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Zone-specific Gene Expression Patterns in Articular Cartilage

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Abstract

Objective—Identify novel genes and pathways specific to superficial (SZ), middle (MZ) and deep zones (DZ) of normal articular cartilage.

Methods—Articular cartilage was obtained from knees of 4 normal human donors. The cartilage zones were dissected on a microtome. RNA was analyzed on human genome arrays. Data obtained with human tissue were compared to bovine cartilage zone specific DNA arrays. 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 were observed between SZ and DZ in both human and bovine cartilage. The MZ was transitional between the SZ and DZ and thereby shared some of the same pathways as well as structural/functional features of the adjacent zones. Cellular functions and biological processes enriched in the SZ relative to the DZ, include most prominently ECM receptor interactions, cell adhesion molecules, regulation of actin cytoskeleton, ribosome-related functions and signaling aspects such as Interferon gamma, IL4, CDC42Rac and Jak-Stat. 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.

Keywords

cartilage zones; gene expression

INTRODUCTION

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

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AUTHOR CONTRIBUTIONS

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 design: Lotz, Grogan, D'Lima

Acquisition of data: Duffy, Pauli

Analysis and interpretation of data: Duffy, Koziol, Grogan, Su, Lotz

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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, are elongated, flattened and are oriented parallel to the cartilage surface (7). Cells within the SZ synthesize and secrete the important joint lubricant superficial zone protein (SZP), which is also known as megakaryocyte-stimulating factor, lubricin, or PRG4 (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 DZ chondrocytes by their lower collagen type II gene expression levels (12–14), 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 surface receptors CD105, CD166, (20), Notch-1 (22, 23) STRO-1 and VCAM-1 (25). SZ cells are strongly positive for alpha 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 contains randomly organized collagen fibrils, high concentrations of aggrecan (28), hyaluronic acid, dermatan sulfate and collagen type II (29–34).

The deep or radial zone contains ellipsoid cells with an extensive PCM amongst radially orientated 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 collagen type X (37), alkaline phosphatase (ALP) 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 to cell based cartilage repair. Successful recapitulation of the zonal organization does not occur during spontaneous 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 of 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 by tissue banks from one female (age 23) and three male (age 24, 44 and 46) donors (approved by Scripps Institutional Review Board) and processed within 24–60 hours post mortem. Osteochondral cores (6.5 mm diameter) were

harvested for RNA isolation from identical locations on the medial and lateral femoral condyles of each knee using ACUFEX Anatomic ACL Guide System (Smith & Nephew, Andover, MA). 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 downwards within 100–200μm calcified cartilage using a microtome (Microm HM 325, Thermo Scientific, Walldorf, Germany). 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 as follows: The upper 10% of zonal slices were allocated to the SZ, the middle 40% were allocated to the MZ and the lower 30% were allocated to the DZ. To avoid overlap between zones, regions of approximately 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 NanoDrop (ND-1000). Sample quality was determined with the Agilent 2100 Bioanalyzer using the RNA 6000 Pico LabChip (Agilent 5065–4473, Santa Clara, CA). Only samples with RNA Integrity Numbers (RIN) of > 6 were used. Five nanograms (5 ng) of total RNA was amplified using NuGEN Ovation Pico WTA System version 1.0 (NuGEN, San Carlos, CA, USA). For the human samples, post amplification was performed by using 4 μg of the purified cDNA product for processing with the NuGEN WT-Ovation Exon Module version 1.0. For both human post exon and bovine post amplification, 5 μg of the purified cDNA product was fragmented and labeled using NuGEN Encore Biotin Module. Pre and post fragmentation products (5 μg) were analyzed on an RNA 6000 Nano LabChip (Agilent 5065–4476) using the mRNA Assay program following the manufacturer's instructions.

Microarrays

Five μg post fragmentation and labeling product was used in the hybridization cocktail and hybridized overnight to either the Affymetrix GeneChip Human Gene 1.0 ST Array v1 (Affymetrix P/N 901086, Santa Clara, CA.) or the Affymetrix GeneChip Bovine Genome Array (Affymetrix P/N 900562). Hybridization and scanning of samples to arrays was performed using the standard NuGEN Hybridization, Cocktail Assembly, and Fluidics Protocols using Affymetrix's GeneChip Hybridization, Wash, and Stain Kit (Affymetrix P/N 900720), according to Appendix V1 of the NuGEN Encore Biotin Module protocol. Chips were scanned using the Affymetrix GeneChip Scanner 3000 7G with default settings and a target intensity of 250 for scaling.

Microarray data deposition

Data have been deposited in the Gene Expression Omnibus with GSE39797 as the reference series and can be viewed at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39797

Array data analysis

Raw data for mRNA transcript probe set intensities were generated in the form of probe cell intensity files (.CEL) using Affymetrix software and subsequently combined to yield expression measures as described below. Data in (.CEL) files were normalized using 2 different methods including Robust Multichip Average (RMA) Express 1.0 (http://rmaexpress.bmbolstad.com) with 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 (RP) (43) to identify differentially expressed transcripts. RP does not depend on an estimate of the gene-specific variance and is therefore robust for use with a small number of replicates. Log2 transformed signal intensities were used to calculate fold-change between pair-wise zonal comparisons. The difference between the average log2 transformed signals for each zone was used to determine the fold change (FC) in expression levels between zones. Magnitude fold-changes (Mag.FC) were determined using the following calculation: (FC=FC where > 1, else FC = −1/FC if FC < 1), thus allowing us to assign both magnitude and direction. Differentially expressed transcripts for each comparison were identified using the following criteria: proportion of false positives (pfp) < 0.15 and |Mag.FC| >1.4. Transcripts for each zonal comparison (i.e. “SZ vs. DZ”, “SZ vs. MZ”, and “DZ vs. MZ”), which met 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 those human targets, which were validated in the bovine data set (i.e. differentially expressed in the same direction in both data sets), or to highly significant human markers, which 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 pathways as well as structural and functional annotations. The GSEA method (44) ranks genes by differential expression levels between samples and then determines if 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). The RMA normalized human data set only was used for gene set enrichment analysis; we filtered out the genes with maximal expression below 40% of the median of all arrays. “Gene_set permutation” was used to obtain the random background distributions for calculation of false discovery rates (FDR). Additional GSEA non-default parameter settings included: “Collapse to gene symbols”, Permutation by gene_set; Enrichment statistic varied from “Classic” (unweighted) to “Weighted_p2” depending on the enrichment category; Metric for ranking = “Diff_of_Classes” using average[log2(signal)]. Enrichment scores (ES) were corrected for multiple hypothesis testing prior to calculation of (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 each of 4 normal human knees and RNA was analyzed on 12 Human Gene 1.0 ST arrays. Array data was normalized using 2 different methods, RMA and dChip. Rank product (RP) analysis of the human cartilage zonal gene expression, with zones being compared pair-wise, yielded differentially expressed

transcripts identified by both normalization methods. In subsequent analyses, only differentially expressed genes identified by both methods were used.

The largest differences were observed between the SZ and DZ with 343 differentially expressed genes, representing 1.7% of the genes on the array. Of these genes, 59% (202/347) were higher in the SZ, while 41% (141/343) were higher in the DZ. We observed 129 (0.6%) genes different between SZ and MZ and 46 (0.2%) between DZ and MZ. Complete lists of all differentially expressed human genes are included in supplementary Table 1.

Identification of zonal markers in human cartilage

Zonally up-regulated genes were defined as those that are significantly higher in that zone in comparison to the other two zones (Table 1). We identified 59 genes unique for the SZ, 1 for the MZ and 20 for the DZ. For genes that are significantly reduced in one zone as compared to the two other zones we identified 47 specifically down-regulated genes for the SZ, 0 for the MZ and 15 for the DZ (Table 1).

Unique gene expression signatures in bovine cartilage zones

To generate an independent data set to compare the human data, similar DNA array analyses were performed with bovine cartilage zones. Rank product analysis of the bovine cartilage zonal gene expression, compared pair-wise, yielded the following numbers of differentially expressed transcripts as identified by both normalization methods. The largest differences were observed between the SZ and DZ with 184 differentially expressed genes, representing 1.6% of the genes on the array. Of these genes, 34% (63/184) were higher in the SZ, while 66% (121/184) were higher in the DZ. 90 genes were different between SZ and MZ and 120 between DZ and MZ. Complete lists of all differentially expressed bovine genes are included in supplementary Table 2.

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, showing the same direction of differential expression between zones (Table 2). This set of 24 genes showing similar changes in both species will 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 the bovine samples are shown in the form of a heat-map (Figure 1).

While the number of genes showing similar changes in both species is small, our calculations as well as probability estimates derived from a Monte Carlo simulation of randomly assorted human and bovine genes to assess expected numbers of shared differentially expressed genes, indicates that the set of 24 genes showing similar zonal differences in both species is indeed significantly higher than would be expected by chance. In fact, we obtained 8.86-fold enrichment for the 19 differentially expressed genes in the SZ vs. DZ comparison at a probability of < 0.0004. Similarly, for the 7 genes showing similar changes in both species in the SZ vs. MZ comparison, we observed 18.65-fold enrichment at a probability of < 0.0004 and for the 3 differentially expressed genes in the DZ vs. MZ comparison, 104.9-fold enrichment at a probability of < 0.002. These calculations underscore the utility of using cross-species comparison in identifying high priority targets.

Further, the rank order of the 19 cross-species shared targets with respect to magnitude of differential expression of the 358 human SZ vs. DZ targets shows that there is a higher

propensity for the cross species shared genes to be higher ranking. Results indicate that 9/19 (47%) of the cross species shared genes rank within the top 10.3% out of the 358 targets with respect to magnitude of differential expression, which is 4.6-fold higher than would be expected by chance.

Pathway analysis

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

KEGG pathways significantly enriched in the SZ compared to DZ include 1) ribosome, 2) ECM receptor interaction, 3) cell adhesion molecules, 4) regulation of actin cytoskeleton, 5) complement and coagulation cascades, 6) cytokine-cytokine receptor interaction and 7) adipocytokine signaling pathway (Figure 2A). The genes in the enriched KEGG pathways are displayed in Figure 2B. Details of each gene represented are provided in supplementary Table 3.

Central to pathways 2–4 (Figure 2A) are integrin interactions with ECM proteins such as collagens, laminins and thrombospondins. Adhesion molecules coordinating cell and ECM interaction and cytoskeleton include syndecans, CD44, ALCAM, ICAM, VCAM, and VCAN (Figure 2B). PGDF mediated cytoskeleton changes are also implicated in “cytokine-cytokine receptor interaction”. An overview (Figure 2B) of the protein interactions for genes identified in KEGG pathways 2–4 for the SZ was created using ‘STRING’ (Search Tool for the Retrieval of Interacting Genes/Proteins; http://string-db.org). Supplementary Table 3 summarizes the GSEA analysis for the various gene set categories for the SZ vs. DZ comparison of human samples.

Enriched Biocarta pathways include IL4, TID, LAIR, IFN, IL10, RAC CYCD; all of which involve JAK-STAT signaling cascades. Indeed, JAK-STAT cascade is enriched (FDR = 0.076). The CDC42 RAC pathway is also enriched and is implicated in cytoskeleton regulation.

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

DISCUSSION

Understanding cellular heterogeneity across zones of mature articular cartilage is important to elucidating mechanisms of cartilage homeostasis and arthritis pathogenesis. 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.

The largest number of differentially expressed genes (n=343) was observed between the SZ and DZ. Among these, 202 genes were significantly higher in the SZ than in the DZ and 141 genes were higher in the DZ than in the SZ. There were approximately 80 gene sets shown to be enriched in the SZ, while only 2 gene sets were enriched in the DZ. Fewer differentially expressed genes were observed between adjacent zones with only 129 between the SZ (n=71) and MZ (n=58) and only 46 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 heat map of all differentially expressed genes (Figure 3).

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 individual genes that could be used individually or in combination to help differentiate zonal phenotypes. These included genes that were known previously to be expressed preferentially in a particular zone, such as SULF1 (46) and VCAM (25) for the SZ or osteopontin (47), bone sialoprotein (38) and Collagen X (37) for 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 IGF signaling and EFEMP1, a fibulin-like ECM protein, which like the chromatin protein HMGB2, maintains immature differentiation status of chondroprogenitor cells (48–50). This is of interest in view of the highest number of progenitor cells in mature cartilage being located in the SZ (25). Asporin, a regulator of TGFβ activity (51) was also identified as a new gene most strongly expressed in the SZ. For the DZ we identified several new signaling molecules that are more highly expressed, including delta/notch-like EGF repeat containing, src kinase associated phosphoprotein 2, neurotrophic tyrosine kinase, receptor, type 2. It will be of interest to ascertain their role in determining the unique DZ cell differentiation status.

The gene for the calcium binding protein S100A4 is the only one that is preferentially downregulated in the MZ relative to both SZ and DZ. Also, of note, is the fact that the 3 related genes IBSP, SPP1 and OGN are not coordinately expressed as one might expect. The bone matrix components IBSP and SPP1 are upregulated in the DZ, whereas OGN, a proteoglycan osteoinductive factor is downregulated in the DZ.

Analyzing the DNA array data based on sets of related genes and pathways, the most notable findings were that “ribosome’ and “extracellular matrix” genes most readily distinguish zonal phenotypes.

Remarkably, 56 out 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. GSEA identified “Extracellular Matrix” as enriched with an FDR < 0.004 and 18 core genes as upregulated in SZ relative to DZ. In fact, there are actually 36 differentially expressed genes within this annotation group, which provide distinction between the cartilage zones. This not only implies that there is a zone-specific ECM composition but also that the interaction of cells with 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 interaction, cell adhesion molecules, and regulation of actin cytoskeleton, which are all involved in ECM-mediated cell signaling. Biocarta also identified CDC42 RAC pathway, which is implicated in ECM to cytoskeleton signaling (52).

Additional KEGG pathways that are differentially represented among zones are “complement and coagulation cascades”, “cytokine-cytokine receptor interaction” and “adipocytokine signaling pathway”. The complement cascade has received increased attention as complement components C5, C6 or the complement regulatory protein CD59a are involved in pathogenesis of experimental OA (53). Cytokine-cytokine receptor interactions include a large number of molecules. Biocarta pathways identified include IL4,

TID, LAIR, IFN, IL10, RAC CYCD; all of which involve JAK-STAT signaling cascades (54). Indeed, the JAK-STAT cascade is enriched as well (FDR = 0.076).

A potential limitation of the present study is that the technique used to resect the cartilage zones may not precisely separate adjacent zones, 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 contamination between MZ and SZ or DZ. We also guarded against collection of 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 may 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 proteomic analyses will address this relationship.

CONCLUSION

This data set represents a baseline for comparison to disease states and in 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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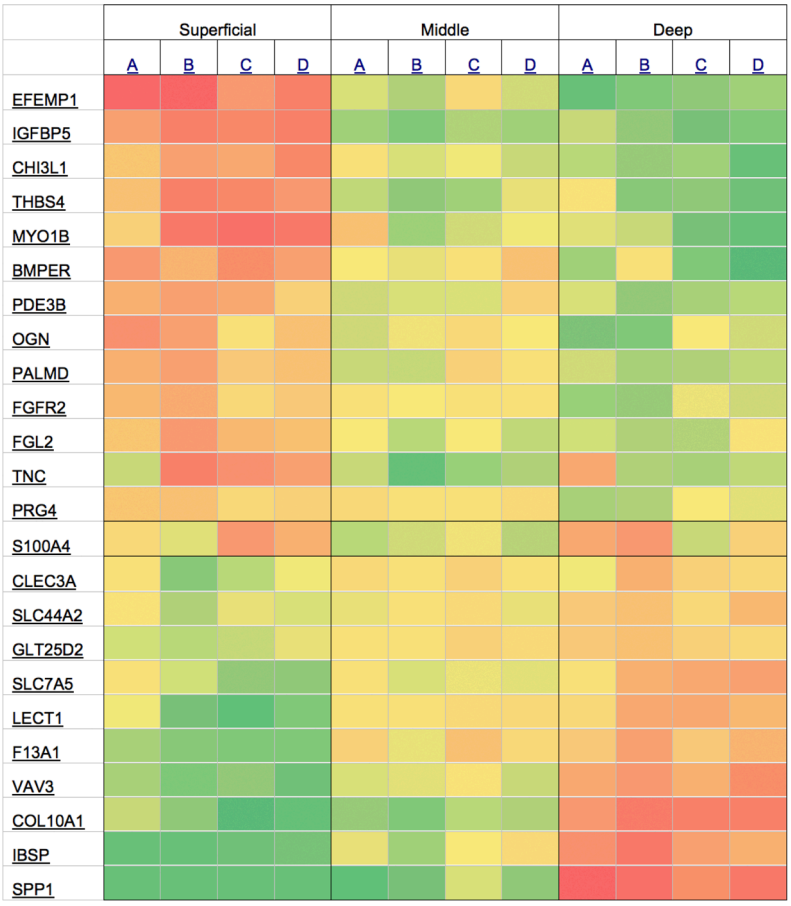

Figure 1.

Genes that are differentially expressed in a zone-specific manner in human cartilage, which were also cross-validated in the bovine data set. Shown are the donor-wise target-normalized signal intensities for the 24 human cartilage zone-specific genes that are significantly different across the 3 zones of the 4 normal human donors (A, B, C, and D).

A

C2_KEGG:	FDR q-val	core genes	size
HSA03010_RIBOSOME	0	56	58
HSA04512_ECM_RECEPTOR_INTERACTION	0.022	36	52
HSA04514_CELL_ADHESION_MOLECULES	0.128	26	54
HSA04810_REGULATION_OF_ACTIN_CYTOSKELETON	0.158	56	128
HSA04610_COMPLEMENT_AND_COAGULATION_CASCADES	0.179	21	29
HSA04060_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	0.206	33	92
HSA04920_ADIPOCYTOKINE_SIGNALING_PATHWAY	0.246	24	50

B

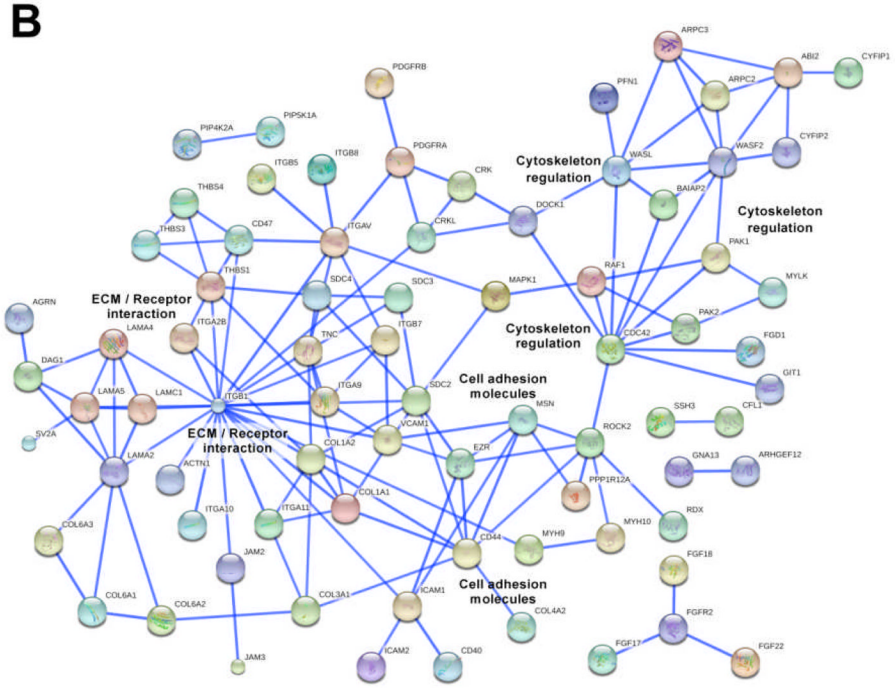

Figure 2.

Pathway analysis A. Significantly enriched KEGG pathways in the superficial zone (SZ) compared to the DZ following GSEA (gene set enrichment analysis). B. Visual representation of the relationship between genes significantly enriched GSEA pathways for the superficial zone using STRING network analysis. Combined view of KEGG pathways for the SZ, including ECM receptor interaction, cell adhesion molecules and regulation of actin cytoskeleton.

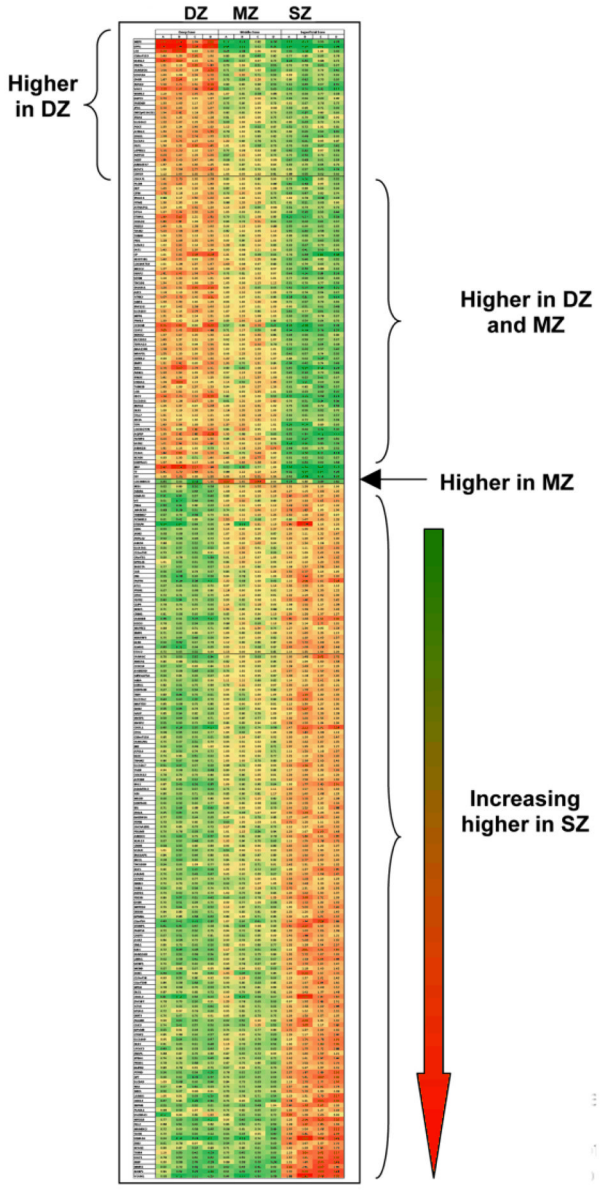

Figure 3.
Heat map of all differentially expressed genes in each zone of human articular cartilage (donors A–D).

Table 1

Zone Specific Genes human cartilage: Fold-Change (Red-Green: Up-regulated - Down-regulated).

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
FAM38B	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
B3GALT1	2.2	3.5

SUPERFICIAL ZONE (UP):		
Gene Symbol	**SZ v MZ**	**SZ v DZ**
KAL1	1.8	3.5
ASPN	1.8	3.4
GJA1	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
CHRDL2	−2.1	−2.2
OCR1	−2.7	−2.2
HSD17B11	−2.0	−2.3
CLEC3A	−1.8	−2.3
PDZRN4	−2.8	−2.4
TTLL7	−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

SUPERFICIAL ZONE (DOWN):		
Gene Symbol	**SZ v MZ**	**SZ v DZ**
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
AQPEP	−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
PCDHB5	−2.5	−2.3
SNORD13P1	−7.5	−2.5
VIT	−2.3	−2.7
ANGPTL7	−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

Table 2

Genes shared between the human and bovine specimens, showing the same direction of differential expression between zones. Values represent fold change.

Gene Symbol	SZ/DZ human	SZ/DZ bovine	Description
CHI3L1	5.8	6.4	chitinase 3-like 1 (cartilage glycoprotein-39)
EFEMP1	5.1	5.9	EGF-containing fibulin-like ECM protein 1
THBS4	4.5	31.6	thrombospondin 4
OGN	3.6	75.3	osteoglycin
BMPER	3.26	26.1	BMP 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	collagen, type X, alpha 1
SPP1	−31.3	−9.2	secreted phosphoprotein 1
IBSP	−34.7	−93.5	integrin-binding sialoprotein

Gene Symbol	SZ/MZ human	SZ/MZ bovine	Description
IGFBP5	5.9	11.3	insulin-like growth factor binding protein 5
MYO1B	2.9	4.4	myosin IB
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

Gene Symbol	DZ/MZ human	DZ/MZ bovine	Description
OGN	−1.9	−69.5	osteoglycin
IBSP	3.3	34.0	integrin-binding sialoprotein
SPP1	13.7	8.1	secreted phosphoprotein 1