

Zone-Specific Gene Expression Patterns in Articular Cartilage

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Objective. To identify novel genes and pathways specific to the superficial zone (SZ), middle zone (MZ), and deep zone (DZ) of normal articular cartilage.

Methods. Articular cartilage was obtained from the knees of 4 normal human donors. The cartilage zones were dissected on a microtome. RNA was analyzed on human genome arrays. The zone-specific DNA array data obtained from human tissue were compared to array data obtained from bovine cartilage. Genes differentially expressed between zones were evaluated using direct annotation for structural or functional features, and by enrichment analysis for integrated pathways or functions.

Results. The greatest differences in genome-wide RNA expression data were between the SZ and DZ in both human and bovine cartilage. The MZ, being a transitional zone between the SZ and DZ, thereby shared some of the same pathways as well as structural/functional features of the adjacent zones. Cellular functions and biologic processes that were enriched in the SZ relative to the DZ included, most prominently, extracellular matrix–receptor interactions, cell adhesion molecule functions, regulation of actin cytoskeleton, ribosome-related functions, and signaling aspects such as the IFN, IL4, Cdc42/Rac, and JAK/STAT signaling pathways. Two pathways were enriched in the DZ relative to the SZ, including PPARG and EGFR/SMRTE.

Conclusion. These differences in cartilage zonal gene expression identify new markers and pathways that govern the unique differentiation status of chondrocyte subpopulations.

Articular cartilage possesses a unique structure that enables it to perform its function as a lubricating and load-bearing surface in the joints. Variations in matrix biochemical composition, cell morphology, cell density, cell metabolism, and the pericellular matrix (PCM) determine the zonal architecture (1).

The superficial zone (SZ) spans the first 10–20% of full-thickness articular cartilage and contains densely packed collagen fibrils and low levels of aggrecan (2–4), although fibril-associated decorin and biglycan are found in higher concentrations in the SZ (2,5,6). Chondrocytes in this zone produce little PCM and are elongated, flattened, and oriented parallel to the cartilage surface (7). Cells within the SZ synthesize and secrete the important joint lubricant superficial zone protein, which is also known as megakaryocyte-stimulating factor, lubricin, or proteoglycan 4 (8–11).

Clusterin, a glycoprotein that regulates complement activation and cell death, is also exclusively expressed in SZ chondrocytes (12). Chondrocytes located in the SZ differ from deep zone (DZ) chondrocytes by their lower type II collagen gene expression levels (12–14) and lower production of keratan sulfate and other proteoglycans (15–19). Recent studies show that the SZ of mature articular cartilage contains cells with phenotypic and functional properties of mesenchymal stem or progenitor cell populations (20–24). These cells are characterized by the expression of the surface receptors CD105, CD166 (20), Notch-1 (22,23), STRO-1, and vascular cell adhesion molecule 1 (VCAM-1) (25). SZ cells are strongly positive for α -smooth muscle actin, a contractile actin isoform (26) that is also present in progenitor cells (27).

The middle zone (MZ), or transitional zone, comprises the next 40–60% of cartilage thickness and

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contains randomly organized collagen fibrils and high concentrations of aggrecan (28), hyaluronic acid, dermatan sulfate, and type II collagen (29–34). The DZ, or radial zone, contains ellipsoid cells with an extensive PCM among radially oriented collagen fibrils that extend into the calcified zone to preserve cartilage and bone integration (35,36). In the calcified zone, which represents the boundary between cartilage and subchondral bone, cells are contained within a calcified matrix and express hypertrophic molecules such as type X collagen (37), alkaline phosphatase, and osteocalcin (1,17,37–39).

Thus, only limited information is available on markers and regulators of cells in the different zones of articular cartilage. Such functional and phenotypic differences between cell populations are of interest for cell-based cartilage repair strategies. Successful recapitulation of the zonal organization does not occur during spontaneous cartilage repair, and remains an elusive goal in tissue engineering (18,40).

Resolving differences in gene expression between chondrocyte subpopulations will provide new insight into the pathogenesis of diseases affecting articular cartilage. For example, in osteoarthritis (OA), zone-specific changes and distinct expression profiles may occur between stages of the disease process (41). Among the earliest changes are loss of cells in the SZ, with activation and abnormal differentiation in the DZ. Cell proliferation typically occurs in areas of cartilage fibrillation and leads to the formation of cell clusters that express a broad spectrum of pathogenic mediators (42). This study used genome-wide RNA expression analysis to reveal novel zone-specific markers and potential regulators of zonal chondrocyte subsets in normal human articular cartilage.

MATERIALS AND METHODS

Cartilage procurement. Normal human knee joints were procured from tissue banks, using joints from 1 female donor (age 23) and 3 male donors (ages 24, 44, and 46); approval for use of these samples was provided by the Scripps Institutional Review Board. The knee joints were processed within 24–60 hours postmortem. Osteochondral cores (6.5 mm in diameter) were harvested for RNA isolation from identical locations on the medial and lateral femoral condyles of each knee, using the ACUFEX Anatomic ACL Guide System (Smith & Nephew). Adjacent osteochondral cores were harvested for histology to verify the cartilage integrity.

Intact bovine knee joints ($n = 2$) from skeletally mature animals (14–30 months of age) were obtained from abattoirs within 48 hours after slaughter. Cartilage surfaces were confirmed to be macroscopically normal. Osteochondral

plugs were cored out from the weight-bearing area of the distal femoral condyles.

Harvesting cartilage zones. The bone part of each osteochondral core was embedded in paraffin in a standard plastic cassette to allow fixation of the plug in the microtome. The entire cartilage was sliced into 50- μm -thick sections, from the cartilage surface downward within 100–200 μm of the calcified cartilage, using a microtome (Microm HM 325; Thermo Scientific). Each section was transferred to one well of a 96-well plate filled with RNAlater (Qiagen). The sections were segregated into zones for RNA isolation. The upper 10% of zonal slices was allocated to the SZ, the middle 40% to the MZ, and the lower 30% to the DZ. To avoid overlap between zones, regions of ~ 200 μm between the SZ and MZ and between the MZ and DZ were discarded.

RNA isolation, quality assessment, and labeling. RNA was isolated from cartilage zones using RNeasy kits (Qiagen) with DNase digestion. Total RNA was quantified using a NanoDrop spectrophotometer (ND-1000). Sample quality was determined with an Agilent 2100 Bioanalyzer using the RNA 6000 Pico LabChip (Agilent 5065-4473). Only samples with RNA integrity numbers of >6 were used. Five nanograms of total RNA was amplified using the NuGEN Ovation Pico WTA System, version 1.0. For postamplification processing of human samples, we used 4 μg of purified complementary DNA (cDNA) product for processing with the NuGEN WT-Ovation Exon Module (version 1.0). For both human samples post-Exon processing and bovine samples postamplification processing, 5 μg of the purified cDNA product was fragmented and labeled using a NuGEN Encore Biotin Module. Pre- and postfragmentation products (5 μg) were analyzed on an RNA 6000 Nano LabChip (Agilent 5065-4476) using the Agilent mRNA Assay program, following the manufacturer's instructions.

Microarrays. Five micrograms of postfragmentation/postlabeling product was used in the hybridization cocktail and hybridized overnight to either an Affymetrix GeneChip Human Gene 1.0 ST Array (version 1, Affymetrix P/N 901086) or Affymetrix GeneChip Bovine Genome Array (Affymetrix P/N 900562). Hybridization and scanning of samples to arrays was performed using standard NuGEN Hybridization, Cocktail Assembly, and Fluidics protocols with an Affymetrix GeneChip Hybridization, Wash, and Stain kit (Affymetrix P/N 900720), in accordance with procedures outlined in Appendix V1 of the NuGEN Encore Biotin Module protocol. Chips were scanned using an Affymetrix GeneChip Scanner 3000 7G, with default settings and a target intensity of 250 for scaling.

Microarray data deposition. Our data have been deposited in the Gene Expression Omnibus, with GSE39797 as the reference series. These data can be viewed at <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39797>.

Array data analysis. Raw data for the signaling intensities of messenger RNA (mRNA) transcript probe sets were generated in the form of probe cell intensity (.CEL) files using Affymetrix software. These data were subsequently combined to yield expression measures, as described below.

Data in the .CEL files were normalized using 2 different methods, a robust multichip average (RMA) method (RMA Express, version 1.0; <http://rmaexpress.bmbolstad.com>), which has quantile normalization, median polish, and background adjustment, as well as the method of Li and

Wong as implemented in the dChip application (www.dChip.org), which uses model-based expression. Both normalized data sets were subsequently analyzed using the rank products method (43) to identify differentially expressed transcripts. Rank products analysis does not depend on an estimate of the gene-specific variance, and is therefore robust for use with a small number of replicated data sets.

The \log_2 -transformed signal intensities were used to calculate the fold change in gene expression between zones, in pairwise comparisons. The difference between the average \log_2 -transformed signals for each zone was used to determine the fold change in expression levels between zones. The magnitude fold change (Mag.FC) was then determined, using one of the following calculations: if fold change >1 , $\text{Mag.FC} = \text{fold change}$; if fold change <1 , $\text{Mag.FC} = -1/\text{fold change}$. These calculations allowed us to assign both the magnitude and direction of the change. Differentially expressed transcripts for each zonal comparison (i.e., SZ versus DZ, SZ versus MZ, and DZ versus MZ) were identified using the following criteria: 1) proportion of false positives must be <0.15 , and 2) $|\text{Mag.FC}|$ must be >1.4 . The differentially expressed transcripts meeting these criteria (using both normalization methods) were identified as higher confidence targets, and thus comprised the final data sets.

Prioritization of zone-specific markers was then given to transcript targets in the human data set that were validated in the bovine data set (i.e., genes differentially expressed in the same direction in both data sets), or to highly significant markers in the human data set that were not present on the bovine array. The validation data set was further evaluated by calculating the number of such genes expected by chance, as well as by performing a Monte Carlo simulation of randomly permuted sets of human and bovine data to determine the probability of obtaining the observed number of validated genes.

Functional mapping. Gene set enrichment analysis (GSEA) was performed to identify enriched pathways, as well as structural and functional annotations. The GSEA method (44) ranks genes by differential expression levels between samples, and then determines whether a specific set of genes in a given pathway is significantly overrepresented toward the top or bottom of the ranked list, relative to randomly permuted samples or gene lists (45). Only the RMA-normalized data obtained from human articular cartilage was used for the GSEA; we filtered out any genes with maximal expression below 40% of the median value of all arrays.

A gene set permutation analysis was used to obtain the random background distributions for calculation of the false discovery rate (FDR). Additional nondefault parameter settings for the GSEA included the following: “collapse to gene symbols,” with permutation by gene set; enrichment statistic, varying from “classic” (unweighted) to “weighted_p2” depending on the enrichment category; and metric for ranking = “Diff_of_Classes,” using the average $[\log_2(\text{signal})]$. Enrichment scores were corrected for multiple hypothesis testing, prior to calculation of the FDR. For screening, an FDR of 0.25 was used.

RESULTS

Unique gene expression signatures in human cartilage zones. The 3 cartilage zones were harvested from the articular cartilage of each of 4 normal human

knees, and RNA samples were analyzed on 12 Affymetrix GeneChip Human Gene 1.0 ST arrays. Array data were normalized using 2 different methods, RMA and dChip. Rank products analysis of the human cartilage zonal gene expression, with zones being compared pairwise, yielded evidence of differentially expressed transcripts between zones in both normalized data sets. In subsequent analyses, only differentially expressed genes identified by both normalization methods were used.

The largest differences observed were between the SZ and DZ for 343 differentially expressed genes, representing 1.7% of the genes on the array. Of these genes, 59% (202 of 343) were higher in the SZ, while 41% (141 of 343) were higher in the DZ. We observed 129 genes (0.6%) that were different between the SZ and MZ, and 46 genes (0.2%) that were different between the DZ and MZ. Complete lists of all differentially expressed human genes are included in Supplementary Table 1 (available on the *Arthritis & Rheumatism* web site at [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131)).

Identification of zonal markers in human cartilage. Zonally up-regulated genes were defined as those whose expression was significantly higher in one zone in comparison to the other two zones (Figure 1). We identified 59 genes unique to the SZ, 1 unique to the MZ, and 20 unique to the DZ. When we analyzed the genes whose expression was significantly reduced in one zone as compared to the other two zones, we identified 47 specifically down-regulated genes in the SZ, none in the MZ, and 15 in the DZ (Figure 1).

Unique gene expression signatures in bovine cartilage zones. To generate an independent data set to compare to the human data set, similar DNA array analyses of the 3 zones were performed in bovine cartilage. Rank products analysis of the bovine cartilage zonal gene expression, with zones compared pairwise, yielded the following numbers of differentially expressed transcripts (as identified by both normalization methods). The largest differences observed were between the SZ and DZ for 184 differentially expressed genes, representing 1.6% of the genes on the array. Of these genes, 34% (63 of 184) were higher in the SZ, while 66% (121 of 184) were higher in the DZ. In total, 90 genes were different between the SZ and MZ, and 120 between the DZ and MZ. Complete lists of all differentially expressed bovine genes are included in Supplementary Table 2 (available on the *Arthritis & Rheumatism* web site at [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131)).

SUPERFICIAL ZONE (UP):		
Gene Symbol	SZ v MZ	SZ v DZ
VCAM1	10.8	9.7
SEMA3A	4.8	7.7
DLX3	4.2	7.6
SULF1	6.5	6.9
IGFBP5	6.1	6.8
STEAP4	2.4	6.7
EFEMP1	3.0	6.4
CHI3L2	3.4	6.2
MMP2	7.2	6.1
PDE1A	5.0	6.0
SLC16A9	2.3	5.9
THBS4	4.1	5.6
MAMDC2	4.5	5.4
HHIP	3.6	5.3
DPT	3.6	5.2
HTRA4	3.3	5.1
CHI3L1	2.1	5.0
PTPRD	2.8	4.6
CLIC2	2.2	4.5
CDC42EP3	3.0	4.3
CAPN6	2.3	4.1
MYO1B	2.6	4.1
RBPMS	3.3	4.0
PAPPA	2.2	3.9
C1orf54	2.3	3.8
CDH13	2.9	3.7
FAM388	2.1	3.7
NTN4	2.0	3.7
LPCAT2	2.8	3.7
UBASH3B	1.7	3.7
OGN	1.9	3.7
F5	3.6	3.6
TC2N	2.2	3.6
FAP	2.7	3.6
DOK5	2.3	3.6
B9GALT1	2.2	3.5
KAL1	1.8	3.5
ASPN	1.8	3.4
GIA1	2.0	3.3
SETBP1	2.1	3.2
PDE3B	2.1	3.1
LAMA4	2.3	3.1
PDGFA	3.1	2.9
CRTAC1	2.2	2.9
RND1	1.8	2.8
PAMR1	2.8	2.7
ZNF814	2.0	2.7
CORIN	2.5	2.7
LOC100130876	2.4	2.6
MYLK	2.2	2.5
EGR2	2.3	2.5
SVEP1	2.1	2.4
FGL2	2.3	2.4
WLS	2.1	2.4
HRH1	2.0	2.3
ZNF778	2.1	2.3
ITGB8	3.0	2.0
ZEB2	2.7	2.0
TNC	4.8	1.9

SUPERFICIAL ZONE (DOWN):		
Gene Symbol	SZ v MZ	SZ v DZ
CHAD	-1.5	-2.1
GMNN	-2.3	-2.2
CHRD12	-2.1	-2.2
OCR1	-2.7	-2.2
HSD17B11	-2.0	-2.3
CLEC3A	-1.8	-2.3
PDZRN4	-2.8	-2.4
TTL7	-2.2	-2.4
OMD	-2.7	-2.5
IGFBP7	-1.7	-2.5
FAT1	-2.0	-2.5
GGH	-2.1	-2.8
BMP5	-2.5	-2.8
SERPINA5	-2.5	-2.9
MFAP3L	-2.6	-2.9
RANBP3L	-2.2	-3.0
ITM2C	-2.2	-3.0
SCUBE1	-2.3	-3.1
KIAA1598	-2.4	-3.3
SCRG1	-2.7	-3.5
LOC402778	-2.3	-3.5
SLC14A1	-2.4	-3.6
INSIG1	-2.8	-3.7
ST8SIA1	-3.0	-4.1
RSPO2	-4.1	-4.2
TLR6	-2.4	-4.4
PAK3	-4.0	-4.5
SLC46A3	-2.9	-4.5
SKAP2	-3.1	-5.1
TFPI	-3.1	-5.2
F13A1	-3.9	-5.7
SATB2	-3.4	-6.5
CP	-3.7	-6.8
DNAH11	-3.3	-6.9
WIF1	-3.7	-7.2
LECT1	-4.5	-7.3
VAV3	-2.7	-7.6
STMN1	-3.6	-7.8
CLVS2	-3.8	-8.8
NTRK2	-5.8	-9.0
FST	-7.9	-9.3
AQPPEP	-4.6	-10.1
TF	-6.0	-10.2
DSC3	-4.7	-10.4
CCDC68	-3.6	-10.9
IBSP	-5.1	-14.7
SPP1	-2.1	-22.4

UP IN MIDDLE ZONE:		
Gene Symbol	MZ v SZ	MZ v DZ
SNORD13P1	3.0	7.5

DEEP ZONE (UP/DOWN):		
Gene Symbol	DZ v MZ	DZ v SZ
SPP1	10.4	22.4
MEPE	9.5	16.9
IBSP	2.9	14.7
RUNX2	3.9	13.8
CCDC68	3.0	10.9
DSC3	2.2	10.4
CLVS2	2.3	8.8
STMN1	2.2	7.8
VAV3	2.8	7.6
WIF1	2.0	7.2
FGF14	3.0	4.0
LYZ	2.6	3.7
GLB1L2	2.8	3.6
SLC7A5	2.3	3.5
PBRM1	2.8	2.8
SPARCL1	2.6	2.8
PDE7A	2.8	2.6
IGF2	1.9	2.3
GP1BB	2.7	2.4
SNORD116-20	2.6	2.2
KIT	-2.2	-2.0
SNORA50	-2.3	-2.1
PCDH5	-2.5	-2.3
SNORD13P1	-7.5	-2.5
VIT	-2.3	-2.7
ANGPT7	-3.9	-3.1
ASPN	-1.9	-3.4
INHBA	-2.2	-3.6
OGN	-1.9	-3.7
COL12A1	-2.7	-3.9
CRISPLD2	-2.3	-4.4
BEX1	-4.2	-4.6
ELMO1	-2.4	-4.7
CHI3L1	-2.4	-5.0
STEAP4	-2.8	-6.7

Figure 1. Zone-specific differential gene expression in human cartilage. Colors indicate the fold change in gene expression for each zonal comparison (ranging from up-regulated [red] to down-regulated [green]). SZ = superficial zone; MZ = middle zone; DZ = deep zone. Color figure can be viewed in the online issue, which is available at [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).

Cross-species comparison and enrichment analysis of human cartilage zone-specific genes. We identified a set of 24 genes shared between the human and

bovine specimens that showed the same direction of differential expression between zones (Table 1). This set of 24 genes showing similar changes in both species

Table 1. Genes shared between the human and bovine specimens showing the same direction of differential expression between zones*

Zonal comparison, gene symbol	Human cartilage	Bovine cartilage	Description
SZ versus DZ			
CHI3L1	5.8	6.4	Chitinase 3-like 1 (cartilage glycoprotein 39)
EFEMP1	5.1	5.9	Endothelial growth factor-containing fibulin-like extracellular matrix protein 1
THBS4	4.5	31.6	Thrombospondin 4
OGN	3.6	75.3	Osteoglycan
BMPER	3.26	26.1	Bone morphogenetic protein-binding endothelial regulator
PDE3B	3.2	13.4	Phosphodiesterase 3B, cGMP-inhibited
FGFR2	2.8	4.6	Fibroblast growth factor receptor 2
PALMD	2.7	7.2	Palmdelphin
PRG4	2.3	165.1	Proteoglycan 4
TNC	2.0	6.6	Tenascin C
SLC44A2	-2.1	-20.9	Solute carrier family 44, member 2
GLT25D2	-2.2	-17.2	Glycosyltransferase 25 domain-containing 2
SLC7A5	-3.1	-12.4	Solute carrier family 7
LECT1	-5.6	-26.3	Leukocyte cell-derived chemotaxin 1
F13A1	-6.5	-30.9	Coagulation factor XIII, A1 polypeptide
VAV3	-7.6	-12.6	Vav-3 guanine nucleotide exchange factor
COL10A1	-8.4	-27.8	Type X collagen, α 1
SPP1	-31.3	-9.2	Secreted phosphoprotein 1
IBSP	-34.7	-93.5	Integrin-binding sialoprotein
SZ versus MZ			
IGFBP5	5.9	11.3	Insulin-like growth factor binding protein 5
MYO1B	2.9	4.4	Myosin 1B
FGL2	2.4	10.2	Fibrinogen-like 2
S100A4	1.9	15.9	S100 calcium-binding protein A4
CLEC3A	-1.9	-4.3	C-type lectin domain family 3, member A
VAV3	-2.5	-8.3	Vav-3 guanine nucleotide exchange factor
LECT1	-3.6	-13.3	Leukocyte cell-derived chemotaxin 1
DZ versus MZ			
OGN	-1.9	-69.5	Osteoglycin
IBSP	3.3	34.0	Integrin-binding sialoprotein
SPP1	13.7	8.1	Secreted phosphoprotein 1

* Values represent the fold change in expression of each gene in the superficial zone (SZ) compared to the deep zone (DZ), SZ compared to the middle zone (MZ), or DZ compared to the MZ.

could serve as a high-priority set of markers for further investigation of zonal differences as they pertain to studies of tissue regeneration and disease states. The relative gene expression profiles of the 24 human cartilage zone-specific genes that were also identified in the bovine samples are shown in the form of a heatmap (Figure 2).

Although the number of genes showing similar changes in both species was small, our calculations, as well as the probability estimates derived from a Monte Carlo simulation of randomly assorted human and bovine genes to assess expected numbers of shared differentially expressed genes, indicated that the set of 24 genes showing similar zonal differences in both species indeed had significantly higher expression than would be expected by chance. In fact, we observed a mean 8.86-fold enrichment for the 19 genes differentially expressed

in the SZ compared to the DZ, at a significance level of $P < 0.0004$. Similarly, we observed a mean 18.65-fold enrichment for the 7 genes showing similar changes in both species in the SZ compared to the MZ ($P < 0.0004$), and a mean 104.9-fold enrichment for the 3 differentially expressed genes in the DZ compared to the MZ ($P < 0.002$). These calculations underscore the utility of using cross-species comparison in identifying high-priority targets.

Furthermore, rank ordering of the 19 cross-species-shared targets, with respect to the magnitude of differential expression of the 358 human transcript targets in the SZ compared to the DZ, showed that there was a greater propensity for the cross-species-shared genes to be higher ranking. Results indicated that 9 (47%) of the 19 cross-species-shared genes ranked within the top 10.3% of the 358 targets with respect to

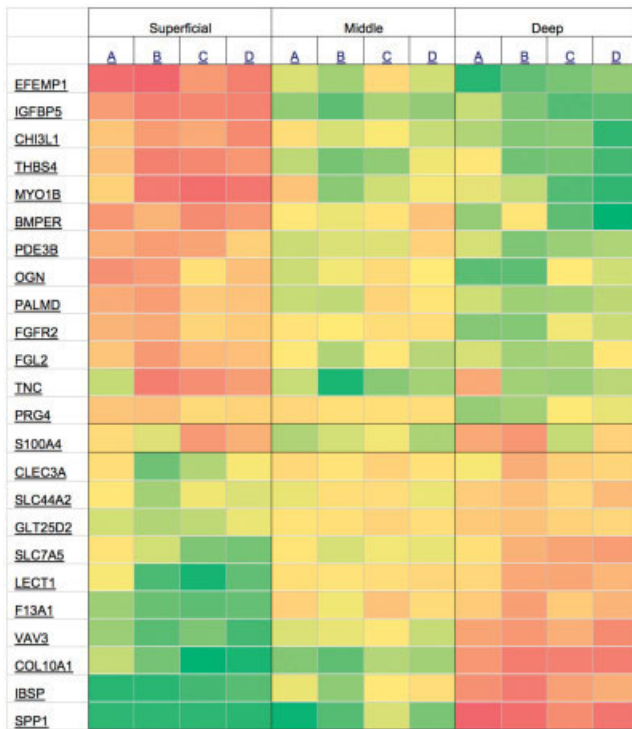


Figure 2. Genes differentially expressed in a zone-specific manner in human cartilage that were also differentially expressed in bovine cartilage. Results are shown as a heatmap of the target-normalized signal intensities for the 24 zone-specific genes in articular cartilage from 4 normal human donors (lanes A, B, C, and D) that were significantly different across the 3 zones (superficial, middle, and deep). Colors indicate the spectrum of gene expression, ranging from up-regulated (red) to down-regulated (green).

magnitude of differential expression, which is a frequency that is 4.6-fold higher than would be expected by chance.

Pathway analysis. GSEA was performed to identify significantly enriched pathways and structural or functional annotation groups associated with the differentially expressed genes across the human cartilage zones. GSEA results were derived from a number of curated gene sets from publicly available databases of metabolic and signaling pathways, such as the Kyoto Encyclopedia of Genes and Genomes (KEGG), BioCarta, and Gene Ontology groups.

GSEA of the KEGG data set revealed that pathways or cellular functions and biologic processes that were significantly enriched in the SZ compared to the DZ included 1) ribosome-related functions, 2) extracellular matrix (ECM)–receptor interactions, 3) cell adhesion molecule functions, 4) regulation of actin cytoskeleton, 5) complement and coagulation cascades,

6) cytokine–cytokine receptor interactions, and 7) the adipocytokine signaling pathway (Figure 3A). The genes in these enriched pathways in the KEGG data set are displayed in Figure 3B. Details of each gene represented are provided in Supplementary Table 3 (available on the *Arthritis & Rheumatism* web site at [http://online.library.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://online.library.wiley.com/journal/10.1002/(ISSN)1529-0131)).

Central to KEGG pathways 2–4 (Figure 3A) are integrin interactions with ECM proteins such as collagens, laminins, and thrombospondins. The adhesion molecules coordinating cell and ECM interactions and cytoskeleton regulation include syndecans, CD44, activated leukocyte cell adhesion molecule, intercellular adhesion molecule, VCAM, and versican (Figure 3B). Platelet-derived growth factor–mediated cytoskeleton changes are also implicated in cytokine–cytokine receptor interactions. An overview of the protein interactions for genes identified in KEGG pathways 2–4 in the SZ (Figure 3B) was created using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) network analysis (see <http://string-db.org>). Supplementary Table 3 (available on the *Arthritis & Rheumatism* web site at [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131)) summarizes the findings from GSEA for the various gene set categories in the SZ compared to DZ of the human articular cartilage.

Findings from GSEA of the BioCarta data set showed that the enriched pathways included IL4, TID, LAIR, IFN, IL10, and Rac CYCD, all of which involve JAK/STAT signaling cascades. Indeed, the JAK/STAT cascade itself was enriched (FDR 0.076). The Cdc42/Rac pathway was also enriched and is implicated in cytoskeleton regulation.

GSEA identified only 2 pathways in the KEGG data set, PPARG and EGFR/SMRTE, that were up-regulated in the DZ relative to the SZ.

DISCUSSION

Understanding cellular heterogeneity across zones of mature articular cartilage is important for elucidating the mechanisms of cartilage homeostasis and the pathogenesis of arthritis. The identification of zone-specific markers and regulatory mechanisms will also be useful in tissue-engineering approaches to recapitulate the native zonal architecture. Current information is limited to zone-specific expression patterns of a small set of genes and proteins. The objective of this study was to use genome-wide mRNA expression analysis to identify genes and pathways that distinguish cartilage zonal cellular phenotypes.

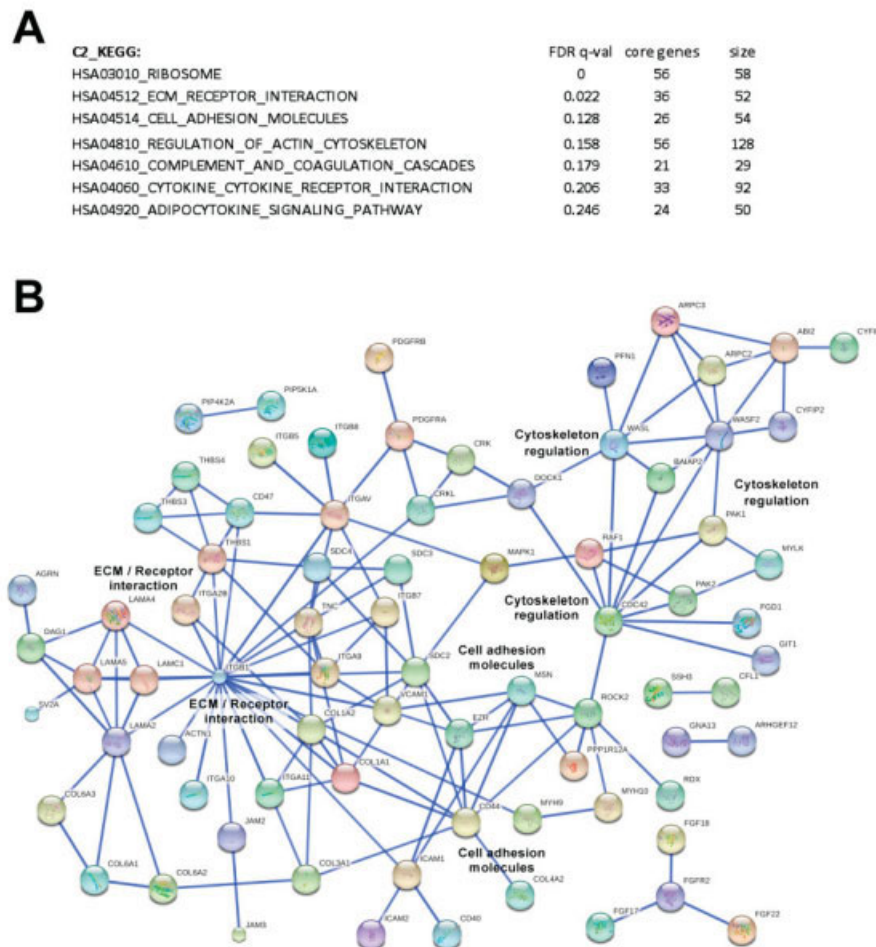


Figure 3. Results of pathway analysis. **A**, Significantly enriched pathways in the superficial zone (SZ) compared to the deep zone of articular cartilage, as identified by gene set enrichment analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) data set. FDR = false discovery rate. **B**, Visual representation of the relationships between the significantly enriched pathways in the SZ, using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) network analysis of the KEGG data set. The combined view of interactions between KEGG pathways includes the pathways of extracellular matrix (ECM)–receptor interactions, cell adhesion molecule functions, and regulation of actin cytoskeleton. Color figure can be viewed in the online issue, which is available at [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).

The largest number of differentially expressed genes ($n = 343$) was observed between the SZ and DZ. Among these, expression of 202 genes was significantly higher in the SZ than in the DZ, and expression of 141 genes was higher in the DZ than in the SZ. There were ~80 genes shown to be enriched in the SZ, while only 2 genes were enriched in the DZ. Fewer differentially expressed genes were observed between adjacent zones, with only 129 genes differentially expressed between the SZ ($n = 71$) and MZ ($n = 58$), and only 46 differentially expressed between the MZ ($n = 16$) and DZ ($n = 30$). These results emphasize that the SZ cells are the most unique zonal population, with the MZ being the least

unique zone. There also appears to be a gradient in gene expression between zones. This trend can be seen in the heatmap of all differentially expressed genes in human cartilage (Figure 4).

DNA array data can be interpreted on the basis of the individual gene expression patterns, as well as by analyzing sets of coordinately expressed genes to identify pathways that are important in regulating the unique zone-specific cellular differentiation status.

We were able to identify specific genes that could be used individually or in combination to help differentiate zonal phenotypes. These included genes that are already known to be expressed preferentially in a par-

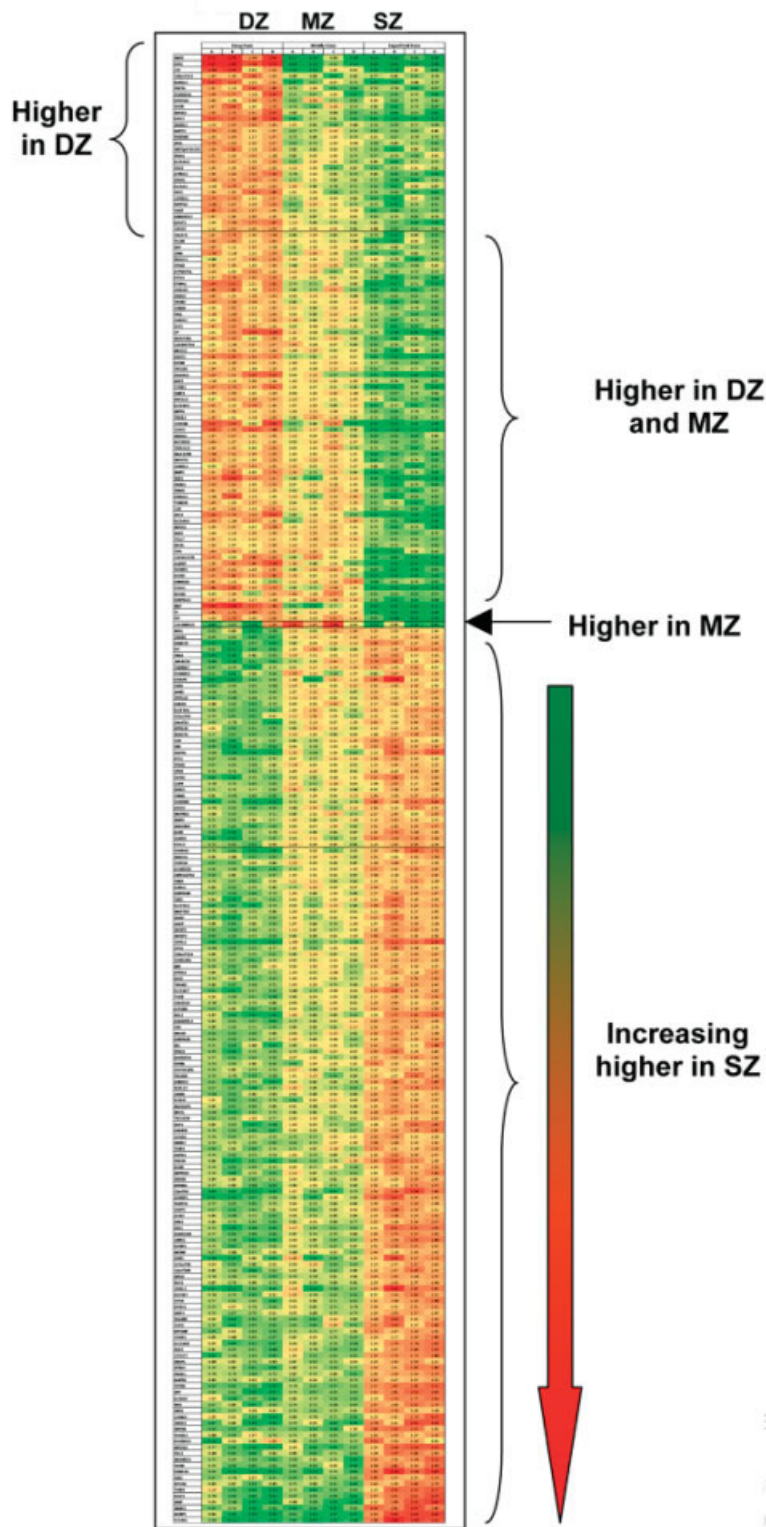


Figure 4. Heatmap of all differentially expressed genes in each zone (deep zone [DZ], middle zone [MZ], and superficial zone [SZ]) of human articular cartilage (from donors A–D). Colors indicate the spectrum of gene expression, ranging from up-regulated (red) to down-regulated (green). Color figure can be viewed in the online issue, which is available at [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).

ticular zone, such as SULF1 (46) and VCAM (25) in the SZ, or osteopontin (47), bone sialoprotein (38), and type X collagen (37) in the DZ.

We also identified sets of new genes with preferential gene expression in a particular zone. Interesting examples include IGFBP5, which implies zonal differences in insulin-like growth factor signaling, and EFEMP1, a fibulin-like ECM protein that, like the chromatin protein HMGB2, maintains the immature differentiation status of chondroprogenitor cells (48–50). This is of interest in view of the fact that the highest numbers of progenitor cells in mature cartilage are located in the SZ (25). Asporin, a regulator of transforming growth factor β activity (51), was also newly identified as a gene that is most strongly expressed in the SZ. In the DZ, we identified several new signaling molecules that are more highly expressed, including Delta/Notch-like epidermal growth factor repeat-containing, Src kinase-associated phosphoprotein 2 and neurotrophic tyrosine kinase receptor type 2. It will be of interest to ascertain their role in determining the unique cell differentiation status of the DZ.

In our DNA array data set, we found that the gene for the calcium-binding protein S100A4 was the only one that was preferentially down-regulated in the MZ relative to both the SZ and DZ. Also of note, the 3 related genes IBSP, SPP1, and OGN were not, as one might expect, coordinately expressed. The bone matrix components IBSP and SPP1 were up-regulated in the DZ, whereas OGN, a proteoglycan osteoinductive factor, was down-regulated in the DZ.

Importantly, in our analyses of the DNA array data based on sets of related genes and pathways, the most notable findings were that ribosome- and ECM-related genes could most readily distinguish the zonal phenotypes. Remarkably, 56 of the core 58 ribosomal genes in the annotated KEGG pathway were significantly enriched in the SZ compared to the DZ. This differential expression pattern may reflect increased turnover or activity of ribosomes in SZ cells.

Our GSEA findings identified ECM-related genes as enriched (FDR <0.004) and 18 core genes as up-regulated in the SZ relative to the DZ. In fact, there are actually 36 differentially expressed genes within this annotation group that provide distinction between the cartilage zones. This implies not only that there is a zone-specific ECM composition, but also that the interaction of cells with the ECM via specific cell surface receptors is a signaling mechanism involved in maintaining zonal cell phenotypes. This notion is supported by the observation that the top KEGG pathways

significantly enriched in the SZ compared to DZ included ECM–receptor interactions, cell adhesion molecule functions, and regulation of actin cytoskeleton, which are all involved in ECM-mediated cell signaling. In addition, in the BioCarta data set, the Cdc42/Rac pathway was also identified as a significantly enriched pathway and is implicated in ECM-to-cytoskeleton signaling (52).

Additional KEGG pathways that were differentially represented among zones included the complement and coagulation cascades, cytokine–cytokine receptor interactions, and adipocytokine signaling pathway. The complement cascade has received increased attention because complement components C5 and C6 or the complement regulatory protein CD59a are involved in the pathogenesis of experimental OA (53). Cytokine–cytokine receptor interactions include a large number of molecules.

The BioCarta pathways identified included IL4, TID, LAIR, IFN, IL10, and Rac CYCD, all of which are involved in JAK/STAT signaling cascades (54). Indeed, the JAK/STAT cascade itself was enriched (FDR 0.076).

A potential limitation of the present study is that the technique used to resect the cartilage zones might not precisely separate each adjacent zone, such as the SZ from the MZ and the DZ from the MZ. Although we controlled for this by discarding 200 μm of tissue thickness between zones, we cannot exclude the possibility that contamination might have occurred between the MZ and SZ or DZ. We also guarded against collection of the calcified zone; the detection of high expression of MEPE and VAV3, inhibitors of mineralization (55,56), in the DZ suggests that we did not include calcified zone.

Application of more sensitive techniques, such as RNA sequencing and analysis of larger sample sizes, might reveal additional genes with zonal expression. It is also expected that there may be differences between the mRNA expression profiles reported here and the zonal protein signatures. Ongoing proteomics analyses will address this relationship.

This gene expression array data set thus represents a baseline for use in comparisons to disease states and in assessing the response of normal cells to various stimuli. Resolving differences in gene expression in chondrocyte subpopulations in normal articular cartilage will guide cell-based repair strategies, enhance our basic understanding of cartilage biology, and identify unique cellular phenotypes, pathways, and transcription factors. The delineation of zone-specific pathways may

help to identify new therapeutic targets, and lead to new therapeutic interventions.

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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. Lotz 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. Grogan, D'Lima, Lotz.

Acquisition of data. Grogan, Duffy, Pauli, Koziol.

Analysis and interpretation of data. Grogan, Duffy, Su, Lotz.

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