Gene Expression Patterns Analysis in the Supraspinatus Muscle after a Rotator Cuff Tear in a Mouse Model

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Rotator cuff tear is a muscle-tendinous injury representative of various musculoskeletal disorders. In general, rotator cuff tear occurs in the tendon, but it causes unloading of the muscle resulting in muscle degeneration including fatty infiltration. These muscle degenerations lead to muscle weakness, pain, and loss of shoulder function and are well known as important factors for poor functional outcome after rotator cuff repair. Given that rotator cuff tear in various animal species results in similar pathological changes seen in humans, the animal model can be considered a good approach to understand the many aspects of the molecular changes in injured muscle. To comprehensively analyze changes in gene expression with time following a rotator cuff tear, we established a rotator cuff tear in mouse supraspinatus tendon of shoulder. At weeks 1 and 4 after the tear, the injured muscles were harvested for RNA isolation, and microarray analysis was performed. Expression patterns of genes belonging to 10 muscle physiology-related categories, including aging, apoptosis, atrophy, and fatty acid transport, were analyzed and further validated using real-time PCR. A total of 39,429 genes were analyzed, and significant changes in expression were observed for 12,178 genes at 1 week and 2,370 genes at 4 weeks after the tear. From the list of top 10 significantly up- and downregulated genes at the 2 time periods and the network evaluation of relevant genes according to the 10 categories, several important genes in each category were observed. In this study, we found that various genes are significantly altered after rotator cuff tear, and these genes may play key roles in controlling muscle degeneration after a rotator cuff tear.

1. Introduction

Identifying the molecular mechanisms underlying changes in the rotator cuff muscle after a rotator cuff tear would be the first step and an important determinant in understanding the rotator cuff tear-related muscle changes and improving outcomes of rotator cuff repair surgery. Several studies have suggested various causes and mechanisms for muscle changes, such as atrophy and degeneration [9, 10, 21–25]; however, despite this, much remains unknown. Progression of muscle degeneration, atrophy, and fatty infiltration is usually caused by abnormal signaling processes in muscle cells [25]. Abnormal muscle cell activities are the results of external stimuli, such as physical damage or aging, which lead to differentiation into...
fat cells or fibrous tissue and ultimately myocyte destruction (instead of normal myocyte differentiation) [26]. Most of these cellular activities are directly related to intracellular gene regulation. Therefore, if we know how genes are regulated in muscle cells in response to external stimuli, it would be possible to understand the mechanism(s) underlying pathologic rotator cuff muscle changes after a rotator cuff tear. Further, this may also provide important information for the treatment of rotator cuff tears by controlling the expression of relevant genes. To date, only a few genes, such as those coding for p38 mitogen-activated protein kinase-activated kinase (MAP-kinase), myogenin, myostatin, and matrix metalloproteinases (MMPs), have been identified in relation to muscle changes after rotator cuff tears [20, 21, 24, 27]. However, to the best of our knowledge, a comprehensive analysis of the time-dependent changes in gene expression patterns after rotator cuff tears has not been reported.

Therefore, this study aimed to comprehensively analyze the patterns of gene expression in rotator cuff muscles with time after a rotator cuff tear (acute or chronic) by categorizing muscle physiology-related genes in a mouse model. In this study, we hypothesized that rotator cuff injury may cause alterations in gene expression regarding pathophysiology of rotator cuff muscle. Our study may improve understanding of molecular events after rotator cuff injury and may help the identification of novel regulation or control way to overcome the poor functional outcome after rotator cuff repair.

2. Materials and Methods

2.1. Animal Experiment. Eight-week-old male C57BL/6 mice (Orient Bio Inc., Seongnam, Korea) were used in this study. Before beginning the experiments, the mice were acclimated to a 12:12-h light/dark cycle at 22 ± 2°C for 1 week and allowed unlimited access to food and water.

We generated the rotator cuff tear model in mice as described previously [10]. Briefly, the supraspinatus tendon of the right shoulder of each mouse was fully exposed and completely transected from the greater tuberosity of the humerus under anesthesia using Zoletile (30 mg/kg; Virbac, Carros, France) and Rompun (10 mg/kg; Bayer Korea Ltd., Seoul, Korea). The supraspinatus of the left shoulder served as a control. A 3-0 nylon suture was used to close the skin, and the mice were allowed unrestricted cage activity. At weeks 1 and 4 after the surgery, mice (n = 4 per time interval) were sacrificed by cervical dislocation, and the supraspinatus muscles of both shoulders were completely harvested from the scapular fossa. The muscle tissue samples were used for total RNA extraction. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Konkuk University (IACUC: KU17122) and were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.2. RNA and Gene Expression Profiling. RNA quality was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, USA), and its quantity was determined using the ND-1000 spectrophotometer (NanoDrop Technologies, USA). The total RNAs from each supraspinatus muscle at different phase (1 or 4 weeks) after injury were pooled and used for microarray. Gene expression analyses were performed using the global Affymetrix GeneChip® Human Gene 2.0 ST oligonucleotide arrays. About 300 ng of each RNA sample was used for the Affymetrix procedure, as recommended by the manufacturer (http://www.affymetrix.com). Briefly, 300 ng total RNA from each sample was converted to double-strand cDNA. Using a random hexamer incorporating a T7 promoter, amplified RNA (cRNA) was generated from the double-stranded cDNA template through an IVT (in vitro transcription) reaction and purified with the Affymetrix Sample Clean-up Module. cDNA was synthesized by random-primed reverse transcription using a dNTP mix containing dUTP. Next, cDNA was digested using the UD G and APE 1 restriction endonucleases, and end-labeled using the terminal transferase reaction incorporating a biotinylated deoxynucleotide. The fragmented end-labeled cDNA was hybridized to the GeneChip® Human Gene 2.0 ST arrays for 16 h at 45°C and 60 rpm, as described in the Gene Chip Whole Transcript (WT) Sense Target Labeling Assay manual (Affymetrix). After hybridization, the chips were stained using Streptavidin Phycoerythrin (SAPE), washed in Genechip Fluidics Station 450 (Affymetrix), and scanned using GeneChip Array Scanner 3000 7G (Affymetrix).

Ten gene categories that have direct or indirect effects on muscle physiology in relation to rotator cuff tears were selected. The categories were as follows: (1) aging, (2) inflammation, (3) apoptosis, (4) neovascularization, (5) extracellular matrix composition, (6) myocyte differentiation, (7) myocyte proliferation, (8) cellular migration, (9) fatty acid transport, and (10) muscle atrophy. Every gene expression values were measured, and among them, those with the fold change of rotator cuff tear group/control group were more than 2 or less than 1/2 with the raw values (log 2) of more than 4 were defined as significant genes and further analyzed. Especially, the aging-, apoptosis-, fatty acid transport-, and muscle atrophy-related categories were analyzed in detail because these categories are known to be closely associated with muscle changes, such as degeneration, fatty infiltration, and atrophy, after rotator cuff tear [10, 22, 23].

2.3. STRING Network. The genes showing significant changes in expression were selected and used as the input for STRING (Search Tool for the Retrieval of Interacting Genes/Proteins; https://string-db.org/). Protein network analyses were performed. The database and web-tool STRING is a meta-resource that integrates most of the available information on protein–protein associations, and scores, weights, and augments it with predicted interactions, as well as with the results of automatic literature mining searches [28]. Using this, we obtained the protein interaction network images associated with functional enrichment [29]. Information regarding the size of each node and edges between nodes is shown in Figure 1 (EBI OGEN Inc., Korea).

2.4. Data Analysis. After the final washing and staining step, the Affymetrix GeneChip® Human Gene 2.0
ST oligonucleotide array was scanned using Affymetrix Model 3000 G7 Scanner and the image data was extracted using the Affymetrix Command Console software v1.1. The raw.cel file generated after the above procedure showed the expression intensity data and was used for the next step. Expression data were generated by the Affymetrix Expression Console software version 1.1. For normalization, the Robust Multi-Average (RMA) algorithm implemented in the Affymetrix Expression Console software was used. In order to find the coexpressing gene groups (which had similar expression patterns), we performed hierarchical clustering in the MultiExperiment Viewer software v4.4 (MEV; www.tm4.org). The web-based tool DAVID (Database for Annotation, Visualization, and Integrated Discovery; http://david.abcc.ncifcrf.gov/home.jsp) was used to perform biological interpretation for the differentially expressed genes. Next, these genes were classified based on the information about their functions in Gene Ontology in the KEGG Pathway database.

2.5. Quantitative Reverse Transcription (qRT) PCR Analysis. To validate the gene expression analysis results obtained using the microarray process described above, we performed qRT-PCR analysis for several representative genes. Total RNA was extracted from the supraspinatus muscles using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions, and used for cDNA synthesis using the Maxime RT PreMix kit (iNtRON Biotechnology, Korea). The qRT-PCR analysis was carried out using Light Cycler 480 System (Roche Diagnostics, Swiss) with 2 × qPCR BIO SyGreen Mix Lo-ROX (PCR Biosystems, London, UK). All expression data were normalized to actin expression.

2.6. Statistical Analysis. Descriptive statistics were used to present the analyzed data in this study.

3. Results
A total of 39,429 genes were analyzed. Among these, 9,696 genes were associated with muscle physiology. Significant changes in expression were observed for 12,178 genes during the acute phase and 2,370 genes during the chronic phase. The number and distribution of genes expressed per category are shown in Figure 2 and Table 1.

From the Venn diagram of genes showing significantly different expression at weeks 1 and 4 after the rotator cuff tear, we could identify 115 genes which showed a reverse expression pattern (genes that increased during the acute phase but decreased during the chronic phase or genes that decreased during the acute phase but increased during the chronic phase) (Figure 3).

Overall, the most highly expressed gene at week 1 was keratin 18, which increased 217.8 times compared to the control. This gene decreased 22.1 times compared to the control at week 4. Signal-regulatory protein beta 1B (Srpib1b) and keratin 8 also displayed higher expression (more than 100 times) than the control at week 1, then subsequently decreased (expression: 4 and 16 times higher than the control, respectively) at week 4. The overall up- and downregulated genes and fold-change values of rotator cuff tear/control at weeks 1 and 4 are listed in Table 2.

3.1. Gene Expression Patterns in the Aging Category. Analysis of the up- and downregulated genes in the 10 categories showed that insulin-like growth factor binding protein 2 (Igfbp2) was the most highly expressed gene in the aging category. It displayed 55.7 times higher expression in the RC tear side than in the control at week 1, and subsequently showed a decreasing trend (expressed 5.2 times higher than control) at week 4. In the string network which depicts the interactions between genes, we observed that IL6, Ccl2, and Vcam1, which are known to be related to inflammation or cell adhesion, actively interacted with each other showing high expression at week 1 after the RC tear. In addition, RAD54L (a DNA repair-related gene) and cyclin-dependent kinase 1 (Cdk1; a cell cycle regulation-related gene) also interacted closely (Figure 4). The top 10 up- and downregulated genes in the aging category at weeks 1 and 4 after the RC tear are listed in Table 3.
3.2. Gene Expression Patterns in the Apoptosis Category. In the apoptosis category, keratin 18 showed the highest expression (217.8 times higher expression than the control group) at week 1. This expression subsequently decreased (22 times higher expression than the control group) at week 4 (Figure 5 and Table 4). Interestingly, Birc5 (55.6 and 1.6 times higher expression than control at weeks 1 and 4, respectively), Rps6ka2 (0.2 times and 0.5 times higher expression than control at weeks 1 and 4, respectively), and Bub1 (37.5 and 0.3 times higher expression than control at weeks 1 and 4, respectively), which also showed reverse expression patterns, presented active interactions with each other revealed by the STRING network analysis results of week 1. These genes are known to be involved in apoptotic processes, such as cell growth, cell cycle, and cell differentiation.

3.3. Gene Expression Patterns in the Muscle Atrophy Category. Among the 39,429 genes analyzed, a total of 11 genes belonged to the muscle atrophy category (Figure 6 and Table 5). Myog (myogenin), which is associated with myogenesis, showed the highest expression at week 1. In contrast, Mstn (myostatin), which is to inhibit myogenesis, showed the lowest expression at week 1. Further, Actin 3 (actin alpha 3), which is coexpressed with myostatin, also showed low expression at week 1 but a high expression at week 4 together with myostatin. This
Table 1: Summary of the rotator cuff tear-induced expression for genes belonging to 10 categories at (a) 1 and (b) 4 weeks.

(a)

| I week | Total | Aging | Angio. | Apop. | Diff. | Migr. | Prolif. | ECM | Infl. | FA tr. | Atro |
|--------|-------|-------|--------|-------|-------|-------|--------|-----|------|--------|------|
| Total gene number | 39,429 | 370 | 443 | 1,100 | 4,701 | 994 | 788 | 683 | 532 | 66 | 19 |
| % of Total | 100 | 0.94 | 1.12 | 2.79 | 11.92 | 2.52 | 2 | 1.73 | 1.35 | 0.17 | 0.05 |
| Up significant (n) | 8,662 | 127 | 174 | 378 | 1,422 | 262 | 249 | 0 | 3 |
| % of Up significant | 22 | 34.3 | 39.3 | 34.4 | 30.2 | 38.8 | 33.2 | 38.7 | 46.8 | 15.2 | 15.8 |
| Down significant (n) | 3,516 | 40 | 30 | 113 | 436 | 75 | 54 | 31 | 13 | 9 |
| % of Down significant | 8.9 | 10.8 | 6.8 | 10.3 | 9.3 | 7.5 | 5.7 | 7.9 | 5.8 | 19.7 | 47.4 |
| Total significant (n) | 12,178 | 184 | 203 | 525 | 1,867 | 463 | 300 | 267 | 278 | 23 | 11 |
| % of Total significant | 30.9 | 45.1 | 46 | 44.6 | 39.5 | 46.4 | 39 | 46.6 | 52.6 | 34.8 | 63.2 |

Angio., angiogenesis; Apop., apoptosis; Diff., cell differentiation; Migr., cell migration; Prolif., cell proliferation; ECM, extracellular matrix; Infl., inflammation; FA tr., fatty acid transport; Atro., muscle atrophy.

Up significant means the genes with the fold change of rotator cuff tear/control of more than 2 and the raw values (log2) of more than 4.

Down significant means the genes with the fold change of rotator cuff tear/control of less than 1/2 with the raw values (log2) of more than 4.

(b)

| 4 weeks | Total | Aging | Angio. | Apop. | Diff. | Migr. | Prolif. | ECM | Infl. | FA tr. | Atro |
|---------|-------|-------|--------|-------|-------|-------|--------|-----|------|--------|------|
| Total gene number | 39,429 | 370 | 443 | 1,100 | 4,701 | 994 | 788 | 683 | 532 | 66 | 19 |
| % of Total | 100 | 0.94 | 1.12 | 2.79 | 11.92 | 2.52 | 2 | 1.73 | 1.35 | 0.17 | 0.05 |
| Up Significant (n) | 1,233 | 21 | 20 | 52 | 203 | 59 | 35 | 31 | 46 | 2 | 1 |
| % of Up Significant | 3.1 | 5.7 | 4.5 | 4.7 | 4.3 | 5.9 | 4.4 | 4.5 | 8.6 | 3 | 5.3 |
| Down Significant (n) | 1,137 | 8 | 9 | 34 | 150 | 31 | 32 | 12 | 15 | 0 | 4 |
| % of Down Significant | 2.9 | 2.2 | 2.1 | 3.1 | 3.2 | 3.1 | 4.1 | 1.8 | 2.8 | 0 | 21.1 |
| Total Significant (n) | 2,370 | 29 | 29 | 86 | 353 | 90 | 67 | 43 | 61 | 2 | 5 |
| % of Total Significant | 6 | 7.8 | 6.5 | 7.8 | 7.5 | 9.1 | 8.5 | 6.3 | 11.5 | 3 | 26.3 |

Angio., angiogenesis; Apop., apoptosis; Diff., cell differentiation; Migr., cell migration; Prolif., cell proliferation; ECM, extracellular matrix; Infl., inflammation; FA tr., fatty acid transport; Atro., muscle atrophy.

Up significant means the genes with the fold change of rotator cuff tear/control of more than 2 and the raw values (log2) of more than 4.

Down significant means the genes with the fold change of rotator cuff tear/control of less than 1/2 with the raw values (log2) of more than 4.

Figure 3: Venn diagram depicting the number of genes up-, down-, and contra-regulated among the significant genes for weeks 1 and 4.
Table 2: The top 10 up- and downregulated genes after rotator cuff tear (at weeks 1 and 4).

(a)  
| Gene symbol | Genbank accession | Fold change | Gene name |
|-------------|-------------------|-------------|-----------|
| **Upregulated** |
| Krt18       | NM_010664         | 217.799     | keratin 18 |
| Sirpb1b     | NM_001137460      | 150.129     | signal-regulatory protein beta 1B |
| Cd300lf     | NM_001169153      | 128.186     | CD300 antigen like family member F |
| Ms4a4c      | NM_029499         | 122.342     | membrane-spanning 4-domains, subfamily A, member 4C |
| Plekhh1     | NM_3801073        | 119.491     | pleckstrin homology domain containing, family H (with MyTH4 domain) member 1 |
| Cd5l        | NM_009690         | 104.575     | CD5 antigen-like |
| Krt8        | AK166854          | 104.209     | keratin 8 |
| Myh8        | AK01482           | 95.091      | myosin, heavy polypeptide 8, skeletal muscle, perinatal |
| LOC10264210 | XM_006544504      | 94.351      | tyrosine-protein phosphatase non-receptor type substrate 1-like |
| **Downregulated** |
| Pitx1       | NM_011097         | 0.025       | paired-like homeodomain transcription factor 1 |
| Adam28      | NM_383366         | 0.025       | a disintegrin and metallopeptidase domain 28 |
| Rnf180      | NM_027934         | 0.027       | ring finger protein 180 |
| Gms860      | NR_040659         | 0.029       | predicted gene 5860 |
| H2-M9       | NM_008205         | 0.03        | histocompatibility 2, M region locus 9 |
| Smco1       | NM_383283         | 0.03        | single-pass membrane protein with coiled-coil domains 1 |
| AU042410    | BB209673          | 0.031       | expressed sequence AU042410 |
| Kcn1        | NM_00112739       | 0.031       | potassium voltage gated channel, Shaw-related subfamily, member 1 |
| 8430426206Rik | NR_077229     | 0.032       | RIKEN cDNA 8430426206 gene |
| Gmi10822    | AK144860          | 0.033       | predicted gene 10822 |

(b)  
| Gene symbol | Genbank accession | Fold change | Gene name |
|-------------|-------------------|-------------|-----------|
| **Upregulated** |
| Gm7325      | NM_00117470       | 45.121      | predicted gene 7325 |
| Plekhh1     | NM_3801073        | 42.851      | pleckstrin homology domain containing, family H (with MyTH4 domain) member 1 |
| Ces2b       | NM_398171         | 28.227      | carboxylesterase 2B |
| Ces2c       | NM_456030         | 25.457      | carboxylesterase 2C |
| Sln         | NM_025540         | 24.155      | sarcolipin |
| Krt18       | NM_010664         | 22.114      | keratin 18 |
| Myh3        | NM_00199635       | 17.984      | myosin, heavy polypeptide 3, skeletal muscle, embryonic |
| Krt8        | AK166854          | 16.550      | keratin 8 |
| Zfp735      | NM_00126489       | 15.836      | zinc finger protein 735 |
| Eph1        | NM_023580         | 15.534      | Eph receptor A1 |
| **Downregulated** |
| Dok6        | NM_001039173      | 0.063       | docking protein 6 |
| Shn11       | NM_395629         | 0.072       | small nucleolar RNA host gene 11 |
| Olfr981     | NM_456286         | 0.087       | olfactory receptor 981 |
| Pit1        | NM_011097         | 0.087       | paired-like homeodomain transcription factor 1 |
| Gmi12       | NM_001195544      | 0.093       | predicted gene 12 |
| Olfr586     | NM_147111         | 0.098       | olfactory receptor 586 |
| Samd11      | XR_881429         | 0.101       | sterile alpha motif domain containing 11 |
| Dnase1      | NM_010061         | 0.111       | deoxycytidinenucleosidase I |
| 1190003K10Rik | NM_001195435    | 0.114       | RIKEN cDNA 1190003K10 gene |
| Gpr176      | NM_201367         | 0.117       | G protein-coupled receptor 176 |
Table 3: Gene expression patterns after rotator cuff tear in the aging category ((a) week 1 after tear; (b) week 4 after tear).

| Gene symbol | Genbank accession | Fold change | Gene name |
|-------------|------------------|-------------|-----------|
| **Upregulated** |                  |             |           |
| Igfbp2      | NM_008342        | 55.685      | insulin-like growth factor binding protein 2 |
| Arg1        | NM_007482        | 54.973      | arginase, liver |
| Cdk1        | NM_007659        | 34.242      | cyclin-dependent kinase 1 |
| Cdkn2a      | NM_009877        | 30.112      | cyclin-dependent kinase inhibitor 2A |
| Aldh3a1     | NM_007436        | 19.548      | aldehyde dehydrogenase family 3, subfamily A1 |
| Ddc         | NM_00190448      | 17.423      | dopa decarboxylase |
| Ccl2        | NM_011333        | 16.814      | chemokine (C-C motif) ligand 2 |
| Inpp5d      | NM_00566         | 15.961      | inositol polyphosphate-5-phosphatase D |
| Mpo         | NM_00824         | 14.836      | myeloperoxidase |
| Rad54l      | NM_009015        | 13.26       | RAD54 like (S. cerevisiae) |
| **Downregulated** |              |             |           |
| Tfcp2l1     | NM_023755        | 0.125       | transcription factor CP2-like 1 |
| Nat1        | NM_008673        | 0.146       | N-acetyltransferase 1 |
| Ppargcla    | NM_008904        | 0.153       | peroxisome proliferative activated receptor, gamma, coactivator 1 alpha |
| Atp8a2      | NM_015803        | 0.157       | ATPase, aminophospholipid transporter-like, class I, type 8A, member 2 |
| Pde4d       | NM_01056         | 0.183       | phosphodiesterase 4D, cAMP specific |
| Endog       | NM_007931        | 0.198       | endonuclease G |
| Cypl1       | NM_009992        | 0.199       | cytochrome P450, family 1, subfamily a, polypeptide 1 |
| KI          | NM_013823        | 0.2         | klotho |
| Cryab       | NM_001289782     | 0.21        | crystallin, alpha B |
| P2ry1       | NM_008772        | 0.216       | purinergic receptor P2Y, G-protein coupled 1 |
| **Upregulated** |              |             |           |
| Slc1a2      | NM_001077514     | 10.851      | solute carrier family 1 (glial high affinity glutamate transporter), member 2 |
| Krtap4-16   | NM_00103823      | 9.991       | keratin associated protein 4-16 |
| Ghrhr       | NM_001003685     | 9.285       | growth hormone releasing hormone receptor |
| Ddc         | NM_00190448      | 8.068       | dopa decarboxylase |
| Aldh3a1     | NM_007436        | 7.897       | aldehyde dehydrogenase family 3, subfamily A1 |
| Cdkn2a      | NM_009877        | 6.885       | cyclin-dependent kinase inhibitor 2A |
| Igfbp2      | NM_008342        | 5.164       | insulin-like growth factor binding protein 2 |
| Retn        | NM_001204959     | 3.729       | resistin |
| Adra1a      | NM_013461        | 3.523       | adrenergic receptor, alpha 1a |
| Cnr1        | NM_007726        | 3.51        | cannabinoid receptor 1 (brain) |
| **Downregulated** |              |             |           |
| Il10        | NM_0010548       | 0.195       | interleukin 10 |
| Cdk1        | NM_007659        | 0.227       | cyclin-dependent kinase 1 |
| Glrx2       | NM_001038592     | 0.325       | glutaredoxin 2 (thioltransferase) |
| Tfcp2l1     | NM_023755        | 0.391       | transcription factor CP2-like 1 |
| Fos         | NM_010234        | 0.438       | FBJ osteosarcoma oncogene |
| Atp8a2      | NM_015803        | 0.459       | ATPase, aminophospholipid transporter-like, class I, type 8A, member 2 |
| Hmgal       | NM_00166546      | 0.462       | high mobility group AT-hook 1 |
| Bcl2        | NM_177410        | 0.464       | B-cell leukemia/lymphoma 2 |
| Srf         | NM_020493        | 0.482       | serum response factor |
| Arntl       | NM_007489        | 0.519       | aryl hydrocarbon receptor nuclear translocator-like |
result was in contrast to that of myogenin, which showed the highest expression at week 1 but low expression at week 4.

3.4. Gene Expression Patterns in the Fatty Acid Transport Category. In the fatty acid transport category, the expression of apolipoprotein E (ApoE; expressed 12.229 times higher than control at week 1), annexin A1 (expressed 5.517 times higher than control at week 1) which is a phospholipid-binding protein, and perilipin 2 (expressed 5.018 times higher than control at week 1) which is an adipose differentiation-related gene was notable. The expression of all these genes decreased at week 4 (ApoE, annexin A1, and perilipin 2 were expressed 2.729, 1.377, and 1.196 times higher than control, respectively). In addition, these genes interacted with the phospholipase groups regulating PPARy expression, which regulates fatty acid storage and glucose metabolism, at weeks 1 and 4 as shown in the STRING network (Figure 7 and Table 6).

3.5. Gene Expression Patterns in Other Categories Related to Muscle Physiology. Gene expression patterns and STRING networks for other categories, such as angiogenesis, inflammation, cell migration, cell proliferation, extracellular matrix,
Table 4: Gene expression patterns in the apoptosis category after rotator cuff tear ((a) week 1 after tear; (b) week 4 after tear).

(a)

| Gene symbol | Genbank accession | Fold change | Gene name |
|-------------|-------------------|-------------|-----------|
| Krt18       | NM_010664         | 217.799     | keratin 18 |
| Cd5l        | NM_009690         | 104.575     | CD5 antigen-like |
| Krt8        | AK166854          | 104.209     | keratin 8  |
| Serpina3g   | NM_009251         | 85.473      | serine (or cysteine) peptidase inhibitor, clade A, member 3G |
| Melk        | NM_010790         | 72.784      | maternal embryonic leucine zipper kinase |
| Birc5       | NM_009689         | 55.603      | baculoviral IAP repeat-containing 5 |
| Gpr65       | NM_008152         | 54.323      | G-protein coupled receptor 65 |
| Gzma        | NM_010370         | 52.943      | granzyme A |
| Top2a       | NM_011623         | 44.234      | topoisomerase (DNA) II alpha |
| Bcl2ald     | NM_007536         | 43.608      | B-cell leukemia/lymphoma 2-related protein A1d |

Downregulated

| Gene symbol | Genbank accession | Fold change | Gene name |
|-------------|-------------------|-------------|-----------|
| Dnase1      | NM_010061         | 0.051       | deoxyribonuclease I |
| Casp14      | NM_009809         | 0.099       | caspase 14 |
| Robo2       | BC055333          | 0.104       | roundabout homolog 2 (*Drosophila*) |
| Camk2a      | NM_009792         | 0.107       | calcium/calmodulin-dependent protein kinase II alpha |
| Il24        | NM_053095         | 0.112       | interleukin 24 |
| Chac1       | NM_026929         | 0.119       | ChaC, cation transport regulator 1 |
| Nrl4a1      | NM_010444         | 0.122       | nuclear receptor subfamily 4, group A, member 1 |
| Mtp1        | NM_026443         | 0.124       | mitochondrial fission process 1 |
| Rps6ka2     | NM_011299         | 0.156       | ribosomal protein S6 kinase, polypeptide 2 |
| Casr        | NM_013803         | 0.158       | calcium-sensing receptor |

(b)

| Gene symbol | Genbank accession | Fold change | Gene name |
|-------------|-------------------|-------------|-----------|
| Krt18       | NM_010664         | 22.114      | keratin 18 |
| Krt8        | AK166854          | 16.55       | keratin 8  |
| Bcl2l14     | NM_025778         | 14.994      | BCL2-like 14 (apoptosis facilitator) |
| Txo3        | NM_127913         | 10.794      | TOX high mobility group box family member 3 |
| Fasl        | NM_010177         | 10.487      | Fas ligand (TNF superfamily, member 6) |
| Pak7        | NM_172858         | 9.86        | p21 protein (Cdc42/Rac)-activated kinase 7 |
| Fgf21       | NM_020013         | 7.87        | fibroblast growth factor 21 |
| Avp         | NM_109732         | 7.357       | arginine vasopressin |
| Nlr4c       | NM_001033367      | 7.471       | NLR family, CARD domain containing 4 |
| Cdkn2a      | NM_009877         | 6.885       | cyclin-dependent kinase inhibitor 2A |

Downregulated

| Gene symbol | Genbank accession | Fold change | Gene name |
|-------------|-------------------|-------------|-----------|
| Dnase1      | NM_010061         | 0.111       | deoxyribonuclease I |
| Cd27        | NM_001033126      | 0.17        | CD27 antigen |
| Fgf8        | NM_010205         | 0.162       | fibroblast growth factor 8 |
| AYO74887    | NM_145229         | 0.168       | cDNA sequence AYO74887 |
| Tnf         | NM_013693         | 0.205       | tumor necrosis factor |
| Naip1       | NM_008670         | 0.217       | NLR family, apoptosis inhibitory protein 1 |
| Cdk1        | NM_007659         | 0.227       | cyclin-dependent kinase 1 |
| Epha7       | NM_010141         | 0.239       | Eph receptor A7 |
| Tiam1       | NM_009384         | 0.253       | T-cell lymphoma invasion and metastasis 1 |
| Bubl        | NM_009772         | 0.284       | budding uninhibited by benzimidazoles 1 homolog (*S. cerevisiae*) |
and cell differentiation, are described in 'Supplementary Materials (available here)'.

3.6. Validation of Gene Expression Using Real-Time PCR Analysis. Cdk1 from the aging category, keratin 8 and 18 from the apoptosis category, and myogenin, myostatin, and actn3 from the muscle atrophy category were selected as representative genes for the validation of gene expression using quantitative real-time PCR analysis. The result of qRT-PCR analyses for all these selected genes was similar to the result of microarray analysis discussed above. The mRNA levels from both microarray and qRT-PCR analyses are depicted in Figure 8.

3.7. Commonly Expressed Genes. There were 2 common genes, namely, Rps6kbl and gelsolin, which displayed commonly regulated expressions in the 3 categories (aging, apoptotic process, and muscle atrophy) of our interest that are known to be associated with muscle degeneration and atrophy after a rotator cuff tear. Rps6kbl in the rotator
Table 5: Gene expression patterns in the muscle atrophy category after rotator cuff tear ((a) week 1 after tear; (b) week 4 after tear).

(a)

| Gene symbol | Genbank accession | Fold change | Gene name |
|-------------|-------------------|-------------|-----------|
| **Upregulated** |                   |             |           |
| Myog        | NM_031189         | 38.535      | myogenin  |
| Gatm        | NM_025961         | 8.862       | glycine amidinotransferase (L-arginine:glycine amidinotransferase):energy metabolism of muscle |
| Cflar       | NM_009805         | 2.442       | CASP8 and FADD-like apoptosis regulator |
| Rps6kb1     | NM_028259         | 1.287       | ribosomal protein S6 kinase, polypeptide 1 |

| **Downregulated** |                   |             |           |
| Ili5         | NM_008357         | 0.727       | interleukin 15 |
| Tbce         | NM_178337         | 0.648       | tubulin-specific chaperone E |
| Gsn          | NM_146120         | 0.439       | Gelsolin:actin-binding protein |
| Trim63       | NM_001039038      | 0.435       | tripartite motif-containing 63 |
| Actn3        | NM_013456         | 0.279       | actin alpha 3:alpha-actin skeletal muscle isoform 3 |
| Ppargc1a     | NM_008904         | 0.216       | peroxisome proliferative activated receptor, gamma, coactivator 1 alpha |
| Mstn         | NM_010834         | 0.108       | myostatin  |

(b)

| Gene symbol | Genbank accession | Fold change | Gene name |
|-------------|-------------------|-------------|-----------|
| **Upregulated** |                   |             |           |
| Myog        | NM_031189         | 11.945      | myogenin  |
| Gatm        | NM_025961         | 1.712       | glycine amidinotransferase (L-arginine:glycine amidinotransferase) |
| Gsn          | NM_146120        | 1.249       | gelsolin  |
| Rps6kb1     | NM_028259         | 1.245       | ribosomal protein S6 kinase, polypeptide 1 |

| **Downregulated** |                   |             |           |
| Ili5         | NM_008357         | 0.98        | interleukin 15 |
| Cflar        | NM_207653         | 0.935       | CASP8 and FADD-like apoptosis regulator |
| Ppargc1a     | NM_008904         | 0.763       | peroxisome proliferative activated receptor, gamma, coactivator 1 alpha |
| Tbce         | NM_178337         | 0.712       | tubulin-specific chaperone E |
| Actn3        | NM_013456         | 0.494       | actin alpha 3 |
| Trim63       | NM_001039048      | 0.387       | tripartite motif-containing 63 |
| Mstn         | NM_010834         | 0.378       | myostatin  |

cuff tear side was downregulated (expressed 0.48 times of the control) during the acute phase, and showed increased expression during the chronic phase (expressed 0.806 times of the control). In addition, Gelsolin in the rotator cuff tear side was also downregulated (0.40 times of the control) during the acute phase and showed increased expression during the chronic phase (expressed 1.17 times of the control).

4. Discussion

The major findings of this study were comprehensive analysis of genetic changes with time following a rotator cuff tear and categorization of those genes associated with 10 muscle physiology, including aging, apoptosis, atrophy, and fatty acid transport.

We regarded week 1 as the acute phase and week 4 as the chronic phase, based on our previous study using the same animal model [10]. In our previous study, we detected acute inflammatory reactions in the rotator cuff muscle with a rapid increase in inflammatory cytokines at week 1; however, the inflammatory reactions almost disappeared 4 weeks after inducing the rotator cuff tear. Conversely, fat deposition with degenerative changes in the rotator cuff muscle started increasing during week 2 and a noticeable increase was observed 4 weeks after the rotator cuff tear [10]. Based on these findings, we defined week 1 after the rotator cuff tear as the acute phase and week 4 as the chronic phase.

Among a total of 39,429 genes, the gene that showed the highest increase in expression (expressed 217.7 times higher than the control) at week 1 after the rotator cuff tear was Krt18, which plays an important role in maintaining cell structure and is a major component of the intermediate filaments of epithelial cells [30]. It is also well-known to be an indicator of the progression of chronic liver diseases because
it is related to apoptosis [31]. In the present study, a rapid increase in Krt18 expression was found during the acute phase after inducing the rotator cuff tear. We considered that the natural apoptotic process in muscle cells started considerably early after the rotator cuff tear, and if the apoptotic process progressed faster than the restoration process of damaged myocytes, a permanent and irreversible damage to the rotator cuff muscle may occur and the outcome may be worse even after a successful rotator cuff repair.

In the apoptosis category, among all genes, the expression of lgbp2 (insulin-like growth factor binding protein 2) was the highest at week 1 after the rotator cuff tear. This suggested that the muscle damage induced by the rotator cuff tear affected the aging process of myocytes. Davalos et al. showed that the IGF-binding proteins were highly expressed in aged fibroblast cells, which supported our results [32]. Particularly, IL-6 and Ccl2, which are adhesion molecules known to be secreted from aged cells, and Vcam1 interacted with each other and showed high expression as shown in the STRING network. In the apoptosis category, Birc5 (baculoviral IAP repeat-containing 5), which is known to be a survivin like krt 18, significantly increased (expressed 55.6 times of the control)
Table 6: Gene expression patterns in the fatty acid transport category after rotator cuff tear ((a) week 1 after tear; (b) week 4 after tear).

| Gene symbol | Genbank accession | Fold change | Gene name |
|-------------|-------------------|-------------|-----------|
| **Upregulated** | | | |
| Mfsd2a | NM_029662 | 12.606 | major facilitator superfamily domain containing 2A |
| Apoe | NM_009696 | 12.229 | apolipoprotein E |
| Drd4 | NM_007878 | 10.607 | dopamine receptor D4 |
| Slc27a6 | NM_001081072 | 7.557 | solute carrier family 27 (fatty acid transporter), member 6 |
| Pla2g4a | NM_008869 | 6.457 | phospholipase A2, group IVA (cytosolic, calcium-dependent) |
| Anxa1 | NM_010730 | 5.517 | annexin A1 |
| Plin2 | NM_007408 | 5.018 | perilipin 2 |
| Pla2g2e | NM_012044 | 3.407 | phospholipase A2, group IIE |
| Pla2g1b | NM_011107 | 3.384 | phospholipase A2, group IB, pancreas |
| Hnfla | M57966 | 2.029 | HNF1 homeobox A |
| **Downregulated** | | | |
| Fabp3 | NM_010174 | 0.131 | fatty acid binding protein 3, muscle and heart |
| Pla2g5 | NM_00122954 | 0.15 | phospholipase A2, group V |
| Cpt1b | NM_009948 | 0.186 | carnitine palmitoyltransferase 1b, muscle |
| Pla2g12a | NM_183423 | 0.262 | phospholipase A2, group XIIA |
| Got2 | NM_010325 | 0.289 | glutamic-oxaloacetic transaminase 2, mitochondrial |
| Slc27a2 | NM_011978 | 0.299 | solute carrier family 27 (fatty acid transporter), member 2 |
| Slc27a1 | NM_011977 | 0.32 | solute carrier family 27 (fatty acid transporter), member 1 |
| Acs1 | NM_007981 | 0.354 | acyl-CoA synthetase long-chain family member 1 |
| Pnpla8 | NM_026164 | 0.372 | patatin-like phospholipase domain containing 8 |
| Pla2g2c | NM_008868 | 0.408 | phospholipase A2, group IIC |
| **Upregulated** | | | |
| Nmur2 | NM_153079 | 3.742 | neumedin U receptor 2 |
| Apoe | NM_009696 | 2.729 | apolipoprotein E |
| Pla2g2d | NM_011109 | 2.704 | phospholipase A2, group IID |
| Pparg | NM_011146 | 1.56 | peroxisome proliferator activated receptor gamma |
| Procal | XM_006532963 | 1.548 | protein interacting with cyclin A1 |
| Pla2g2e | NM_012044 | 1.539 | phospholipase A2, group IIE |
| Pla2g4a | NM_008869 | 1.485 | phospholipase A2, group IVA (cytosolic, calcium-dependent) |
| Anxa1 | NM_010730 | 1.377 | annexin A1 |
| Pla2g2a | NM_001082531 | 1.286 | phospholipase A2, group II A (platelets, synovial fluid) |
| Mfsd2a | NM_029662 | 1.268 | major facilitator superfamily domain containing 2A |
| **Downregulated** | | | |
| Pla2g1b | NM_011107 | 0.428 | phospholipase A2, group IB, pancreas |
| Slc27a5 | NM_0099512 | 0.456 | solute carrier family 27 (fatty acid transporter), member 5 |
| Pla2g3 | NM_172791 | 0.511 | phospholipase A2, group III |
| Hnfla | M57966 | 0.529 | HNF1 homeobox A |
| Fabp3 | NM_010174 | 0.587 | fatty acid-binding protein 3, muscle and heart |
| Pla2g2f | NM_012045 | 0.592 | phospholipase A2, group IIF |
| Abcc4 | NM_001033336 | 0.592 | ATP-binding cassette, sub-family C (CFTR/MRP), member 4 |
| Pnpla8 | NM_026164 | 0.614 | patatin-like phospholipase domain containing 8 |
| Ppard | NM_011145 | 0.628 | peroxisome proliferator activator receptor delta |
| Pla2g5 | NM_00122954 | 0.661 | phospholipase A2, group V |
during the acute phase. This survivin is known to influence cell division and cause cell death inhibition. Hence, it is related to tissue injury and healing [33]. In addition, this gene interacts with Rps6ka2 [34], which is related to cell survival; caspase 14 [35] and Cdk1 [36], which play important roles in cell growth and apoptosis; and MELK [37], which is related to cell proliferation, apoptosis, RNA processing, and embryonic development. Birc5, which is known to play an important role in apoptosis, seemed to play a similar role in the rotator cuff muscle after the rotator cuff tear. Only 11 among the 39,429 genes analyzed belonged to the muscle atrophy category.

Among these, Myog (myogenin), which is known to play an important role in the differentiation of myocytes, showed the highest expression during the acute phase after rotator cuff tear as expected. On the contrary, Mstn (myostatin), which is known to play an inhibitory role during myogenesis, exhibited the lowest expression. These results suggested that myogenin rapidly increases during the early phase after the rotator cuff tear in order to regenerate damaged muscles (with low myostatin expression) and decreases as muscle atrophy and degeneration progressed with time. It was interesting that Gatm (L-arginine: glycin amidinotransferase),
which is known to be related to obesity associated with creatine metabolism [38], showed the highest expression after myogenin. In addition, two new genes, namely, Cflar and Ppargc1a, which showed reverse patterns of expression between the acute and chronic phases (Cflar: CASP8 and FADD-like apoptosis regulator, expression decreased from 2.442 times during the acute phase to 0.935 times during the chronic phase; Ppargc1a, expression changed from 0.216 times during the acute phase to 0.763 times during the chronic phase), although not significant, can be studied as novel targets associated with changes in muscle atrophy after rotator cuff tear or repair. In the fatty acid transport category, the expression of ApoE (Apolipoprotein E) was noticeable. ApoE is known to play a pivotal role in lipid homeostasis [39], and was also highly expressed among the cell proliferation category genes. This gene can be analyzed in future studies for understanding its role in the reversal of fatty infiltration after rotator cuff repair. In this study, we observed two genes (Rps6kb1 and gelsolin) which showed common expression patterns across 3 categories, namely aging, apoptosis, and muscle atrophy. Rps6kb1 is known to play an important role in anabolic signaling by increasing lipid accumulation in

Figure 8: Validation of microarray gene expression patterns using qRT-PCR analysis. (a) Cdk1, (b) keratin 8, (c) keratin 18, (d) myogenin, (e) myostatin, and (f) actn3.
the adipose tissue and inducing skeletal muscle hypertrophy [40], whereas gelsolin is known to be a physiological effector of apoptotic morphological changes after being cleaved by caspase 3 [41]. In this study, low expression of Rps6kb1 during the acute phase (expressed 0.48 times of the control) and high expression during the chronic phase (expressed 0.81 times of the control) suggested muscle damage recovery over time. Conversely, low expression of gelsolin during the acute phase (expressed 0.40 times of the control) and increased expression during the chronic phase (expressed 1.17 times of the control) potentially indicates ongoing apoptotic processes in rotator cuff muscles against the force to restore the damaged muscle. These two key genes may link and control muscle degeneration and sarcopenia after the rotator cuff tear.

The strength of our study is that this is the first study which comprehensively analyzed time-dependent expression of genes belonging to different categories associated with muscle physiology after making a rotator cuff tear. This finding may help to understand the rotator cuff tear-related muscle changes and, further it may improve various detrimental outcomes of rotator cuff repair surgery by regulation of the potent key molecule or its signal pathway.

Nevertheless, this study has several limitations that require consideration. First, this study was an animal-based study. Differences in anatomic features, different injury and healing reactions, and genetic variations between humans and rats limit generalization of the results. In this study, we established rotator cuff tear (RCT) model by injuring supraspinatus to make the most similar condition to the real clinical situation because most tears occur in the supraspinatus tendon in clinical situation. However, we humbly admit that 2 tendons (supraspinatus + infraspinatus) tear model may be better to mimic the muscle degenerative changes compared to the 1 tendon model, and there could exist a likelihood of self-healing in a mouse supraspinatus tear model. Thus, we have to be cautious while interpretation of results. Second, we investigated gene expression patterns in the rotator cuff muscles up to 4 weeks after making the tear, based on the results of our previous study [10]; however, there can be more biological and genetic changes during later time points (more than 4 weeks). Finally, molecular pathways underlying changes in muscle physiology after a rotator cuff tear were not identified in this study. This should be the next step of the study.

5. Conclusions

(i) Rotator cuff tear induces specific genes associated with changes in muscle physiology such as aging, apoptosis, muscle atrophy, and fatty acid transport.

(ii) Several genes which are significantly altered after rotator cuff tear may play key roles in controlling muscle degeneration after a rotator cuff tear.

(iii) Mouse rotator cuff tear model could be a good approach to understand the many aspects of the molecular changes in injured muscle.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Supplementary Materials

“Supplementary Figures” and “Supplementary Table” show gene expression patterns and the top 10 up- and downregulated genes in an additional muscle physiology-related category after rotator cuff tear, respectively. (Supplementary Materials)

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