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Time-Series Transcriptional Profiling Yields New Perspectives on Susceptibility to Murine Osteoarthritis

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Objective. **Chronological age is a powerful epidemiologic risk factor for osteoarthritis (OA), a multifactorial disease that is characterized by articular cartilage (AC) degradation. It is unclear from a molecular perspective how aging interacts with OA to produce this risk to AC integrity. To address this key question, we used in vivo time-course analysis of OA development and murine interstrain variability in natural susceptibility to OA to examine changes in non–OA-prone CBA mice versus OA-prone STR/Ort mice, which develop disease that bears significant histologic resemblance to human OA. Through global transcriptome profiling, we attempted to discover the molecular signature linked with both OA vulnerability and progression.**

Methods. **Affymetrix Mouse Gene 1.0 ST Array profiles were generated from AC samples derived from CBA and STR/Ort mice at 3 different ages, corresponding to the stages prior to, at, and late after the natural onset of OA in the STR/Ort mice.**

Results. **We found that the OA in STR/Ort mice exhibited a molecular phenotype resembling human OA, and we pinpointed a central role of NF-κB signaling and the emergence of an immune-related signature in OA cartilage over time. We discovered that, strikingly, young healthy AC has a highly expressed skeletal muscle gene expression program, which is switched off during maturation, but is intriguingly retained in AC during OA development in STR/Ort mice.**

Conclusion. **This study is the first to show that AC chondrocytes share a high-abundance gene-expression program with skeletal muscle. We show that failure to switch this program off, as well as the restoration of this program, is associated with inappropriate expression of NF-κB signaling pathways, skeletal muscle–related genes, and induction and/or progression of OA.**

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Dr. Timmons has filed applications for patents related to the impact of age on muscle function and muscle responses to exercise.

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Osteoarthritis (OA) is a complex age-related, multifactorial, polygenic disease characterized by degradation and consequent loss of the extracellular matrix (ECM) of the articular cartilage (AC) (1). Chondrocytes, the only resident cells of AC, are vital for maintaining the integrity of normal joints, but they also contribute to the initiation and progression of the loss of AC in OA (2,3). These dual roles in healthy ECM remodeling and in pathologic changes in the AC have made it difficult to identify targets by which to limit OA development or protect against increased susceptibility during aging.

The study of OA currently relies upon in vivo animal models, partly because it is difficult to obtain AC samples during specific stages of OA in humans, as well as healthy site- and age-matched samples for comparison. Preclinical models of OA are also needed for drug development (4). Alternative comparative approaches in humans have relied mostly upon using normal AC samples and samples obtained during late-stage OA or

samples taken from various proximities to OA lesions (5). This has meant that specific factors that are crucial in determining OA development remain unidentified. This challenge is exacerbated by a lack of AC samples obtained before the onset of OA from individuals with known susceptibility to the disease.

It is therefore necessary to use models in which OA susceptibility and time course are defined in order to identify the predisposing and etiologic factors that lead to OA. We and other investigators (6,7) have previously used gene array analysis of AC after OA induction in surgical models of the disease to identify potential disease regulators. It is apparent that although relevant to posttraumatic OA, such models do not necessarily mirror natural primary OA and responses to surgery may complicate the analysis and results. Moreover, these models are not necessarily useful for identifying genes that *predispose* joints to the development of OA, but rather, those that are involved in AC remodeling and pathology.

Different mouse strains exhibit distinct age-related OA vulnerability (8). The inbred STR/Ort mouse strain develops histologic OA spontaneously. Genetic and microarray analyses and studies into the roles of obesity, patellar dislocation, matrix metalloproteases (MMPs), and inflammatory cytokines have yet to define the cause of OA in STR/Ort mice. Their OA develops over a relatively predictable time course, closely resembling primary OA in humans, with characteristic AC proteoglycan loss and active ECM degradation by MMPs, and likely involves genetic and mechanical factors (9–11). In contrast, CBA mice show very low levels of OA with age (9) and therefore represent a model of healthy AC aging.

In the present study, we used AC from both strains of mice, at ages corresponding to time points before, at, and after the overt development of OA in STR/Ort mice, to address whether specific AC gene expression patterns characterize OA risk. Our primary hypothesis was that differential gene expression prior to disease onset would identify molecular markers of OA risk. Second, we hypothesized that age-related changes specific to OA-prone AC would pinpoint a molecular program that contributes to OA progression. This would provide new candidate genes for future studies that attempt to discover, using targeted genetics (12), why aging interacts with AC to produce OA risk in humans. We contrasted the transcription profiles of chondrocytes during normal AC aging (CBA mice) and during OA (STR/Ort mice). We robustly demonstrated that normal healthy AC has a signature characterized by an age-related decline in the transcription of a muscle-selective gene repertoire, which is lacking in AC that is prone to OA. The importance of NF-κB pathway signaling in OA development in a mouse model of naturally occurring disease is highlighted.

MATERIALS AND METHODS

Animals and sample collection. Male CBA (Charles River) and STR/Ort (bred in-house) mice were kept in polypropylene cages, at a temperature of 21°C (±2°C), with 12-hour light/dark cycles, and were fed standard RM1 maintenance diet ad libitum. All procedures complied with the Animals (Scientific Procedures) Act 1986 and with guidelines of the local ethics committee. Mice were used in experiments at ages 8–10 weeks (young; 5 CBA and 6 STR/Ort mice), 18–20 weeks (mature; 5 CBA and 7 STR/Ort mice), and 40–42 weeks (aged; 9 CBA and 14 STR/Ort mice). Left knee tibial and femoral surfaces were exposed, and AC from each condyle was isolated using a Friedman-Pearson micro-rongeur (extra-fine tip, 0.7-mm cup width; Fine Science Tools) (7,13), and samples were kept in RNAlater (Qiagen). Right knees were processed for histologic examination.

RNA extraction and microarray process. A sample of AC from each STR/Ort and CBA mouse (n = 4 for each strain at each age) was immersed in QIAzol (Qiagen), homogenized, and total RNA was isolated with a Qiagen Mini Kit. RNA quality was assessed using an Agilent 2000 Bioanalyzer. Samples were stored at −80°C. In vitro transcription was achieved with a BioArray high-yield RNA transcript labeling kit (Affymetrix). Unincorporated nucleotides were removed using an RNeasy column (Qiagen). Hybridization to a total of 24 Affymetrix Mouse Gene 1.0 ST Arrays (1 sample per array; n = 4 samples per age group for each mouse strain), washing, staining, and scanning of arrays were performed according to the manufacturer's instructions (see array design online at https://www.affymetrix.com). Standard quality assessment (housekeeper 5′:3′ ratios, scaling factors) and individual array quality were controlled by hierarchical clustering and by NUSE to identify outliers (2 samples were removed from analyses; 1 sample each from 8-week-old and 40-week-old CBA mice). Raw data were submitted to the Gene Expression Omnibus (GEO) database (accession no. GSE33754; online at www.ncbi.nlm.nih.gov/geo).

Array analysis. Microarray data were normalized using Robust Mean Analysis and log2-file routines and analyzed in an R environment using Significance Analysis of Microarrays (SAM) (http://www-stat.stanford.edu/~tibs/SAM/ [14]), which provides a list of statistically significant genes and an estimate of the false discovery rate (FDR). SAM analysis calculates statistically significant genes by determining the percentage of genes discovered by chance through permutations of repeated measures from a candidate gene list. This generates a robust estimation of FDR. A gene list set at an appropriate FDR was then obtained. Genes were considered significantly changed when delta values corresponding to the number of false significant genes were <5% (q value) and an average 1.5-fold change was achieved. A 1.3-fold change was used for comparison to other studies. We used Ingenuity Pathways Analysis

(IPA; http://www.ingenuity.com) to discover networks regulated by aging and OA vulnerability, onset, or progression. Only networks with high confidence limits are reported. Gene lists for each comparison selected by SAM analysis were input into IPA, Core analysis was run, and Benjamini and Hochberg test was used to calculate *P* values for each function (highest *P* value found in the category). Array profiles of tissues obtained from the GEO database were used for comparing muscle gene expression levels (tibialis anterior muscle, accession no. GSE11105; heart, accession no. GSE12420; liver, accession no. GSE21060; testes, accession no. GSE16853; ovary, accession no. GSE16853; and duodenum, accession no. GSE18074).

Real-time quantitative polymerase chain reaction (qPCR). We used real-time qPCR to measure gene expression levels in 40-week-old CBA and STR/Ort mice (n = 6 per strain). Reverse transcription was performed on 1 μg of RNA (20-μl reaction volume) using a high-capacity RNA-to–complementary DNA (cDNA) kit (Applied Biosystems) run at 37°C for 60 minutes and at 85°C for 5 minutes. To detect messenger RNA (mRNA) transcripts, primers (Supplementary Table 1, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131) were premixed with SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich), and 10-μl aliquots were applied to 384-well optical plates (Bio-Rad). The cDNA was diluted 1:10, and triplicate 2-μl aliquots were added. Thermal cycling conditions were as follows: 10 minutes at 95°C, with 40 cycles of 15 seconds at 95°C, 30 seconds at 62°C, and 20 seconds at 72°C, on a Bio-Rad CFX384 Real-Time System. The cDNA was diluted (200×), and 18S levels were measured by TaqMan assay according to the manufacturer's protocol (Applied Biosystems).

Histologic assessment. Right knees were fixed and decalcified (Immunocal; Quartett), and coronal wax sections were cut (15). Serial sections taken at regular 120-μm intervals across the entire joint were stained with toluidine blue, and AC lesions were scored using a previously described system (15), and a mean ± SEM grade per joint was calculated. Briefly, changes in the AC were graded on a scale of 0–6, where 0 = normal, 1 = rough AC surface/lesions in superficial zone, 2 = lesion down to intermediate AC, 3 = lesions down to tidemark or loss of AC, 4 = AC loss across 20–50% of the condylar surface, 5 = AC loss across 50–80% of the condylar surface, and 6 = loss of AC with exposed subchondral bone. Osteophyte formation was also scored as described previously (16), using a scale of 0–3, where 0 = none, 1 = predominantly cartilaginous, 2 = cartilage and bone with chondrocyte hypertrophy and bone formation, and 3 = predominantly bone with marrow.

Sections of joints from representative 8-week-old CBA and STR/Ort mice (n = 4 per strain) were dewaxed, pepsin-digested (3 mg/ml in 0.02*M* HCl for 45 minutes at 37°C), blocked with 10% normal goat serum (for 1 hour), and p65 antibodies (recognizing the p65 subunit of NF-κB) were applied overnight at 4°C (1:10 dilution; Santa Cruz Biotechnology). After washing, sections were incubated for 1 hour with biotinylated goat anti-rabbit secondary antibody (1:200 dilution; Dako) and for 30 minutes with a Vectastain elite ABC kit (Vector). Sections were stained with a diaminobenzidine substrate kit (Vector).

Flow cytometry. Flow cytometry was performed as described elsewhere (17). Briefly, a splenic suspension was prepared by teasing the spleens (n = 4 per age group per strain), and the residual erythrocytes were removed by lysis using 0.85% NH_4Cl. Spleen cells were washed in cold phosphate buffered saline (PBS), counted, and incubated with combinations of fluorochrome-conjugated monoclonal antibodies for 1 hour at 4°C: anti-CD4, anti-CD44, anti-CD62L, anti-CD86, anti-CD45R (B220), and isotype controls (all from eBioscience). After 2 washes in PBS, cells were fixed in 1% paraformaldehyde and analyzed using FACSCanto II instrument (Becton Dickinson). Data were processed using FlowJo software (Java Software).

RESULTS

Confirmation of validity of known OA targets by analysis of cartilage in diseased STR/Ort mice. STR/Ort mice are a well-known spontaneous model of OA. There was increased severity of AC lesions with age in these mice, which demonstrated early OA at 18 weeks and late-stage disease at 40 weeks (Figures 1A–G). In contrast, CBA mice showed only very mild, if any, AC lesions at 18 weeks and no age-related progression (Figures 1A–G). In addition, only mild osteophytes were detected in AC samples from 40-week-old CBA mice (grade 1 in 4 of 9 mice), whereas STR/Ort mice developed mild osteophytes from 18 weeks (grade 1 in 3 of 7 mice) and had increased maturation scores at 40 weeks (grade 3 in 6 of 14 mice).

Our main objective was to use microarray analysis to define risk and disease genes and to plot how these alter with aging. First, we contrasted gene expression in all nondisease samples (8-week-old STR/Ort and all CBA mice) with all OA samples (18-week-old and 40-week-old STR/Ort mice). Such merging of data from the 2 strains effectively limits any differences attributable solely to strain-specific variation, which might be unrelated to OA, and provides the greatest statistical power. This analysis revealed 390 up-regulated and 272 down-regulated genes in the OA samples (Supplementary Table 2, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN) 1529-0131).

A significant number of genes described in a recent editorial (7,18–22) as being OA targets based on the results of microarray analysis of human OA and a rat model were also identified (Figure 1I), providing further confirmation of the relevance of our preclinical STR/Ort mouse model to human OA. Among these genes, levels of mRNA for Col2a1, tissue inhibitor of metalloproteinases 1 (TIMP-1), MMP-13, and ADAMTS-4 were further investigated by qPCR and showed increased levels in AC samples from 40-week-old STR/Ort mice as compared to those from age-

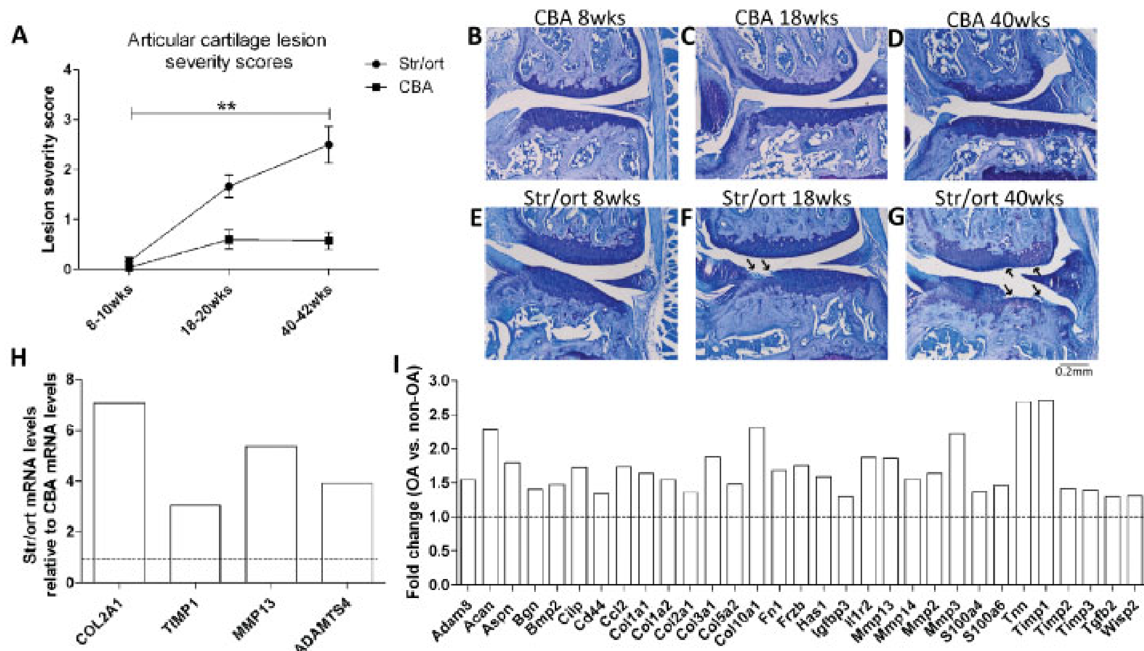

Figure 1. Increased osteoarthritis (OA) severity and expression of selected markers of extracellular matrix (ECM) turnover in STR/Ort mice. **A,** Grading of knee joint articular cartilage (AC) lesions in CBA mice and STR/Ort mice at ages 8–10 weeks (n = 5 or more mice per strain), 18–20 weeks (n = 5 or more mice per strain), and 40–42 weeks (n = 9 or more mice per strain) shows increased OA severity in STR/Ort, but not CBA mice. Values are the mean ± SEM. ** = $P < 0.01$. **B–G,** Toluidine blue staining of knee joint sections from STR/Ort mice, but not CBA mice, obtained at ages 8 weeks, 18 weeks, and 40 weeks shows increasing OA severity over time (**arrows**). **H,** Levels of mRNA for the ECM turnover markers COL2A1, tissue inhibitor of metalloproteinases 1 (TIMP-1), matrix metalloproteinase 13 (MMP-13), and ADAMTS-4 show increased expression in AC samples from STR/Ort mice at 40 weeks of age as compared with samples from CBA mice (n = 6 mice per strain). **I,** Genes demonstrating increased expression in samples of OA cartilage from STR/Ort mice that have also been identified in human OA or animal models are shown. Their selection was based upon their increased expression in previous array studies (7,18–22). Only selected genes with q values <5% and a >1.3-fold change are shown. Broken horizontal line in **H** and **I** shows mean control levels in CBA mice against which the levels in STR/Ort mice were corrected. Values in **H** and **I** are the ratio of the mean. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

matched CBA mice (Figure 1H). Bibliometric pathway analysis (Ingenuity) found that the top molecular or disease functions associated with these genes included cancer ($P = 2.45^{-13}$), reproductive system diseases ($P = 8.43^{-13}$), and cell-to-cell interaction and signaling ($P = 2.13^{-12}$) and that NF-κB pathway signaling (see below) was the most common molecular network upon which all of these were centered. This comparison revealed that many of the known OA targets are also differentially expressed by AC in STR/Ort mice and that these are centered upon the NF-κB pathway.

Gene expression changes related to OA predisposition, onset, and progression. To identify genes that characterize OA risk, we determined chondrocyte gene expression prior to OA onset by comparing samples from 8-week-old STR/Ort mice with those from age-matched CBA mice. This analysis showed that 139 genes were up-regulated and none were down-regulated in STR/Ort mice relative to CBA mice (Figure 2 and Supplementary Table 3, available on the *Arthritis &*

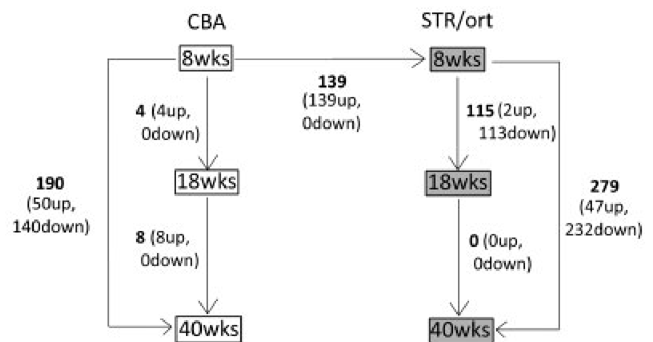

Figure 2. Schematic representation of the number of genes found to be differentially up-regulated and down-regulated across several comparisons. Numbers are restricted to those showing >1.5 fold differential expression with a q value <5%. Adjustment for additional multiple testing was not made for secondary comparisons. Our primary comparisons included differential expression between the CBA and the STR/Ort mouse strains at baseline and comparison of youngest to oldest age groups (8 weeks versus 40 weeks) between the two strains (n = 4 mice per strain, except for CBA mice at 8 weeks and 40 weeks, which were n = 3 after quality control was performed).

Rheumatism web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131). Among those that were up-regulated, were several ECM genes, including types VIII and XII collagen, tenascin-C, MMP-19, HTRA-1, and TIMP-1. Pathway analysis suggested that their main functions included genetic and connective tissue disorders ($P = 8.32^{-06}$), such as arthritic diseases and Dupuytren's contracture, and cell death ($P = 4.19^{-05}$).

As found in healthy versus OA samples, pathway analysis also revealed that the top networks into which these "risk" genes were centered featured significant NF-κB complex involvement (Figure 3A). The likely importance of this NF-κB pathway was confirmed by immunohistochemical labeling, which disclosed increased levels of p65 NF-κB subunit expression in chondrocytes from STR/Ort mice as compared to those from CBA mice (Figures 3B–E). This immunologic/inflammatory signature found in STR/Ort mouse chondrocytes before the appearance of overt OA prompted examination of their immunologic phenotype. These analyses strengthened this connection by showing that 8-week-old STR/Ort mice had increased spleen cellularity, decreased naive T cell numbers, and increased activated T cell and B cell numbers (Figures 4A–D); consistent with heightened inflammatory status prior to OA onset and modified NF-κB pathway signaling as a potential common molecular etiology for OA and immune phenotypes in STR/Ort mice.

Gene changes associated with OA onset were defined by comparing AC samples from 8-week-old STR/Ort mice with those from 18-week-old STR/Ort mice. This showed 2 up-regulated and 113 down-regulated genes (Figure 2 and Supplementary Table 4, available on the *Arthritis & Rheumatism* web site at http://online

Figure 3. Linkage of osteoarthritis (OA) susceptibility of articular cartilage (AC) in STR/Ort mice to the NF-κB complex. **A,** Network involving the NF-κB interactions (blue) formed by genes that are up-regulated in STR/Ort mice as compared to CBA mice at 8 weeks of age (before OA onset). Genes up-regulated in STR/Ort mice (pink/red) and other genes (white) are included based on known interactions with the NF-κB network. **B–E,** Immunohistochemistry for p65 in 8-week-old CBA mice and STR/Ort mice. Levels of p65 protein were increased in AC chondrocytes from STR/Ort mice. Images in **C** and **E** are higher-magnification (×20) views of the boxed areas in **B** and **D** (×10), respectively. Results are representative of 4 mice per group. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

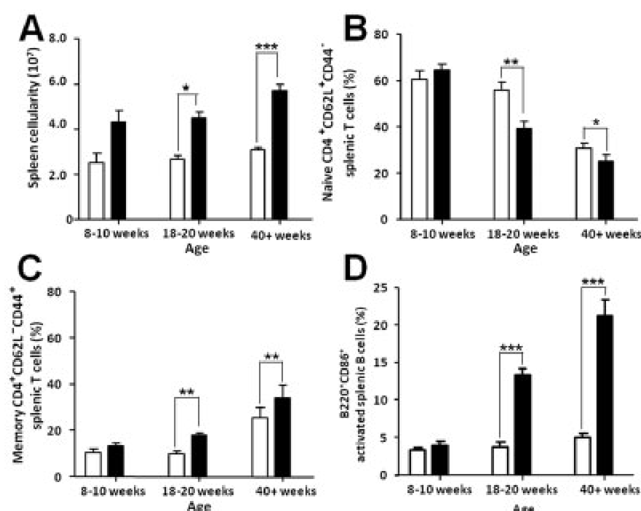

Figure 4. Increased numbers of total and activated T cells and B cells in the spleen of STR/Ort mice. At both 18–20 and ≥40 weeks of age, STR/Ort mice (solid bars) show increased splenic cellularity (**A**), decreased naive T cell numbers (**B**), increased activated memory T cells (**C**), and increased B cell activation (**D**) as compared to the CBA mice of the same age groups (open bars). Values are the mean ± SEM of 4 mice per group. $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$ by two-way analysis of variance comparing all variables, with Bonferroni multiple-comparison post-test.

library.wiley.com/journal/10.1002/(ISSN)1529-0131), none of which had previously been ascribed roles in OA. Pathway analysis revealed that their top functions included lymphocyte proliferation ($P = 6.96^{-05}$) and differentiation and activation ($P = 1.19^{-04}$) and that their top network was again focused on NF-κB signaling. Interestingly, no further changes in AC gene expression were observed at later stages of OA (18-week-old versus 40-week-old STR/Ort mice) (Figure 2).

Discovery of a myogenic signature in young AC that is lost during normal aging but not in the presence of OA. The gene expression profile linked with healthy AC chondrocyte aging but not with OA has not been described in mice. Using CBA mice as a model of healthy AC aging, since they never exhibit overt OA, we found 190 genes (50 up-regulated and 140 down-regulated) that were differentially expressed between young (8 weeks) and aged (40 weeks) AC from non–OA-prone CBA mice (Figure 2 and Supplementary Table 5, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN) 1529-0131). These genes did not include previously defined OA targets, but did include cartilage intermediate-layer protein (CILP) and histone deacetylase 5 (HDAC-5), suggesting that deviation in their levels is associated with normal AC aging. Replication of this age-related change in gene expression in normal human AC is required before the potential OA relevance is fully recognized.

Ontology analysis (Ingenuity) and visual inspection revealed that most down-regulated genes in this category were, surprisingly, skeletal muscle–specific isoforms of various structural, metabolic, and matrix-related proteins (Table 1); their age-dependent changes

Table 1. Identity of main functional categories linked to genes differentially expressed in CBA and STR/Ort mice between 8 weeks and 40 weeks of age*

Category, functional annotation	No. of molecules	P
CBA mice		
Skeletal and muscular system development and function		
Contraction of muscle	32	1.43^{-28}
Contraction of skeletal muscle	11	8.17^{-13}
Contraction of striated muscle	9	1.03^{-10}
Development of muscle	9	2.33^{-02}
Tissue morphology		
Contraction of muscle	32	1.43^{-28}
Contraction of skeletal muscle	11	8.17^{-13}
Contraction of striated muscle	9	1.03^{-10}
Skeletal and muscular disorders		
Myopathy	16	2.48^{-13}
Skeletal and muscular disorder	60	1.29^{-11}
Huntington's disease	27	1.42^{-11}
Muscular dystrophy	7	4.32^{-05}
Duchenne's muscular dystrophy	4	1.05^{-03}
STR/Ort mice		
Cellular growth and proliferation		
Proliferation of blood cells	29	2.33^{-06}
Proliferation of lymphocytes	27	2.33^{-06}
Proliferation of B lymphocytes	15	1.78^{-05}
Proliferation of normal cells	41	1.05^{-04}
Proliferation of eukaryotic cells	48	6.04^{-04}
Hematologic system development and function		
Proliferation of lymphocytes	27	2.33^{-06}
Proliferation of B lymphocytes	15	1.78^{-05}
Differentiation of B lymphocytes	11	1.93^{-04}
Quantity of B lymphocytes	12	5.29^{-04}
Quantity of B-1 lymphocytes	6	6.37^{-04}
Humoral immune response		
Proliferation of B lymphocytes	15	1.79^{-08}
Differentiation of B lymphocytes	11	4.53^{-07}
Quantity of B lymphocytes	12	1.42^{-06}
Quantity of B-1 lymphocytes	6	2.77^{-06}
Quantity of pre–B lymphocytes	6	3.98^{-06}

* Analysis revealed regulation of muscle-specific genes during healthy aging in only the CBA mice. Genes closely linked to hematopoietic cell lineage functions were revealed in aging osteoarthritis-prone STR/Ort mice. Ingenuity Pathway Analysis was used to identify the top 3 categories and their main functional annotations (selection based on containing >4 molecules and significance at $P < 0.05$ after Benjamini and Hochberg correction). The full list of molecules associated with these functions is given in Supplementary Table 7, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

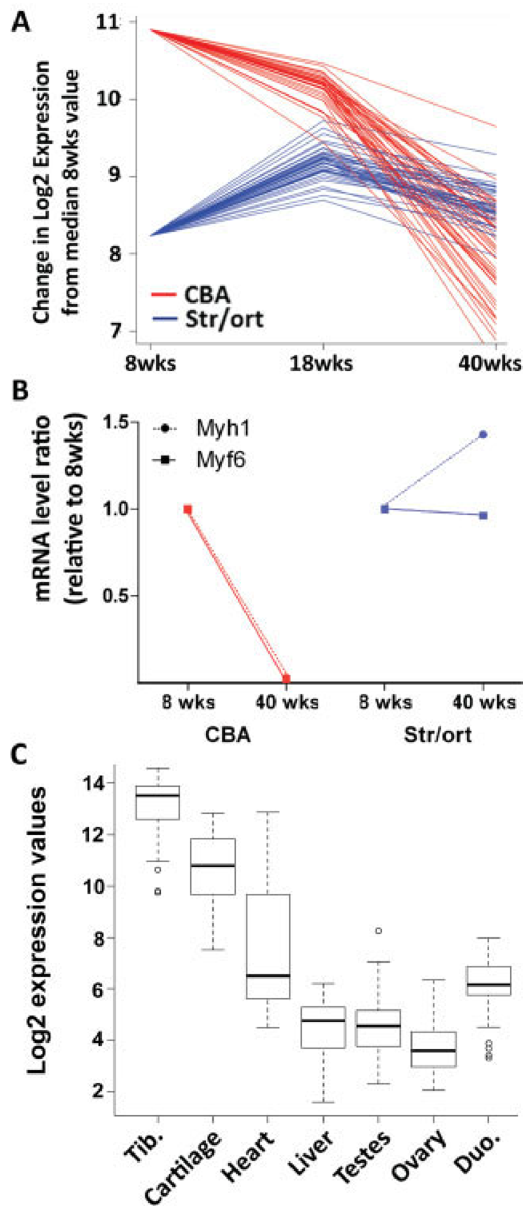

Figure 5. Muscle-specific gene expression in articular cartilage (AC) samples from CBA mice and STR/Ort mice over time. **A,** Myogenic gene profiling disclosed decreases in expression in CBA mice, but not STR/Ort mice with age (n = 4 mice per group; 42 probe sets). **B,** Ratio of mRNA expression of myosin heavy chain 1 (Myh1) and myogenic factor 6 (Myf6), as determined by quantitative polymerase chain reaction techniques, in AC samples at 40 weeks of age relative to 8 weeks of age confirmed decreases in the CBA mice, but not the STR/Ort mice (n = 3 mice per group). **C,** Microarray-derived gene profiles of the "skeletal muscle" signature in cartilage more closely resembles murine skeletal muscle (Tib), than heart, liver, testes, ovary, or colon (Duo) (n = 3 mice per group, except for ovary, which was 2 mice per group). Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and the 90th percentiles. Circles indicate outliers.

in expression are plotted in Figure 5A. Two features are striking. First, their level of expression in AC samples from CBA mice was higher than in samples from STR/Ort mice. Second, the muscle "program" is switched off, at least in part, in aged CBA mice, but not STR/Ort mice (Figure 5A), which retain AC expression throughout the aging/disease process. These aging-related patterns of muscle-specific gene expression were indeed confirmed by qPCR for myogenic factor 6 and myosin heavy chain 1 (Figure 5B). Pathway analysis of the genes differentially regulated between 8 weeks and 40 weeks in AC samples from STR/Ort mice revealed their relatedness to cellular growth and proliferation and to lymphocyte development and function (Table 1 and Supplementary Table 6, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131). It is most critical to note that the expression of these muscle-specific genes is not ubiquitous and that their expression is higher in AC than in heart muscle and is remarkably close to that found in adult murine skeletal muscle (higher than in liver and testes) (Figure 5C). Our data also showed that AC retained some aspect of the muscle gene expression program throughout life at a remarkably high level of expression (Supplementary Figure 1, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).

Using IPA, 13 of the nonmuscle genes that were down-regulated in both CBA and STR/Ort mice between 8 weeks and 40 weeks of age, which are most likely related to non-OA healthy aging, were found to serve known roles in hematologic disorders and function ($P = 6.67^{-05}$). It is possible that genes found only in the AC of STR/Ort mice are specifically linked to the OA that occurs at these ages and include 43 up-regulated and 209 down-regulated genes that are unmodified in CBA mice between 8 and 40 weeks of age. These genes are involved in the inflammatory response ($P = 2.94^{-12}$), cell death ($P = 6.45^{-09}$), and cell growth and proliferation ($P = 1.13^{-0.7}$) pathways, all of which are centered on NF-κB signaling. Unusually, none had previously identified links with OA development.

These findings indicate that AC from normal, but not OA, mice has a myogenic signature in the young that diminishes in its expression during healthy aging AC and that NF-κB pathway signaling might act as a hub in driving gene expression changes in OA-prone mice.

DISCUSSION

In this study, we used a model of natural, spontaneous OA in STR/Ort mice as compared to age-

matched non–OA-prone CBA mice to determine the AC gene expression profiles specific to OA susceptibility, early and late-stage disease, and healthy aging. We found that AC from STR/Ort mice with overt OA displays an expression profile consistent with that previously described in human OA and in animal models of OA (including types II and X collagen, MMP-3, and TIMPs 1–3), confirming these mice as a valuable model of disease pathology. This study also showed that chondrocytes had a strong myogenic signature that was partially down-regulated with age in healthy AC, but was retained or reexpressed in aging OA cartilage from STR/Ort mice. We found that chondrocytes also possess an inherent inflammatory gene phenotype involving the NF-κB pathway, which is likely linked to early OA in these mice, and that early and late OA profiles implicate similar processes. This study therefore confirms several known targets by use of a spontaneous murine model of OA, defines new targets, including those converging upon NF-κB pathway signaling, and defines a myogenic program, which may be used to monitor, attenuate, or even treat OA.

STR/Ort mice develop OA spontaneously and are a model of natural (primary) OA (9–11,23–29). Their OA develops relatively predictably, with first signs presenting at 18 weeks of age and progressing toward loss of AC and exposure of subchondral bone at 40 weeks. Comparison of diseased AC (from STR/Ort mice at 18 and 40 weeks of age) with AC lacking overt features of OA (from CBA mice of all ages and from STR/Ort mice at 8 weeks of age) revealed almost all changes found in previous arrays using samples from animal models and humans with OA (7,18–22). This supports the suitability of STR/Ort mice as a model of chondrocyte involvement in OA-related AC pathology. Our analyses also revealed several unexplored or novel processes that may represent targets for therapeutic evaluation of OA development.

Watters et al (30) performed microarray analysis of OA joints from STR/Ort mice using matched tissue from younger STR/Ort mice, thus removing any chance of detecting age-related gene changes or differences based on genetic background. They described changes associated with osteophyte development that correlated with AC lesion severity and found peroxisome proliferator–activated receptor γ (PPARγ) and PPARα to be central to OA in STR/Ort mice. However, by not including AC samples from non–OA-prone mice, their analysis did not specifically identify the muscle expression program noted herein. In our study, we found that NF-κB likely played a central role in OA. Indeed, NF-κB was the top network with regard to diseased versus nondiseased joints, OA predisposition in 8-week-old CBA mice versus STR/Ort mice, and during early OA and late OA. The divergence in the outcomes between these studies could also rely on the fact that Watters et al used samples containing both AC and bone, whereas our samples were limited to AC alone (7).

These data suggest that the role of these PPARs might be relatively restricted to bone; however, it should be noted that cartilage-specific PPAR signaling is emerging as an OA target (31,32) and that, like NF-κB, it also integrates into inflammatory signaling and regulates NF-κB function in various tissues (33,34). It is possible, therefore, that the informatics methods applied to the identification of transcription signatures and the timing of these analyses during OA development underpin the differences seen in their outcomes.

Cytokine signaling has also been associated with severe OA in humans (18), confirming the potential modulation of the NF-κB signaling pathway as a therapeutic target. This has also been recently discussed in various review articles (35,36), and inhibition of NF-κB p65 expression was shown to decrease OA development in a rat surgical model (37). The NF-κB network can be activated by various cytokines, by degradation products of ECM components such as fibronectin (38,39), and by mechanical stimuli (40), all of which are increased in STR/Ort mice during OA (24,25,41). NF-κB activation also plays a role in chondrocyte hypertrophy (42), which is increased in OA and, consequently, may lead to MMP and cytokine expression. Levels of MMP and cytokine expression were also found to be increased in our study and are known to further increase ECM degradation, thus contributing to OA progression. The increased splenic weight and numbers of activated immune cells found in young STR/Ort mice also supports a general non–tissue-specific proinflammatory state that may reflect the genetic etiology underpinning OA susceptibility in these particular mice.

It is also pertinent to consider whether these signatures are causally connected via NF-κB or whether they are separate products with a common molecular etiology. Clarifying these relationships is crucial, as this may reveal alternative strategies for OA prevention or treatment. Nevertheless, it is important to note that OA in STR/Ort mouse joints occurs without overt inflammation or cell infiltration (11). Modulating NF-κB would also have to be achieved in a cell-specific or tissue-specific manner due to its role in immune and other skeletal processes. However, given the potential to directly target large OA surfaces with intraarticular injections, proof-of-concept studies are plausible.

It has previously been proposed that early and

late OA exploit different molecular processes. Indeed, although the total numbers of differentially regulated genes were few (14 in total), microarray studies have shown that chondrocyte gene profiles differ in mild and severe OA in humans (18). In contrast, our study showed no difference in gene expression in early and late OA occurring in STR/Ort mice, which suggests that progression is linear in this spontaneous model and implies that baseline AC gene signatures may be especially important. The discrepancy with human data may be explained by distinct classification criteria: early OA in humans might represent fibrillation without AC loss, whereas mild AC loss is seen in early OA in mice. Alternatively, it may point to the need for larger sample sizes for such studies if they are to detect these discrete processes.

CBA mice have been used as controls for the STR/Ort strain for decades, as they do not develop overt OA (9). Indeed, very mild AC lesions occur only rarely in CBA joints and often fail to advance significantly with age. This underpins their selection as an appropriate model of healthy AC. We found that changes in gene expression in CBA mouse AC were limited and, again, rather gradual over time. In fact, most genes, except for CILP, that have been implicated in OA did not change significantly with age in CBA mice, which bolsters the likelihood that the OA gene changes we describe are specific to OA and are unrelated to aging alone. These analyses also revealed a new chondrocyte signature that integrated many skeletal muscle gene isoforms, which is usually associated with contractile cells. This signature was surprisingly enriched in the AC as compared with other tissues, was highly expressed in young AC, and showed age-related declines. This age-related decline in myogenic gene expression was not, however, evident in OA chondrocytes from aged STR/Ort mice, in which this arguably inappropriate signature was retained or reexpressed. Whether this signature signals an attempt to repair AC damage or whether it contributes to OA development in skeletally mature mice is unknown.

During the time we were preparing the manuscript describing our study, Loeser et al (43) reported that a change in muscle-specific gene expression was among the genes that were selectively regulated in older, but not young, mice subjected to surgical destabilization of the medial meniscus. Although the lack of any solely age-related decline in muscle-specific gene expression in those studies may be due to their inclusion of non-AC tissues (43), their findings support a role for a muscle signature in OA. The role of this program clearly requires further interrogation.

The presence of myogenic gene expression in young chondrocytes most likely relates to their common origin with muscle cells during development, yet it is very surprising to observe high absolute levels of expression in nonmuscle tissue into adulthood. Indeed, while chondrocytes, brown fat cells, and myocytes are formed from mesoderm-derived mesenchymal stem cells (44–46), the myogenic isoforms of contractile, ECM, and energy metabolism were not thought to be found in mature tissues not containing striated muscle. Appearance of this program in OA chondrocytes might indicate that they exhibit some dedifferentiation and is consistent with a reexpression of type IIA collagen (47) and an increase in mesenchymal progenitor cells (48) in OA joints. The findings therefore further extend the repertoire of factors reflecting recapitulation of developmental mechanisms in OA (49).

As with other microarray studies in animal models, some caveats remain. First, OA in STR/Ort mice does not rely on external induction and may thus show greater variability even within this inbred population. This is similar to any studies with human OA cartilage, but it adds further complexity and increases the scope for assessing the genes involved. Second, the appropriateness of using CBA mice as a control strain is questionable. This choice makes our study consistent with previous work and is based on their relatively low incidence of age-related OA. The CBA mouse is also the commercially available strain that is most closely related to the STR/Ort strain (50), and its use in this study attempts to additionally adjust for age-related changes. Care should be taken when comparing different strains, as this may exaggerate differences in gene expression that may be unrelated to disease status. Third, use of 40-week-old mice as representative of "aged" AC is debatable. Until aging is more completely understood, however, many factors, including chronology, relationship to skeletal or sexual maturation, or even total likely lifespan, may affect the interpretation of these data. We chose 40 weeks since at this age, AC is still available in adequate quantities from the OA joints of STR/Ort mice.

In conclusion, we found that NF-κB signaling is central to the gene changes that take place in AC chondrocytes during natural OA development in STR/Ort mice. Our studies also revealed a new chondrocyte gene expression signature in which muscle-related genes are down-regulated with normal aging, but stay highly expressed in OA. This study highlights these two pathways as possible targets for slowing OA progression in nontraumatic, natural OA, as well as possible markers of disease status.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Pitsillides had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Poulet, Beier, Pitsillides.

Acquisition of data. Poulet, Ulici, Pead, Gburcik, Constantinou, Palmer.

Analysis and interpretation of data. Poulet, Ulici, Stone, Gburcik, Palmer, Beier, Timmons, Pitsillides.

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