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Exercise-Driven Metabolic Pathways in Healthy Cartilage

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SUMMARY

Contributions

ADB, JN, PP, SA. Study conception, design, acquisition, analysis and interpretation of data, writing of the article, approval of final version. RG, MP. Analysis and interpretation of data, help in drafting of the article, approval of final version. AMC, mechanical loading of cartilage, interpretation of data, writing of the article. NLW, BSL, TEH, BL, TAB, study conception, design, analysis and interpretation of data, help in writing of the article, approval of final version.

Competing Interests

All author of this manuscript do not have any financial and personal relationships with other people or organizations that could potentially and inappropriately influence (bias) this work and conclusions. There are no conflicts of interest of any author.

The raw data has been deposited in a MIAME compliant database GEO (accession number GSE74898).

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Objective—Exercise is vital for maintaining cartilage integrity in healthy joints. Here we examined the exercise-driven transcriptional regulation of genes in healthy rat articular cartilage to dissect the metabolic pathways responsible for its potential benefits.

Methods—Transcriptome-wide gene expression in the articular cartilage of healthy Sprague-Dawley female rats exercised daily (low intensity treadmill walking) for 2, 5, or 15 days was compared to that of non-exercised rats, using Affymetrix GeneChip arrays. Database for Annotation, Visualization and Integrated Discovery (DAVID) was used for Gene Ontology (GO)-term enrichment and Functional Annotation analysis of differentially expressed genes (DEGs). Kyoto Encyclopedia of Genes and Genome (KEGG) pathway mapper was used to identify the metabolic pathways regulated by exercise.

Results—Microarray analysis revealed that exercise-induced 644 DEGs in healthy articular cartilage. The DAVID bioinformatics tool demonstrated high prevalence of Functional Annotation Clusters with greater enrichment scores and GO-terms associated with extracellular matrix (ECM) biosynthesis/remodeling and inflammation/immune response. The KEGG database revealed that exercise regulates 147 metabolic pathways representing molecular interaction networks for Metabolism, Genetic Information Processing, Environmental Information Processing, Cellular Processes, Organismal Systems, and Diseases. These pathways collectively supported the complex regulation of the beneficial effects of exercise on the cartilage.

Conclusions—Overall, the findings highlight that exercise is a robust transcriptional regulator of a wide array of metabolic pathways in healthy cartilage. The major actions of exercise involve ECM biosynthesis/cartilage strengthening and attenuation of inflammatory pathways to provide prophylaxis against onset of arthritic diseases in healthy cartilage.

Introduction

Exercise is vital for maintaining cartilage integrity/homeostasis in healthy joints[1–4]. However, the mechanistic details of its actions on healthy cartilage remain elusive. Here, our objective was to examine the exercise-driven regulation of gene expression and consequent metabolic pathways that are responsible for its benefits on healthy cartilage.

Exercise/physical activity transcriptionally activates/inhibits genes associated with various cellular functions in cartilage[5–8]. Chondrocytes within the cartilage are mechanosensitive cells that can perceive and respond to mechanical signals by regulating molecular networks. These cells synthesize glycosaminoglycans (GAGs) and collagen type II-rich extracellular matrix (ECM) that are essential for the maintenance, strengthening, and regeneration of healthy cartilage[9–12]. During inflammation, upregulation of proinflammatory genes compromises the ability of chondrocytes to synthesize ECM, leading to loss of cartilage integrity, initiation of cartilage destruction, and onset of osteoarthritis (OA)[13–15]. Adequate exercise has been shown to be beneficial in human and experimental OA: regular aerobic exercise decreases plasma and articular levels of cytokines and their receptors, decreases pain and increases mobility of joints in OA patients and in experimental models of OA[5, 16, 17].

In addition to its positive effects on inflamed joints, exercise is considered to be an anabolic therapy for healthy cartilage. Exercise is shown to increase GAG contents in synovial fluids

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and plasma, improve joint function and muscle performance, and delay joint symptoms in patients with high risk of OA[5, 18]. Nevertheless, there is a clear paucity of mechanistic studies on the molecular networks/metabolic pathways regulated by exercise in healthy joints, which eventually prevents onset of arthritic diseases. This knowledge gap has limited our ability to exploit the therapeutic potential of exercise and maximize its effectiveness in healthy subjects. Understanding the exercise-mediated mechanisms of actions on cartilage would allow identification and functional understanding of key molecules that could be developed as tools to measure exercise effectiveness, and also to develop precisely targeted exercise regimens for both healthy individual and for those at risk of OA. For example, genome wide association studies (GWAS) have recently identified several genes associated with human OA[19–21]. Whether exercise prevents expression of these genes and thus onset of OA is as yet unknown, but this knowledge is critical for the prevention and physiotherapeutic management of OA.

In this study, we utilized a rat model to examine the effects of exercise on healthy articular cartilage. The advantages of this model were that rats could easily be trained to exercise on treadmills, provided sufficient cartilage for analysis from individual specimens, and were relatively docile in cages. The transcriptome-wide microarray analysis followed by analyses with bioinformatics tools, Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 and Kyoto Encyclopedia of Genes and Genome (KEGG) pathway mapper demonstrated that exercise transcriptionally activates/represses 2.6% ± 0.17% of the entire transcriptome of rat femoral cartilage, driving 147 discrete but interdependent metabolic pathways[22–25]. More importantly, the major metabolic pathways regulated by exercise are associated with ECM biosynthesis and inflammation/immune responses in healthy cartilage, including those that are associated with OA[19, 20].

Materials and Methods

Experimental Design

The study was conducted according to guidelines for the Use of Laboratory Animals in Research and was approved by the Institutional Animal Care and Use Committee at The Ohio State University (#2009A0138-R2). Healthy Sprague Dawley rats (n=20 rats, 5/group, 12–14 week-old females, 250–300 grams, Harlan Labs, IN) were housed as three animals/cage in individually ventilated cages with sterile Aspen bedding, and standard environmental enrichment. The cages were maintained at 12-h light/12-h dark cycle at 21°C in a pathogen free unit. All rats had ad libitum access to water and food and were allowed normal cage activity. All rats were trained to walk on treadmills for 3 days to exclude untrainable animals. Fifteen days following the training, animals randomly assigned to each group and were either not exercised (Controls) or subjected to low intensity exercise between 8–11 AM daily (treadmill walking, 12 m/min, 45 min/day) for 2, 5, or 15 days. Three hours following the last exercise regimen, animals were euthanized by CO_2 asphyxia[26]. Full thickness articular cartilage from the distal femoral heads of both legs was carefully dissected on ice under a stereomicroscope (10–15 mg/femur), and immediately placed on dry ice and pulverized (3×30 seconds at 2500 RPM) in a Mikrodismembrator S (Sartorius, Germany). The cartilage from individual rats was analyzed separately. RNA was extracted using Trizol

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reagent (Invitrogen, CA), and RNA quality was verified by a Bioanalyzer 2100 (Agilent, CA)[13]. The Whole Transcript (WT) cDNA Synthesis and Amplification Kit and WT Terminal Labeling Kit (Affymetrix, CA) were used for cDNA synthesis and labeling from 300 ng RNA template as recommended by the manufacturers. Labeled samples from three different rats from each group were hybridized to Affymetrix GeneChip Rat Gene 1.0 ST Arrays and gene chips scanned with GeneChip Scanner 3000 7G System (Microarray Shared Resource Facility, OSU Comprehensive Cancer Center)[13].

Identification of DEGs

Partek Genomic Suite version 6.4 software (Partek Inc., MO) was used to analyze intensity scans from three independent rats per group. The normal distribution of the overall gene expression was confirmed by the histogram showing data distribution within three Σ with the kurtosis and skewness z values within |2| (Supplementary Fig. S1). Principal Component Analysis (PCA) was used to confirm expression uniformity within each group and the data were subjected to both hierarchical and partition clustering by Partek Genomic Suite [Fig. 1(A,B)]. As compared to non-exercised controls, the DEGs showing either up- or down-regulation of a specific gene on all three days, with +2.0 or −2.0 fold or greater change on one or more days were included for the biological interpretation of the exercise-driven molecular/metabolic networks [Fig. 1(C), Tables I,II]. The raw microarray data has been deposited in a MIAME compliant database GEO (accession number GSE74898).

Validation of salient genes differentially expressed in microarray analysis

The expression of select DEGs from microarray analysis was confirmed by quantitative real time polymerase chain reaction (qrt-PCR)[26]. Briefly, RNA samples (n=5/group, including samples used in microarray analysis) were subjected to qrtPCR analysis. Extracted RNA was subjected to first strand cDNA synthesis using the Superscript III Reverse Transcriptase Kit (Invitrogen, CA). Gene expression was assessed by amplifying the cDNA with commercially obtained primers (Qiagen, MA) or are provided in Supplementary Table S1. The mRNA levels were calculated by normalizing to GAPDH using ΔΔCt method.

Functional Annotation and molecular network analysis

To assess biologic relevance, the data set obtained from rats exercised daily for 15 days was compared to non-exercised rats using GO analysis. DAVID Gene ID conversions tool was used to identify *Rattus norvegicus* gene IDs. GO screening and Functional Annotation Clustering were performed by DAVID Bioinformatics tool v6.7 [http://david.ncifcrf.gov/home/jsp; GOTERM_BP_FAT (biological process); GOTERM _MF_FAT (molecular function); GOTERM_CC_FAT (cellular component)] [22, 23]. KEGG Pathway mapper (http://www.genome.jp/kegg/pathway.html) was used to reveal the higher-order biological functions and metabolic pathways regulated by exercise[24, 25]. The pathway terms identified by KEGG Mapper containing two or more DEGs were included in the analysis. Both the *kgml* format downloaded from KEGG website and the manual search function in the online pathway maps were used.

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Statistical Analysis

The statistical significance of the differences in the microarray data between control cartilage and experimental cartilage from rats exercised for 2, 5 or 15 days was tested by ANOVA using Partek genomic suit (n=3 independent sample/group) and only significantly regulated transcripts ($p<0.05$) were further analyzed. SPSS v17 was used to determine the significance levels of qrtPCR data (n=5/group) by ANOVA with Tukey's honest significant difference post hoc test. The data is expressed as the mean ± standard error of the mean (SEM), with $p<0.05$ regarded as significant.

Results

Temporal regulation of gene expression by exercise

All experimental animals remained healthy without adverse effects from exercise, and little change in weights (±2%). The transcriptome wide microarrays analysis identified a pool of DEGs in cartilage from rats subjected to daily exercise for 2, 5, or 15 days, in comparison to non-exercised controls (n=3 out of 5 animals/group). PCA analysis of the DEGs revealed relatively uniform distribution of overall global gene expression in the samples following 2 or 5 days of daily exercise as compared to non-exercised controls. However, differences in gene expression on day 15 were observed, as demonstrated by the average F ratio (signal to noise ratio) of 18.8 [(Fig. 1(A)]. Hierarchical clustering analysis of the differentially regulated genes ($p<0.05$) indicated temporal regulation of distinct sets of genes following each day of exercise [Fig. 1(B)]. A total of 27,342 transcripts were detectable by the Affymetrix Rat GeneChip arrays. Following exclusion of predicted/ambiguous *Rattus norvegicus* genes, the DAVID gene ID conversion tool revealed that in comparison to controls, 774 (2.8%), 677 (2.5%), and 644 (2.4%) of the transcripts were differentially and significantly ($p<0.05$ for each gene) up- or down-regulated by more than 2-fold on days 2, 5, and 15, respectively. Therefore, 644 DEGs from day 15 dataset that were similarly/consistently regulated by exercise on days 2, 5 and 15, were selected for further analysis [Fig. 1(C)]. Among 644 DEGs from the day 15 dataset, 254 transcripts were upregulated and 428 transcripts were downregulated by exercise [(Fig. 1(C)], and were subjected to DAVID database and KEGG pathway mapper to investigate the overall exercise-dependent modification in the molecular networks in the articular cartilage.

The quantification of the DEGs by qrtPCR demonstrated results that were consistent with microarray analysis, albeit the magnitudes were often higher than those observed in the microarray analysis. The RNA samples (n=5/group, inclusive of those used in microarray analysis) were analyzed by qrtPCR for genes from ECM synthesis/(*Fgf2, Cilp, Cytl1)* remodeling *(Mmp8, Mmp9, and Mmp3*) and inflammation (*Ptgs2, Lyz, and Hmgb2*) categories [Fig. 1(D)].

DAVID GO-term Functional Annotation Enrichment Analysis

The GO Clustering of 644 DEGs provided a preliminary description of the potential functions of DEGs and their effects on cells. The GO terms with at least 2 genes and false discovery rate (FDR) of <1.0E-03 as significance threshold were selected to perform Functional Enrichment Analysis. This analysis generated 86 clusters with enrichment scores

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from 10.33 to 0.99 and GO-terms associated with GOTERM_MF_FAT, GOTERM_BP_FAT, and GOTERM_CC_FAT. These annotation clusters contained GO-terms enriched in inflammation/inflammatory responses, signal transduction and ECM biosynthesis/regulation [Fig. 2(A,B,C)]. Additional GO-terms in these clusters were associated with responses to cell division/differentiation/growth, intermediate metabolism, muscle function/differentiation, cell motility/migration, cell adhesion/communication, ion channel regulation, cytoskeletal organization, extracellular communication, and bone development/mineralization.

KEGG pathway analysis of molecular interaction and reaction networks

Further interpretation of the higher-level systemic functions of the 644 DEGs (superimposed on the *Rattus norvegicus* (rno) reference pathway) by KEGG mapper demonstrated that exercise regulated a total of 147 metabolic pathways in the articular cartilage, 93 pathways at cellular levels and 54 at the organismal level (Tables I, II)[24, 27]. Only pathways with two or more DEGs were included in this analysis. Exercise regulated a wide array of metabolic pathways representing the KEGG defined molecular interaction and reaction networks as follows:

i. *Metabolism*. Exercise regulated pathways involved in intermediate metabolism, such as synthesis of carbohydrates, energy, lipids, nucleotides, amino acids, glycans, co-factors/vitamins, terpenoids/polyketides, and xenobiotics.

ii. *Genetic Information Processing*. While exercise regulated pathways involved in DNA repair, translation, folding, sorting and degradation of proteins, and DNA repair, it suppressed DNA replication pathways. For example, exercise suppressed several genes integrally associated with the cell cycle such as *S100a9* and cyclins, the regulators of the cell cycle and mitosis. Furthermore, *Igf1, Igfbp6* and *Pdgfb*, growth factors important in the regulation of cell division, were also suppressed[28, 29].

iii. *Environmental Information Processing.* Surprisingly, exercise upregulated *Per2, Cry1* and *Cry2* genes that regulate circadian rhythms[30].

Another major target regulated by exercise was signal transduction pathways and interactions of signaling molecules, where exercise regulated 31 different signaling cascades. Furthermore, DEGs regulating PI3K, Ras, MAPK, Rap-1, cAMP, sphingolipid, cGMP, NF-kB, and FoxO signaling cascades were markedly over represented in comparison to other metabolic pathways (Table I). Activation of these signaling pathways by exercise in turn regulated multiple cellular processes. For example, exercise regulated PI3K-AKT signaling, which showed association with 18 different signaling cascades regulated by DEGs *Bcl2, Fgf3, Fgf14, Fgf2, Gng11, Hsp90, Itga5, Prkca, IL-1,* and *Thbs2* (Fig. 3).

iv. *Cellular Processes*. Exercise regulated pathways that control cellular transport and catabolism including such activities as Fc-gamma mediated phagocytosis, endocytosis, and lysosome and peroxisome functions. Exercise also regulated

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cell motility, growth and death, and cell communication by regulating the actin cytoskeleton, cell cycle, apoptosis, cell adherence and cell junctions.

v. *Organismal Systems.* Surprisingly, exercise regulated many of the pathways involved in organismal functions in cartilage. For example, exercise regulated more than 20 pathways in immune (soluble and cellular immune responses), 11 in endocrine (renin-angiotensin, insulin, thyroid stimulating hormone, lipolysis, etc), 8 in digestive (protein, fat, carbohydrate absorption, digestive secretions), 8 in nervous (long term depression, several synapses,), 3 in circulatory (smooth & cardiac muscle contraction) systems, 2 in sensory (inflammatory mediator regulation, olfactory transduction), development (osteoclastogenesis), and environmental adaptation (circadian entrainment) (Table II).

vi. *Human Disease*. KEGG mapper also revealed that exercise regulated genes associated with diseases, likely due to involvement of signaling cascades, growth factors and metabolic pathways. We did not explore these genes in detail, but a list of these diseases and pathways is provided in Supplementary Table S2.

Examination of Major Exercise-Driven Pathways in Cartilage

Functional Annotation Clusters identified a preponderance of GO-terms enriched in immune responses, signal transduction and ECM [Fig. 2(A)]. Similarly, KEGG mapper demonstrated exercise-dependent regulation of glycan biosynthesis and inflammation/immune responses (Tables I, II). Since signal transduction pathways are common in many pathways regulated by exercise, we next focused on cartilage specific pathways involved in ECM biosynthesis and immune response. Furthermore, the manual search function in the online pathway maps was used to identify DEGs involved in ECM biosynthesis and immune responses.

ECM biosynthesis and metabolism

Interestingly, DAVID database revealed that the most highly enriched functional clusters contained GO-terms associated with ECM biosynthesis. These GO-terms also contained greater numbers of DEGs than other clusters. These DEGs were involved in GO-terms representing major biosynthetic functions of chondrocytes, such as peptide secretion, ECM synthesis, inhibitors of peptidases, response to mechanical forces, GAG synthesis, peptidases, metalloproteases, etc [Fig. 2(B)]. Similarly, manual examination also demonstrated that exercise upregulated DEGs required for biosynthesis of ECM, and inhibitors of ECM degrading enzymes, but suppressed proteolytic enzymes and non-cartilaginous proteins. For example, exercise upregulated DEGs involved in ECM biosynthesis such as *Dcn*, *Cilp, Eln*, *Vcan*, *Chst1, Chst3*, *Hs3st1*, *Cytl1* (proteoglycan synthesis), and *Fgf2*, while suppressing non-cartilaginous genes such as *Col24a1* (fibrillogenesis), *Col9a2*, *Col9a3* (fibrillar collagen), *Col1a2* (bone collagen), *Ctsk* and *Post* (bone), *Tnn* (nerve protein), *Matn3* and *Matn4* (unknown function) (Table III). Additionally, exercise suppressed synthesis of matrix-metalloproteinases (*Mmp8, Mmp9, Mmp14*), and proteoglycanolytic enzymes (*Adamts3, Adamts14*) involved in ECM degradation (Table III) and upregulated gene expression for inhibitors of proteolytic enzymes such as *Timp4,*

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Serpina1, Serpina3n, Mug1, Mug2, and *Agt*. Furthermore, *Serpinb1a, Serpinf1,* and *Serpinb6b,* clotting factors thrombin and kallikrenin, and serine proteases were all suppressed during the entire 15 days of exercise regimens [Fig. 2(B)], Table III).

Immune Function

DAVID functional enrichment analysis revealed that at least 187 GO-terms enriched in 12 different functional annotation clusters were associated with immune function, indicating that this was the most exercise-regulated function in chondrocytes [Fig. 2(C)]. The GO-terms in these functional annotation clusters were associated with defense response, cytokine activity, chemotaxis, B cell regulation, regulation of ossification, antigen processing, leukocyte proliferation, immune effector mechanisms, phagocytosis, acute inflammatory response, innate immune response, defense response to virus, and antimicrobial activity, further indicating that exercise regulated diverse aspects of inflammation/immune responses [Fig. 2(C)]. KEGG pathway mapper also indicated that exercise regulated 20 different inflammation/immune response (both soluble and cellular components) associated pathways and 14 different signaling pathways that regulate immune responses (Tables I, II). The DEGs showed that exercise upregulated decoy receptors *Il6ra* and *Il1r2,* blocking cellular responses to cytokines IL-6 and IL-1; *Cxcl13*, a chemokine that localizes B cells in follicles; *IL-16*, a chemoattractant and an inhibitor of HIV replication; *Thbs2*, a thrombospondin with anti-angiogenic properties, and *Lbp* in Toll-like receptor signaling pathway. Exercise also induced DEGs encoding protease inhibitors (*Agt, Mug1, Mug2, Cpvl, Serpina1, Serpina3n*), and clotting factors (*Thbs2* and *Plat*), while suppressing gene expression for *C3* and *C7*, chemokine receptors *Ccr1, Cxcl12,* and enzymes involved in prostaglandin/lipoxygenase synthesis *Ptgs2,* and *Alox15.* Additionally, exercise activated and deactivated *Pde3a, Pld5, Plce1, Pla1a, Pde3b, Pla2g2a*, enzymes involved in hydrolysis of phospholipids in response to cytokine and endotoxin signaling pathways, such as *PI3K-AKT, NF-κB, Ras, Rap, MAPK, cAMP, and Ptgs2* (Table IV).

Exercise Regulates OA associated genes

The observations that exercise suppresses proinflammatory pathways led us to investigate whether our dataset includes human OA-associated genes identified by HuGe Navigator [19, 21, 31]. Indeed, several OA-associated DEGs were regulated by exercise in healthy cartilage. For example, exercise suppressed expression of genes upregulated in OA such as *Ptgs2, Mmp9, Mmp8, Igf1, ColIa1, Adamts3, Adamts14,* and *Vdr*, which encode proteins involved in ECM degradation, bone formation, and initiation of pro-inflammatory cascades. Conversely, exercise upregulated many of the genes involved in ECM synthesis that are downregulated in OA such as *Chrdl2, Tnfrsf11b, Timp4, Thbs2, Tgfb1, Mmp3, Il1r1, Il1r2Cilp and Bmp5* (Fig. 4; Supplementary Table S3).

Discussion

We demonstrate for the first time that low intensity exercise is a potent transcriptional activator/repressor of genes that are involved in improving overall cartilage health and contributes to prophylaxis against inflammation, such as those observed during onset of OA. Since conducting studies on healthy human cartilage is not possible, here we used rats as an

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experimental model. The full thickness articular cartilage provided an ideal tissue to critically analyze the complex exercise-driven gene-regulation *in vivo*, specifically in chondrocytes without interference of other cell types[12, 32]. Furthermore, because immobilization itself alters gene expression, cartilage from non-exercised rats with normal cage activity provided a suitable control for comparing DEGs in exercised rat cartilage[33]. The Affymetrix-gene-chip analysis of articular cartilage followed by DAVID gene ID conversions demonstrated that as compared to non-exercised controls, daily exercise differentially and significantly regulated 2.6%±0.17% of the genes in the entire transcriptome: 774 DEGs on day 2, 677 DEGs on day 5, and 644 DEGs on day 15. Furthermore, exercise consistently up- or down-regulates the same 644 DEGs at each time point, indicating that these genes are likely crucial for its actions in cartilage. Therefore, these DEGs from day 15 dataset were selected for biological interpretation of their higher level systemic functions.

A major function of chondrocytes is to maintain the homeostasis of ECM for mechanical support of the joints[12, 32]. The functional annotation clustering of these GO-terms further confirmed that exercise regulates a wide range of cellular functions. However, the prevalence of functional clusters and their higher enrichment scores associated with ECM biosynthesis/remodeling and immune response identified that a major consequence of exercise is maintenance of cartilage integrity.

The regulation of 147 metabolic pathways in KEGG pathway analysis demonstrated that exercise regulates a wide range of chondrocyte functions sub-categorized as those involved in intermediate metabolism, genetic information processing (functional regulation of RNA/DNA), environmental information processing (perceiving and responding to extracellular stimuli, physical and biochemical), cellular processes (cellular functions such as transport, growth, apoptosis, communication), organismal systems (regulation of various body functions), and diseases. Interestingly, exercise regulated as many as 33 or as few as 2 DEGs (Tables I,II) in these pathways to control their activation/suppression. Moreover, many of these DEGs regulated more than one metabolic pathway at both cellular and organismal levels. For example, exercise regulated PI3K-AKT pathway which in turn regulates 18 different pathways including glycolysis, cell cycle, NF-κB and p53 metabolic pathways, revealing the complexity of the gene regulation by exercise (Fig. 3).

The KEGG mapper also demonstrated that exercise upregulates biosynthesis of the basic building blocks of proteoglycans, GAGs including hyaluronan, keratan sulfate, chondroitin sulfate, heparan sulfate, and dermatan sulfate, glycosphingolipids and proteases that are involved in the degradation of GAGs. Similarly, manual identification of DEGs demonstrated that exercise-induced biosynthesis of ECM structural proteins, growth factors (*Fgf2*, *Fgf13*, and *Fgf14*), and signaling molecules, while inhibiting expression of non-cartilaginous DEGs (*Col9a2, Col9a3, Col1a2,* and *Ctsk*)[29, 31, 34–38]. In fact, exercise negatively regulated expression of enzymes involved in ECM degradation, while upregulating expression of inhibitors of proteases involved in ECM degradation (*TIMP14, Serpina 1, Serpina 3a,* and *Mug1 & 2*)[39]. Thus our findings support the earlier observations that exercise strengthens healthy joints, and further provide molecular evidence that exercise activates metabolic pathways critical for maintaining ECM production, limiting cartilage

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destruction and maintaining the cartilage-specific tissue phenotype to protect its integrity and structural strength[1, 40–42].

The second major function demonstrated by the functional annotation clusters with more than 187 GO-terms were enriched in functions associated with inflammatory and immune responses (Fig. 2). These GO-terms and clusters also suggested that exercise regulates both soluble and cellular components of the immune system. KEGG pathway analysis also demonstrated that exercise regulates at least 20 different pathways involved in inflammation/immune function (Table II). Exercise is shown to be anti-inflammatory and to suppress proinflammatory gene induction in inflamed cartilage *in vivo* and *in vitro*[7, 10, 11, 43, 44]. Our data further demonstrates that exercise also significantly controls these pathways in healthy cartilage. However, many of the genes regulated by exercise in an inflammatory state are distinct from those regulated by exercise in healthy cartilage[26, 44–47]. For example, exercise in inflamed cartilage directly attenuates NF-κB activity to suppress induction of cytokines (*Il1β, Tnfα, Il8*) and matrix-metelloproteinases. In healthy cartilage exercise upregulates soluble/decoy receptors for cytokines and chemokines, their receptor antagonists (*Il6ra* and *Il1r2,* and cytokine-cytokine receptor interactions, chemokines that regulate lymphocytes mobility (*Cxcl13*, localizes B cells in follicles, and *Il16*, a chemoattractant for CD4+ cells), and genes encoding protease inhibitors (*Agt, Mug1, Mug2, Cpvl, Serpina1, Serpina3n,* and *Plat*). Exercise downregulates Toll-like receptor-4 (*Tlr4*) expression in circulating lymphocytes[10, 11, 48]. Our data demonstrates that exercise suppresses ligands for TLRs (*Hmgb2* and *S100a8)* in TLR signaling pathway. Strikingly, exercise prevents blood clot formation by upregulating *Plat* but suppressing *F5,* limits expression of complement components (*C1qr1, C3* and *C7)*, chemokines and their receptors (*Ccr1* and *2*, *Cxcr4, Cxcl12),* neutrophilic proteins (*Defa, Lyz, Npg*), and the major proinflammatory enzymes *Ptgs2* and *Alox15* to inhibit production of prostaglandin and leukotrienes to collectively suppress major inflammatory cascades[49, 50]. These observations suggest that exercise-mediated negative regulation of pathways for proinflammatory signaling may be a powerful mechanism to protect cartilage from inflammation and onset of arthritic diseases.

Analysis of DEGs demonstrated that exercise suppresses expression of several genes in healthy cartilage that are upregulated in OA such as pathways involved in ECM degradation, activation of proinflammatory signaling cascades, genes that code for non-cartilaginous proteins and the genes associated with osteoclast activation. Correspondingly, exercise upregulates several genes that are beneficial for ECM synthesis but are suppressed in OA (Fig. 4). This differential regulation of genes explains the putative beneficial effects of exercise in preventing/delaying the onset and progression of OA.

Although our studies have provided an overall mechanistic view of the metabolic changes induced by exercise, we have used the entire femoral cartilage without distinguishing between load-bearing and non-load-bearing sites. Nevertheless, both exercised and non-exercised control rats were allowed normal cage activity, except for 45 minutes/day of exercise in the experimental groups, therefore all differences in gene expression between groups can be attributed to exercise. Furthermore, it may be challenging to extrapolate these findings to humans due to biomechanics during walking. However, the fact that normal homeostasis was maintained in the rats with applied low intensity exercise suggests that this

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amount of loading was appropriately analogous to the humans, where moderate walking exercise in a healthy joint promotes homeostasis of the healthy joints.

In conclusion, on the molecular basis, we have demonstrated that exercise is a robust approach to preserve healthy cartilage. Exercise regulates the metabolic responses at both cellular and systemic levels to interdependently synchronize a wide range of anabolic pathways that are important in protecting cartilage strength and phenotype. More importantly, exercise provides prophylaxis against OA by potentially altering expression of genes involved in its onset. Thus, the findings identify a sophisticated regulatory paradigm whereby exercise synchronizes numerous gene regulatory networks to ensure cartilage health and prevent onset of arthritic diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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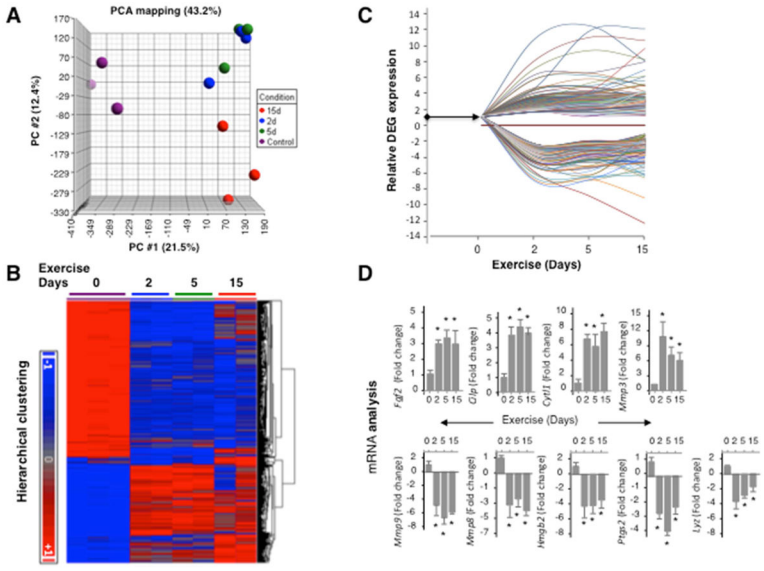

Fig. 1. Regulation of transcriptome-wide gene expression by exercise

The rats were either not exercised or exercised daily for 2, 5 or 15 days. Subsequently, the differentially expressed genes (DEGs) in the distal femoral cartilage were identified by transcriptome-wide microarray analysis. (A) Principal component analysis demonstrating overall gene expression levels in non-exercised control rats and those subjected to daily exercise for 2, 5 or 15 days (n =3/group) and relatively uniform distribution of the datasets within each group. (B) Hierarchical clustering of the transcripts that were significantly ($p<0.05$) and differentially up- or down-regulated by more than two-fold at one or more time points. The cluster map represents the gene expression profiles of the articular cartilage from exercised rats compared to non-exercised rats, demonstrating that distinct gene sets were temporally regulated during each day of exercise. (C) Exercise-driven regulation of transcripts that were, as compared to controls, differentially and significantly ($P<0.05$) up- or down regulated on days 2, 5, or 15, with +2.0 or −2.0 fold or greater change on one or more days. Out of the 644 DEGs that were similarly/consistently regulated by exercise 254 were up- and 428 were down-regulated. Arrow indicates control value of each DEG. (D) Validation of exercise-driven DEGs by qrt-PCR of salient genes associated with ECM biosynthesis (*Fgf2, Cilp, Cytl1*) and remodeling (*Mmp3, Mmp9, Mmp8*), and inflammation (*Hmgb2, Ptgs2, Lyz*). The levels of amplification of these genes in microarray analysis are indicated by * in Tables III & IV. The data in graphs represents mean ± standard error of the mean (SEM). Significant differences with respect to Control are indicated by * ($P<0.05$ by Tukey's post hoc test).

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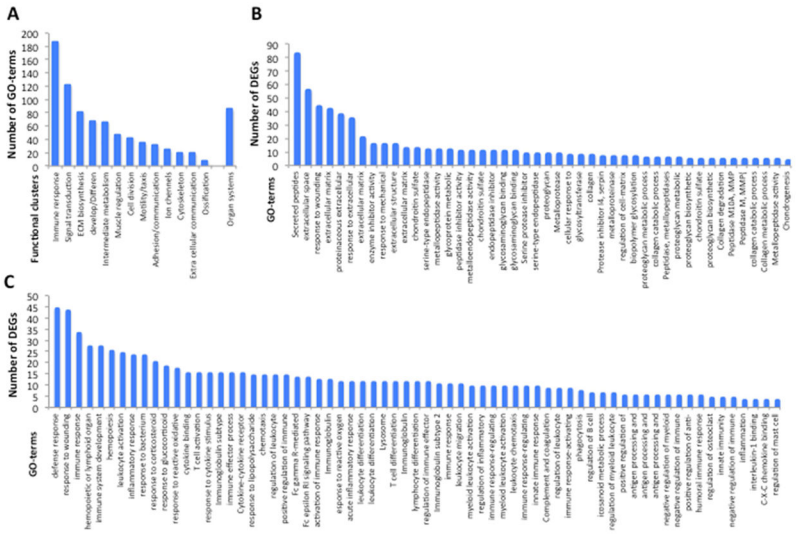

Fig. 2. Functional annotation analysis of GO (gene ontology)-terms involved in cellular functions regulated by exercise

The 644 DEGs were subjected to DAVID database for GO-term functional annotation analysis. (A) The graph shows number of GO-terms enriched in major functional clusters. (B) The number of DEGs involved in each major GO-term associated with ECM biosynthesis/remodeling. (C) The number of GO-terms and number of DEGs involved in each major cellular function associated with inflammation/immune responses. (A–C) FDR < 1.0E-03.

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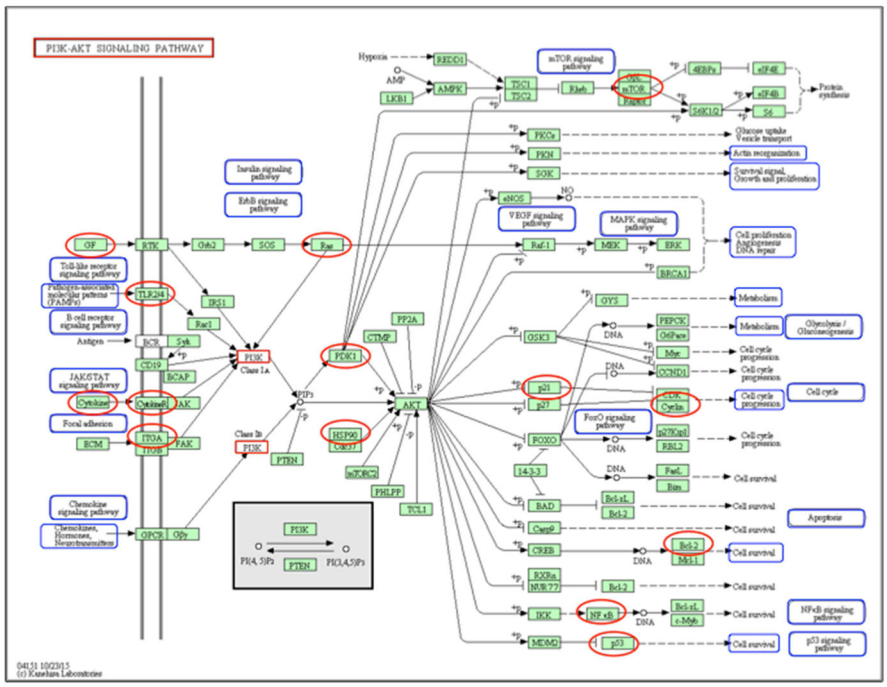

Figure 3. KEGG map displaying regulation of exercise-driven pathways via PI3K-AKT signaling cascade

The metabolic map of PI3K-AKT signaling cascade demonstrating potential points regulated by exercise, providing an example of interdependent regulation of signaling cascades by exercise. The blue rectangles highlight the KEGG identified pathways that are regulated by exercise. Red ovals indicate the DEGs regulated by exercise. Pathways and genes that are regulated by exercise in each pathway are provided in Table I.

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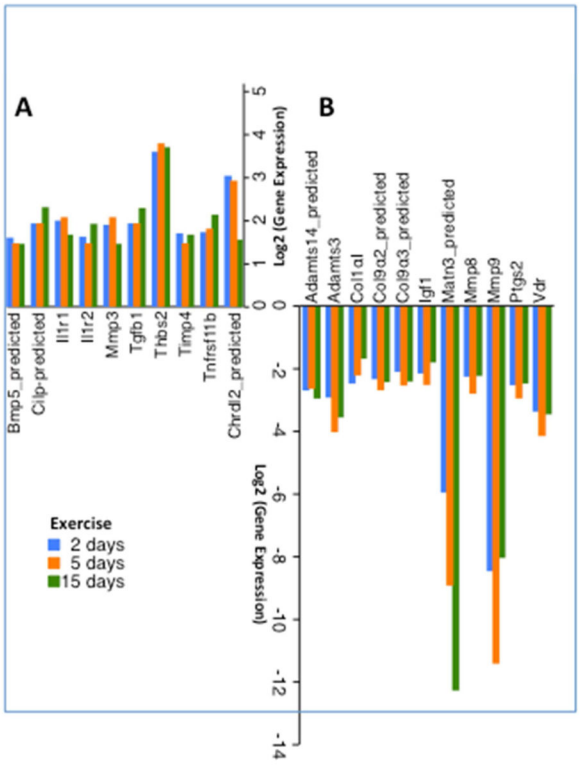

Figure 4. Regulation of OA associated genes by exercise in healthy cartilage

The dataset from microarray analysis was examined for the exercise-driven regulation of OA candidate genes identified by Human Genome Epidemiology Navigator from GWAS. The graphs show (A) genes that are suppressed in OA and show upregulation by exercise in healthy cartilage, and (B) genes that are upregulated in OA and are suppressed following exercise in healthy cartilage.

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TABLE I

KEGG pathway analysis of intracellular pathways regulated by exercise in healthy articular cartilage.

1. Metabolism
1.0 Global Overview
Metabolic pathways (62)
Biosynthesis of antibiotics (19)
Biosynthesis of amino acids (8)
Carbon metabolism (5)
Fatty acid metabolism (2)
1.1 Carbohydrate metabolism
Fructose and mannose metabolism (7)
Glycolysis/Gluconeogenesis (6)
Amino sugar and nucleotide sugar metabolism (5)
Galactose metabolism (3)
Starch and sucrose metabolism (3)
1.2 Energy metabolism
Oxidative phosphorylation (4)
Nitrogen metabolism (3)
1.3 Lipid metabolism
Glycerophospholipid metabolism (8)
Arachidonic acid metabolism (6)
Ether lipid metabolism (6)
Sphingolipid metabolism (5)
Glycerolipid metabolism (5)
Linoleic acid metabolism (3)
1.4 Nucleotide metabolism
Purine metabolism (7)
Pyrimidine metabolism (2)
1.5 Amino acid metabolism
Glycine, serine and threonine metabolism (3)
Glutathione metabolism (3)
Valine, leucine and isoleucine degradation (2)
Lysine degradation (2)
Alanine, aspartate and glutamate metabolism (2)
Tyrosine metabolism (2)
Arginine and proline metabolism (2)
1.7 Glycan Biosynthesis and metabolism
Mucin type O-Glycan biosynthesis (4)
Other types of O-glycan biosynthesis (4)
Glycosaminoglycan biosynthesis-keratan sulfate (2)
N-Glycan biosynthesis (2)
Glycosaminoglycan degradation (2)

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Glycosphingolipid biosynthesis - globo series (2)

1.8 Metabolism of cofactors and vitamins

One carbon pool by folate (4)

Porphyrin and chlorophyll metabolism (2)

1.11 Xenobiotics biodegradation and metabolism

Metabolism of xenobiotics by cytochrome P450 (4)

Drug metabolism - cytochrome P450 (3)

2. Genetic information processing

2.3 Folding, sorting and degradation

Protein processing in endoplasmic reticulum (5)

RNA transport (4)

Ubiquitin mediated proteolysis (2)

2.4 Replication and repair

DNA replication (3)

3. Environmental information processing

3.1 Circadian rhythm (3)

3.2 Signal transduction

PI3K-AKT signaling pathway (28)

Ras signaling pathway (24)

Rap1 signaling pathway (23)

MAPK signaling pathway (17)

cAMP signaling pathway (14)

Sphingolipid signaling pathway (12)

cGMP-PKG signaling pathway (11)

HIF-1 signaling pathway (10)

Oxytocin signaling pathway (10)

Inositol phosphate metabolism (10)

Thyroid hormone signaling pathway (10)

Calcium signaling pathway (10)

FoxO signaling pathway (9)

Phosphatidylinositol signaling system (9)

Estrogen signaling pathway (9)

Wnt signaling pathway (9)

GnRH signaling pathway (9)

NF-kappa B signaling pathway (9)

p53 signaling pathway (8)

AMPK signaling pathway (7)

Jak-STAT signaling pathway (6)

Neurotrophin signaling pathway (6)

VEGF signaling pathway (5)

Glucagon signaling pathway (5)

mTOR signaling pathway (5)

ErbB signaling pathway (4)

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TGF-beta signaling pathway (4)

Hippo signaling pathway (4)

Prolactin signaling pathway (4)

PPAR signaling pathway (2)

Notch signaling pathway (2)

3.3 Signaling molecules and Interactions

Cytokine-cytokine receptor interaction (18)

ECM-receptor interaction (9)

Cell adhesion molecules (CAMs) (9)

Neuroactive ligand-receptor interaction (7)

4. Cellular processes

4.1 Transport and catabolism

Fc gamma R-mediated phagocytosis (18)

Phagosome (12)

Lysosome (10)

Endocytosis (8)

Peroxisome (2)

4.2 Cell motility

Regulation of actin cytoskeleton (15)

Axon guidance (10)

4.3 Cell Growth and death

Cell cycle (12)

Oocyte meiosis (10)

Apoptosis (2)

4.4 Cellular community

Focal adhesion (17)

Gap junction (10)

Tight junction (9)

Signaling pathways regulating pluripotency of stem cells (6)

Adherens junction (5)

Footnote: The pathways identified by KEGG mapper following 15 days of daily exercise. The number of DEGs regulated by exercise in each pathway is given in the parentheses. The pathways with at least two DEGs regulated by exercise have been included in this table.

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Table II

KEGG pathway analysis of pathways regulated by exercise at organismal level in healthy articular cartilage

5. Organismal Systems
5.1 Immune system
Cytokine-cytokine receptor interaction (20)
Chemokine signaling pathway (19)
Fc gamma R-mediated phagocytosis (18)
B cell receptor signaling pathway (15)
Hematopoietic cell lineage (15)
Leukocyte transendothelial migration (15)
Platelet activation (14)
Fc epsilon RI signaling pathway (13)
Phagosome pathways (12)
Lysosome pathways (11)
Natural killer cell mediated cytotoxicity (9)
TNF signaling pathway (8)
Complement and coagulation cascades (8)
T cell receptor signaling pathway (7)
Antigen processing and presentation (7)
NOD-like receptor signaling pathway (5)
Toll-like receptor signaling pathway (5)
Cytosolic DNA-sensing pathway (3)
Intestinal immune network IgA production (3)
Adipocytokine signaling pathway (2)
5.2 Endocrine system
Progesterone-mediated oocyte maturation (10)
Renin secretion (10)
Insulin secretion (8)
Melanogenesis (8)
Aldosterone synthesis and secretion (7)
Insulin signaling pathway (6)
Insulin resistance (6)
Thyroid hormone synthesis (6)
Regulation of lipolysis in adipocytes (5)
Ovarian steroidogenesis (4)
Renin-angiotensin system (3)
5.3 Circulatory system
Vascular smooth muscle contraction (11)
Adrenergic signaling in cardiomyocytes (11)
Cardiac muscle contraction (7)
5.4 Digestive system
Salivary secretion (14)

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Protein digestion and absorption (10)
Pancreatic secretion (10)
Gastric acid secretion (9)
Bile secretion (6)
Carbohydrate digestion and absorption (5)
Fat digestion and absorption (4)
Mineral absorption (3)
5.5 Excretory system
Endocrine and other factor-regulated calcium reabsorption (7)
Aldosterone-regulated sodium reabsorption (6)
Collecting duct acid secretion (4)
Proximal tubule bicarbonate reclamation (4)
5.6 Nervous system
Long-term depression (10)
Dopaminergic synapse (11)
Serotonergic synapse (9)
Glutamatergic synapse (8)
Retrograde endocannabinoid signaling (8)
Long-term potentiation (7)
Cholinergic synapse (6)
GABAergic synapse (4)
5.7 Sensory system
Inflammatory mediator regulation – TRP channels (10)
Olfactory transduction (3)
5.8 Development
Osteoclast differentiation (14)
5.9 Environmental adaptation
Circadian entrainment (11)
6. Human diseases (Supplementary Table S2)

Footnote: Table shows pathways identified by KEGG mapper following 15 days of daily exercise. The number of differentially expressed genes regulated by exercise in each pathway is provided in parentheses.

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Table III

Temporal regulation of salient DEGs associated with ECM biosynthesis.

ECM structural proteins (Gene name)	Gene symbol	Day 0	Day 2	Day 5	Day 15
Decorin	*Dcn*	1	2.04	2.62	4.18
*Fibroblast growth factor 2	*Fgf2*	1	2.65	2.84	2.73
Integrin 5	*Itga5*	1	1.76	1.97	2.09
Phospholipase C	*Plce1*	1	4.18	4.02	4.21
Protein kinase C, alpha	*Prkca*	1	1.75	1.97	1.94
Syndecan	*Sdc4*	1	2.05	1.87	2.23
Chondroitin sulfate N-acetylgalactosaminyltransferase 2	*Csgalnact2*	1	2.24	2.39	1.98
Chondroitin 6	*Chst3*	1	2.38	2.47	2.50
Keratan sulfate Gal-6 sulfotransferase 1	*Chst1*	1	2.44	3.52	2.67
ST6 beta-galactosamide alpha-2,6-sialyltranferase 1	*St6gal1*	1	2.85	2.81	2.29
Heparan sulfate 3-O-sulfotransferase 1	*Hs3st1*	1	2.82	3.08	3.67
Versican	*Vcan*	1	3.47	3.14	4.01
Carbohydrate (Keratan Sulfate Gal-6) Sulfotransferase 1	*Chst1*	1	2.44	3.52	2.67
Carbohydrate (Keratan Sulfate Gal-6) Sulfotransferase 3	*Chst3*	1	2.38	2.47	2.50
Heparan sulfate (glucosamine) 3-O-sulfotransferase 1	*Hs3st1*	1	2.82	3.08	3.67
*Cytokine-like 1	*Cytl1*	1	4.52	5.06	5.71
Cartilage intermediate layer protein	*Cilp*_pred	1	1.99	2.08	2.47
*Cartilage intermediate layer protein 2	*Cilp2*_pred	1	1.76	1.90	2.35
CS N-acetylgalactosaminyltransferase-1	*Csgalnact2*	1	2.24	2.40	1.97
Matrilin 4	*Matn4*	1	−2.90	−3.11	−2.82
Collagen, Type XXIV, Alpha 1	*Col24a1*	1	−2.12	−2.02	−2.32
Collagen, Type IX, Alpha 2	*Col9a2*	1	−2.16	−2.35	−2.23
Proteoglycan 2	*Prg2*	1	−4.56	−3.87	−4.30
Matrilin 3	*Matn3*_pred	1	−5.95	−8.91	−12.28
Tenascin N	*Tnn*	1	−2.98	−2.19	−1.63
Collagen type I alpha 2	*Col1a2*	1	−2.03	−2.00	−1.41
Collagen, type IX alpha 3	*Col9a3*_pred	1	−1.95	−2.23	−2.18
Periostin, osteoblast specific factor	*Postn*_pred	1	−4.11	−3.70	−1.97

Osteoarthritis Cartilage. Author manuscript; available in PMC 2017 July 01.

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Inhibitors of ECM Degradation	Gene symbol	Day 0	Day 2	Day 5	Day 15
Serpin peptidase inhibitor, Clade A (antitrypsin)	*Serpina1*	1	4.86	12.15	12.36
Serpin Peptidase Inhibitor, Clade A memb3	*Serpina3n*	1	4.79	4.37	5.05
Murinoglobulin 1	*Mug1*	1	2.69	2.74	3.00
Murinoglobulin 2	*Mug2*	1	2.57	3.28	3.01
Mannan-Binding Lectin Serine Peptidase 1	*Masp1*	1	2.60	2.85	2.59
TIMP metallopeptidase inhibitor 4	*Timp4*	1	1.51	1.46	1.71
Serpin Peptidase Inhibitor, Clade A	*Serpinb1a*	1	−4.62	−4.04	−2.40
Alpha-2-macroglobulin	*A2m*	1	−2.17	−2.13	−2.47

Inhibitors of ECM Degradation	Gene symbol	Day 0	Day 2	Day 5	Day 15
Cathepsin K	*Ctsk*	1	−2.86	−3.58	−2.48
ADAM metallopeptidase thrombospondin type1 motif, 3	*Adamts3*	1	−3.00	−4.36	−4.98
*Matrix metallopeptidase 8 (neutrophil collagenase)	*Mmp8*	1	−2.42	−3.17	−2.37
Dipeptidyl-peptidase 4	*Dpp4*	1	−3.81	−3.87	−4.03
*Matrix metallopeptidase 9 (gelatinase B)	*Mmp9*	1	−8.20	−11.32	−7.84
ADAM metallopeptidase thrombospondin type1 motif, 14	*Adamts14*_pred	1	−2.47	−2.36	−2.47
ADAM metallopeptidase thrombospondin type1 motif, 3	*Adamts3*	1	−2.48	−3.90	−3.31
*Matrix Metallopeptidase 3	*Mmp3*	1	2.06	2.13	1.53
Matrix Metallopeptidase 14	*Mmp14*	1	−1.92	−2.50	−2.02
Heparanase	*Hpse*	1	1.27	1.05	2.01

Footnote: Salient transcripts showing two fold or greater up- or down-regulation of DEGs following 2, 5, or 15 days of exercise as compared to unexercised controls.

*Represents genes analyzed by microarray analysis for comparison with qrtPCR dataset in Fig 1D.

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Table IV

Temporal regulation of inflammation/immune system associated transcripts by exercise.

Gene name	Gene symbol	Day 0	Day 2	Day 5	Day 15
Chemokine (C-X-C motif) ligand 13	*Cxcl13*	1	2.25	4.37	9.85
Interleukin 16	*Il16*	1	2.62	3.07	2.69
Interleukin 1 receptor, type II	*Il1r2*	1	1.72	1.65	2.01
Interleukin 17B	*Il17b*	1	1.83	2.24	2.02
Interleukin 1 receptor, type I	*Il1r1*	1	2.10	2.04	1.77
Interleukin 6 receptor	*Il6ra*	1	2.18	2.61	1.92
*Lipopolysaccharide binding protein	*Lbp*	1	1.80	1.89	3.00
Thrombospondin 2	*Thbs2*	1	3.77	3.99	3.95
Plasminogen activator, tissue	*Plat*	1	2.06	2.37	4.25
FK506 binding protein 5	*Fkbp5*	1	6.50	6.70	8.11
Clusterin	*Clu*	1	2.71	2.71	3.20
Phosphodiesterase 10A	*Pde10a*	1	2.58	2.34	2.61
Phosphodiesterase 3A	*Pde3a*	1	2.04	2.14	2.16
Phospholipase D5 (inactive)	*Pld5*	1	11.19	12.71	8.19
Phospholipase C1	*Plce1*	1	4.18	4.02	4.21
Phospholipase a1	*Pla1a*	1	2.25	2.70	3.14
Phosphodiesterase 3B	*Pde3b*	1	2.62	2.28	1.74
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	*Pfkfb3*	1	2.29	1.76	1.56
Phospholipase A2, group IIA	*Pla2g2a*	1	1.03	1.87	9.74
Cathepsin S	*Ctss*	1	1.05	1.27	2.86
Heat shock 22kDa protein 8	*Hspb8*	1	3.47	3.51	3.73
Heat shock protein 90kDa alpha (cytosolic), class A 1	*Hsp90aa1*	1	2.09	2.10	2.11
Heat Shock protein 105	*Hsph1*	1	3.38	3.17	3.00
Heat shock 70kDa protein 1B	*Hspa1b*	1	1.54	1.99	3.43
Tumor necrosis factor receptor superfamily, member 11b	*Tnfrsf11b*	1	1.84	1.83	2.27
Plasminogen activator, tissue	*Plat*	1	2.06	2.37	4.25
Serpin peptidase inhibitor, clade A	*Serpina1*	1	4.86	12.15	12.36
Serpin peptidase inhibitor, clade A member 3N	*Serpina3n*	1	4.79	4.37	5.05

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Gene name	Gene symbol	Day 0	Day 2	Day 5	Day 15
Complement component 1, q subcomponent, C chain	*C1qc*	1	−1.34	−1.10	2.14
Chemokine (C-X-C motif) receptor 4	*Cxcr4*	1	−3.30	−3.10	−2.65
Arachidonate 15-lipoxygenase	*Alox15*	1	−2.32	−2.06	−2.81
*Prostaglandin-endoperoxide synthase 2	*Ptgs2*	1	−2.24	−2.57	−2.15
Complement component 3	*C3*	1	−3.50	−2.81	−2.05
Complement component 7	*C7*	1	−2.66	−3.69	−2.12
Neutrophilic granule protein	*Ngp*	1	−7.07	−6.77	−3.86
Interferon regulatory factor 8	*Irf8*	1	−2.31	−2.78	−1.72
Defensin, alpha 1	*Defa*	1	−7.36	−6.30	−1.81
CD93 molecule	*C1qr1*	1	−2.24	−2.65	−1.35
*Lysozyme	*Lyz*	1	−4.23	−4.31	1.19
Chemokine (C-X-C motif) receptor 2	*Il8rb*	1	−2.07	−1.76	−1.82
Chemokine (C-C motif) receptor 1	*Ccr1*	1	−2.29	−3.04	−1.79
Chemokine (C-X-C motif) ligand 12	*Cxcl12*	1	−2.35	−2.97	−1.82
Serpin Peptidase Inhibitor, Clade A	*Serpinb1a*	1	−4.62	−4.04	−2.40
Coagulation factor V (proaccelerin, labile factor)	*F5*	1	−1.86	−2.53	−1.65
*High mobility group box 2	*Hmgb2*	1	−3.83	−3.59	−3.40
S100 calcium binding protein A8	*S100a8*	1	−4.54	−4.32	−1.98
Phospholipase A2, Group VII	*Pla2g7*	1	−2.43	−3.27	−3.20
Phospholipase A2 Group IVa	*Pla2g4a*	1	−1.88	−2.06	−1.83

Footnote: Salient transcripts showing two fold or greater up- or down-regulation of DEGs following 2, 5, or 15 days of exercise as compared to unexercised controls.

*Represents genes analyzed by microarray analysis for comparison with qrtPCR dataset in Fig 1.

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