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Multifidus muscle changes after back injury are characterized by structural remodeling of muscle, adipose and connective tissue, but not muscle atrophy: Molecular and morphological evidence

[1]Paul W Hodges PhD, [1]Gregory James PhD, [1]Linda Blomster PhD, [1]Leanne Hall PhD, [1]Annina Schmid PhD, [2]Cindy Shu PhD, [2]Chris Little PhD, [2,3]James Melrose PhD

1. Uni. of Queensland, Centre of Clinical Research Excellence in Spinal Pain, Injury & Health, School of Health & Rehabilitation Sciences, Brisbane. Australia

2. Uni. of Sydney, Raymond Purves Bone and Joint Research Laboratories, Kolling Institute of Medical Research, The Royal North Shore Hospital, Sydney, Australia

3. Graduate School of Biomedical Engineering, University of New South Wales, Sydney, Australia.

Address for correspondence

Dr Paul Hodges,

School of Health and Rehabilitation Sciences,

The University of Queensland, Brisbane QLD 4072, Australia

Tel: +61 404 854 589 e-mail: p.hodges@uq.edu.au

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Abstract

Study Design: Longitudinal case controlled animal study.

Objective: To investigate putative cellular mechanisms to explain structural changes in muscle, adipose and connective tissues of the back muscles after intervertebral disc(IVD) injury.

Summary of Background Data: Structural back muscle changes are ubiquitous with back pain/injury and considered relevant for outcome, but their exact nature, time-course, and cellular mechanisms remain elusive. We used an animal model that produces phenotypic back muscle changes after IVD injury to study these issues at the cellular/molecular level.

Methods: Multifidus muscle was harvested from both sides of the spine at L1-2 and L3-4 IVDs in 27 castrated male sheep at 3(n=10) or 6(n=17) months after a surgical anterolateral IVD injury at both levels. Ten control sheep underwent no surgery(3 months n=4; 6 months n=6). Tissue was harvested at L4 for histological analysis of cross sectional area(CSA) of muscle, adipose and connective tissue(whole muscle), plus immunohistochemistry to identify proportion and CSA of individual muscle fiber types in the deepest fascicle. Quantitative PCR measured gene expression of typical cytokines/signaling molecules at L2.

Results: Contrary to predictions, there was no multifidus muscle atrophy(whole muscle or individual fiber). There was increased adipose and connective tissue(fibrotic proliferation) CSA and slow-to-fast muscle fiber transition at 6, but not 3 months. Within the multifidus muscle increases in the expression of several cytokines(TNF□ and IL-1□) and molecules that signal trophic/atrophic processes for the three tissue types(e.g. growth factor pathway [IGF-1, PI3k, Akt1, mTOR], potent tissue modifers [calcineurin, PCG-1α, and myostatin]) were present.

Conclusions: This study provides cellular evidence that refutes the presence of multifidus muscle atrophy accompanying IVD degeneration. Instead, adipose/connective tissue increased in parallel

with the expression of the genes that provide putative mechanisms for multifidus structural remodeling. This provides novel targets for pharmacological and physical interventions.

Keywords: Gene expression, cytokines, muscle atrophy, back pain/injury, adipogenesis, myofibroblast, inter-vertebral disc, muscle fiber transformation, fibrosis, fatty infiltration

Level of Evidence: **N/A**

Mini Abstract/Precis

Rather than muscle atrophy, intervertebral disc lesion induces dramatic structural remodeling of multifidus muscle, characterized by increased adipose and fibrotic tissue and muscle fiber changes underpinned by evidence of modified cellular processes within the muscle tissue.

Key Points

- Contrary to contemporary opinion, IVD injury does not induce atrophy of the multifidus muscle, instead the muscle undergoes dramatic remodeling of muscle fiber populations, and adipose/connective tissue structure.
- Multifidus gene profiling identified a number of molecular markers with apparent roles in multifidus reorganization.
- Improved understanding of the nature and mechanisms for structural changes in the multifidus muscle provide novel targets for physical and pharmacological interventions to prevent and reverse these changes that are likely to impact muscle function.

Introduction

Back muscle changes(reduced size[1-4], muscle fiber-type transformation[5,6] and fatty infiltration[4,7,8]) are an ubiquitous sequelae of low back pain(LBP)/injury. Changes are most commonly reported for the deepest back muscle, the multifidus. They have relevance for motor[9,10] and sensory[11] function, and could contribute to LBP recurrence/perpetuation[10,12], but underlying cellular changes and mechanisms are poorly understood. Until resolved, optimal methods for prevention and resolution remain elusive.

Variable presentation, spatial distribution and nature of multifidus structural changes between studies and time-points remains unexplained. Within 3 days of intervertebral disc(IVD) injury in animals[4], or pain onset in humans[1], multifidus cross-sectional area(CSA) is reduced at a single side and spinal level. Yet, chronic LBP is characterized by diffusely reduced CSA and increased non-contractile tissue(attributed to adipose)[2,13], but not in all studies[3]. Intermediate measures(~28 days after onset) reveal greater adipose and total CSA, without reduced muscle CSA[14]. Increased adipose/connective tissue has been suggested qualitatively at day 3 and week 12, but not intermediate timepoints after IVD injury in pigs[4] and rabbits[15], respectively, without muscle fiber changes[15]. Multiple mechanisms with different timecourse might be responsible.

Skeletal muscle retains considerable plasticity regulated by signaling pathways controlling cell and protein turnover[16]. Multiple stressors(e.g. injury,(un)loading, cytokines) affect these pathways[17]. Recent work is unraveling cellular changes and mechanisms. At 6 months after IVD lesion in sheep, multifidus muscle undergoes transformation from slow-(fatigue resistant postural function[18]) to fast-(fatigue sensitive/high torque) muscle fibers in conjunction with increased pro-inflammatory cytokine gene expression(TNF□/IL-1□)[6]. Key issues to resolve are the molecular link between cytokines and structural changes(muscle and non-contractile) and their timecourse.

Mechanically induced IVD degeneration, in our animal model, is accompanied by muscle changes of a similar phenotype to human LBP - rapid localized muscle changes[4] transitioning to diffuse chronic distribution[6]. This creates a platform for detailed cellular analysis. Fig. 1 presents putative mechanisms for multifidus changes, including signaling pathways for muscle atrophy, adipogenesis, and muscle fibrosis. Table 1 details evidence for specific molecules.

We aimed to resolve three fundamental issues of muscle changes after IVD injury/degeneration. First, we undertook detailed histological characterization of structural changes in muscle, adipose and connective tissue at sub-acute and chronic time-points. Second, we probed putative molecular mechanisms for structural multifidus changes by analysis of gene expression for signaling molecules/cytokines. Third, we investigated the relationships between structural changes and molecular markers.

Materials and Methods

Animals

We studied 27 castrated male merino sheep(3-4yr) from a concurrent study of IVD injury[19]. Muscle harvested at 6 months was used in earlier analyses[6]. Procedures were approved by the University of Sydney animal care and ethics care committee.

Surgical procedure, IVD lesion and postoperative care

Ten age- and weight-matched sheep were assigned to a control group without surgery or medication. Surgical procedures for remaining animals were performed as previously described[6]. In brief, 17 animals were anesthetized and received lesions(20mm wide x 6mm deep to penetrate annulus fibrosis without entering nucleus pulpous) to the left annular IVD at L1-2, L3-4 and L5-6 via an extraperitoneal approach. Animals were held in pens for 10 days to monitor wound healing and lameness. As a control for a separate experiment, animals underwent a second surgery at four

or 12 weeks to inject inert phosphate buffered saline solution(0.2ml) into the IVD's right side. Sheep were subsequently housed in an open paddock with unrestricted exercise.

Tissue Harvesting

Four control and 6 lesion animals were culled 3 months after surgery. Remaining animals(6 controls; 11 lesion animals) were culled at 6 months. A transverse section of multifidus muscle was harvested adjacent to the L2 and L4 spinous processes and processed as previously described[6].

Whole muscle histology

CSA of muscle, adipose and connective tissue was identified from microtome sections(8μm) of paraffin embedded whole muscle(L4) stained using standard Masson's Trichrome and Van Gieson's staining methods. The entire multifidus muscle was imaged(Scanscope AT Turbo, Aperio, USA) and each tissue was outlined(Fig. 2) and measured using ImageJ software(NIH, USA).

Muscle fiber analysis

Muscle fiber analysis(L4) of the deep multifidus fascicle(which changes most after IVD injury) was conducted using immunohistochemical staining assays as described previously[6]. In brief, muscle cells were identified as fast or slow based on reaction to specific antibodies(mouse anti-slow and -fast Myosin(Sigma, USA)), and intermediate if they reacted to both[20](Fig. 2). Muscle fiber CSA was measured using ImageJ from a random selection of 5-10 muscle fibers of each type from twelve 620x400μm images(20x magnification)(60-120 fibers/type/animal/side).

Muscle gene expression

PCR reactions quantified gene expression using established procedures[6]. Table 1 justifies included molecules. Total ribonucleic acid(RNA) was extracted from the deep multifidus fascicle (L2) using the RNeasy mini kit(Qiagen, USA), followed by reverse transcription into

cDNA(Invitrogen, USA). Gene expression was quantified with the Rotor-Gene 6000 Real-Time PCR Machine(Qiagen, USA). PCR reaction was performed using IQ SYBR Green Mastermix(Bio-Rad Laboratories, USA) with primer pairs outlined in Table 2. Levels were expressed as a percentage of the housekeeping gene GAPDH and converted to fold difference relative to the control muscle mean.

Statistical Analysis

Muscle structural features(muscle, adipose and connective tissue CSA; individual muscle fiber CSA; proportion of muscle fiber types) and gene expression levels were compared separately for 3- and 6-month samples between muscle samples from control(C) animals, and those from the injured(I) and non-injured(NI) side of injured animals with analysis of variance(ANOVA) and post hoc testing using Bonferroni's test. Relationships between structural parameters and gene expression were analysed with Pearson's r correlation coefficient and interpreted according to standard criteria(weak-r<0.35; moderate-r=0.36-0.67; strong-r=0.68-0.90; very strong-r>0.90[21]). Significance was set at P=0.05.

Results

Structural changes in muscle, adipose and connective tissue

Histological data show extensive structural remodeling of all multifidus tissue types, but no muscle atrophy (Fig. 3). Muscle sections at 6 months demonstrated increased CSA of adipose(P=0.038; post hoc-Cvs.I P=0.01; Cvs.NI P=0.68) and connective tissue(P=0.035; post hoc-Cvs.I P=0.01; Cvs.NI P=0.03), but not muscle(P=0.315) or individual muscle fibers of any type(all P>0.605). Adipose and connective tissue accounted for 8.7(2.4)% and 8.4(2.1)% of total muscle area on the injured side, respectively, and 7.8(2.3)% and 7.5(1.5)% in controls.

As reported previously[6], slow fiber proportion was less(P<0.001) on both the injured(Cvs.I P<0.05) and non-injured sides(Cvs.NI P<0.001), intermediate fiber proportion was greater(P<0.001) on both sides(both P<0.01), and fast fiber proportion was greater(P=0.043) on the injured side(Cvs.I P=0.05; Cvs.NI P=0.17). Consistent with increased connective tissue CSA, the expression of Collagen-1 increased on the injured side at 6 months(Table 3). Collagen-3 expression was unchanged(Table 3). On the injured side there was a moderate to strong correlation between connective tissue CSA and Collagen-1(r=0.66) and Collagen-3(r=0.74) expression. Samples at 3 months revealed no change in CSA of any tissue(all: P>0.114) or individual muscle fibers(all: P>0.445) and proportion of muscle fiber types(all P>0.153).

Cytokine gene expression and relationship to muscle, adipose and connective tissue

Table 3 and Fig. 4 show statistical results for gene expression. Expression of pro-inflammatory cytokines tumor necrosis factor-alpha(TNF□□□injured and non-injured sides) and interleukin 1 beta(IL-1□□□injured side only) increased at 6 months, but not 3 months. Connective tissue CSA correlated strongly with TNF□(r=0.56, P=0.12), and moderately with IL-1□(r=0.37, P=0.32). Adipose CSA correlated moderately with expression of TNF□(r=0.54, P=0.13), and weakly with IL-1□□(r=0.26, P=0.50). TNF□ correlated very strongly with Collagen-1(r=0.90, P<0.01) and -3(r=0.92, P<0.01) gene expression. IL-1□ strongly correlated with Collagen-3(r=0.83, P<0.01). Correlations between muscle fiber CSA and cytokines were weak(all r<0.34, P>0.34).

Expression of transforming growth factor-beta(TGF-□□□ did not differ between groups(Table 3). This was explained by variation on the injured side, which related to variation in adipose/connective tissue CSA; TGF-□□ expression correlated moderately with connective tissue(r=0.66, P=0.05) and adipose(r=0.62, P=0.07) CSA, and very strongly with Collagen-1 and -3 gene expression(both r=0.95, P<0.001).

Ubiquitin-Proteasome pathway gene expression

There were few differences in expression of the genes that compromise the ubiquitin-proteasome pathway for muscle atrophy(Table 3). Only Forkhead box O3(Foxo3) expression was upregulated at 3 and 6 months on the injured side.

Growth factor pathway gene expression

There was significant upregulation of the genes involved in the growth factor pathway for muscle hypertrophy in multifidus of the injured side at 6, but not 3 months. Mammalian target of Rapamycin(mTOR) expression correlated weakly with adipose CSA($r=0.56$, $p=0.11$).

Gene expression of molecules with putative role on muscle tissues

Calcineurin(CN) gene expression was increased in injured side muscle(Table 3) at 3 and 6 months. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha(PCG-1α) and myostatin gene expression increased at 6 months on the injured side. Myostatin gene expression moderately correlated with adipose CSA($r=0.71$, $p<0.04$).

Discussion

These detailed data of multifidus cellular/molecular changes after IVD injury discount muscle atrophy as a direct result of injury. This does not preclude a reduced contribution of multifidus to spine control[10,22], but implies mediation by structural remodeling of its constituent tissues rather than muscle fiber atrophy. Increased adipose, fibrosis and slow-to-fast muscle fiber transition and multifidus gene profiling provide putative remodeling mechanisms.

Muscle fiber remodeling, but not atrophy follow IVD injury

From clinical[1-3] and experimental[4] observations we presumed our analysis would reveal whole muscle/single fiber atrophy, and upregulation of atrophic molecular signaling pathways, but it did not. This concurs with augmented non-contractile element CSA rather than muscle in

human disc herniation(imaging)[14] and rabbit IVD injury(muscle fiber histology)[15]. How then, can evidence of reduced multifidus CSA[1-3] be explained? First, immediate large CSA reduction after selective IVD lesion in animals (~17%[4]) and within days after onset of unilateral LBP in humans (~31%[1]) might have a different mechanism(e.g. vascular effects; reflex inhibition), although large protein loss follows immobilization/dennervation[23]. Second, diffuse multifidus CSA reduction and fiber atrophy could either pre-exist(potential predisposition to LBP) or result from disuse/deconditioning[24,25] rather than a direct consequence of injury. Indications of whole muscle[3] and single fiber[26] atrophy involves cohorts of longer LBP duration(e.g. 240 months[26]). Present data of no atrophy at subacute(3 months) to early-chronic(6 months) phases, when IVD degeneration had peaked[19], indicate that if atrophy does develop later (after 6 months), it is not responsible for the earlier IVD degeneration, and unlikely to be mediated by acute effects of injury(e.g. reflex inhibition from IVD lesion, although that may explain a separate very early process[4,27]). Multifidus disuse, either by generalized deconditioning[25], or selective disuse secondary to altered motor patterns[22] are possible. Third, denervation of multifidus from nerve root compromise[28,29], causes atrophy[30] and has been confirmed by histological features[31]. This may be a longer-term consequence of IVD degeneration. Fourth, ultrasound cannot clearly delineate adipose/connective tissue from muscle. Increased ultrasound measures of multifidus CSA in chronic LBP[32] might have included adipose; whereas reduced CSA[1] might not. Fifth, reduced CSA may be a consequence of reference values. Reference to contralateral muscle[1] may exaggerate observations if reference muscle is larger for reasons other than pain/injury[33]. Comparison against a control group[3,13] or normative data[33] depends on the comparability of groups. Our data resolve many of these issues (clear histological delineation of tissue types; case controls with similar body size, genetic lineage and physical activity exposure) to conclude that IVD injury does not induce muscle atrophy in the sub-acute to early chronic period despite IVD

degeneration. Normal muscle fiber CSA also precludes muscle atrophy at this timepoint and agrees with other work[15].

Molecular data are also inconsistent with atrophy(Fig. 5); expression of the genes in the ubiquitin-proteosome pathway(major atrophy pathway[16]) were unchanged, and gene expression of the hypertrophic growth factor pathway increased, rather than decreased as would be predicted from atrophy[16](Fig. 5). The absence of hypertrophy, despite upregulation of genes in the growth factor pathway can be explained by reported inhibitory effects of TNF□□on the hypertrophic effects of signaling molecules(e.g. Protein Kinase B(Akt1); Phosphoinositide 3-kinase(PI3k)[34]). Studies using TNF□-blocking agents could test this theory

Slow-to-fast muscle fiber transformation of multifidus deep fascicles was bilateral, but more advanced on the injured side, as per whole muscle analysis[6]. New data show no transformation at 3 months, which indicates changes are not simply associated with surgical IVD exposure. This agrees with rabbit data at 12 weeks post-injury[15]. Unlike muscle, IVD pathology(loss of disc height, biomechanics, histopathology, and biochemistry) is maximal at 3 months in this model[19]. Muscle changes either develop more slowly than histopathological IVD changes at 3 months, or are secondary to later events.

Slow muscle fiber proportion reduces by selective slow fiber loss[35], fast fiber proliferation[36] and muscle fiber transformation[37]. Greater intermediate fiber proportion implies transformation in this model. Molecular mechanisms remain under-investigated, but may involve cytokines(Fig. 5). Although humoral TNF□ causes generalized muscle atrophy via the ubiquitin-proteasome pathway[38](which was unchanged), muscle-synthesized TNF□ promotes preferential fast fiber differentiation[39]. Further, myotubules preferentially develop into fast fibers when connective tissue expresses TGF-□1[36]. The correlation between TGF-□1 and adipose/connective tissue CSA supports this hypothesis, despite no difference in TGF-□1 between groups. Parallel

timecourses and pattern of cytokine and muscle changes(bilateral TNF□ and muscle fiber transformation) imply mediation by a slowly developing cytokine driven inflammatory process. Longitudinal human data show progressive elevation of serum pro-inflammatory cytokines as LBP persists[40].

Some data do not fully support this proposal. TNF□ affects muscle fiber transformation via NF-□B[39], but this molecule's expression was unchanged. This does not preclude increased availability and phosphorylation/activation of the molecule. Although PCG-1α prevents slow fiber atrophy and induce fast-to-slow transformation[41], action of PCG-1□ involves inhibition of Atrogin1[41](which was unchanged).

As an alternative, fiber transformation can be mediated by mechanotransduction mechanisms(see[42]) such as increased transcription of fast heavy chain myosin genes in absence of muscle stretch or force[43,44]. Multifidus stretch/load may be limited by modified movement[45] and muscle activity[22,27,46,47] as observed in human LBP, or reduced IVD height in this animal model[19]. Investigation of potential changes in loading is required.

A further consideration is increased CN and Foxo3 gene expression at 3 months, i.e. before multifidus structural remodeling. CN drives muscle regeneration after injury/unloading, particularly for slow fibers[48] and opposes adipose[49]/fibrosis[50] development(Table 1). Foxo3 inhibits fibrosis, but controls protein degradation[51] in muscle atrophy and regeneration[52]. These processes are inconsistent with the structural remodeling at 6 months, but may contribute to earlier muscle regeneration(Fig. 6).

Adipose remodeling following IVD lesion

Histological/molecular findings support increased back muscles adiposity in imaging[7], but to a lesser degree than more chronic human data (sheep: ~17% vs. human: ~31%[14]), and provide putative molecular mechanisms. Although a relationship to age rather than LBP/injury is

argued[53], our case-controlled data confirm an association to IVD injury. Although isolated to the injured side at 6 months, diffuse changes might develop later, as in humans[7](Fig. 6). Fatty infiltration has been argued as a response to muscle atrophy[54]; this is refuted by our evidence of increased adipose without atrophy. Others report rapid adipogenesis within days of IVD injury in pigs, and then no changes at 6 weeks in rabbits, but qualitative increase at 12 weeks in rabbits[15]. Consistent with this time variation, muscle injury induces adipogenesis within 3 days, that resolves by day 10[55]. Taken with present data, this implies multiple coexisting mechanisms for adipose changes, with different timecourses(Fig. 6).

What explains fatty infiltration at 6 months? Adipocytes have two primary sources – differentiation from progenitor mesenchymal stem cells[56], and transdifferentiation from myoblasts[57], under the influence of a variety of transcription factors(Fig. 5). These factors mediate shoulder muscle fatty infiltration after tendon injury[58] with Akt/mTOR signaling(Fig. 5), consistent with our data. Adipose CSA correlated with cytokine expression, particularly TNF□(major regulator of adipogenesis[59])(Fig. 5). Again the mechanism is complicated as TGF-□1[60], myostatin[61] and Akt1[62] can antagonize adipogenesis, and TNF□ can have a contradictory anti-adipogenic effects depending on concentration, timepoint and receptor expression[63], indicating further elucidation of the underlying molecular mechanisms is required. Mechanical loading is also implicated in adipogenesis. Unloading/decreased stretch upregulates adipogenic transcription factors[64] and reduces expression of factors that inhibit myoblast transdifferentiation to adipocytes[65](Fig. 5).

Connective tissue remodeling following IVD injury

Histological and molecular evidence of increased multifidus connective tissue extends previous qualitative description[15]. Muscle fibrosis is age associated[66] and necessary for muscle repair[67]. Excessive collagen may explain increased muscle fiber stiffness[15] and would influence

its contractile potential/loading, with consequences for adipogenesis and muscle fiber transformation(Fig. 5).

Molecular studies support putative mechanisms in fibrogenesis. Myogenic cells derived from satellite cells[68] transdifferentiate into profibrotic cells when TGF-□1 increases[67](Fig. 5). Strong correlation between TGF-□1 and connective tissue/adipose CSA supports this hypothesis. Myostatin promotes fibrosis by inducing fibroblasts to express extra-cellular matrix components such as Collagen-1 via the PI3k/Akt pathway[69] as found in our data(Fig. 5).

Myofibroblastic activation is required after muscle injury, but its role following injury to an adjacent IVD is unclear. The latency of development of fibrosis implies mediation by slowly developing pro-inflammatory cytokines. TNF□ causes myofibroblast accumulation and collagen deposition in other tissues[70]. Alternatively, long-term unloading increases fibrosis, and the converse of repetitive muscle activation induces delayed increases in Collagen-1 and TGF-□1 expression[71]. Changes in muscle load in our sheep model require investigation.

Methodological considerations

Sheep spine anatomy/biomechanics differ from humans and results may not directly apply to human LBP/injury. Similarities in pathology and gene expression of IVD degeneration[19] and muscle changes[6] between humans and our model suggest we have developed a good proxy for understanding consequences of IVD injury, although we cannot be certain of the pain experienced in animals. This study investigated gene expression of multiple molecular pathways with potential roles in muscle remodeling. Investigation of proteins and activation of the mediating pathways is now required.

Conclusions and clinical implications

Taken together with other data, our findings provide the foundation for a new understanding of multifidus structural remodeling after IVD injury; mediated by several

mechanisms with different timecourses(Fig. 6). Our findings argue for a sub-acute to chronic phase characterized by a probable pro-inflammatory cytokine mediated affect on slow-to-fast muscle fiber transformation and adipose/connective tissue proliferation, overlaid on separate very acute effects possibly involving reflex inhibition[27] and muscle regenerative processes(early adipogenesis[55] plus activation of hypertrophy/regeneration pathways[48]), and a later phase that may involve atrophy secondary to disuse(local or general) and/or denervation[26].

There are clinical implications. Optimal treatments may vary across the timecourse. Absence of muscle atrophy in the sub-acute to chronic period questions the role of exercise for hypertrophy(e.g.[72]) in this phase. Specific loading with exercise may have a therapeutic role to signal molecular mechanisms promoting structural improvements[42,58], as supported by evidence of muscle changes induced by exercise[73,74]. Molecular mechanisms expose pharmacological targets to prevent/reverse structural remodeling either generally(e.g. targeting cytokines) or in a tissue-specific manner(e.g. mTOR-mediated adipose changes[58]). The model of overlapping but unique mechanisms highlights the need to targeting the right treatment to the right patient and the right time.

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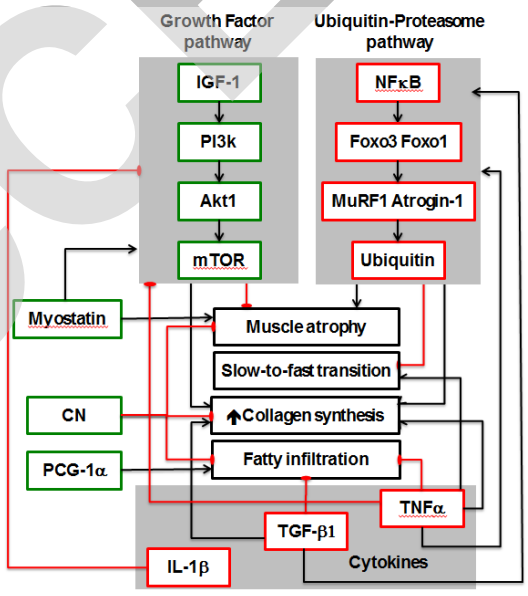

Fig. 1 Key putative mechanisms for structural remodeling of muscle(atrophy and slow-to-fast fiber transformation), fat(fatty infiltration) and connective tissue(fibrosis). Key cytokines and

signaling molecules are shown. Red lines indicate inhibition and black lines indicate facilitation.

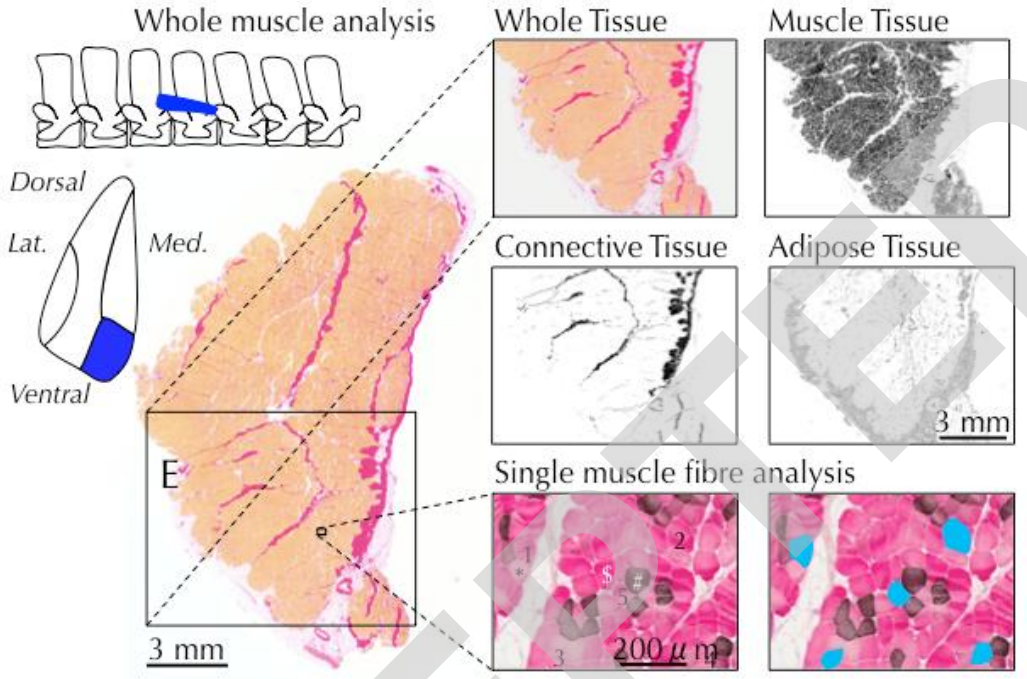

Fig. 2 Methods for histological analysis of muscle tissue. Whole muscle sections were mounted(left panel) and strained with Masson's Trichrome and Van Giesen's stain(shown) to delineate tissue types(top right panels). Image processing with photoshop aided separation of tissue types for measurement of cross-sectional area(CSA). Single muscle fiber types were identified based on immunohistochemical techniques(#-slow fiber; $ - fast fiber; * - intermediate fiber). Region of deep fascicle of multifidus selected for analysis is shown anatomically and in transverse section(top left). For analysis of CSA five-ten fibers of each type were identified(see bottom right for example numbered 1-5) in each of 6 randomly selected 620x400μm images in the deep fascicle of the muscle.

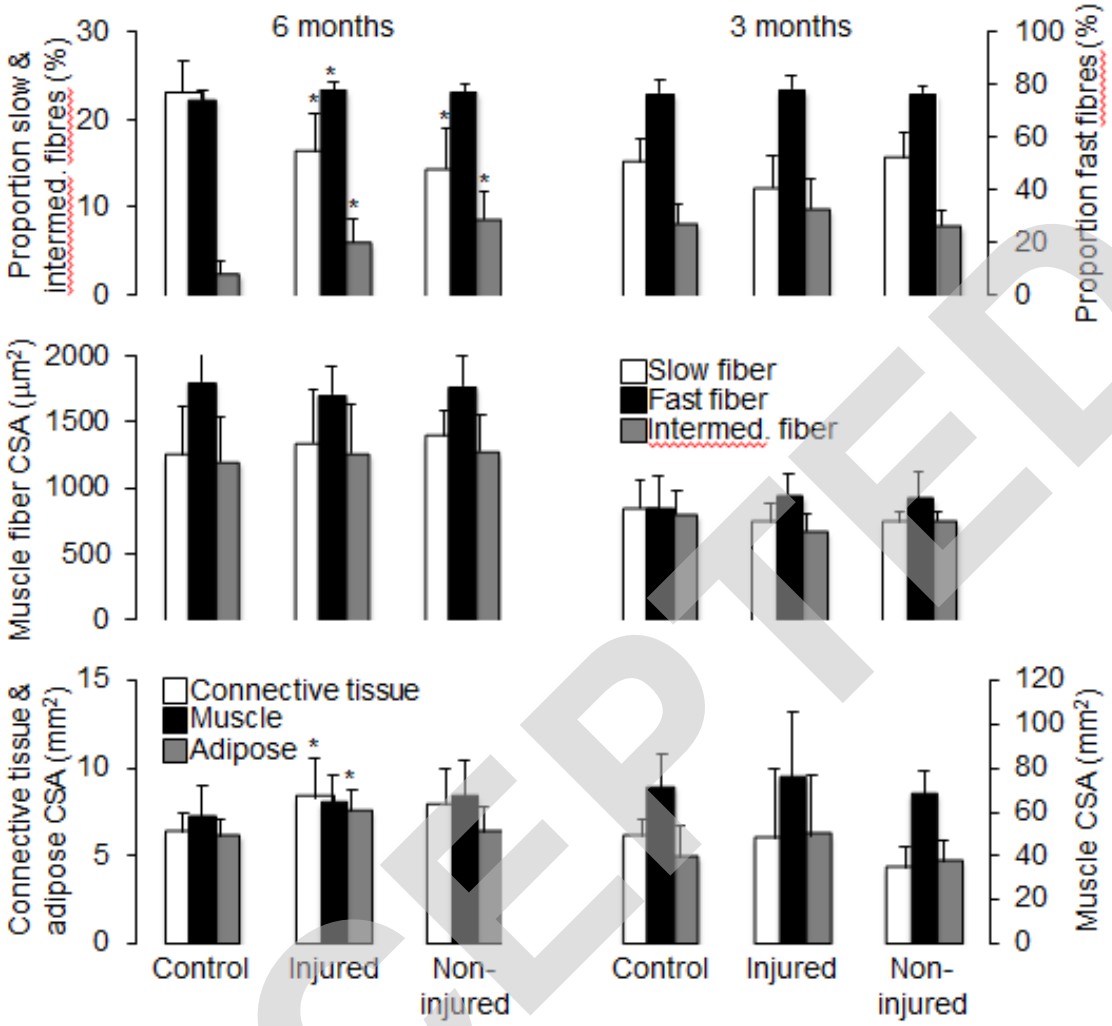

Fig. 3 Results of analysis of whole muscle and single fiber histology. Group mean and SD are shown for CSA of each tissue type, along with CSA and proportion of each muscle fiber type. * - $P<0.05$ relative to muscle from the control animals. Data are shown for 3 and 6 months. Note the different scale for proportion of fast fibers in the top panel and muscle CSA in the bottom panel. Intermed – intermediate, CSA – cross sectional area.

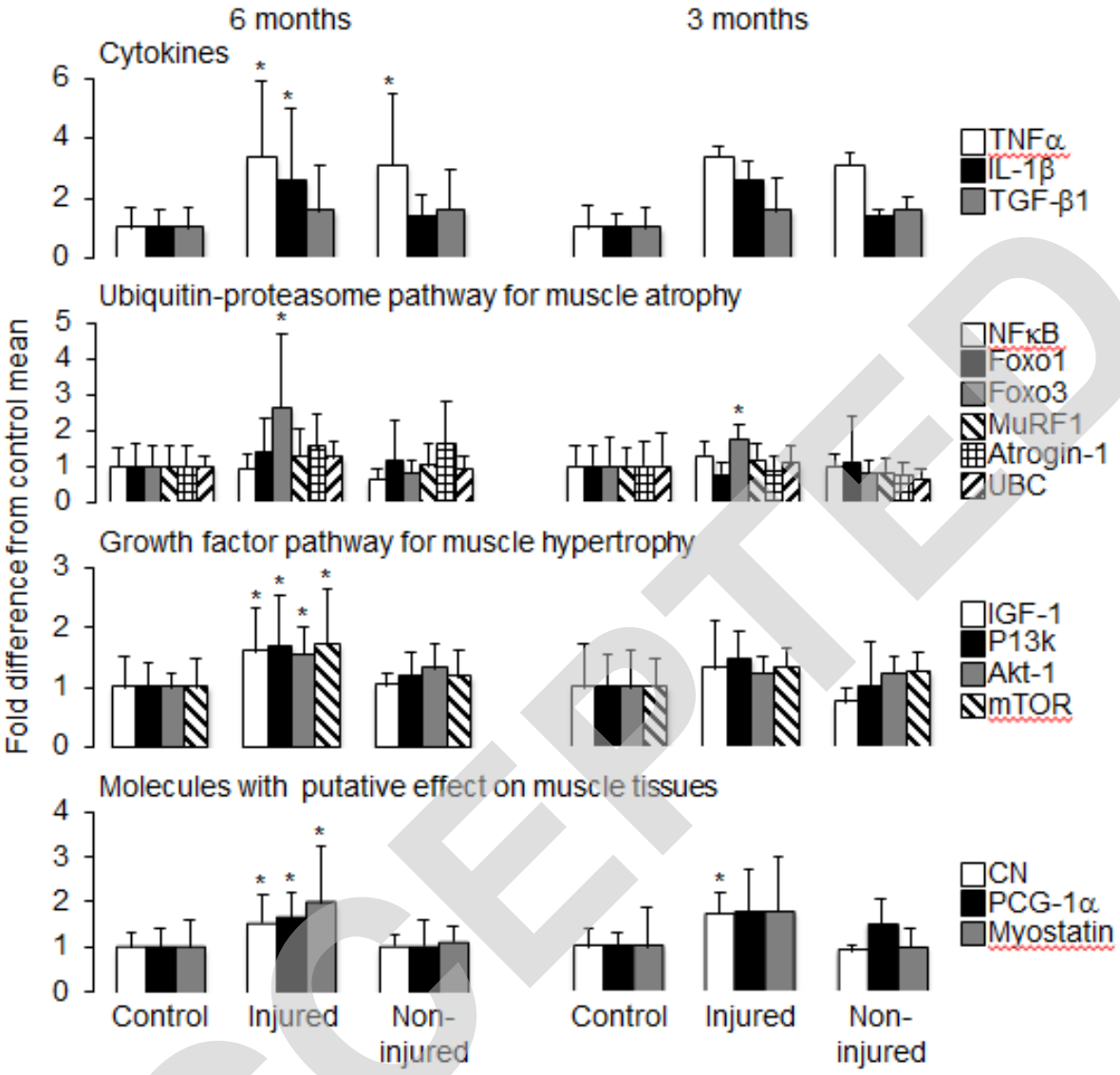

Fig. 4 Results of analysis of gene expression levels. Data are shown as mean and SD for each animal group. * - $P<0.05$ relative to muscle from the control animals. Data are shown for 3 and 6 months. See Table 1 for definition of abbreviations.

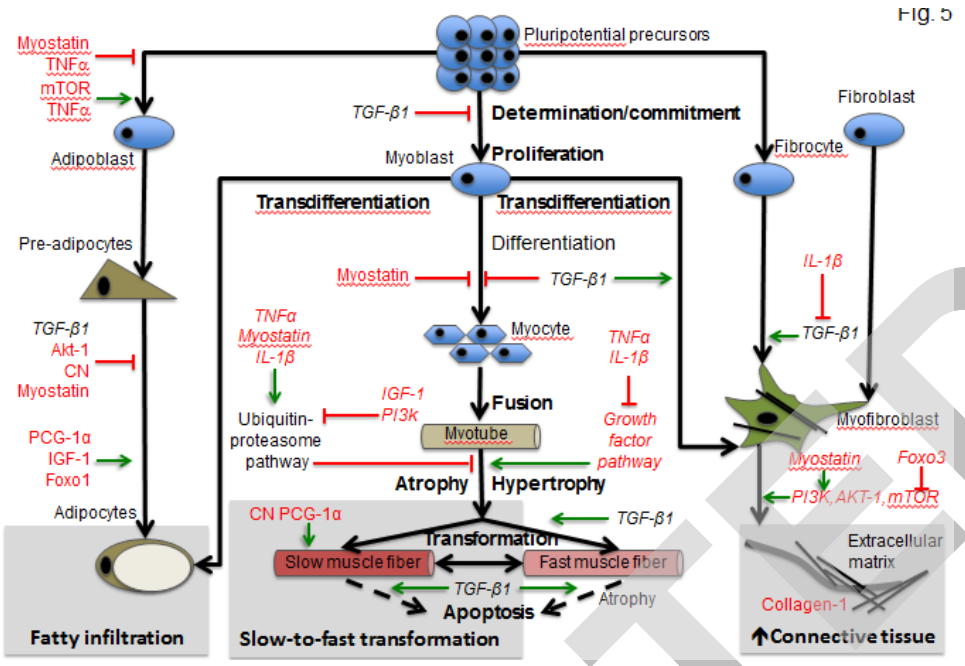

Fig. 5 Summary of mechanisms based on study findings. Mechanisms for structural remodeling of each tissue type are shown along with the cytokines and signaling molecules with upregulated gene expression. See Table 1 for abbreviation definitions. Molecules and pathways that differed between control and injury groups are presented in "red", and those presented in black did not differ between groups, but have relevance for the interpretation of effects.

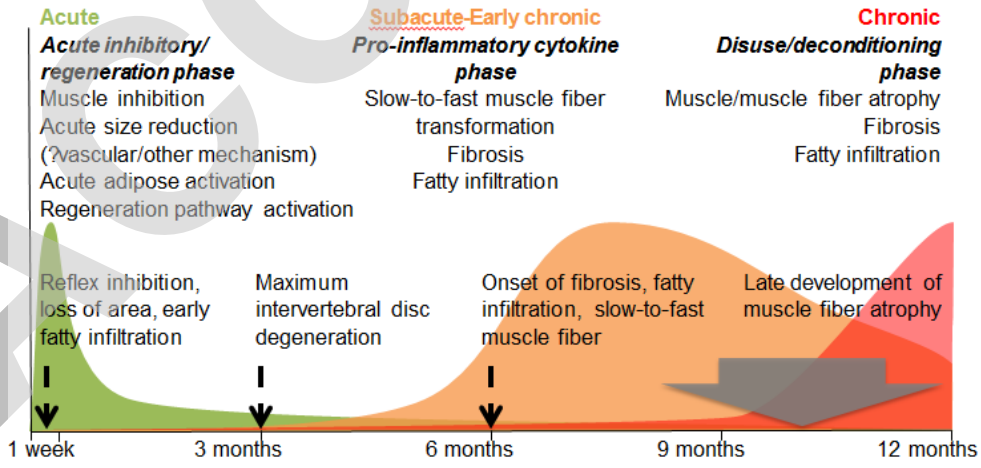

Fig. 6 Conceptual model of the interaction and overlap between the multiple putative mechanisms for structural remodeling of multifidus after intervertebral disc injury. Each

mechanism have a different time course, different physiological basis and different consequences for multifidus structure.

Table 1 Cytokines and signaling molecules involved in muscle, adipose and connective tissue regulation

Molecule	Description	Effect on muscle	Effect on adipose	Effect on connective tissue
CYTOKINES				
TNFα	Pro-inflammatory cytokine	**(Hypertrophy/atrophy)** - Chronically elevated humoral TNF□ regulates muscle atrophy via the ubiquitin pathway[38] - Muscle-synthesized TNF□ promotes muscle adaptation and fast muscle fiber expression[39] - Regulates IGF mediated hypertrophy [75] - Concentration dependent role in myogenesis promotion/inhibition [76]	**(□/□ Adipogenesis)** Promotes[77] and inhibits[63] adipogenesis through ERK1/2 pathway - Regulates expression of PPARγ(major regulator of adipogenesis) [59]	**(□ Fibrosis)** - Increases intestinal myofibroblast proliferation and Collagen-1 accumulation[70] - Blocking TNF□ reduces fibrosis in mouse model of Duschene muscular dystrophy[78]
Interleukin-1beta(IL-1β)	Pro-inflammatory cytokine	**(Hypertrophy/atrophy)** - Role in early phases of myogenesis [79] - Inhibits IGF-1 induced myoblast differentiation and downstream signaling[80]	**(Unknown)**	**(□ Fibrosis)** - Reduction → fibrosis - Inhibits TGF-□1-mediated myofibroblast formation and inc. Collagen-1 expression in fibroblasts[81]
Transforming Growth Factor Beta(TGF-β1)	Cytokine, member of TGF superfamily	**(Atrophy/slow-to-fast transformation)** - Inhibits satellite cell proliferation and myofiber fusion[82] - Causes myogenic cell apoptosis[67] - Causes myotubes to mature into fast muscle fibers[36]	**(□ Adipogenesis)** - Antagonizes adipose cell differentiation[60] - Promotes chondrocyte/ myofibrocyte differentiation at expense of adipocytes[83]	**(□ Fibrosis)** - Major pro-fibrotic growth factor - Induces differentiation of myocytes into myofibroblasts[67], up-regulating collagen synthesis
UBIQUITIN-PROTEASOME PATHWAY FOR MUSCLE ATROPHY				
Nuclear factor kappa	Transcription factor	**(Atrophy /fast-to-slow transformation)** - Mediates ubiquitin–	**(□ Adipogenesis)** - Mediates TNFα role in adipogenesis[59]	**(□ Fibrosis)** - Inhibits Collagen-1 transcription in cell

B(NF□B)		proteasome system in cachexia induced atrophy[84] - Fast fiber atrophy → fast-to-slow transformation[17] - Reduction → inc. muscle regeneration[85]		culture[86]
Forkhead box O1(Foxo1)	Transcription factor for muscle atrophy	**(Atrophy/slow-to-fast transformation)** - Regulates protein degradation by activating Atrogin-1[87] - Overexpression → reduces muscle fiber CSA and slow fiber number[35]	**(□ Adipogenesis)** - Overexpression → prevents differentiation of preadipocytes into adipocytes[88]	**(□ Fibrosis)** - Component of the AMPK pathway that increases collagen-1 expression in myocytes after EtOH exposure [89]
Forkhead box O3(Foxo3)	Transcription factor for muscle atrophy	**(Atrophy)** - Regulates protein degradation in disuse atrophy - denervation/ immobilization[90] via proteasomal/autophagic systems[51]	**(Unknown)**	**(□ Fibrosis)** - Inhibits fibroblast proliferation, in a PI3k/Akt pathway dependent manner[91]
Muscle RING-finger protein-1(MuRF1 or TRIM63)	E3 ubiquitin ligase	**(Atrophy)** - Promotes muscle protein degradation(atrophy) downstream of NFκB[92]	**(Unknown)**	**(□ Fibrosis)** - Reduction → dysregulates Calcineurin function leading to fibrosis of cardiac muscle[93].
Atrogin-1/Muscle atrophy F-box protein(MAFbx)	E3 ubiquitin ligase	**(Atrophy)** - Activated by TNFα signaling pathway - Mediates protein degradation and muscle atrophy[94] - Loss protects muscle from atrophy[95]	**(Unknown)**	**(□ Fibrosis)** - Increased expression in fibrotic muscle when treated with TGF-β1[96]
GROWTH FACTOR PATHWAY FOR MUSCLE HYPERTROPHY				
Insulin-like Growth Factor 1(IGF-1)	Primary mediator of growth hormone	**(Hypertrophy)** - Regulates muscle growth/development/ regeneration though PI3k/Akt1 pathway[34] - Down-regulated in muscle wasting[97]	**(□ Adipogenesis)** - Increases pre-adipocyte replication and differentiation[49]	**(□ Fibrosis)** - Accelerates regeneration of injured skeletal muscle by limiting fibrosis[98]
Phosphoinositide 3-kinase(PI3	Enzyme in the PI3k/Akt	**(Hypertrophy)** - Activates muscle hypertrophy in regenerating	**(□ Adipogenesis)** - Activation required for pre-adipocyte	**(□ Fibrosis)** - Promotes fibrosis in acute muscle injury through

k)	signalling pathway	muscles by inhibiting ubiquitin/proteasome pathway[35] - Reduction → protein degradation and reduced protein synthesis[35]	differentiation[99] - Reduced expression prevents adipogenesis[99]	Akt/mTOR pathway and Myostatin's effect on fibrosis - Blocking reduces fibrosis[100]
Protein Kinase B(Akt-1)	Protein kinase	**(Hypertrophy)** - Over expression → hypertrophy by preventing MuRF1 induction[35] - Reduction → protein degradation/ reduced protein synthesis(atrophy)[35] - Role in myoblast differentiation	**(□ Adipogenesis)** - Activation reduces adipose [62] - Regulates Foxo1 during adipogenesis[88]	**(□ Fibrosis)** - Upregulated after muscle injury, promoting fibrosis with PI3k/mTOR[100] - Regulates Myostatin's effect on fibrosis[101]
Mammalian target of Rapamycin (mTOR)	Serine/threonine protein kinase	**(Hypertrophy/?slow-to-fast transformation)** - Increases protein synthesis in muscle cells – hypertrophy - Reduction → fast-to-slow fiber type transformation and inhibited protein synthesis(atrophy)[102-104] - Conflicting data of role in myogenesis[105]	**(□ Adipogenesis)** - Mediates fatty infiltration in muscle via SREBP-1 and PPAR□ [58] - Role in adipogenesis of MSCs by interaction with PPARγ[106]	**(□ Fibrosis)** - Expression increased after injury, promoting fibrosis with PI3k/Akt-1[100] - Regulates Collagen-1 expression in dermal fibroblasts[107]
MOLECULES WITH PUTATIVE EFFECT ON MUSCLE TISSUES				
Calcineurin(CN)	$Ca2^{+}$-dependent serine/ threonine phosphatase	**(Hypertrophy/fast-to-slow transformation)** - Downstream role in muscle hypertrophy[108] - Regulates muscle fiber type specification(fast-to-slow transformation)[109]	**(□ Adipogenesis)** - Inhibits adipocyte differentiation(prevents expression of transcription factors required for adipocyte differentiation(PPAR□; C/EBP□)[49]	**(□ Fibrosis)** - Attenuates fibrosis/degeneration of muscle if present[50] - Mediates fibrosis in conjunction with MuRF1 in cardiac muscle[93]
Peroxisome proliferator-activated receptor gamma coactivator 1-alpha(PCG-1α)	Transcriptionco-activator	**(Hypertrophy/fast-to-slow transformation)** - More in slow muscle fibers → slow fiber development/hypertrophy[110] - Prevents slow fiber atrophy by inhibiting Atrogin1/MAFbx expression[41] - Overexpression → fast-to-slow fiber transformation[111]	**(□ Adipogenesis)** - Co-activates PPARγ, the master regulator of adipogenesis[112]	**(Unknown)**
Myostatin	Member	**(Atrophy)**	**(□ Adipogenesis)**	**(□ Fibrosis)**

	of TGF superfamily	- Major role to suppress skeletal muscle growth and development - Enhances muscle protein breakdown via ubiquitin-proteasome system[87] - Reduces myoblast differentiation/ myotube size by blocking PI3k/Akt/mTOR pathway[113]	- Inhibits adipogenesis in human MSC/preadipocytes [61]	- Stimulates muscle fibroblast proliferation through - PI3k/Akt pathway → increases Collagen in fibroblasts[69]

AMPK - AMP-dependent protein kinase; C/EBP□□□□ CCAAT/enhancer-binding proteins; CSA – Cross sectional area; ERK1/2 - extracellular signal-regulated kinase 1/2; MCS – Mesenchymal stem cells; PPAR□□□□ peroxisome proliferator–activated receptors; SREBP-1 - sterol regulatory element binding protein 1.

Table 2 Genes, primer sequences, and amplicon sizes

Gene	Primer Sequence	T (°C)	Size (bp)
COLLAGEN			
Collagen-1	F: 5'- GACATCCCACCAGTCACCTG -3' R: 5'- GGGACTTTGGCGTTAGGAC -3'	56	161
Collagen-3	F: 5'- GGTCAGCCTGGCGTCATGGG -3' R: 5'- GACCTCCAGGGCCACCTCGT -3'	58	88
CYTOKINES			
TNF	F: 5'- AACAGGCCTCTGGTTCAGACA -3' R: 5'- CCATGAGGGCATTGGCATAC -3'	60	133
IL-1β	F: 5'- TCACAGGAAATGAGCCGAGAA -3' R: 5'- CAGCTGCAGGGTCGGTGT-3'	60	150
TGF-β1	F: 5'- TTACAACAGTACCCGCGACC -3' R: 5'- GAGCTCGGACGTGTTGAAGA -3'	58	181
UBIQUITIN-PROTEASOME PATHWAY FOR MUSCLE ATROPHY			
NFκB	F: 5'- GTCTCCTGGAGCCTCAAACC -3' R: 5'- CCTCGTAGAACCGAACCTCG -3'	58	131
Foxo1	F: 5'- TACCATTAGCGGGAGGCTCT -3' R: 5'- GGTTCTCCATGTTCTCGGGG -3'	58	155
Foxo3	F: 5'- AGAAGTTCCCCAGCGACTTG -3' R: 5'- TCCCCACGTTCAAACCAACA -3'	58	159
MuRF1	F: 5'- AGGTTGATACAGCTGCCCAC -3' R: 5'- CTCGGTCACCGTCCCTTATG -3'	58	122
Atrogin-1	F: 5'- AGGCTGGACTTCTCAACTGC -3' R: 5'- CCACTCAGGGATGTGAGCTG -3'	56	101
Ubiquitin C	F: 5'- TCGTCTTAGGGGTGGCTGTT -3'	56	76

	R: 5’- GCTAGAGTGCAGAACGATGC -3’		
GROWTH FACTOR PATHWAY FOR MUSCLE HYPERTROPHY			
IGF-1	F: 5’- GGATGCTCTCCAGTTCGTGT -3’ R: 5’- TGAGAGGCGCACAGTACATC -3’	56	161
PI3k	F: 5’- CGGCTTTTTCAACCCTTTTTAAA -3’ R: 5’- CATGCCGATAGCAAAACCAAT -3’	58	93
Akt1	F: 5’- CTGGATGCGTTTGGGTGGAA -3’ R: 5’- GCGTCTGGAGAACTGGATGA -3’	58	167
mTOR (FRAP1)	F: 5’- CGGCAACTTGACCATCCTCT-3’ R: 5’- TGCTGGAAGGCGTCAATCTT-3’	56	101
MOLECULES WITH PUTATIVE EFFECT ON MUSCLE TISSUES			
CN	F: 5’- GGATTTGATGGAGCAACGGC -3’ R: 5’- CAGCGTCAGCACACTTTCAC -3’	58	123
PCG-1α	F: 5’- AATGCAGTGGCCTCAGTACC -3’ R: 5’- TTGAGAAGCTCTGAGCACGG -3’	56	157
Myostatin	F: 5’- ACCCAGGCACTGGTATTTGG -3’ R: 5’- TCTCCTGGTTCTGGGAAGGT -3’	58	148
HOUSEKEEPING GENE			
GAPDH	F: 5’- CCTGGAGAAACCTGCCAAGTATG -3’ R: 5’- GGTAGAAGAGTGAGTGTCGCTGTTG -3’	58	139

Table 3 P-values for statistical analysis of gene expression

	6 month		**3 month**	
	ANOVA	**Post-hoc**	**ANOVA**	**Post-hoc**
COLLAGEN				
Collagen-1	0.024	C vs. I: 0.02 C vs. NI: 0.52	0.273	
Collagen-3	0.803		0.147	
CYTOKINES				
TNF□	0.017	C vs. I: 0.03 C vs. NI: 0.05	0.824	
IL-1β	0.047	C vs. I: 0.05 C vs. NI: 1.00	0.310	
TGF-β1	0.408		0.623	
UBIQUITIN-PROTEASOME PATHWAY FOR MUSCLE ATROPHY				
NFκB	0.115		0.462	
Foxo1	0.619		0.686	
Foxo3	0.004	C vs. I: 0.01 C vs. NI: 1.00	0.025	C vs. I: 0.10 C vs. NI: 1.00

MuRF1	0.585		0.440	
Atrogin-1	0.263		0.647	
Ubiquitin C	0.128		0.468	
GROWTH FACTOR PATHWAY FOR MUSCLE HYPERTROPHY				
IGF-1	0.026	C vs. I: 0.04 C vs. NI: 1.00	0.331	
PI3k	0.035	C vs. I: 0.04 C vs. NI: 1.00	0.282	
Akt1	0.007	C vs. I: 0.01 C vs. NI: 0.19	0.594	
mTOR (FRAP1)	0.05	C vs. I: 0.03 C vs. NI: 0.41	0.324	
MOLECULES WITH PUTATIVE EFFECT ON MUSCLE TISSUES				
CN	0.010	C vs. I: 0.02 C vs. NI: 1.00	0.006	C vs. I: 0.02 C vs. NI: 1.00
PCG-1α	0.010	C vs. I: 0.03 C vs. NI: 0.26	0.139	
Myostatin	0.026	C vs. I: 0.03 C vs. NI: 0.60	0.245	