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Insights into osteoarthritis progression revealed by analyses of both knee tibiofemoral compartments

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SUMMARY

Objective: To identify disease relevant genes and pathways associated with knee Osteoarthritis (OA) progression in human subjects using medial and lateral compartment dominant OA knee tissue.
Design: Gene expression of knee cartilage was comprehensively assessed for three regions of interest from human medial dominant OA ($n = 10$) and non-OA ($n = 6$) specimens. Histology and gene expression were compared for the regions with minimal degeneration, moderate degeneration and significant degeneration. Agilent whole-genome microarray was performed and data were analyzed using Agilent GeneSpring GX11.5. Significant differentially regulated genes were further investigated by Ingenuity Pathway Analysis (IPA) to identify functional categories. To confirm their association with disease severity as opposed to site within the knee, 30 differentially expressed genes, identified by microarray, were analyzed by quantitative reverse-transcription polymerase chain reaction on additional medial ($n = 16$) and lateral ($n = 10$) compartment dominant knee OA samples.
Results: A total of 767 genes were differentially expressed ≥two-fold ($P \leq 0.05$) in lesion compared to relatively intact regions. Analysis of these data by IPA predicted biological functions related to an imbalance of anabolism and catabolism of cartilage matrix components. Up-regulated expression of IL11, POSTN, TNFAIP6, and down-regulated expression of CHRDL2, MATN4, SPOCK3, VIT, PDE3B were significantly associated with OA progression and validated in both medial and lateral compartment dominant OA samples.
Conclusions: Our study provides a strategy for identifying targets whose modification may have the potential to ameliorate pathological alternations and progression of disease in cartilage and to serve as biomarkers for identifying individuals susceptible to progression.

Introduction

Although the progression of osteoarthritis (OA) is generally currently unpredictable, altered biomechanical and biochemical properties of the joint organ facilitate progression of disease[1–4]. It is widely accepted that the molecular homeostasis of the joint depends on both the structural integrity of articular cartilage and the appropriate biomechanical stresses[5,6]. A detailed examination of

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molecular changes in chondrocytes, the only cell type of articular cartilage during OA disease progression, is of pivotal importance for choosing molecules that could potentially be targeted to achieve a therapeutic benefit.

Joint tissues readily available for research are generally acquired at the time of knee joint replacement and suffer from full-thickness or severe cartilage loss of the medial weight-bearing compartment. This level of OA severity is often associated with an abnormal external knee adduction moment and imbalanced load distribution on the medial compartment of the knee[7]. Therefore, results of molecular analyses of advanced medial compartment knee OA tissue may not only represent direct effects of disease, but also site-related effects driven by altered mechanical loading. In addition, few studies have profiled human tissues for genes associated with OA progression; this is largely due to difficulties obtaining joint tissues with either early- or intermediate-stages of OA severity[8,9]. Another drawback to using patient tissues for gene expression profiling is large inter-patient differences due to variability in genetic background, duration of disease, age and gender.

To overcome these obstacles, we have pursued a two-pronged approach. First we have profiled cartilage gene expression changes relative to a gradient of histological OA severity across the tibial plateau in knees with medial compartment dominant disease[10,11]. This model system was based on evaluation of regions of cartilage and bone across the knee joint from the uninvolved (lateral compartment of the knee) to the involved surfaces (medial compartment of the knee), evaluating for disease severity and site associated gene expression changes. Because initiation of OA usually occurs in a focal subregion where the articular surface is affected most by asymmetric mechanical loading[12], other regions of the articular cartilage in the joint remain macroscopically and structurally normal or much less damaged. With disease progression, regions adjoining the damaged cartilage are affected until the entire joint surface is involved in advanced OA[13]. In our previous study we demonstrated that specific regions across the tibial plateau yielded a gradient of disease severities; intra-individual comparisons of these regions provided a means of overcoming the inter-individual background variation in gene expression studies[10]. This model system also provided a means to overcome the difficulty of obtaining early stage disease tissue by providing relatively normal tissue from the lateral compartment for comparison with the more diseased regions medially.

For the second aspect of this approach, we now profile cartilage gene expression changes relative to a gradient of histological OA severity across the tibial plateau in knees with lateral compartment dominant disease (LOA). Whereas medial compartment dominant knee OA (MOA) is often associated with an abnormal external knee adduction moment and imbalanced load distribution on the medial compartment of the knee, load plays less of a role in development of OA in lateral compartment disease[14,15]. This may be due to the fact that even in valgus knees, the medial compartment remains relatively overloaded until the valgus deformity exceeds 15°[16]. This overcomes a third drawback described above by enabling us to examine gene expression changes relative to worsening OA severity in the lateral compartment that has been shown to be relatively disassociated from loading stress.

For the current study, we hypothesized that this model system, encompassing a full range of histological severity across the tibial plateau, could enable us to identify gene expression changes directly associated with knee OA progression. We hypothesized that the pattern of gene expression for genes directly associated with disease severity, as opposed to joint site or mechanical load, could be the "mirror image", i.e., in medial compartment dominant disease the greatest gene expression changes associated with OA progression should occur in the most degenerative medial cartilage whereas the inverse should be true for lateral compartment disease. Because load may play less of a role in development of lateral compartment dominant disease than medial compartment OA[7,15], the identification of the severity associated genes expressed in common between medial and lateral compartment dominant disease may more readily identify genes related to biological factors directly associated with OA progression that are independent of load.

Therefore, to identify gene expression patterns directly associated with effects of disease and to differentiate them from patterns of expression that are driven by external mechanical loading and/or site-specific alternations, we evaluated whether OA-related genes identified in medial compartment dominant knee OA joints could be validated in lateral compartment dominant knee OA joints. In the present study, we performed whole genome transcriptome analysis of three regions of interest of articular cartilage in OA and non-OA knee joints and validated gene expression of the 30 most differentially regulated genes in additional independent medial ($n = 16$) or lateral ($n = 10$) compartment dominant knee OA joint cartilage specimens. Ingenuity Pathway Analysis (IPA) was used to discern the gene expression patterns most reflecting the different stages of OA progression. This study identified molecular targets that are involved in the homeostasis of cartilage integrity and provides potential pathways for therapeutic druggable targets or biomarkers in OA.

Materials and methods

Human knee joint tissues

A total of 42 human tibial plateaus were obtained during total knee joint replacement surgery from patients with medial or lateral compartment dominant knee OA ($N = 26$ medial OA, 10 for microarray analysis and 16 for validation of gene expression, mean age 68.9 ± 7.4 years, 70% female; $N = 10$ lateral OA, mean age 73.9 ± 11.45 years, 50% female) and non-OA joints acquired at the time of tumor surgery and above the knee amputation ($N = 6$ non-OA, mean age 39 ± 11.4 years, 50% female). The anatomic orientation was indicated on the freshly isolated specimens by marker pen to ensure consistency of sampling at prespecified regions of interest. All specimens were stored immediately in liquid nitrogen. The study was approved by the institutional review board of all the participating hospitals and Academia Sinica, Taiwan, Written informed consent was obtained from all of the participants.

Regions of interest, cartilage harvest and RNA isolation

The processes of cartilage harvest, sectioning, grinding, and RNA extraction were performed as previously described in Ref. 10. Briefly, regions of interest were sectioned and powdered under liquid nitrogen; 100 mg of articular cartilage powder was used for RNA isolation with 5 ml of Trizol (Invitrogen, CA). The RNA concentration and quality (RNA integrity number, RIN and 28S/18S ratio) were determined by a Nano-Drop (NanoDrop Technologies, DE) and the RNA 6000 Nano Assay on an Agilent 2100 Bioanalyzer (Agilent Technologies, CA), respectively. An adjacent section was preserved for histological evaluation using the OARSI grading system[17] by a scorer blinded to the results of the molecular analyses.

Microarray analysis

400 ng of total RNA per sample was used for one round of cRNA synthesis and amplification. Cyanine 3-labeled cRNAs were purified and hybridized to Agilent whole human genome 44k microarray chips (Agilent Technologies, Santa Clara, CA, USA). All procedures were carried out according to the manufacturer's instructions. The

array signal intensities were further analyzed by the Agilent GeneSpring GX software (version 11.5). All data sets were normalized by quantile normalization and probes with low signal intensities less than 72 were excluded. Significant differentially expressed genes between samples in OA patients were identified by Repeated Measures ANOVA. Probes with a threshold of $\geq$2 fold-change and a *P*-value $\leq$0.05 in gene expression were further analyzed by the hierarchical clustering method, with Euclidean distance and centroid linkage according to the manufacturer's instructions.

Pathways analysis

Significant differentially expressed genes were uploaded into IPA (Ingenuity Systems; www.ingenuity.com) to identify functional annotations and predict biological interactions. The biological interaction scores were defined by the IPA statistical algorithm. The analysis was based on the z-score and *P*-value (red dots), which were calculated by the IPA regulation z-score algorithm and the Fischer's exact test. A positive or negative z-score of more than 2 or less than 2, and *P*-value less than 0.05 ($-\log_{10} \geq 1.3$) indicates that a biological function is significant and predicts that the biological process or disease is trending towards an increase (z-score $\geq$ 2) or decrease (z-score ≤ -2). In our study, some predictions with the z-score between 1.5 and 2.0 (positive or negative) were also considered due to their biological functions relevant to joint tissue metabolism. *P* values were corrected for multiple comparisons with the Benjamini–Hochberg test.

qRT-PCR validation

To validate results from microarray analysis, 16 medial compartment and 10 lateral compartment dominant OA joint tissue samples were analyzed by qRT-PCR for expression of 30 genes and GAPDH (endogenous control) (Supplementary Table I) across the three regions of interest. The qRT-PCR was performed using the Taqman probes (Invitrogen, CA) according to the manufacturer's instructions and using the methods of data analysis as described in our previous study[11].

Results

Pathological evaluation of regions of interest

In specimens with medial compartment dominant OA selected for microarray analysis, histological evaluation confirmed a gradient of disease severity across the tibial plateau (least degeneration laterally and most degeneration medially) [Fig. 1(A)]. By the OARSI histological scoring system, the mean severity ranged from 4.4 ± 0.8 at the minimally damaged outer lateral tibial plateau (oLT), to 14.8 ± 3.4 at the moderately damaged inner lateral tibial plateau (iLT), and 19 ± 1.7 at the severely damaged inner medial tibial plateau (iMT). In contrast, OARSI scores of specimens from non-OA donors were 2.7 ± 2.1, 4.0 ± 0, and 4.3 ± 0.8 in oLT, iLT and iMT regions, respectively. These data further demonstrate the histological similarity of cartilage from macroscopically normal regions of OA joints (oLT) and all the regions of the non-OA samples. Thus, for the purposes of these analyses, the oLT region of the knee with medial compartment dominant OA could be considered normal or a very early stage of OA. The histological scores of each sample used in the microarray analyses are shown in Fig. 1(D).

Analysis of gene expression

Whereas gene expression profiles in non-OA tissue could not be segregated by region, gene expression profiles in OA tissue varied significantly by region. Significant numbers of genes ($N = 767$) were differentially expressed greater than two-fold in the iLT and iMT regions compared with the oLT region: 156 in iLT only, 272 in iMT only, and 339 genes were differentially expressed in both regions A total of 222 genes (144 upregulated and 78 downregulated) were expressed in a gradient pattern across the tibial plateau, i.e., they were up or down regulated at an intermediate level in iLT regions and further up or down regulated in iMT regions. We considered this subset of genes to be the most likely candidates associated with OA progression [Fig. 1(B) and (C), Supplementary Table II, and with annotations by IPA in Supplementary Table III].

Unsupervised hierarchical clustering of OA and non-OA samples was performed on the gene expression data of the differentially regulated genes to identify mechanistic pathways of disease development and progression. Except for two OA-iLT samples that clustered with the OA-MT samples, OA-oLT, OA-iLT, and OA-iMT samples grouped as distinct and separate clusters. In contrast, non-OA samples did not cluster by sample site but were more akin to the OA-oLT samples than to the OA-iLT and OA-iMT samples [Fig. 1(D)].

Predicted biological functions

To more comprehensively explore the effects of gene expression changes associated with damaged cartilage and to predict the potential biological associations, differentially regulated genes with changes greater than 1.5-fold were further examined by IPA. Figure 2 shows predicted biological functions associated with OA including molecular and cellular regulation and physiological system disease. Connective tissue and vessel development were the most strongly predicted functional pathways activated in iLT cartilage regions (z-score $\geq$ 1.5 or ≤ -1.5); these included adhesion of connective tissue (z-score = 3.63, *P*-value = 1.41×10^{-6}), ossification of bone (z-score = 2.0, *P*-value = 2.08×10^{-7}), and vessel development (five functional annotations with z-scores over 2.0 and one below −2.0). Biological functions predicted to be inhibited in cartilage (z-score ≤ -1.5) were related to cell signaling, cellular metabolism, movement, and quantity and growth; these included binding of endothelial cells (z-score = −2.59, *P*-value = 3.18×10^{-7}), metabolism of acylglycerol (z-score = −2.27, *P*-value = 8.96×10^{-6}), migration of cells (z-score = −2.4, *P*-value = 8.88×10^{-25}), quantity of leukocytes (z-score = −2.1, *P*-value = 1.08×10^{-9}), and proliferation of chondrocytes (z-score = −1.88, *P*-value = 1.10×10^{-5}). All of these predicted biological functions point to an imbalance of anabolism and catabolism of cartilage matrix components.

In iMT regions, biological functions predicted to be activated in damaged cartilage (z-score $\geq$ 1.5) were related to tissue morphology, cellular growth and tissue development; these included morphology of cells (z-score = 2.51, *P*-value = 4.36×10^{-10}), formation of connective tissue cells (z-score = 2.31, *P*-value = 2.14×10^{-6}) and adhesion of connective tissue cells (z-score = 3.88, *P*-value = 2.35×10^{-7}). These predictions support our histological results characterizing the structural degeneration of OA cartilage. In contrast, biological functions predicted to be inhibited in damaged cartilage (z-score ≤ -1.5) were related to cellular movement, a connective tissue disorder, immunological disease, metabolism, cell cycle and cell death, proliferation of chondrocytes (z-score = −1.82, *P*-value = 1.67×10^{-7}), and development of body axis (z-score = −2.32, *P*-value = 3.92×10^{-15}). These countermeasures might indicate that cells in damaged cartilage try to protect against the invasion of other cells and reduce immune responses, but the growth rate of major cells (chondrocytes) was inhibited as well.

In addition to surveying the most differentially regulated genes, we also examined our microarray data for the set of protease genes (581) listed in the Degradome database (online at http://degradome.

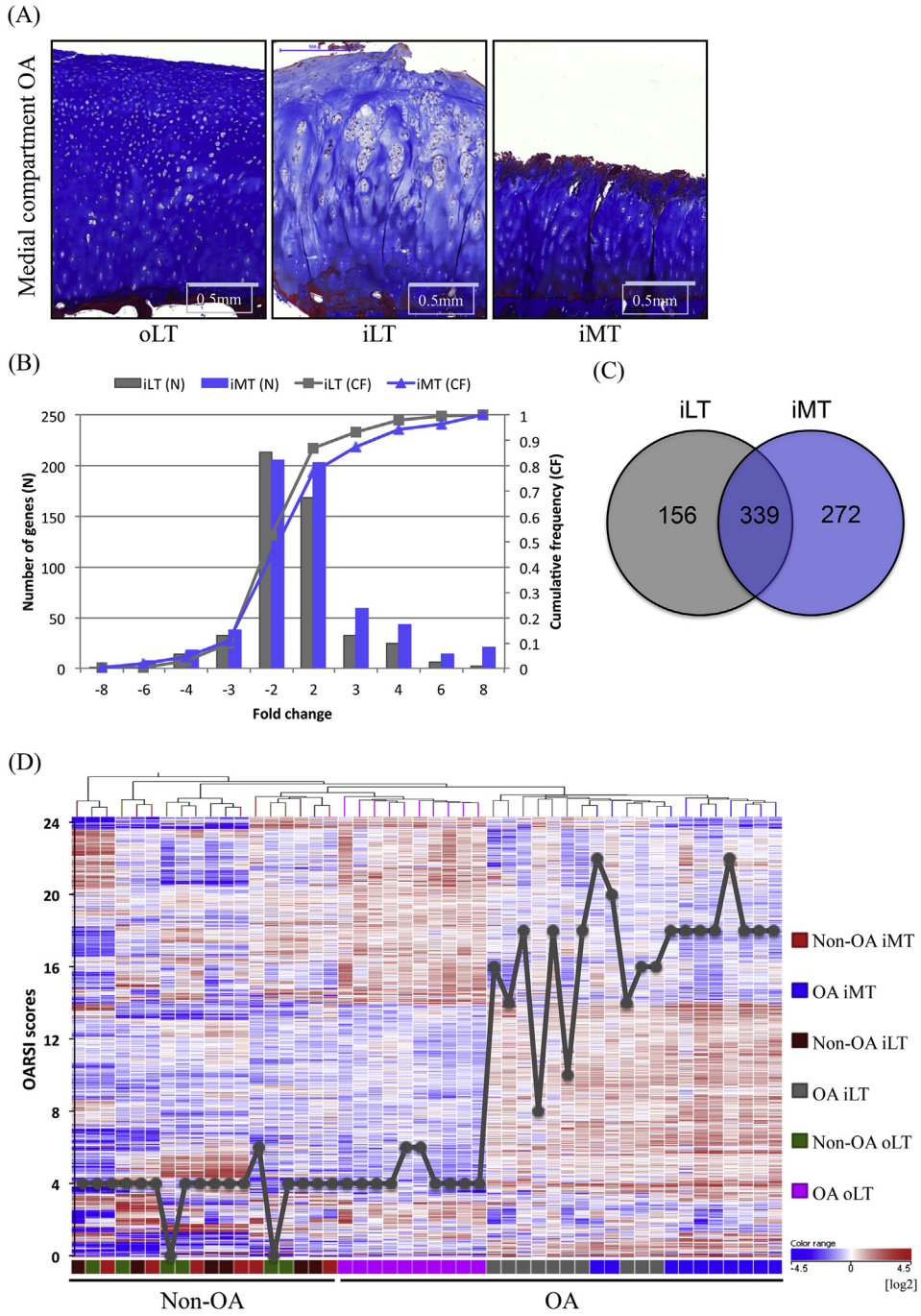

Fig. 1. Microarray gene expression analysis of OA and non-OA articular cartilage. (A) Representative histological images of cartilage for RNA isolation from the oLT, iLT and iMT from specimens with medial compartment dominant OA. (B) Cumulative frequency distribution of the number of differentially expressed genes with a minimum of two-fold, three-fold, four-fold, six-fold, and eight-fold changes from iLT and iMT samples (gray and blue lines). Fold change (*x*-axis) is plotted against the number of differentially expressed genes (bars) and also against the cumulative frequency (lines). (C) Venn diagram indicating the overlap of differentially expressed genes from iLT and iMT samples. (D) Heat map and unsupervised hierarchical clustering of OA and non-OA samples were performed for genes whose differential expression exceeded two-fold (767 genes from 894 probes). Distances between samples were detected with a Euclidean algorithm and clustered with an average linkage method. The OA-oLT (pink), OA-iLT (gray) and OA-iMT (blue) samples clustered separately from one another and non-OA samples, except for two iMT samples. Non-OA-oLT (green), non-OA-iLT (brown) and non-OA-MT samples manifested a mixed pattern. The cartilage integrity of each sample was determined by the OARSI histological grading system and these scores were merged with the heatmap (gray dots and line). Samples with scores under six clustered into a subgroup (e.g., OA-oLT and all non-OA samples) separate from OA-iLT and OA-iMT samples.

uniovi.es/dindex.html) (Supplementary Table IV). We observed 46 protease or protease inhibitor genes that were differentially regulated more than two-fold in cartilage at intermediate and late stages of OA. Pathway analysis of the 30 most differentially regulated genes (15 up- and 15 down-regulated) and the 46 differentially regulated protease genes identified a novel signaling network in OA entitled "post-translational modification, protein degradation, and protein synthesis". Interestingly, except for VIT, all of the most up- and down-regulated genes could be directly or indirectly linked to this network (Supplementary Fig. 1). This pathway provides a comprehensive network encompassing regulation of protein quality and quantity in either cells or cartilage in OA progression.

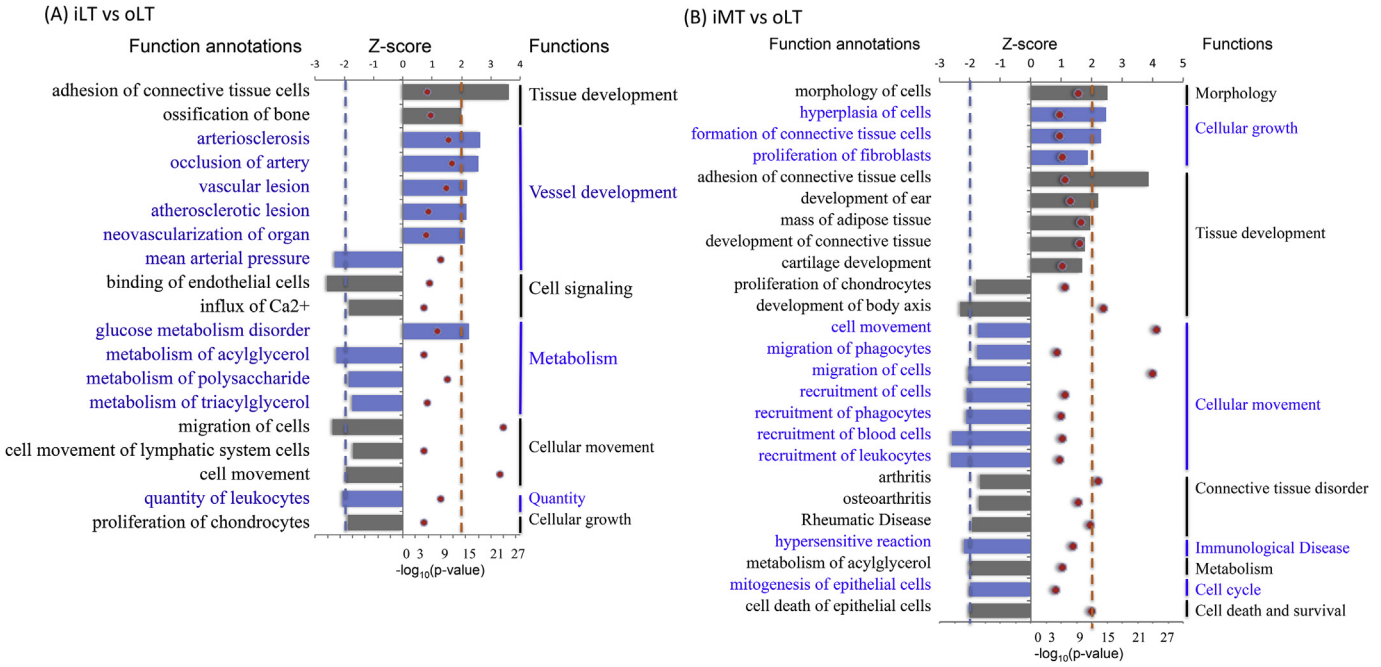

Fig. 2. Representative molecular and cellular functions identified in iLT and MT regions compared with oLT regions. To predict potential biological associations of the differentially expressed genes, microarray data from iLT (A) and iMT (B) regions of medial compartment dominant OA were further examined by IPA (http://www.ingenuity.com). Predicted biological functions included molecular and cellular regulation and physiological system disease. The analysis was based on the z-score and *P*-value (red dots), which were calculated by the IPA regulation z-score algorithm and the Fischer's exact test. A positive or negative z-score with more than 2 or less than 2 fold difference, respectively and *P*-value less than 0.05 ($-\log_{10} \geq 1.3$) indicate that a biological process or disease increases (z-score ≥ 2, represented by orange dotted lines) or decreases (z-score ≤ -2, represented by blue dotted lines) significantly.

We further investigated the network specifically associated with the early events in OA, those differentially expressed in regions of intermediate OA severity; IPA analysis identified changes of extracellular matrix properties and microenvironment, which may induce bone remodeling, bone cell differentiation and neovascularization reaching to the deep layer of cartilage (Supplementary Fig. 2). In addition to the predicted biological functions of differentially regulated genes, several predicted canonical pathways also showed consistent changes in expression with OA progression across early, intermediate and late stages of cartilage degradation; these pathways included a major inflammatory response pathway (IL-6 and acute phase response signaling) in intermediate stage cartilage, a bone development pathway (Wnt/β-catenin and BMP signaling pathways) in late stage cartilage damage, and changes in the quality of cartilage and bone (role of osteoblasts, osteoclasts, and chondrocytes) in both intermediate and late stage cartilage damage (Supplementary Table V). All of these data reveal that these differentially regulated genes, identified through our model system, are plausibly associated with the pathogenesis of OA and disease progression.

qRT-PCR validation of differentially regulated genes in medial (MOA) and lateral compartment OA (LOA)

Based on the results of the histological and biological function evaluation of the initial samples with medial compartment dominant OA, we defined oLT, iLT and iMT regions as early (E), intermediate (IM), and late (L) stages of OA, respectively [Fig. 3 (A) and (C)]. This disease severity pattern was mirrored for lateral compartment dominant disease [Fig. 3 (B) and (D)]. We hypothesized that gene expression effects specific to disease, as opposed to a site within the joint, would track with the histological severity of disease; by this reasoning, we would expect that disease specific gene regulation would be maximal in the compartment of maximal histological damage, independent of site (medial or lateral compartment). To test this hypothesis, human osteoarthritic tibial plateaus with either medial (MOA $n = 16$) or lateral (LOA $n = 10$) compartment dominant OA were obtained during total knee joint replacement surgery from knee OA patients. In the MOA samples, the mean histological severity ranged from 4.6 ± 1.2 at the minimally damaged oLT region, to 14.5 ± 3.5 at the moderately damaged iLT region, and 19.3 ± 1.8 at the severely damaged iMT region. In LOA samples, the mean severity ranged from 5.8 ± 2.4 at the minimally damaged outer medial tibial plateau (oMT), to 11.2 ± 2.7 at the moderately damaged iMT, and 18.4 ± 3.8 at the severely damaged iLT. Except for BHLHE22 (due to failure of the qPCR probe design), all the genes with the greatest up- and down-regulated expression in microarray could be validated in independent MOA samples by qRT-PCR (Table I). Analysis of LOA samples demonstrated that the expression pattern of three up-regulated genes and five down-regulated genes reproduced results with MOA samples (i.e., in a "mirror image pattern"), and their expression occurred in a gradient pattern corresponding to the gradient of disease severity; these included up-regulated IL11, POSTN, TNFAIP6, and down-regulated CHRDL2, MATN4, SPOCK3, VIT, PDE3B. Additionally, up-regulated CDH10 and GALNTL1 could be validated in moderately damaged cartilage [Fig. 3(E)], whereas up-regulated TNFSF11, LOXL2, and down-regulated LMO3, DACT1, MMP3, RSPO3, ADCY1, C4BPA and NRXN2 could be validated in severely damaged cartilage [Fig. 3(F)].

Upstream regulators of differentially regulated genes

Upstream regulator analysis may identify proximal transcriptional control mechanisms responsible for OA-related gene regulation (differentially regulated genes) and forecast upstream targets

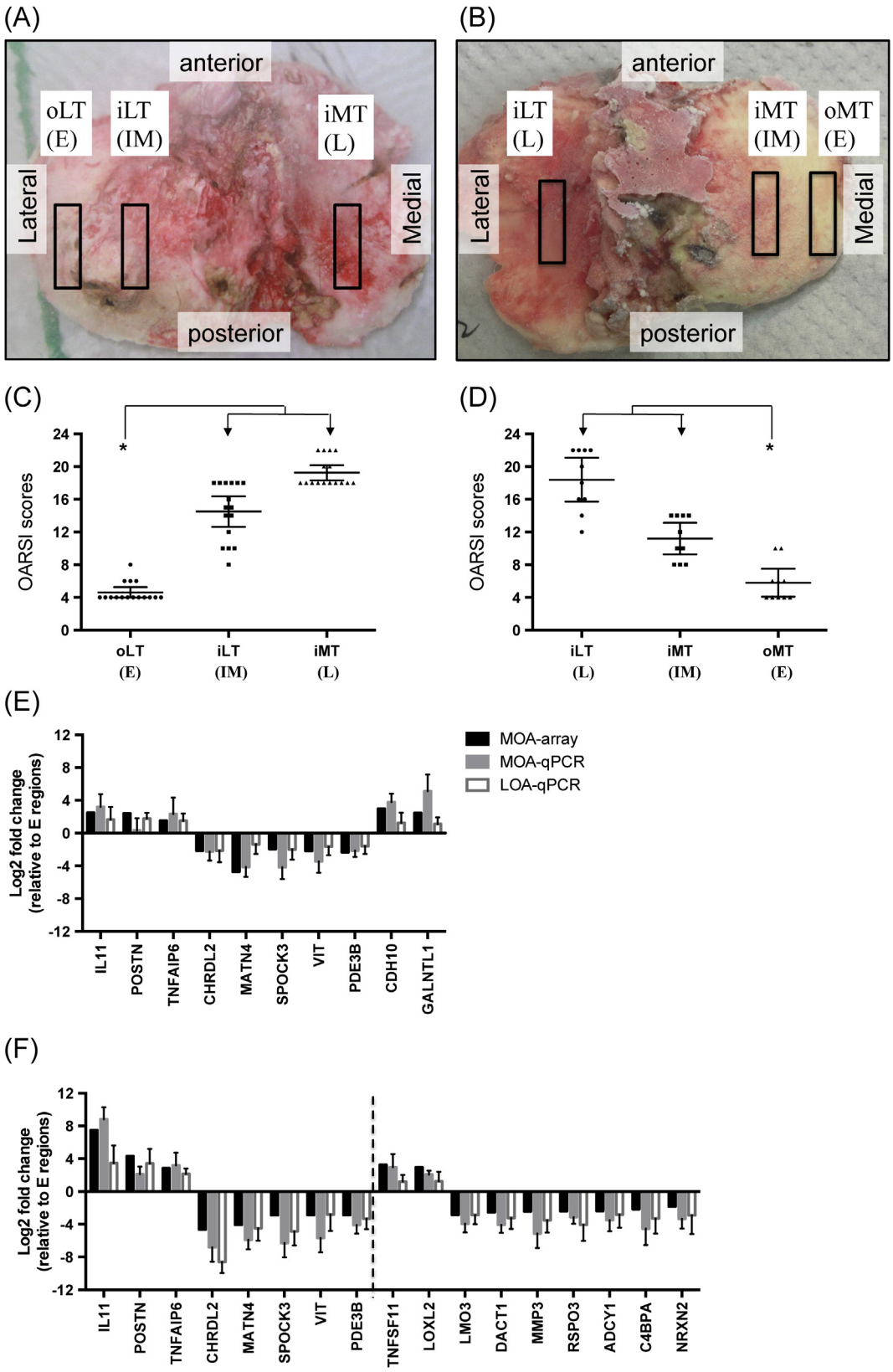

Fig. 3. Validation of top OA genes in medial and lateral compartment dominant knee OA. A total of three regions of interest were chosen for RNA isolation and histological analysis—indicated by the black-boxed areas. The macroscopic appearance and histological scores of tibial plateau regions of interest are shown for human medial compartment dominant knee OA (MOA, $N = 16$, panels A and C) and lateral compartment dominant knee OA (LOA, $N = 10$, panels B and D). The cartilage from the oLT, iLT and iMT from MOA knees were designated early (E), intermediate (IM) and late (L) stages of OA, respectively based on their histological scores. The cartilage from the outer medial tibial plateau (oMT), iMT, and iLT from LOA knees were designated E, IM and L, respectively. The histological severity scores across MOA and LOA regions were significantly different. (non-parametric repeated measures test, $P < 0.001$ for MOA, $P < 0.001$ for LOA). (E–F) 10 and 17 genes were validated in IM and L stage of OA samples, respectively in both MOA and LOA ($n = 16$ MOA, $n = 10$ LOA). The eight genes shown left of the dashed line were validated in both IM and L stages of OA in both MOA and LOA; error bars represent 95% confidence intervals.

Table I
Top up- and down-regulated genes comparing outer lateral tibial cartilage (intact cartilage) to inner lateral or medial tibial cartilage (damaged cartilage)

Accession no.	Gene symbol	Gene name	*P*	Medial compartment OA						Lateral compartment OA	
				iLT (OA)	iMT (OA)	iLT (N)	iMT (N)	iLT (OA)	iMT (OA)	iMT (OA)	iLT (OA)
				Microarray (FC)		Microarray (FC)		qRT-PCR (FC), $n = 16$		qRT-PCR (FC), $n = 10$	
Genes with increased expression in inner lateral or medial tibial cartilage											
NM_000641	IL11	interleukin 11	4.69E-06	5.63	180.87	1.28	1.99	63.93§	450.07§	3.14*	11.07¶
NM_139072	DNER	delta/notch-like EGF repeat containing	1.02E-07	42.53	67.22	10.82	4.60	19.93§	23.71§	1.61	−1.15
NM_003108	SOX11	SRY (sex determining region Y)-box 11	2.79E-05	14.54	29.84	9.52	2.20	7.45§	5.57*	2.80*	−1.28
NM_006475	POSTN	periostin	7.52E-05	5.31	19.70	2.90	1.09	2.31	4.30§	3.42§	10.83¶
NM_004878	PTGES	prostaglandin E synthase	4.44E-05	5.51	12.73	1.30	1.87	6.00§	8.02§	1.97¶	1.87*
NM_000066	C8B	complement component 8	1.65E-04	7.55	10.81	1.23	1.30	3.06¶	2.94¶	−1.77	−6.09§
NM_024812	BAALC	brain and acute leukemia, cytoplasmic	1.16E-07	3.80	10.75	1.86	1.51	7.29§	10.87§	1.76	−1.30
NM_006727	CDH10	cadherin 10, type 2	4.48E-05	7.89	10.60	1.24	1.46	15.84§	18.91§	2.39*	−1.03
NM_001001557	GDF6	Homo sapiens growth differentiation factor 6	2.61E-03	5.50	9.87	1.49	1.29	1.88	4.61*	1.43	−1.07
NM_003701	TNFSF11	tumor necrosis factor (ligand) superfamily, member 11	1.78E-05	4.07	9.50	1.39	1.17	3.99	7.69¶	1.40	2.31¶
NM_020692	GALNTL1	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 1	1.63E-05	5.57	9.17	2.36	1.18	27.11§	23.55§	2.15*	1.08
NM_001495	GFRA2	GDNF family receptor alpha 2	8.70E-06	4.07	8.99	1.45	1.53	7.50§	7.84§	1.84	1.12
NM_007329	DMBT1	deleted in malignant brain tumors 1	2.06E-05	3.95	8.39	1.10	1.72	4.77§	4.40¶	1.50	−2.09
NM_002318	LOXL2	lysyl oxidase-like 2	9.73E-05	4.73	7.73	1.76	1.64	3.32§	4.21§	1.58	2.39*
NM_007115	TNFAIP6	tumor necrosis factor, alpha-induced protein 6	3.11E-03	2.86	7.14	−1.00	−1.08	5.01*	9.18§	2.85§	4.42§
Genes with decreased expression in inner lateral or medial tibial cartilage											
NM_015424	CHRDL2	chordin-like 2	5.79E-05	−4.39	−24.62	−3.52	−1.62	−23.23§	−112.37§	−4.40¶	−383.46§
NM_003833	MATN4	matrilin 4	1.23E-05	−26.28	−16.73	−2.50	−1.74	−31.68§	−36.39§	−2.57*	−22.55§
NM_001204352	SPOCK3	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 3	1.18E-03	−3.83	−7.45	−14.34	−5.88	−59.51§	−78.33§	−3.94	−29.07§
NM_053276	VIT	vitrin	1.10E-05	−4.50	−7.34	−5.15	−3.23	−23.24§	−51.19§	−3.13¶	−7.04*
NM_152414	BHLHE22	basic helix-loop-helix family, member e22	6.55E-03	−4.01	−7.33	−1.48	−4.08	NA	NA	NA	NA
NM_000922	PDE3B	phosphodiesterase 3B, cGMP-inhibited	6.49E-07	−5.03	−7.32	−3.35	−2.38	−8.85§	−17.15§	−3.02¶	−9.98§
NM_018640	LMO3	LIM domain only 3(rhombotin-like 2)	1.69E-04	−3.88	−7.19	−3.41	−2.41	−11.25§	−15.30§	−2.52	−7.33§
NM_016651	DACT1	antagonist of beta-catenin, homolog 1	3.19E-06	−5.20	−5.81	−1.79	−1.60	−11.27§	−16.80§	−1.23	−9.39§
NM_002422	MMP3	matrix metallopeptidase 3	3.48E-04	−3.46	−5.42	−4.41	−2.46	−9.41¶	−34.96§	−1.33	−11.35§
NM_032784	RSPO3	R-spondin 3	1.11E-05	−3.28	−5.24	−1.84	−1.62	−4.16	−8.71§	−1.75	−16.73¶
NM_021116	ADCY1	adenylate cyclase 1	2.06E-05	−3.66	−5.17	−1.37	−2.26	−6.25§	−11.16§	−1.48	−7.09¶
NM_153692	HTRA4	HtrA serine peptidase 4	1.90E-02	−4.20	−4.77	1.01	1.45	−1.97	−3.66	1.01	−1.47
NM_001205315	STEAP4	STEAP family member 4	7.79E-05	−4.20	−4.53	−1.17	−2.01	−7.21§	−11.09§	−1.55	−3.00
NM_000715	C4BPA	complement component 4 binding protein, alpha	3.32E-05	−4.75	−4.46	−3.25	−2.47	−16.04§	−23.12¶	−1.46	−9.74¶
NM_138732	NRXN2	neurexin 2	3.16E-06	−4.27	−3.57	−1.51	−1.17	−7.47¶	−10.09§	−2.86	−7.56*

iLT = inner lateral tibail plateau, iLT = inner lateral tibial plateau, iMT = inner medial tibial plateau, *N* = non-OA sampels, FC = Fold change, NA = not analyzed, §: $P < 0.001$, ¶: $P < 0.01$, *: $P < 0.05$.

that may be involved in the pathogenesis of OA. This analysis may identify potential therapeutic and biomarker targets. A total of 105 significant upstream regulators were predicted by IPA based on significant z-scores (Supplementary Table VI). Table II shows the top upstream regulators, currently available drugs targeting these upstream regulators and potential expressing cells identified by IPA analysis and their predicted activation state. These analyses revealed that TNF, TGFB1, PPARG, ERBB2 and SMAD4 would be potential upstream regulators at intermediate and late stages of OA severity. These regulatory molecules might be released by chondrocytes in cartilage, immune cells (e.g., macrophages) in synovial fluid, synovium, or bone cells (e.g., osteoblasts) in underlying subchondral bone.

Discussion

For this study we evaluated histological and transcriptomic changes of three regions of interest across the tibial plateau encompassing a wide range of disease severities. All the most strongly differentially expressed genes except one, identified by microarray in medial compartment dominant disease, could be validated by qRT-PCR in independent medial compartment dominant knee OA specimens. Eight of these most differentially expressed genes in medial compartment dominant knee OA were mirrored in lateral compartment dominant knee OA; namely, independent of site, each of these genes was expressed in a gradient pattern across the tibial plateau, either positively or negatively with respect to histological disease severity. The genes expressed in the lateral compartment dominant disease mirrored the pattern of gene expression in the medial compartment dominant disease suggesting that these genes are disease severity related rather than site dependent. Although some of these genes, according to the available literature, have not previously been associated with OA progression in humans, their involvement is plausible given their known functions. For instance, although its role in OA progression is still unclear, IL-11 is a member of the IL-6-type cytokine family whose up-regulation is associated with anti-inflammation in human cartilage[18]. The role of POSTN in OA progression is also unknown; POSTN is a matricellular glutamate-containing protein that plays multiple roles in bone metabolism and is an important

Table II
Summary of representative predicted upstream regulator identified in iLT and MT regions compared with oLT regions

Regulator	Type	iLT		iMT		Drug(s)	Expressing cells/tissues				
		z-score	*P*-value	z-score	*P*-value		P/S	S/SF	OB	C	M
CEBPA*	TR	−2.42	4.4E-08							x	x
CEBPB	TR	−2.26	5.9E-08				x		x		x
COMMD1	T	−2.24	8.3E-05								x
IL13	CK	−2.17	1.5E-04			x	x	x			
tretinoin	CH	−2.03	4.4E-09								
NCOA1	TR	−2.01	1.1E-06								x
HOXD3	TR	−2.00	7.2E-04								
TGFB1	GF	3.08	1.1E-14	5.68	5.2E-26		x	x			x
TNF	CK	2.57	5.2E-19	3.80	4.1E-28	x	x	x		x	x
ERBB2	K	2.19	1.7E-10	2.57	1.3E-14	x	x				
PIN1	E	2.00	1.2E-02	2.24	4.4E-03						x
SMAD4	TR	2.08	4.5E-04	2.79	7.9E-08						x
MTTP	T	2.00	1.6E-03	2.00	2.7E-05	x					
ENPP2	E	2.00	8.9E-05	2.00	1.9E-04			x			x
TGM2	E	−2.53	3.8E-02	−2.78	2.2E-05						x
NCOA3	TE	−2.42	4.6E-04	−2.21	2.7E-04						x
PPARG*	LNR	−2.41	1.0E-11	−2.16	1.1E-15	x		x		x	x
CXCL12*	CK	−2.38	4.2E-03	−2.03	5.7E-03		x	x	x	x	x
MEDAG*	other	−2.24	3.8E-07	−2.00	4.3E-05						
PARP2	E	−2.22	8.4E-07	−2.00	7.5E-05	x					
IDH1	E	−2.00	5.0E-05	−2.00	1.3E-03		x	x			x
IGF2	GF			3.23	2.6E-10		x			x	
HIF1A	TR			2.83	1.7E-07			x			x
CTNNB1	TR			2.47	6.7E-14						x
IGF1*	GF			2.75	2.8E-11		x		x	x	x
GDF2	GF			2.71	8.0E-06						
F2	P			2.51	6.1E-08	x	x				
EGFR	K			2.48	2.7E-12	x	x		x	x	
prostaglandin E2	CH			2.47	5.6E-10						
TGFB2	GF			2.37	4.9E-05	x	x				
WNT3A	CK			2.36	5.2E-11						
progesterone	CH			2.31	1.9E-19						
BMP2*	GF			2.31	3.0E-07				x	x	
FGF2	GF			2.29	6.2E-12	x	x	x		x	
BMP6*	GF			2.21	3.8E-05						
NCOR2	TR			2.18	4.3E-09		x				
MAPK3	K			2.16	7.9E-07					x	
PDGF BB	CO			2.10	3.0E-08						
EGF	GF			2.07	1.0E-08		x				
let-7a-5p	mR			−3.25	2.7E-03						
NOG*	GF			−2.43	3.6E-06						x
miR-16-5p	mR			−2.43	2.3E-03						
APC	E			−2.21	6.8E-07		x				
CR1L	other			−2.16	4.3E-07		x				

TR = transcription regulator, T = transporter, CK = cytokine, CH = chemical, GF = growth factor, K = kinase, E = enzyme, LNR = ligand-dependent nuclear receptor, P = peptidase, CO = complex, mR = mature microRNA, P/S = Plasma/Serum, S/SF = Synovium/Synovial Fluid, OB = Osteoblasts, C = Cartilage/Chondrocyte, M = Macrophage, * = FC $\geq$ 2 or $\leq$ −2.

regulator of bone formation[19] suggesting that POSTN may be an important initiator of subchondral bone turnover. TNFAIP6 (tumor necrosis factor, alpha-induced protein 6) is a third example of a gene whose role in OA progression is unclear. TNFAIP6 is a secretory protein that contains a hyaluronan-binding domain; increased levels of this protein are found in the synovial fluid of patients with OA and OA progression[20]. Therefore, the present study provides strong proof of the concept that this model system recapitulates states of OA progression and provides disease-relevant molecular signatures.

To date, gene expression studies have been conducted in several animal models to identify genes associated with progressive degeneration of articular cartilage[21–26]. Surgical destabilization of the medial meniscus (DMM) was performed in mice lacking ADAMTS-5 activity to identify OA initiation and progression candidates involved in cartilage destruction independent of ADAMTS-5[27]. Identified genes included *Il-11*, *Mmp3*, *Ptgs2*, *Crlf1*, *Inhba*, *Capn2*, *Press46*, *Klk8 and Phdla2*. With the exception of *Press46* and *Klk8*, all of these genes are among the differentially expressed genes in our study. Moreover, both IL-11 and MMP3 are among the top-dysregulated genes that could be validated in both LOA and MOA joint cartilage. Interestingly, some of the genes significantly associated with disease progression in the current study (such as ADAMTS1, ASPN and TNFSF11) were previously identified to be among our 19 human genes associated with the interaction of articular cartilage and subchondral bone[10].

Of the eight genes that could be validated as disease associated in both MOA and LOA samples, seven genes have been implicated in the organization of cartilage extracellular matrix, cartilage development or OA pathogenesis[18–20,28–31]. The single exception, the PDE3B gene, has not been implicated previously in OA. Nevertheless, its involvement in OA is plausible based on previous data showing that activation of PDE3B likely serves as a regulatory feedback mechanism of cAMP and calcium related responses and plays an important role in regulating energy homeostasis[32].

Although several candidate biomarkers have been investigated and some potential structure-modifying OA drugs (SMOADs) and disease-modifying OA drugs (DMOADs) have shown efficacy in OA models, currently, the practical use of these biomarkers is still limited and no DMOADs have been proved to slow or stop disease progression or repair damaged cartilage[33,34]. A discovery of upstream regulators of differentially regulated genes could be a way of aiding development of pharmaceutical interventions. Based on IPA analysis, 105 molecules were predicted to be upstream regulators mediating the observed phenotypic or functional outcomes of cartilage damage. The molecules regulating gene expression changes in chondrocytes from intermediate stage cartilage ($N = 28$) may potentially be associated with driving a catabolic and increased joint tissue turnover phenotype in chondrocytes. These same molecules represent potential candidate biomarkers and candidates whose inhibition might engender new DMOAD development. Drugs designed to target upstream regulators identified from late stage cartilage could be potential DMOADs to suppress the dominant catabolic phenotype and disease progression before irreversible joint failure. Moreover, the upstream regulators involving both intermediate and late stage cartilage may affect the homeostasis of whole joint tissue and represent additional interesting targets. For example, TGFβ (z-scores 3.08 in IM, 5.68 in L) and SMAD4 (z-scores 2.08 in IM and 2.79 in L) were predicted as significant upstream regulators in our study. TGFβ could be released from underlying subchondral bone cells in response to OA progression in early stages; this is plausible given that TGFβ signaling is associated with initiation of pathological OA changes including turnover of subchondral bone, a high level of angiogenesis and cartilage loss[35]. Activated TGFβ could result in phosphorylation of SMAD4 and its translocation to the nucleus to modify gene expression to change the balance between cartilage matrix catabolism and anabolism in chondrocytes[36]. Therefore, drugs or biomarkers designed to target TGFβ/SMAD4 signaling cascades could be potential chondroprotective therapeutics.

Our study provides a comprehensive gene expression profile related to the pathogenesis of OA and OA disease progression. Overall, our study provides a strategy, using a novel model system, for identifying targets whose modification may have the potential to ameliorate pathological alterations and progression of disease in cartilage and to serve as biomarkers for identifying individuals susceptible to progression.

Authors' contributions

CH Chou carried out the RNA isolation, qRT-PCR, microarray data analysis, statistical analysis, histological evaluations and drafted the manuscript. CC Wu, HC Shen, CH Lee, IW Song, and LS Lu participated in sample collection. CC Wu, JY Wu, YT Chen, VB Kraus, MTM Lee conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final version to be published. CC Wu 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.

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Conflict of interest

The authors declare that they have no competing interests.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2014.12.020.

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