiltration). This cell surface marker does not differentiate between the two localities.

The pennation angle was measured three times at different locations and the mean of these measurements used for comparison in the longitudinal sections of the ISP muscle in the

Picrosirius Red stained sections at 20x magnification. The H&E sections underwent semi quantitative analysis. The cross section of the SSP muscle was divided into four quadrants and four images at 20x magnification were taken from each quadrant and analyzed for the frequency of inflammatory cell infiltrate, degenerative cells (hypereosinophilic staining, cell swelling, fragmentation, presence of retraction caps), regenerative cells (rows of myoblast nuclei, cytoplasmic basophilia, internal nuclei), muscular atrophy (rounded to angular cells, hypereosinophilc sarcoplasm, crowded nuclei), fibrosis and fat deposition by a veterinary pathologist who was blinded to the sample group.

Gene expression

The entire SSP samples for Real Time qPCR (RTqPCR) were stored in RNAlater at -20°C until RNA extraction. The TrizolPlus Kit (Life Technologies) was utilized for RNA extraction. The samples were homogenized in 1ml Trizol per 100mg tissue using a MixerMill (Qiagen). After homogenization RNA was isolated by phase separation with 0.2ml chloroform and incubation. The upper phase, containing the RNA, was then transferred to a new tube and one volume 70% Ethanol was added. The solution was then transferred to the Spin Cartridges for binding and washing as per standard manufacturers protocol, which included DNase digestion. The purified RNA was then eluted in 30µl RNase free water. The relative amount of RNA was measured with a NanoDrop spectrophotometer (Thermo Scientific) and equal amounts of RNA were then reverse transcribed to cDNA with a RNA-to-cDNA Kit (Life Technologies) as per standard manufacturer's protocol. RTqPCR was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems, USA) using TaqMan probes with Fast Advanced Mastermix for the expression of inflammatory (NF-κB, IL1-ß, TNFα, IL-6), apoptotic (Caspase3, AIF), atrophic (FOXOS1, MuRF, Atrogin1, Ube2b, Ube3a), regenerative (AKT, $MyoD_1$, Myf-5), fibrotic ($TGFß_1$ and MSTN) and fatty infiltration (PPARγ, Fabp4) genes. GADPH serves as the housekeeping gene and relative

levels of gene expression are measured with the ΔΔCt method relative to the contralateral uninjured side.

MRI

We acquired T1 weighted images using a RARE sequence (Rapid Acquisition with Relaxation Enhancement) for the anatomic depiction. For the fat quantification in-phase and out-of-phase sequences were performed [33]. The sequences included the following scanning parameters: In-phase (flip angle: 50°; echo time: 2.9 ms; repetition time: 200 ms), Out-of-phase (flip angle: 50°; echo time: 2.2 ms; repetition time 200 ms) and RARE T1 (flip angle: 180°; echo_time: 10 ms; repetition time: 1000 ms). All data was acquired on a 4.7-T PharmaScan (Bruker Corporation, Billerica, MA, USA). A linear polarized hydrogen whole-body mouse radiofrequency coil was used. The mice were laid head first and in prone position on an animal bed. We fitted the bed with a pad with continuous flow of warm water in order to avoid cooling of the animals. The animals were anesthetized during the acquisition with isoflurane (Attane, Minrad I, Buffalo, NY) and ophthalmic ointment (Vitamin A Crème, Bausch & Lomb, Steinhausen, Switzerland) was applied to protect the mice from dry eyes. With the acquired data a region of interest (ROI) analysis was done using in house Matlab routines (The MathWorks, Natick, MA) for the fat quantification.

Statistics

Statistical analysis included analysis of variance (ANOVA) and post Hoc tests to reveal differences between the subgroups with the Fisher's LSD test or Mann-Whitney test for non-parametric measurements. Linear correlation was measured with the Pearson product-moment correlation coefficient. The level of significance was set to $p<0.05$. Data is reported as the mean ± standard error of the mean (SEM).

Results

All animals survived the surgical procedure with no postoperative complications. All mice used their operated left forelimb less than the contralateral side, and the expected gait abnormality secondary to diminished use of the affected limb continued until euthanasia. There was no evidence of any adverse effects (e.g. developmental or reproductive abnormalities) on examination of the PARP-1 KO mice [43].

Macroscopic Analysis

All mice in both groups showed retraction of the tendon and muscle of the SSP and ISP at 1 week, with further retraction evident at 6 weeks post combined tenotomy and neurectomy.
The retraction and atrophy remained unchanged in the WT group at 12 weeks post surgery.
In contrast, the PARP-1 KO mice had less retraction and almost normal muscle volume at the 12 weeks time point. Sample images are shown in **Fig. 2:**A.

Retraction was quantified on MRI scans (**Fig. 2**B and **Fig. 2**D) at the 12 week time point. Both tendon and muscle retraction was significantly lower in the PARP-1 KO mice compared to the WT mice ($p = 0.012$ and $p = 0.081$ respectively, **Fig. 2**D and Table 1). The correlation between muscle and tendon retraction reached statistical significance. (PARP-1 KO: $r = 0.91$, $p = 0.001$; WT: $r = 0.98$, $p = 0.0001$).

The wet weight of the SSP muscle decreased significantly in both the PARP-1 KO and in the WT mice (relative decrease compared to uninjured contralateral side in **Fig. 2**C and effective weight in Table 1) in the first 6 weeks post combined tenotomy and neurectomy compared to the uninjured contralateral sideAt 12 weeks post surgery the wet weight of the SSP in PARP-1 KO mice was almost normal in comparison to the contralateral side whilst it remained significantly lower in the WT mice (difference $p < 0.0001$, **Fig. 2**C and Table 1).

Histology

In comparison to the uninjured contralateral side of all animals (Control: 23.9 ± 0.9°; **Fig. 3**E and Table 2) there was a statistically significant increase in pennation angle in the WT mice (1 week: 31.1 ± 2.4°; p = 0.016, 6 weeks: 36.1 ± 4.9°; p = 0.0002, 12 weeks: 34.4 ± 5.9°; p = 0.0014 respectively). Conversely, after an initial increase in the pennation angle in the PARP-1 KO mice it remained unchanged at the 6 and 12 weeks time points and did not reach statistical significance when compared to the controls (1 week: 30.0 ± 3.5°; p = 0.088, 6 weeks: 28.1 ± 4.9°; p = 0.155, 12 weeks: 28.5 ± 3.9°; p = 0.103 respectively). There was a statistically significant correlation between the pennation angle, and the tendon and muscle retraction measurements in the PARP-1 KO mice (r = 0.93, p = 0.008 and r = 0.9, p = 0.014 respectively) but not in the WT mice (r = -0.38, p = 0.517 and r = -0.36, p = 0.546 respectively).

H&E staining of the SSP cross sections showed a higher inflammatory cell infiltrate at 1 week post injury in the WT mice (**Fig. 3**A). This was followed by an increase in degenerative changes in both groups, with muscle fibers undergoing degradation and atrophy at 6 weeks. PARP-1 KO mice had a higher number of regenerating fibers at this time point. After 12 weeks almost no degenerative changes were observed in either group. Muscles of the PARP-1 KO group had less fibrosis and better muscle architecture compared to the WT group (**Fig. 3**D)**.**

Fatty infiltration

Both groups had no fatty infiltration at 1 week (data not shown). Fatty infiltration was present in both groups at 6 weeks with an average grade of 2.7 ± 0.49 in the PARP-1 KO mice and 2.3 ± 0.49 in the WT mice (difference: p = 0.818 **Fig. 3**B and C). This almost significantly decreased in the PARP-1 KO mice to 1.4 ± 0.25 at 12 weeks post surgery (p = 0.082), which

was significantly lower than in the WT mice (2.8 ± 0.37; difference p = 0.032). Intramuscular fat was also quantified in the In-Phase and Opposed-Phase of the MR scans. The relative amount of intramuscular fat was significantly lower in the PARP-1 KO group (12.5 ± 1.82%) compared to the WT group (19.6 ± 1.96%; difference p = 0.027).

Gene Expression Analysis

Gene expression analysis of various *inflammatory* genes revealed that TNFα mRNA was upregulated at 1 and 12 weeks after injury in both PARP-1 KO and WT mice without reaching statistical significance (p = 0.775 and p = 0.390 respectively, **Fig 4**A). IL1-ß expression was upregulated at 1 and 6 weeks post surgery in the WT group without reaching statistical significance when compared to the PARP-1 KO mice (1 week: p = 0.197, 6 weeks: p = 0.110). There was a significant upregulation of NF-κB and the *proapoptotic* factor AIF at the 1-week time point in the WT group (p < 0.0001 and p = 0.005 respectively). The mRNA of the *proliferative factors* TGFß$_1$ and MSTN were also significantly upregulated in the WT group at 1 week (p < 0.0001 and p = 0.0038 respectively, **Fig. 4**B). The *muscle atrophy* related Ubiquitin ligases MuRF1 and Atrogin-1 were present at significantly (p = 0.048 and p = 0.0018 respectively) higher levels in the WT group consistent with the higher levels of Ubiquitin ligase Ube3a mRNA at the 1-week time point (p < 0.0001, **Fig. 4**C). The mRNA level of regulatory protein FOXO1 was also significantly upregulated in the WT mice at 6 weeks (p = 0.013, **Fig. 4**C). The main regulator of *muscle regeneration* AKT was equally upregulated in the PARP-1 KO and WT group at 1 and 12 weeks (p = 0.447 and p = 0.990 respectively, **Fig. 4**D). Both MyoD and Myf-5 mRNA was upregulated at week 1 and week 6 post surgery in both groups. The upregulation of both factors was significantly higher at week 1 in the WT group compared to the PARP-1 KO group (p = 0.0053 and p = 0.012 respectively, **Fig. 4**D). The mRNA levels of genes regulating *fatty infiltration* were significantly upregulated at 6 weeks in the WT group (PPARγ: p = 0.012 and Fabp4: p =

270 0.0124 **Fig.** **4**E).

Discussion

Disturbed muscular architecture, complete atrophy and fatty infiltration remain irreversible in chronic rotator cuff tears even after repair. The complex interplay of molecular and cellular mechanisms, which leads to potentially irreversible structural alterations in skeletal muscle have been described [22]. Poly (ADP-ribose) polymerase-1 (PARP-1), also known as ADP-ribosyl-transferase (ARTD1), is a key transcription factor involved in the maintenance of cellular homeostasis [21]. PARP-1 has shown to be a key regulator of inflammation, apoptosis, muscle atrophy, muscle regeneration and adipocyte development [7; 14; 40]. Our study is the first to show that the absence of PARP-1 leads to a reduction in muscular architectural damage in the mice' supraspinatus and infraspinatus muscle. PARP-1 may be the upstream regulator that orchestrates the molecular and cellular mechanisms that leads to these potentially irreversible structural alterations after RCT.

Macroscopic analysis showed different degrees of tendon and muscle retraction in both WT and PARP-1 KO mice at 1 and 6 weeks post combined tenotomy and neurectomy. After 12 weeks retraction of the tendon and muscle was significantly lower in the PARP-1 KO mice compared to the WT mice measured in MRI scans. In a 2006 sheep study, Meyer et al. also showed that the tendon retracts more than muscle in experimental chronic tears of the rotator cuff. This results in an apparently shortened tendon [31]. In our study, despite the degree of fatty infiltration being less than 50% of the muscle volume (< Goutallier stage 3) in all animals the degree of tendon retraction was consistently much greater than muscle retraction.

Liu et al. observed significant and consistent muscle atrophy after rotator cuff tendon transection in a mouse model [25]. Furthermore they found that denervation significantly increased the amount of muscle atrophy after a rotator cuff tear in a mouse model [25]. Muscle atrophy persisted in the WT group in our study whilst the PARP-1 KO mice had almost

normal muscle volume at the 12 weeks time point. This occurrence was further supported by near normalization of the wet weight of the SSP in PARP-1 KO mice, whilst it remained low in the WT mice after the initial decrease in both groups. Only after continuous elongation and subsequent refixation do retracted, fatty infiltrated and atrophied rotator cuff muscles in sheep, achieve partial reversal of muscle atrophy but not fatty infiltration [10].

Meyer et al. [30] described the pathomechanical concept of the pennation angle to explain muscle loss and fatty infiltration following RCT. Geometric modeling showed that the increase of the pennation angle separates the muscle fiber bundles mechanically like limbs of a parallelogram. Infiltrating fat cells fill the created space between the reoriented muscle fibers, which may be quantitatively calculated without affecting the structural properties of the muscle cells. Our histological data was consistent with the macroscopic findings. Both groups in our study demonstrated an increase in the pennation angle at 1 week following combined tenotomy and neurectomy. There was a further increase in the pennation angle in the WT mice at 6 weeks and it remained high at 12 weeks. Whilst in the PARP-1 KO mice the pennation angle remained unchanged at 6 and 12 weeks. In contrast to the WT group, the increase in pennation angle in the PARP-1 KO mice did not reach statistical significance when compared to the controls at any time point.

Fatty infiltration was present in both groups at 6 weeks. The infiltration decreased in the PARP-1 KO mice to 1.4 ± 0.25 at 12 weeks post surgery, which was significantly lower than in the WT mice where the grading conversely increased to 2.8 ± 0.37 from the 6 week time point. The MRI measurement of relative intramuscular fat was also significantly lower in the PARP-1 KO group at 12 weeks. Gerber et al. demonstrated an arrest of fatty infiltration after continuous elongation and refixation in a sheep model [10]. In a sheep study, neither an anabolic steroid nor IGF contributes to regeneration of the muscle once degenerative changes are

established. The findings demonstrated that muscle cells lose reactiveness to an anabolic steroid and IGF once retraction has led to fatty infiltration and atrophy of the muscle [11] Treatment of mice with Tamoxifen , a competitive estrogen receptor inhibitor, has shown to cause less atrophy and inflammation after RCT but fatty infiltration remained unchanged [4]. To date there is only one other study in the literature that has demonstrated reversal of fatty infiltration; through local administration of adipose-derived stem cells (ADSCs) into repaired rabbit SSC muscle, Oh et al. demonstrated improvement in fatty infiltration and tendon healing [34]. As we have significantly less fatty infiltration and atrophy at 12 weeks in the PARP-1 KO group, one may speculate that outcome post fixation of the RCT in this group may have an improved surgical outcome.

Results of the gene expression analysis further support the hypothesis that PARP-1 may be an instrumental upstream regulator that orchestrates potentially irreversible structural alterations after RCT. Regeneration and degeneration are in harmony during normal muscle homeostasis. RCT incite an inflammatory response that begins with inflammatory cell infiltration and subsequent release of proinflammatory cytokines [32]. Intramuscular macrophages release TNFα and IL1-ß and thereby stimulate the up-regulation of NF-κB. NF-κB has an integral role in influencing muscle degeneration [23; 42]; (1) it co-regulates the expression of inflammatory and proapoptotic cytokines that cause muscle damage, (2) promotes muscular atrophy and degradation directly via activation of MuRF1 or indirectly via up-regulation of other cytokines and (3) it inhibits myogenic differentiation and regeneration. PARP-1 has been shown to be an important co-factor for NF-κB dependent transcription of various genes [14] and the disturbance of this interaction leads to a lower inflammatory reaction to injury [14]. Studies have shown that inactivation or deletion of PARP-1 protects tissues from damage (review in Kraus and Hottiger, 2013) [21].

Our results show an inflammatory response at 1 week post combined tenotomy and neurectomy of the SSP and ISP muscles in the PARP-1 KO and the WT mice. TNFα and IL-1ß are extracellular inflammatory cytokines that induce intracellular inflammatory cascades. They were upregulated in both groups but only lead to a significant upregulation of NF-κB in the muscles of WT mice. This may be explained by a dampened inflammatory response and subsequent reduction in proinflammatory cytokine expression in the muscles of PARP-1 KO mice [13]. Only the WT mice had significantly higher levels of the pro-apoptotic AIF, which is activated by PARP-1 and promotes caspase independent apoptosis. AIF translocates into the nucleus where it triggers apoptosis [44]. The increase in pro-apoptotic gene expression suggests higher apoptosis rates in WT mice leading to a more pronounced cell death in this group.

Unloading or denervation of the musculotendinous unit initiates complex pathways that eventually result in muscle ubiquitination and degradation [41]. Ubiquitination requires ligase's to form complexes with Ube3a (Ubiquitin-protein ligase E3A) that allows recognition and proteosome mediated degradation of muscle fibers [41]. The most important of these ligase's are MuRF1 (muscle RING finger 1) and Atrogin-1 (FBX032) [3]. Their transcription is upregulated by inflammatory, profibrotic, proadipogenic and the forkhead box 0 (FOX0) transcription factors [23; 29; 36; 38]. The key elements involved in the process of ubiquitination and muscle degradation, Ube3a, MuRF1 and Atrogin-1, were all significantly upregulated in the WT mice at 1 week post combined tenotomy and neurectomy.

During muscle regeneration satellite cells and mesenchymal stem cells (MSC) are activated and undergo proliferation and differentiation (Review in [45]). This process is orchestrated by the myogenic regulatory factors (MRF), such as MyoD and Myf-5, which are activated through the AKT/mTOR pathway [45]. Additionally it has been shown that NF-κB has a direct inhibitory effect on muscular regeneration by inhibiting the MRF's, specifically MyoD [13].

This inhibition of myogenic differentiation and regeneration is also a major effect of NF-κB in muscle degeneration. We interpret the significant up regulation of MyoD in WT mice after 1 week as a failed attempt of the muscle to induce regeneration through stimulation of satellite cells and MSCs. Meanwhile in the absence of PARP-1 in the knock-out group, NF-κB is not effective in inhibiting MyoD and less muscle fibers were damaged during the initial inflammatory response. Upregulation of MyoD, like in the WT group, is not needed and low levels of MyoD may be sufficient for regeneration of the muscle fibers leading to a normalization of the muscle weight after 12 weeks.

Both factors, TGFß$_1$ and Myostatin, were significantly upregulated in the WT group at 1 week. The inflammatory cell infiltrate triggers the release of TGFß$_1$ and Myostatin from the fibroblasts in the ECM [18; 24]. Both factors belong to the Transforming Growth factor superfamily [20]. Members of this TGF superfamily have been shown to induce fibrosis and regulate muscle mass [28]. Specificially Myostatin inhibits myogenic differentiation by downregulating the expression of MyoD and Myogenin [39]. PARP-1 modulates TGF-ß$_1$ activity via negative and positive feedback mechanisms allowing fine-tuning of these pathways [6; 26]. Our data suggests that the significant early activation of TGF-ß$_1$ transcription in WT mice directs the balance towards fibrosis and degeneration.

Our study showed fatty infiltration in both mice groups at 6 weeks but significantly less fatty infiltration in PARP-1 KO mice after 12 weeks. Both proadipogenic factors (Peroxisome proliferator-activated receptor-γ = PPARγ and Fatty Acid Binding Protein = FABP4) revealed a significantly higher expression in the WT mice compared to PARP-1 KO group at 6 weeks post-injury. These proadipogenic genes are key factors in fat accumulation in between free inter- and intramyofibrillar spaces and also decrease the expression of MRF [16]. In addition Myostatin and TGFß reduce the expression of the proadipogenic factors [12]. This may be the

reason why PPARγ is only upregulated at 6 weeks - after the inhibitory effect of Myostatin and TGFß has dissipated. Furthermore, absence of PARP-1 directly inhibits the function of PPARγ [7; 17] and is a crucial regulator of adipogenic differentiation [8].

There are limitations in this study. It could be suggested the differences observed in our study were due to reinnervation. This is not plausible for three reasons. Firstly, a 2 mm length of the nerve was transected from the main branch at its entrance into the scapular notch extending beyond its branches to the SSP ad ISP in both the WT and PARP-1 KO mice. Secondly, if the nerves were to reinnervate by chance, then we would expect more outliers in our data - all of our data, including muscle weight measurements, demonstrate no outliers with a narrow standard deviation. Thirdly, why should the reinnervation phenomena be confined to the PARP-1 KO group only and not occur in the WT group? Another possible criticism could be that we analyzed gene expression and not effective protein levels and their activity. This does limit our ability fully interpret the molecular mechanisms at play. We surgically transected the tendons of the SSP and ISP from its origin at the humeral head. This may not accurately mimic degenerative RCT seen in the human population, but to our knowledge there are no degenerative RCT mouse models. There are other animal models of chronic rotator cuff tears, but this would not allow us to use the PARP-1 knockout model. This study relays on gene expression analysis and does not investigate the exact interactions between PARP-1 and the described proteins on a molecular level. Further molecular biological methods would be needed to describe these mechanisms. The first time point of 1 week may be perceived as a bit delayed to assess inflammation, but we were still able to observe significant differences between the PARP-1-KO and WT mice in all the various modes of analyses.

Conclusion

Our study is the first to show that the absence of PARP-1 leads to a reduction in muscular architectural damage, early inflammation, apoptosis, atrophy and fatty infiltration after combined tenotomy and neurectomy of the rotator cuff muscle. PARP-1 is one of the upstream regulators that orchestrates the molecular and cellular mechanisms that leads to potentially irreversible structural alterations after RCT. It plays an important role in modulating the muscles reaction to RCT by promoting the immediate inflammatory response. This inflammatory response leads to apoptosis and damage to the muscle fibers and initiates muscular degeneration and atrophy. Architectural changes and loss of myocytes hinders the muscles ability to regenerate and ultimately leads to fatty infiltration. In the absence of PARP-1, the initial inflammatory response is dampened leading to less myocyte degeneration. Although the macroscopic muscles reaction to injury is similar in the first 6 weeks, its ability to regenerate is much greater in the PARP-1 KO group leading to a near normalization of the muscle substance and muscle weight, less retraction, and less fatty infiltration after 12 weeks. We conclude that PARP1 is a molecular regulator of muscular deterioration after RCT.

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Figure legends:

Fig. 1: Flow chart of the experimental design including the time points of surgery and sacrifice.

Fig. 2: Results of the macroscopic and MRI measurements. A: Representative macroscopic images showing less retraction of the tendon in PARP-1 KO mice compared to WT mice. The arrow indicates the distance of the tendon stump to the humeral head. B: Representative images of the radiological retraction measurements in the MR scans. The arrow indicates the distance of the tendon stump to the humeral head. C: Muscle weight measurement. The relative weight to the contralateral uninjured side of the PARP-1 KO and Wild Type mice is shown in the bar graph. D: Bar graphs of the retraction measurements. Statistical significant differences are shown * $p<0.05$, ** $p<0.01$ and **** $p<0.0001$.

Fig. 3: Representative histological slides and results of the fat quantification and pennation angle measurement. A: Representative histological cross sections of the SSP stained with H&E after 1, 6 and 12 weeks. B: Representative histological cross sections stained with an antibody against Fabp4. C: Fat quantification in the SSP muscles. Relative fat quantification in the MR scans with a 2-Point Dixon Method on a 4.7T small animal MRI scanner and histological grading of the endo- and perimysial fat content in the cross sections oft he SSP muscles stained with Fabp4. D: Representative histological cross sections of the SSP stained with Picrosirius Red to visualize the connective tissue. E: Pennation angle measurements in the Picrosirius Red stained longitudinal sections of the ISP muscles of PARP-1 KO and WT mice and bar graphs indicating the degree of the angle. The contralateral side of both groups acted as an uninjured control measurement. Statistical significant differences are shown * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Fig. 4: Results of the gene expression analysis with real time RT-PCR. The increase of mRNA levels is shown as fold expression compared to the uninjured contralateral side with

607 the ΔCt method. A: Genes of the inflammatory cascade (TNFα, IL-1ß and NF-κB) and
608 apoptosis (AIF). B: Proliferative factors of the TGFß superfamily represented by TGFß1 and
609 Myostatin. C: Genes involved in the degeneration of muscle fibers. Foxo1 is the upstream
610 regulator of the Ubiquitin-Ligases MuRF1 and Atrogin-1, which bind to Ube3a. D: Genes for
611 muscular regeneration. AKT is the upstream regulator of the MRFs here represented by
612 MyoD1 and Myf-5. E: Genes regulating fatty infiltration (PPARγ) and binding of fatty acids
613 (Fabp4). Statistical significant differences are shown * $p<0.05$, ** $p<0.01$ and **** $p<0.0001$.
614 **Fig. S 1:** Supplemental results of the gene expression analysis with real time RT-PCR. The
615 increase of mRNA levels is shown as fold expression compared to the uninjured contralateral
616 side with the ΔCt method. A: Genes of the inflammatory cascade (IL6) and apoptosis
617 (Casp3). B: Proliferative factors TGFß3. C: Genes involved in the degeneration of muscle
618 fibers Foxo3 and Ube2b. D: Gene for muscular regeneration, Myogenins. E: Genes regulating
619 fatty infiltration Leptin. Statistical significant differences are shown with * $p<0.05$ and ****
620 $p<0.0001$.
621