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Gene expression profiling of articular cartilage reveals functional pathways and networks of candidate genes for osteochondrosis in pigs

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Rangkasenee N, Murani E, Schellander K, Cinar MU, Ponsuksili S, Wimmers K. Gene expression profiling of articular cartilage reveals functional pathways and networks of candidate genes for osteochondrosis in pigs. *Physiol Genomics* 45: 856–865, 2013. First published August 6, 2013; doi:10.1152/physiolgenomics.00055.2013.—Osteochondrosis (OC) is a joint disorder that frequently causes leg weakness in growing pigs, resulting in welfare problems and economic losses. We aimed to detect molecular pathways relevant to the emergence of the disease and to identify candidate genes for the liability to the disorder. Therefore, we compared microarray-based expression patterns of articular cartilage with ($n = 11$) and without ($n = 11$) histologically diagnosed OC lesions obtained from discordant sib-pairs. A total of 1,564 genes were found with different transcript abundance [differentially expressed (DE) genes] at $q \leq 0.05$. To further identify candidate genes, we integrated data from quantitative trait loci (QTL) and genome-wide association (GWA) studies with the expression analysis. We detected 317 DE genes within the QTL confidence intervals, of which 26 DE genes also overlapped GWA regions. Ingenuity Pathway Analysis suggests a pathogenic role of immune response, angiogenesis, and synthesis of extracellular matrix pathways for OC. These processes could facilitate the emergence of defects. But they may also promote the degradation of articular cartilage and the worsening of the disease. A functional network was derived that comprised genes with functional and positional clues of their role in bone and cartilage metabolisms and development, including extracellular matrix genes (e.g., LOX, OGN, and ASPN), angiogenesis genes (e.g., ANGPTL4 and PDGFA), and immune response genes (e.g., ICAM1, AZGP1, C1QB, C1QC, PDE4B, and CDA). The study identified molecular processes linked to OC and several genes with positional, genetic-statistical, and functional evidence for their role in the emergence of articular cartilage lesions and the liability to OC.

leg weakness; QTL; microarray; functional genomics; genome wide association

OSTEOCHONDROSIS (OC) IS A common joint disorder that occurs in growing domestic animals as well as in children. It is characterized by a failure of endochondral ossification of the articular-epiphyseal cartilage and the physeal growth cartilage (74). OC is regarded as a frequent cause of leg weakness in growing pigs and thus economic losses from culling of breeding pigs (10, 72). The rapid growth rates are suspected of predisposing pigs to the development of pathogenic disorder of the osteochondrotic system. An increase in muscle mass and muscle forces affects the bone strength and the development of articular cartilage, epiphyseal plates, and subchondral bone tissues and finally fosters the incidence of OC (2, 67). In pigs, OC lesions mainly occur at the distal epiphysis of ulna, the medial condyles of humerus, and femur joints (41).

OC is generally considered as a complex disorder with multiple risk factors including rapid growth, anatomic conformation, trauma, dietary imbalances, and genetic disposition, all of which may hamper adequate chondrocyte differentiation and blood supply to the growing cartilage and may provoke the formation of a fragile cartilage and subchondral bone necrosis (22, 74). The heritability of OC or leg weakness was estimated as being low to moderate in different pig breeds (21, 22, 34, 49). Linkage studies in pigs identified numerous quantitative trait loci (QTL) for OC lesions or leg weakness-related traits in pigs (28, 31, 66). However, knowledge of the molecular processes of the pathogenesis underlying the OC disorder remains limited. The integral analysis of differentially expressed (DE) genes can be performed to obtain the biological functions and regulatory pathways involved in the pathogenesis of diseases. So far, expression studies on horses with articular cartilage lesions have been published (37, 38, 54). The aim of this study was to compare expression patterns of articular cartilage with or without OC lesions, to get a snapshot of the gene activity that is indicative of molecular pathways and biological functions relevant to OC pathogenesis. Therefore, we performed a holistic microarray expression analysis in OC-negative and OC-positive articular cartilage obtained from a porcine experimental Duroc × Pietrain crossbreed population. We further integrated data of the expression study with the results of a previous QTL study, conducted in the same Duroc × Pietrain population (28), as well as with a genome-wide association study (GWAS) performed with animals of commercial Large White herds. Assignment of genes with different transcript abundance to QTL regions aids in identifying functional and positional candidate genes associated with the development of articular cartilage OC lesions.

MATERIALS AND METHODS

Tissue collection and RNA isolation. Animal care and tissue collection procedures followed the guidelines of the German Law of Animal Protection, and the experimental protocol was approved by the Animal Care Committee of the Leibniz Institute for Farm Animal Biology. Samples were obtained from animals of a Duroc × Pietrain crossbreed population that were kept and performance tested at the Frankenforst experimental research farm of the university of Bonn according to the performance test guidelines of the German umbrella organization of pig production (Zentral Verband der Deutschen Schweinproduktion) (77). Piglets were weaned at 28 days, and male piglets were castrated. The pigs were fed ad libitum during the whole testing period starting at 30 kg and were slaughtered at ~105 kg body wt. The average age at slaughter was 177.6 ± 15.6 days. Articular cartilage samples of 292 animals were obtained from the medial part

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of condylus humeri (CMH) or condylus medialis femoris (CMF), where serial sections of cartilage were histopathologically inspected for OC lesions, as previously described (29, 47, 48). In brief, slices of the articular cartilage surface of macroscopically nonaffected areas and affected regions, if present, were stored at −80°C for RNA isolation and at −20°C for histopathological assessment, to consider the criteria of appearance and texture of cartilage surface, hyperplasia and vascularization of the hyaline cartilage, as well necrosis and ossification of the hyaline cartilage. For lesion scoring, histological sections of 7 μm were prepared and stained in duplicate after fixation and demineralization. Representative sections were scored on a scale from 1 (= normal, subsequently designated "OC negative") to 4 (= severe, subsequently designated "OC positive"). (*Scores 1–4* denote 1 = smooth surface, homogenous cartilage matrix, columns of chondrocytes visible; 2 = homogenous cartilage matrix, low-grade fraying of the surface; 3 = surface erosions, fraying in deeper cartilage layers; 4 = surface erosions, fraying in deeper cartilage layers and cavitation). In this study, we selected 22 articular cartilage tissues from seven discordant sib-pairs (4 pairs of females and 3 pairs of castrates) with histological OC lesion scores of 1 and 4, respectively, at either only CMH (3 sib-pairs, 6 samples) or both CMF and CMH (4 sib-pairs, 16 samples) for RNA isolation.

Total RNA was isolated with TRI reagent (Sigma-Aldrich, Taufkirchen, Germany), treated with DNase, and cleaned up with the RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA samples were visualized on 1% agarose gels containing ethidium bromide to check RNA integrity. RNA was quantified by spectrometry with a NanoDrop ND-1000 spectrophotometer (PEQLAB, Erlangen, Germany). In addition, absence of DNA contamination was checked by using the RNA as a template in standard polymerase chain reactions (PCR) amplifying fragments of the GAPDH gene. All RNAs were stored at −80°C for further analysis.

Microarray and data analysis. Microarray experiments were performed using the Affymetrix GeneChip Porcine Genome Arrays (Affymetrix, Santa Clara, CA), which contains 24,123 probe-sets. For each hybridization, 500 ng of a total RNA was reverse-transcribed into cDNA, and this was transcribed into cRNA to generate biotin-labeled antisense cRNA targets according to Affymetrix protocols. The cRNA targets were fragmented and individually hybridized onto Affymetrix GeneChip Porcine Genome Arrays. After the staining and washing steps, the arrays were scanned following the manufacturer's recommendation. Data were analyzed with Affymetrix GCOS 1.1.1 software using the global scaling to a target of 500 for background correction and probe summarization.

Then data were imported into the Expression Console software (Affymetrix) for subsequent analysis. First, the data were processed with the MAS5 algorithm to generate probe cell intensity values (using default settings with detection P values of <0.04 for "present," ≥ 0.04 and ≤ 0.06 for "marginal," <0.06 for "absent"; only present calls were used). Then quantitative expression levels were estimated of those transcripts with present calls in at least 50% of the samples of either affected or nonaffected CMH or CMF by using the probe logarithmic intensity error (PLIER) algorithm. PLIER values were log-transformed and used for further statistical analysis of expression levels. All microarray data are MIAME-compliant and have been deposited in a MIAME-compliant database, the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo, accession number GSE44991).

Subsequently, the significance of differences in transcript abundance was evaluated by a linear mixed model performed with the procedure "Mixed" of the JMP Genomics 5.0 software (SAS Institute, Cary, NC), including the fixed effect of sex; family was used as a random effect and slaughter weight as covariate ($P \leq 0.05$). Corresponding q values were calculated according to Storey and coworkers (58, 59) to estimate the proportion of false positives among all significant hypotheses. A cut-off of $q \leq 0.05$ was set for further considering probe-sets in Ingenuity Pathway Analysis (IPA). Gene identity was assigned to the Affymetrix probe-sets based on BLAST comparisons to the porcine genome sequence [Ensembl_Sscrofa10.2; according to Naraballobh et al. (42), updated]; 20,689 of the 24,123 probe-sets on the Affymetrix Porcine GeneChip were localized and annotated. Genes encoding transcripts with significant different abundance between the groups of healthy and affected samples (DE genes) were assigned to the QTL regions for OC lesion scores that were previously obtained in F2 Duroc × Pietrain crossbreed pigs of the same resource population (28). In brief, the association mapping revealed QTL on the confidence region of chromosomes (SSC) 2, 3, 6, 10, and 14 influencing OC scores at articular cartilage. The confidence intervals of QTL regions are given as physical distances in megabase pairs (Mb) (Ensembl_Sscrofa10.2).

Gene ontology and network analysis. DE genes were investigated for gene function and membership of functional networks by IPA. For a more stringent characterization between OC-negative and OC-positive cartilage, a 1.5-fold difference in transcript abundance was set as the threshold among DE genes. The list of DE genes was analyzed, referring to predefined pathways and functional categories of the Ingenuity Knowledge Base (IKB). Also, canonical pathways were identified from the IKB library. The significance was determined by Fisher's exact test with a cut-off set at 0.05. P values from Fisher's exact test were adjusted for multiple testing with the Benjamini-Hochberg multiple testing correction. To summarize the large amount of data generated by IPA, we focused and discussed the top most affected biological functions and canonical pathways that related to the pathogenesis of the OC disorder.

Quantitative real-time PCR. Different transcript abundance of selected genes was validated using quantitative real-time polymerase chain reactions (qPCR). In brief, total RNA was reverse-transcribed into cDNA using random primer and an oligo dT primer in the presence of Superscript III reverse transcriptase (Invitrogen, Karlsruhe, Germany). qPCR was performed on LightCycler480 system using the LightCycler 480 SYBR Green I Master (Roche, Mannheim, Germany). The primers were designed from Affymetrix core sequence with Primer3 (http://frodo.wi.mit.edu/primer3). The list of primer sequences is provided in Table 1. The amplification was conducted in duplicate according to manufacturer's instructions with 10 μM of each primer. Reactions were performed in a final volume of 10 μl using 5.0 μl of LightCycler 480 SYBR Green I Master (Roche), 2.0 μl of distilled water, 10 μM (0.5 μl) of each primer, and 40 ng (2 μl) of cDNA. The temperature profiles comprised an initial denaturation step at 95°C for 10 min and 40 cycles consisting of denaturation at 95°C for 15 s, annealing at 60°C for 10 s, and extension/fluorescence acquisition at 72°C for 15 s. For all the assays, threshold cycles were converted to copy numbers by a standard curve generated by amplifying serial dilutions of an external PCR standard (10^7–10^2 copies). Two common housekeeping genes, *HPRT1* and *RPL13A,* were used for the data normalization. The $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ methods were used to calculate relative expression of each target gene. For the $2^{-\Delta Ct}$ method, mean Ct values of target genes in each sample were normalized to housekeeping gene values (32). The $2^{-\Delta\Delta Ct}$ method was used to analyze changes in gene expression between OC-negative and OC-positive tissues, where the ΔCt value in the OC-positive tissues was normalized to the ΔCt value in the OC-negative cartilage to give the ΔΔCt value. Statistical analysis was performed using the Proc "Mixed" of SAS version 9.3 (SAS Institute, Cary, NC) with a generalized linear model including the fixed effect of sex, the random effect of family, and the covariate slaughter weight. Differences were considered significant at $P \leq 0.05$.

RESULTS

Differential gene expression analysis. To address molecular pathways, functional networks, and genes that are relevant in the pathogenesis of OC we compared the abundance of transcripts in articular cartilage samples obtained from OC-negative and OC-positive joints. Out of 24,123 probe-sets of the

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Table 1. *Primers used for qPCR*

Gene Symbol	Gene Name	Primer Sequence (5′-3′)	Accession No.
ASPN	asporin	F: TGATGCTGAAGGACATGGAA R: AATGTTGCTTGGGACAGAGG	NM_001243889.1
IGFBP6	insulin-like growth factor binding protein 6	F: GCCCAGCACTCTACCTTCTG R: ACGCCAACACCAACAATCTT	NM_001100190.1
MED13L	meditor complex subunit 13-like	F: CCCGAAAGTTGATTGAGGAA R: ACTGCCACAGGGAAATCATC	XM_001925106.3
OGN	osteoglycin	F: TTGCAAGCCATGACCATCTA R: CAGAGGCACAAACAGGAACA	XM_003481114.1
PDGFA	platelet-derived growth factor alpha polypeptide	F: GAAACGCTGTACTGGCTGCT R: CCTGACGTATTCCACCTTGG	XM_003124238.3
HPRT	hypoxanthine phospho-ribosyltransferase	F: GTGATAGATCCATTCCTATGACTGTAGA R: TGAGAGATCATCTCCACCAATTACTT	NM_001032376.2
RPL13A	ribosomal protein L13A	F: AGAGGAAGGAGAAGGCCAAG R: GAATCCATGGGTCTTGAGGA	XM_003127305.1

qPCR, quantitative real-time RT-PCR; F, forward; R, reverse.

24K microarray, a total of 12,292 probe-sets were found present in at least 50% of the samples of either affected or nonaffected CMH or CMF and used for further analysis. ANOVA revealed 1,977 probe-sets differentially expressed between samples of OC-negative and OC-positive cartilage at q values ≤ 0.05 (Supplementary Table S1).[1] Among these, 1,391 had low-abundance transcripts and 586 had high-abundance transcripts in OC-positive cartilage relative to OC-negative cartilage. According to the annotation of probe-sets to the porcine genome sequences (Ensemble_Sscrofa10.2) 1,564 unique DE genes were represented. The absolute expression differences of transcript abundance between OC negative and OC positive cartilage ranged from 1.03- to 4.82-fold.

Biological functions and canonical pathways of DE genes. A total of 112 probe-sets with low or high transcript abundance >1.5-fold difference were used to identify biological function and pathway association by IPA tool (Supplementary Table S2). Among these, 94 DE genes were eligible for gene ontology category based on their functional annotation of IKB. Of these DE genes, 38 genes demonstrated high abundance and 56 genes demonstrated low abundance transcripts in OC-negative and OC-positive cartilage.

The top 10 of significantly enriched IPA categories and main functions related to cellular and physiological processes pertained to proliferation, differentiation, migration and recruitment of cell types that promote inflammatory response to tissue damage, i.e., engulfing cellular debris, angiogenesis, and remodeling. The main results from functional analysis with IPA are reported in Table 2 (more details are reported in Supplementary Table S3). Through network functions, 9 networks were derived from IPA and detailed in Supplementary Table S4. Three networks were enriched for biological functions related to regeneration (organismal, organ and cell developmental) and healing (cell-to-cell signaling and inflammatory response). Accordingly, we also found canonical pathways enriched for DE genes were related to response to tissue damage (Fig. 1, Supplementary Table S5).

DE genes under QTL regions. A previous QTL study (28) found that OC lesion scores were affected by 5 QTL located on SSC2, 3, 6, 10 and 14. Out of 1,977 probe-sets representing genes with different transcript abundance there were 370 probe-sets (representing 317 genes) within the confidence intervals of the five QTL regions [SSC2, 0–103.0 cM (0–140.1 Mb); SSC3, 0–69.5 cM (0–85.5 Mb); SSC6, 29.0–150.0 cM (9.8–143.0 Mb); SSC10, 8.5–97.0 cM (6.4–58.8 Mb); SSC14, 0–43.0 cM (0–62.5 Mb)] (Supplementary Table S6). Genes represented by probe-sets with >1.5-fold difference in transcript abundance between OC-negative and OC-positive cartilage mapped within the confidence intervals of QTL on SSC 2, 3, and 6 are listed in (Table 3); whereas under the QTL on SSC10 and 14 there were no genes represented by probe-sets with ≥1.5-fold divergence of transcript frequency. Based on these 17 genes, IPA revealed a functional network containing 14 genes, which are related to embryonic development, organismal development, and cardiovascular system development and function (Fig. 2). In addition, when combining the results of the QTL study with our previous GWAS for OC lesions (48), significant single nucleotide polymorphism (SNP) marker association was evident within the QTL regions of SSC 2, 3, 10, and 14. In proximity (~2 Mb intervals) of the trait associated SNPs on SSC 2, 10 and 14 there were 26 DE genes (Table 4).

Quantitative real-time PCR validation. To validate the expression data, five genes (ASPN, IGFBP6, MED13L, OGN, and PDGFA) that displayed differential expression in microarray data were selected to perform qPCR assays. These genes were chosen based on their biological functions and their positions within previously detected QTL for OC lesion scores. All genes were validated as DE genes at $P \leq 0.05$ (Fig. 3*A*). Three of them represented high-abundance transcript in OC-positive cartilage: ASPN [fold change (FC) = 1.70, P = 0.017], MED13L (FC = 1.53, P = 0.027) and OGN (FC = 1.78, P = 0.009), whereas the other DE genes represented low-abundance transcript in OC positive cartilage: IGFBP6 (FC = −1.73, P = 0.018), and PDGFA (FC = −2.44, P = 0.040) (Fig. 3*B*).

DISCUSSION

Although the causes of OC disorder are multifactorial and include traumas, vascular abnormalities, and mechanical factors, recent findings strongly support the contribution of genetics to disease pathogenesis. Genetic differences in susceptibil-

[1] The online version of this article contains supplemental material.

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Table 2. *Top IPA categories and main functional annotations of genes (Benjamini-Hochberg P value ≤ 0.01) represented by probe-sets with >1.5-fold difference in transcript abundance between OC-negative and OC-positive cartilage*

IPA Category	Main Functional Annotation	B-H *P* Value	DE Genes, *n*
Cellular movement	cell movement	1.91E-10	38
	migration of cells	1.91E-10	36
Immune cell trafficking	leukocyte migration	3.22E-08	24
	cell movement of myeloid cells	2.52E-05	15
Hematological system development and function	cell movement of leukocytes	3.49E-07	21
	proliferation of lymphocytes	1.69E-05	18
Cardiovascular system development and function	angiogenesis	3.19E-06	18
	vasculogenesis	2.40E-05	17
Cell-to-cell signaling and interaction	recruitment of cells	3.73E-06	13
	recruitment of phagocytes	1.06E-04	9
Organismal development	development of blood vessel	8.45E-06	19
	mineralization of bone	4.01E-05	8
Cellular development	proliferation of blood cells	8.45E-06	20
	proliferation of chondrocytes	4.44E-03	4
Cellular growth and proliferation	proliferation of immune cells	1.23E-05	19
	proliferation of connective tissue cells	4.13E-03	11
Organismal functions	healing of wound	1.69E-05	9
	neovascularization of wound	1.79E-03	2
Tissue morphology	size of lesion	5.26E-04	8
	quantity of cells	1.29E-03	23

OC, osteochondrosis; IPA, Ingenuity Pathway Analysis; DE, differentially expressed. *P* values are Benjamini-Hochberg (B-H) multiple testing corrected.

ity to develop OC are obvious from association analyses for traits related to the incidence of leg weakness and OC scores in pigs (9, 44). Indeed, many loci have been associated with OC. However, the functional characterization of gene networks and pathways underlying OC pathogenesis remains to be performed. In particular, few studies have assessed global gene expression in individuals affected with OC. In this study, we applied whole-genome microarrays to uncover differences in transcript abundance in OC-negative and OC-positive articular cartilage of pigs. This approach enabled us to identify genes associated with emergence of the disease and to investigate the underlying biological functions and molecular pathways related to the disorder. Furthermore, we integrated data from previous QTL studies and GWAS with our expression analysis to identify powerful candidate genes.

Biological functions and canonical pathways of DE genes. To gain insight into the pathological process of OC, DE genes with ≥1.5-fold difference between OC-negative and OC-positive cartilage were assigned to functional categories and pathways as defined in the IKB. The majority of DE genes were associated with cellular movement, immune cell trafficking, and hematological system development and function. These categories are related to the inflammatory and immune response as for the main functional annotations that are associated with leukocytes or immune cells (e.g., lymphocytes and myeloid cells) (Table 2).

Although the molecular mechanisms of immune response involvement in OC pathogenesis remain unknown, recent studies indicate that immune system factors might contribute to the development of OA (53, 69). Notably, the progression of OC

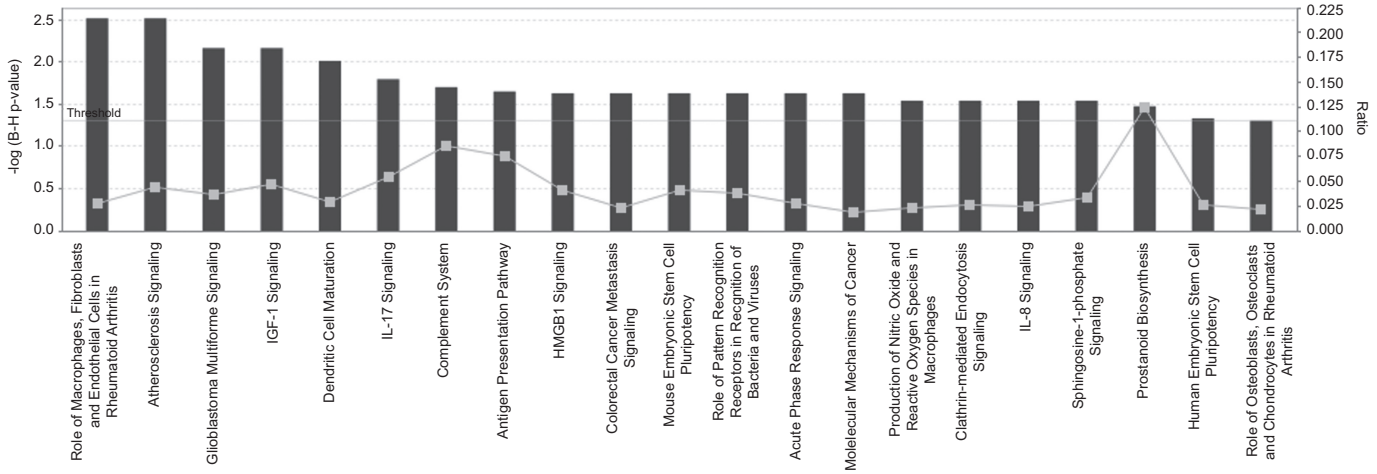


Fig. 1 Assignment of differentially expressed (DE) genes to canonical pathway according to Ingenuity Pathways Analysis. Genes were considered that were represented by probe-sets with >1.5-fold difference in transcript abundance between osteochondrosis (OC)-negative and OC-positive cartilage. The *left y*-axis corresponds to the bars giving the negative logarithm of *P* value after Benjamini-Hochberg (B-H) multiple testing correction [−log(B-H *P* value)]. The threshold was set at [−log(B-H *P* value) = 1.3, corresponding to B-H *P* value = 0.05]. The *right y*-axis corresponds to the line graph representing the ratio of the molecule numbers in a given pathway that meet cutoff criteria, divided by total number of molecules that make up that pathway. A corresponding list of DE genes is given in Supplementary Table S5.

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Table 3. *Genes represented by probe-sets with >1.5-fold difference in transcript abundance between OC-negative and OC-positive cartilage that map within QTL for OC scores*

SSC	QTL (cM)	QTL (Mb)	Gene Symbol	Gene Name	Gene Position SSC (Mb)
2	14 (0–103.0)	7.4 (0–140.1)	MDK	midkine (neurite growth-promoting factor 2)	2 (17.47)
			PIK3C2A	phosphoinositide 3-kinase, class 2, alpha polypeptide	2 (40.01)
			ICAM1	intercellular adhesion molecule 1	2 (69.43)
			ANGPTL4	angiopoietin-like 4	2 (71.27)
			TLE2	transducin-like enhancer of split 2 [E(sp1) homolog, Drosophila]	2 (76.03)
			LYSMD3	LysM, putative peptidoglycan-binding, domain containing 3	2 (100.76)
			LOX	lysyl oxidase	2 (130.89)
3	13 (0–69.5)	8.5 (0–85.5)	PDGFA	platelet-derived growth factor alpha polypeptide	3 (0.15)
			AZGP1	alpha-2-glycoprotein 1, zinc-binding	3 (7.12)
			OGN	osteoglycin	3 (43.25)
			ASPN	asporin	3 (43.35)
6	61 (29.0–150.0)	29.4 (9.8–143.0)	ARHGEF1	Rho guanine nucleotide exchange factor (GEF) 1	6 (46.03)
			CA11	carbonic anhydrase XI	6 (49.74)
			CDA	cytidine deaminase	6 (73.01)
			C1QC	complement component 1, q subcomponent, C chain	6 (74.61)
			C1QB	complement component 1, q subcomponent, B chain	6 (74.62)
			PDE4B	phosphodiesterase 4B, cAMP-specific	6 (134.87)

Positions of quantitative trait loci (QTL) for OC scores in cM according to Laenoi et al. (28) and in Mb taken from marker positions in the porcine genome sequence (Ensembl_Sscrofa10.2).

lesions can lead to osteoarthritis (OA) (40, 74); cartilage affected by OC is not in a steady state but may improve or worsen. Wang et al. (69) showed that the development of OA is an inflammatory process driven by the complement system. The complement system, part of the innate immune system, comprises a group of anaphylatoxins that promote the inflammatory response. Several components of the complement system, including complement component 1q subcomponents B chain (*C1QB*) and C chain (*C1QC*), were significantly associated with OC in this study. C1QB and C1QC proteins are secreted by normal and OA chondrocytes, but their potential roles in matrix homeostasis and chondrocyte metabolism are

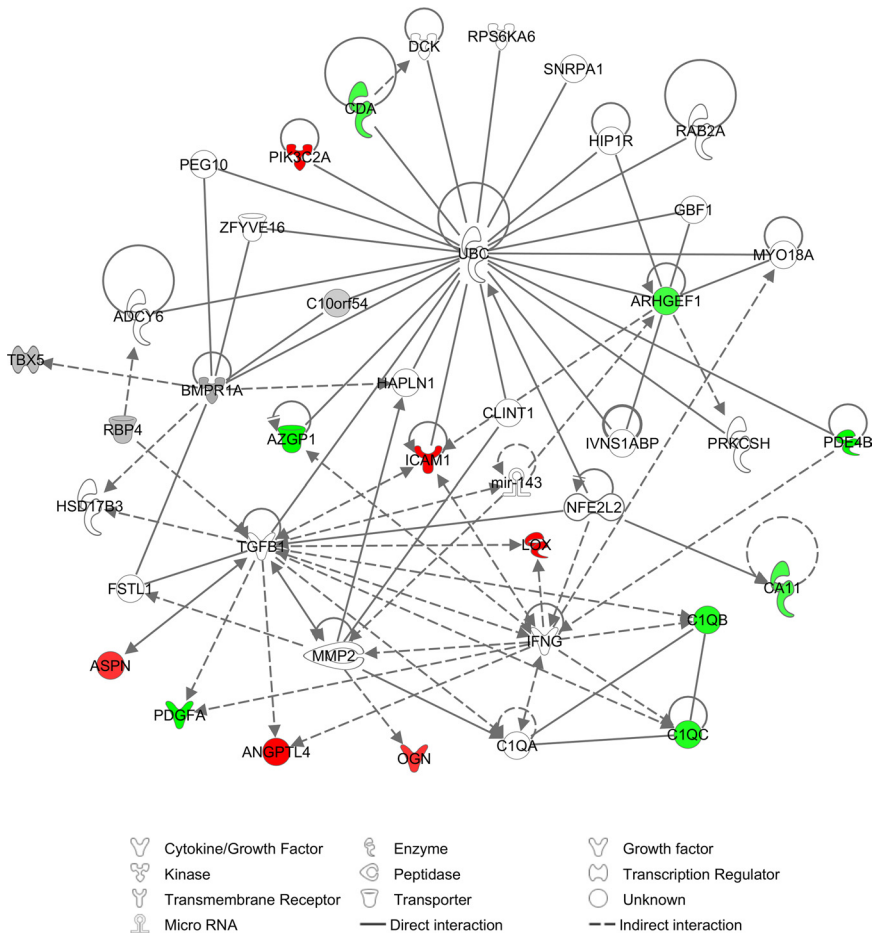


Fig. 2. DE genes within the leg weakness quantitative trait locus (QTL) regions assorted to a functional network that is associated with embryonic development, organismal development, and cardiovascular system development and function, by Ingenuity Pathways Analysis. Red, high-abundance transcripts; green, low-abundance transcripts with 1.5-fold difference; gray, regulated genes with <1.5-fold difference of transcript abundance.

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Table 4. *Genes represented by probe-sets with significantly different transcript abundance between OC-negative and OC-positive cartilage that map within genomic intervals around significantly trait-associated SNPs*

Gene Symbol	Gene Name	Gene Positions (Mb)	GWAS Interval (Mb)
LYSMD3	LysM, putative peptidoglycan-binding, domain containing 3	2 (100.76–100.78)	2 (99.92–110.78)
TTC37	tetratricopeptide repeat protein 37	2 (105.83–105.86)	
DMXL1	Dmx-like 1	2 (128.40–128.54)	2 (130.97–131.24)
TNFAIP8	tumor necrosis factor, alpha-induced protein 8	2 (128.72–128.72)	
SLC35A4	solute carrier family 35, member A4	2 (129.33–129.33)	
CD14	CD14 molecule	2 (129.35–129.35)	
SRFBP1	serum response factor binding protein 1	2 (130.80–130.86)	
LOX	lysyl oxidase	2 (130.89–130.90)	
CEP120	centrosomal protein 120 kDa	2 (132.18–132.16)	
IARS2	isoleucyl-tRNA synthetase 2, mitochondrial	10 (11.93–11.96)	10 (14.08)
WDR26	WD repeat domain 26	10 (15.13–15.17)	
PYCR2	pyrroline-5-carboxylate reductase family, member 2	10 (15.94–15.94)	
LPL	lipoprotein lipase	14 (3.82–3.85)	14 (3.90)
SLC39A14	solute carrier family 39 (zinc transporter), member 14	14 (7.07–7.12)	
ZCCHC8	zinc finger, CCHC domain containing 8	14 (30.25–30.25)	14 (27.90)
C12orf49	chromosome 12 open reading frame 49	14 (37.90–37.92)	14 (39.31–41.49)
MED13L	mediator complex subunit 13-like	14 (38.64–38.66)	
TBX5	T-box 5	14 (40.21–40.25)	
RAB35	*RAB35*, member RAS oncogene family	14 (42.68–42.70)	
TRIAP1	TP53 regulated inhibitor of apoptosis 1	14 (42.88–42.88)	
GATC	glutamyl-tRNA(Gln) amidotransferase, subunit C homolog (bacterial)	14 (42.88–42.89)	
SRSF9	serine/arginine-rich splicing factor 9	14 (42.89–42.90)	
COQ5	coenzyme Q5 homolog, methyltransferase (*S. cerevisiae*)	14 (42.93–42.95)	
DYNLL1	dynein, light chain, *LC8*-type 1	14 (42.95–42.95)	
ACADS	acyl-coenzyme A dehydrogenase, C-2 to C-3 short chain	14 (43.11–43.12)	
SPPL3	signal peptide peptidase like 3	14 (43.16–43.34)	

Positions of intervals of 2 Mb up-/downstream single nucleotide polymorphisms (SNPs) flanking a region of significantly trait-associated SNPs according to Rangkasenee et al. (48) (Ensembl_Sscrofa10.2). GWAS, genome-wide association study.

largely unknown (4). Moreover, infiltrating immune cells such as lymphocytes, macrophages, and mast cells are often found in the OA synovial tissue (6, 39, 50). Similarly, dendritic cell (DC) maturation was among the canonical pathways to which a significant number of DE genes were assigned. DC are key antigen presenting cells and important initiators of the immune response (57). E et al. (8) suggested that DCs may play a critical role in the early stage of OA, as inflammation initiates the destruction of articular cartilage.

Ingenuity functional categories and canonical pathways detected here implicate immune responses in the pathogenesis of OC. However, it remains to be clarified whether the involvement of inflammatory and immune responses is causal to the disease or is secondary to tissue destruction. Interestingly, pigs selected for high immune response were shown to be prone to inflammatory diseases of the joints (19). Together with the fact that inflammatory and immune responses are involved in the worsening of OC to OA (8), it could be speculated that low to moderate inflammatory and immune responsiveness is associated with tolerance to slight mechanical injuries of articular cartilage.

Our analyses also identify functional annotations that were significantly associated with angiogenesis and development of blood vessels (Table 2, Supplementary Table S3). Angiogenesis describes the growth of new blood vessels from the existing vascular network. Vascularization plays a role in wound healing and response to bone trauma and can also promote chondrocyte hypertrophy and endochondral ossification during physiological changes in articular cartilage (23). In this context, it is notable that failure of blood supply to reach growing cartilage contributes to OC development (43, 74–76). These findings also related to our identification of atherosclerosis signaling within the top canonical pathways. Previous studies demonstrated that angiogenesis may play an important role in the development of atherosclerosis (7, 55). Remarkably, atherosclerosis is related to progression of OA because of its

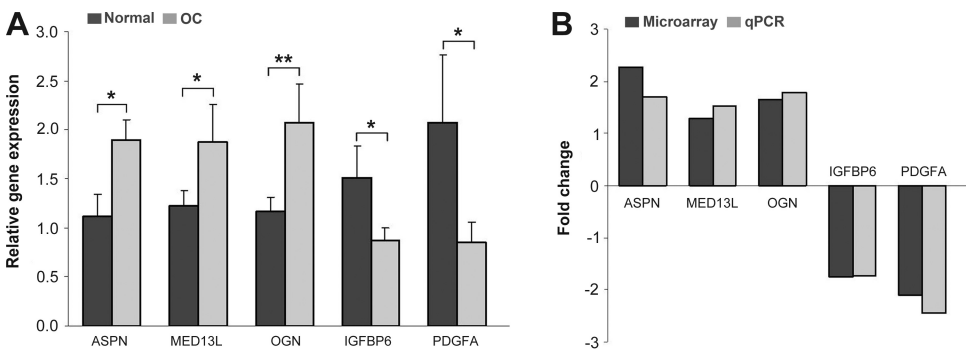


Fig. 3. Validation of microarray results by quantitative real-time RT-PCR (qPCR) for the selected genes. *A*: gene expression in OC-negative and OC-positive cartilage tissue obtained by qPCR. Relative gene expression was determined by the $2^{-\Delta\Delta}$ method (32). Data represent means ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$. *B*: comparison of microarray and qPCR expression ratios between OC-negative and OC-positive cartilage.

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inflammatory pathophysiology (14). Several DE genes in both angiogenesis and atherosclerosis likely affect expression of genes contributing to OC, such as metalloproteinase 3 (*MMP3*) and platelet-derived growth factor alpha polypeptide (*PDGFA*). MMP3 is one of the most highly expressed proteases in cartilage and is proposed to be involved in the breakdown of extracellular matrix in joint diseases. Decreased expression of *MMP3* in OC-positive cartilage in our study is consistent with findings of previous studies of OA cartilage (25, 61). PDGF, associated with indirect stimulation of vascular endothelial cell proliferation and angiogenesis (15), plays a pivotal part in vascular invasion of cartilage and endochondral ossification. Its identification in our dataset further links the process of vascularization with cartilage disease.

We identified several DE genes assigned to Ingenuity Pathway categories of mineralization of bone, proliferation of chondrocytes, and proliferation of connective tissue cells; their protein products have described function in these pathways. For example, bone morphogenetic protein receptor, type 1A (BMPR1A) is essential for chondrogenesis; loss of BMPR1A function leads to deficient early-stage joint formation and thus results in a complete failure to form joints (51, 73). Insulin-like growth factor 1 (IGF1) and insulin-like growth factor binding protein 6 (IGFBP6) have known roles in articular cartilage homeostasis. IGF1 is important in the regulation of normal articular cartilage growth and development through its capacity to stimulate proteoglycan synthesis, promote chondrocyte survival, and oppose the activities of catabolic cytokines (60, 70). IGF1 action was shown to be modulated by IGF-binding proteins (IGFBP-1–6) (16). The effects of IGFBP6 on bone development are not as well characterized, but it is generally believed to have inhibitory effects (71).

Network analysis of candidate genes within the QTL regions. We integrated data from the microarray analysis and a previous QTL study for OC lesion scores (28). We identified 17 DE genes with 1.5-fold difference in transcript abundance between OC-negative and OC-positive cartilage within QTL regions on SSC2, 3, and 6 (Table 3). These regions are consistent with the QTL for OC-related traits of other studies (31, 66). Among these overlapping genes, a functional network of 14 eligible genes was associated with embryonic development, organismal development, and cardiovascular system development and function (Fig. 3). Among these genes, lysyl oxidase (*LOX*), *PDGFA*, osteoglycin (*OGN*), and asporin (*ASPN*) regulate cartilage extracellular matrix synthesis and chondrogenesis (15, 17, 27, 56). Moreover, we found that many DE genes in this network are directly or indirectly related to bone and cartilage metabolism, including interferon-γ (*IFNG*) and transforming growth factor-beta 1 (*TGFβ1*). IFNG inhibits the synthesis of cartilage-specific matrix macromolecules (45), while TGFβ1 stimulates synthesis of proteoglycan in articular chondrocytes (68).

Angiopoietin-like 4 (*ANGPTL4*), intercellular adhesion molecule 1 (*ICAM1*), and *LOX*, located within the QTL on SSC2, have indirect interactions in IFNG and TGF-β1 signaling pathways. Expression of ANGPTL4 and ICAM1 are induced by the effect of cytokines, such as IFNG, TNF-α, and IL-1α (5, 33). *ANGPTL4* encodes a secreted protein of the angiopoietin family involved in angiogenesis (18). *ANGPTL4* transcript localizes to stromal fibroblast-like cells adjacent to blood vessels in the mouse arthritic tissue (14). *ICAM1* is expressed by many cell types, including fibroblasts, endothelial cells, some leukocytes, and also on the surface of osteoblasts (62). ICAM1 plays a role in the immune response; however, it might also be involved in the regulation of joint diseases (24, 30). LOX is a copper-dependent enzyme that catalyzes cross-linking of collagen and elastin, which plays an important role in biological functions of extracellular matrix (56). Phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 alpha (PIK3C2A) has unknown function in cartilage degradation, but it plays a role in signaling pathways involved in cell proliferation, oncogenic transformation, cell survival, cell migration, and intracellular protein trafficking (52).

Within the functional network shown in Fig. 2, *PDGFA*, alpha-2-glycoprotein 1, zinc-binding [*AZGP1*, also named zinc alpha-2-glycoprotein 1 (*ZAG*)], *OGN*, and *ASPN* map to SSC3. As mentioned above, PDGFA plays a role in angiogenesis. AZGP1 is known to stimulate lipid degradation in adipocytes (3). This protein seems to have a role in the immune response because its structural organization and folding are similar to MHC-I molecules (12). However, its functional role in joint degeneration is unknown. OGN and ASPN belong to a family of small leucine-rich proteoglycans that contribute to the regulation of collagen fibrillogenesis and cellular growth, differentiation, and migration (63). OGN in combination with TGF-β1 or 2 is effective in inhibiting osteoclast formation (27). ASPN is involved in cartilage extracellular matrix formation and binds to TGF-β1 to inhibit TGF-β-induced expression of cartilage matrix genes and chondrogenesis (26). It is also associated with susceptibility to OA (17). These findings suggest that OGN and ASPN are important cartilage matrix proteins that interact with TGF-β1 during cartilage degradation.

Six DE genes that were assigned to this network are located on SSC6. These included *C1QB* and *C1QC,* which contribute to the immune response. Phosphodiesterase 4B, cAMP-specific (*PDE4B*) is a member of the type IV, cyclic AMP (cAMP)-specific, cyclic nucleotide phosphodiesterase (PDE) family that has distinct functional roles in inflammatory and immune response (1, 20). Previously, Tenor et al. (64) suggested that PDE4 inhibitors suppress IL-1β-induced nitric oxide release in human osteoarthritis. These proteins also interact with IFNG or TGF-β1. Cytidine deaminase (*CDA*) encodes the cytidine deaminase enzyme that is released from dead or damaged polymorphonuclear leukocytes, a type of inflammatory cell also found in inflamed joints (65). CDA is a marker of the inflammatory response in rheumatoid arthritis and possibly in other joint disease, including OA (11, 35, 46). Rho guanine nucleotide exchange factor (GEF) 1 (*ARHGEF1*) and carbonic anhydrase XI (*CA11*) have no known function related to cartilage damage, but their networks are directly or indirectly related to the ubiquitin C (*UBC*) gene that is involved in protein degradation (36).

Candidate genes in proximity to significant trait-associated markers of a GWAS. In addition to relating results of the microarray expression study with a previous QTL study performed in the same population, we investigated DE genes within these QTL regions that were close to significantly trait-associated SNPs identified in a GWAS of commercial herds (Table 4). The GWAS revealed multiple significant SNPs on SSC2, SSC10, and SSC14 that were overlapping with the QTL for OC lesion scores (48). For SSC2, *LYSMD3* and

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LOX are highlighted above because these genes exhibited >1.5-fold difference in transcript abundance between OC-negative and OC-positive cartilage. *WDR26* on SSC10 is involved in a variety of cellular process, including cell cycle progression, signal transduction, apoptosis, and gene regulation; in contrast, for *IARS2* and *PYCR2* no obvious functional link to OC has been reported. On SSC14, there were three genes that overlapped with expression data and GWAS data, including chromosome 12 open reading frame 49 (*C12orf49*), mediator complex subunit 13-like (*MED13L*), and T-box 5 (*TBX5*). We have previously described an association of the SNP *TBX5* c.54T>C with OC lesion scores in pigs (48). Moreover, *TBX5* was also differentially expressed between OC-negative and OC-positive cartilage [according to real-time RT-PCR reported in Rangkasenee et al. (48): FC = −1.63, $P < 0.05$; according to microarray analysis reported here: FC = −1.41, $P < 0.05$]. This may indicate that *TBX5* is a noteworthy candidate gene for OC lesions in pigs. Furthermore, in functional networks (Fig. 2), we found that *TBX5* is indirectly related to *BMPR1A*, which was is functionally significant during chondrogenesis (73).

Conclusions

The holistic comparison of transcript abundance of healthy and OC articular cartilage provided a glimpse at the various functional pathways involved in the expression of the disease. The functional genomics analyses confirmed the involvement of immune response and angiogenesis and synthesis of extracellular matrix in the pathogenesis of OC. These biofunctions could facilitate the emergence of defects and promote the degradation of articular cartilage and worsening of the disease. We generated an integrative list of candidate genes from the microarray expression data and QTL data. Furthermore, previous GWAS data were also combined in this study. We identified several genes with genetic-statistical and functional evidence for their role in the emergence of articular cartilage lesions and their contribution to the predisposition for OC development.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: N.R., M.U.C., and S.P. performed experiments; N.R., E.M., M.U.C., S.P., and K.W. analyzed data; N.R., E.M., S.P., and K.W. interpreted results of experiments; N.R. prepared figures; N.R. drafted manuscript; N.R., E.M., K.S., M.U.C., S.P., and K.W. approved final version of manuscript; E.M., S.P., and K.W. edited and revised manuscript; K.S. and K.W. conception and design of research.

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