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ORIGINAL ARTICLE

Wnt pathway genes in osteoporosis and osteoarthritis: differential expression and genetic association study

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Abstract
Summary In comparison with hip fractures, increased expression of genes in the Wnt pathway and increased Wnt activity were found in bone samples and osteoblast cultures from patients with osteoarthritis, suggesting the involvement of this pathway in subchondral bone changes. No consistent differences were found in the genetic association study.
Introduction This study aims to explore the allelic variations and expression of Wnt pathway genes in patients with osteoporosis and osteoarthritis.
Methods The expression of 86 genes was studied in bone samples and osteoblast primary cultures from patients with hip fractures and hip or knee osteoarthritis. The Wnt-related activity was assessed by measuring *AXIN2* and in transfection experiments. Fifty-five SNPs of the LRP5, LRP6, FRZB, and SOST genes were analyzed in 1,128 patients.
Results Several genes were differentially expressed in bone tissue, with the lowest values usually found in hip fracture and the highest in knee osteoarthritis. Overall, seven genes were consistently upregulated both in tissue samples and in cell cultures from patients with knee osteoarthritis (*BCL9, FZD5, DVL2, EP300, FRZB, LRP5*, and *TCF7L1*). The increased expression of *AXIN2* and experiments of transient transfection of osteoblasts with the TOP-Flash construct confirmed the activation of Wnt signaling. Three SNPs of the *LRP5* gene and one in the *LRP6* gene showed marginally significant differences in allelic frequencies across the patient groups, but they did not resist multiple-test adjustment.
Conclusions Genes in the Wnt pathway are upregulated in the osteoarthritic bone, suggesting their involvement not only in cartilage distortion but also in subchondral bone changes.

Keywords Frizzled · Gene expression · LRP5 · Osteoarthritis · Osteoporosis · Wnt

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Introduction

Osteoporotic fractures and osteoarthritis (OA) cause significant morbidity in the middle-age and elderly population. Decreased bone mass and increased fragility are the hallmarks of osteoporosis. On the other hand, cartilage alterations are critical in OA. However, the bone tissue may also be involved in the pathogenesis of OA, playing more than just a passive role. Subchondral sclerosis and the

formation of osteophytic new bone are well-known characteristics of OA. The resulting increase in bone stiffness might decrease its ability to act as a shock absorber, thus favoring the damage of the overlying cartilage. Osteoblasts in the subchondral bone of patients with OA may have an increased metalloproteinase 13 expression, which could facilitate the degradation of the cartilage [1]. Moreover, several studies have shown that patients with OA may have, apart from the local changes in the affected joints, a generalized increase in bone mass [2]. Thus, osteoporosis and OA might be seen as opposite processes, because bone mass decreases in osteoporosis, whereas it tends to increase in OA.

The Wnt pathway has emerged as an important regulator of bone remodeling. It has multiple elements exerting pleiotropic and somewhat redundant effects, including ligands, receptors, co-receptors, and inhibitors. Nineteen Wnt ligands have been described, which bind a membrane receptor complex formed by a frizzled protein (FZD) and a lipoprotein receptor related protein (LRP). Ten different frizzled genes exist, as well as two LRPs, LRP5 and LRP6. The best known signaling mechanisms constitute the so-called canonical pathway, which lead to the accumulation of ß-catenin, but alternative non-canonical pathways exist. Some extracellular Wnt inhibitors act as decoy receptors that bind Wnt ligands. Several secreted proteins, similar to membrane frizzled but encoded by different genes, belong to this group and are known as secreted frizzled-related proteins (SFRP). Other molecules, such as the dickkopf (DKK) family, inhibit Wnt by interacting with its membrane receptors.

Loss-of-function mutations of the LRP5 gene result in osteoporosis, whereas activating mutations are associated with increased bone mass [3]. Those natural experiments and data obtained in animal models and cell cultures have shown that the activation of the canonical Wnt pathway has a bone anabolic effect, as a consequence of the stimulation of bone formation and perhaps the inhibition of bone resorption [4, 5].

The Wnt pathway may be involved in the pathogenesis of OA [6, 7]. Wnt ligands increase the expression of matrix metalloproteases by human synovium and stimulate the chondrocyte matrix catabolic action in a rabbit model of OA [8, 9]. It has been recently reported that targeted deletion of the *FRZB* gene (which codes for the Wnt-binding protein SFRP3) increases the injury-associated loss of articular cartilage in mice, in association with increased cortical bone thickness and density [10]. Thus, it has been suggested that the Wnt pathway may represent a "bone and joint connection" [7]. On the other hand, an association between certain polymorphisms of Wnt-related genes and the risk of OA has been reported [11–16]. Those data prompted us to study the expression of an array of Wnt pathway genes in the bone tissue of patients with osteoporotic fractures and OA and to explore the possible differences in the allelic frequencies of a SNP set capturing the common variations of Wnt receptors and inhibitors in both skeletal disorders.

Materials and methods

Subjects

We studied patients undergoing hip or knee prosthetic replacement surgery due to severe hip or knee OA or to hip fracture. The study was approved by the ethics committee of the Hospital U.M. Valdecilla and informed consent was obtained from the participants. Patients with secondary OA, secondary osteoporosis, or fractures due to high-impact trauma were not included.

Bone samples

Bone samples were obtained from the femoral heads or the tibiae discarded during arthroplasty. Trabecular bone cylinders of the central part of the head were obtained with a trephine, cut in small samples, washed extensively in phosphate-buffered saline, and either snap-frozen in liquid nitrogen and stored at −70°C or used to set up osteoblast cultures. Samples of the trabecular bone of the upper part of the tibia were obtained and handled in a similar way.

The primary explant technique was used to obtain osteoblast cultures [17]. In brief, bone fragments were seeded into T-75 plastic flasks containing Dulbecco´s modified Eagle's medium, antibiotics, and 10% fetal bovine serum. This allowed osteoblastic precursor cells to migrate from the fragments and proliferate. After confluence, cells were trypsinized and cultured in the same medium. When sub-confluence was reached again, the medium was aspirated and fresh medium with 10% serum or 0.1% bovine serum albumin was added. Forty-eight hours later, the medium was aspirated, cells were rinsed with phosphate-buffered saline, and the RNA was extracted.

Quantitation of gene expression

Gene expression was analyzed quantitatively by reverse transcription (RT) real-time polymerase chain reaction (PCR). RNA was isolated from cell cultures and from bone tissue samples by using Trizol, following manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). After extraction, RNA was further purified using the RNeasy kit (Qiagen, Hilden, Germany). The purity and integrity of RNA was checked by absorbance and analysis in an automated gel electrophoresis system (Experion, Bio-Rad,

Hercules, CA, USA). Contaminant DNA was removed by treating the samples with DNAse (Ambion, Austin, TX, USA), following manufacturer's instructions.

Aliquots of RNA (250 ng) were reverse-transcribed with the Superscript III kit (Invitrogen), using random hexamers as primers. After RT, gene expression was determined by real-time PCR in an ABI7300 apparatus (Applied Biosystems, Foster City, CA, USA). The reactions were performed in dedicated 96-well plates containing specific primers for 86 genes related to the Wnt pathway and five housekeeping control genes (Superarray, Frederick, MD, USA). Duplicate plates were used for most samples. The total reaction volume was 10 µl and included aliquots of the cDNA obtained by reverse transcription and a master mix containing Taq-polymerase, nucleotides, and SYBR Green (Superarray). The amount of PCR product was monitored by fluorescence and the threshold cycle (Ct) for each well was determined.

Assessment of Wnt activity

In order to determine the overall activity of the Wnt pathway in bone samples we measured the expression of the *AXIN2* gene by real-time PCR using specific primers and Taqman probes (Applied Biosystems) in triplicate wells. Also, we transfected primary cultures with the Super8XTOP-Flash construct (a generous gift of Dr. Randall Moon, Howard Hughes Medical Institute, University of Washington). This is a luciferase reporter of β-catenin-mediated transcriptional activation which contains eight TCF/LEF binding sites. Subconfluent osteoblastic cells were trypsinized and co-transfected with 0.5 µg of Super8XTOP-Flash construct and 0.3 µg of RSVgal internal control plasmid to normalize for transfection efficiency, using an "inverse transfection" protocol and SureEffect reagent, according to the manufacturer's instructions (Superarray). Cells were cultured for 48 h in standard medium, in the presence or the absence of 20 mM lithium chloride (an inhibitor of glycogen synthase kinase that increases Wnt signals). Then luciferase and galactosidase activities were measured with standard protocols. Reporter activity was determined in arbitrary units as the ratio of luciferase to galactosidase activity.

Genotyping

The genotype analysis included 1,128 individuals, 608 with OA (348 undergoing hip replacement surgery and 260 knee surgery) and 520 with hip fractures. Among the OA group 266 (44%) were male and the mean age was 71±1 8 years. On the other hand, males comprised 17% of patients with hip fractures and the mean age of the group was 81±7 years. DNA was isolated from peripheral blood or buccal swabs by using standard column-based commercial methods and quantified by using the Qubit procedure (Invitrogen). We explored the Hapmap database searching SNPs with a minimum allelic frequency of 10% in the Caucasian population of two Wnt receptors (*LRP5* and *LRP6*) and two Wnt inhibitors (*FRZB* and *SOST*, the gene coding for sclerostin). Since *SOST* and *FRZB* are relatively small genes with little representation in Hapmap, we included in the search the 3' and 5' nearby regions. Then tag SNPs covering most gene variation were selected using Haploview software [18]. In addition, we included potentially functional SNPs in those four genes identified by using the PupaSuite web tool [19]. The 55-SNP set (*LRP5*, 27; *LRP6*, 11; *FRZB*, 11; and *SOST*, six) were distributed into three plexes and genotyped using a Sequenom platform at the Centro Nacional de Genotipado in Santiago de Compostela.

Data analysis

Gene expression and activity

The Normfinder software, included in the Genex package (http://www.multid.se/genex.html) was used to explore the variability of housekeeping genes and select the most suitable controls. The results were then normalized to the average expression of the *GAPDH, ACTB*, ß2-microgloulin, and *RPL13A* housekeeping genes. The specific gene expression in bone, relative to a universal reference RNA (Stratagene, La Jolla, CA, USA), was calculated using the formula:

$$2^{\Delta Ct2-\Delta Ct1}$$

ΔCt1 is the difference between the gene of interest threshold cycle and the average housekeeping threshold cycle in the bone (or cell culture) sample and ΔCt2 is the difference in the reference RNA. Fold-changes were estimated. To test the statistical significance of between-group differences, the data were log2-transformed and compared by *t*-test or ANOVA models by using HDBStats software [20], with adjustment for multiple comparisons by computing the false discovery rate assuming dependence (FDRD) [21, 22]. The housekeeping gene TBP (TATA box binding protein) was used to normalize the results of *AXIN2* expression, as previously published [23].

Genetic association study

We applied a Monte Carlo extension of the Fisher exact test to study the differences in genotype distribution across patients groups, by using the SPSS software (SPSS, Chicago, IL, USA). Post hoc power analysis was done with G*Power 3 software [24]. The SNPSpD web tool was

Table 1 Genes showing differential expression in trabecular bone samples

Gene	Hip OA	Knee OA	nominal p-value	FDRD
FZD6	1.46	3.44	1.38E−08	2.90E−06
FRZB	4.58	14.79	1.22E−08	5.15E−06
SENP2	1.65	2.90	1.25E−07	1.32E−05
SFRP1	5.43	6.68	2.54E−07	1.53E−05
FZD8	2.13	4.50	1.14E−07	1.60E−05
WNT10A	6.04	3.50	2.00E−07	1.68E−05
FZD3	1.64	3.47	2.45E−07	1.72E−05
CXXC4	2.90	8.84	5.84E−07	3.08E−05
RHOU	1.57	3.00	8.36E−07	3.91E−05
FZD5	2.31	3.47	1.57E−06	6.60E−05
EP300	2.00	2.21	2.17E−06	8.31E−05
FZD7	1.90	3.40	2.73E−06	9.59E−05
FBXW4	1.58	2.71	2.99E−06	9.68E−05
CTNNBIP1	1.79	3.98	4.20E−06	1.26E−04
CCND2	1.59	2.77	7.12E−06	2.00E−04
BCL9	2.32	4.71	1.00E−05	2.34E−04
TLE1	1.82	2.69	9.52E−06	2.36E−04
LRP5	1.88	3.74	8.96E−06	2.36E−04
WIF1	3.29	10.71	1.09E−05	2.41E−04
TCF7L1	1.52	4.75	1.50E−05	3.15E−04
PYGO1	1.53	3.30	1.67E−05	3.35E−04
WNT4	4.02	2.77	1.87E−05	3.42E−04
FZD4	1.91	5.00	1.85E−05	3.53E−04
DVL2	1.42	2.48	2.66E−05	4.66E−04
CTNNB1	1.06	2.09	5.13E−05	8.64E−04
DIXDC1	1.03	2.13	5.54E−05	8.98E−04
LEF1	2.52	3.00	7.93E−05	1.24E−03
GSK3B	1.35	2.14	8.72E−05	1.31E−03
KREMEN1	2.22	1.96	1.02E−04	1.48E−03
LRP6	1.51	3.09	1.07E−04	1.50E−03
SOX17	1.00	3.40	1.15E−04	1.56E−03
WNT6	2.75	4.62	1.23E−04	1.62E−03
SLC9A3R1	3.25	1.43	1.62E−04	2.06E−03
BTRC	2.31	2.17	1.85E−04	2.29E−03
FBXW2	1.27	1.65	2.06E−04	2.41E−03
APC	1.71	3.28	2.04E−04	2.46E−03
SFRP4	2.32	4.99	2.57E−04	2.92E−03
JUN	2.07	7.64	2.85E−04	3.16E−03
MYC	0.77	2.66	3.18E−04	3.44E−03
CSNK2A1	1.30	2.28	3.53E−04	3.72E−03
CCND1	1.12	2.67	3.95E−04	4.05E−03
AES	1.26	2.04	5.42E−04	5.43E−03
PPP2R1A	0.96	1.52	6.57E−04	6.43E−03
FBXW11	1.39	1.81	7.04E−04	6.74E−03
TCF7	1.94	2.53	7.82E−04	7.16E−03
WNT2B	0.81	2.22	7.65E−04	7.16E−03
TLE2	1.43	2.78	8.78E−04	7.87E−03
DAAM1	2.24	2.42	1.07E−03	9.36E−03
FOSL1	0.18	0.20	2.98E−03	2.56E−02
CSNK1D	1.38	1.91	3.31E−03	2.74E−02
FRAT1	2.36	2.79	3.29E−03	2.77E−02
NLK	1.41	1.61	6.06E−03	4.73E−02
WNT11	1.80	2.55	5.86E−03	4.75E−02
CTBP1	1.46	1.60	6.02E−03	4.78E−02
PPP2CA	0.70	1.08	6.51E−03	4.98E−02

The fold-changes in comparison with values in bone samples from patients with hip fractures (which were given a value of 1) are shown. Statistical significance in ANOVA global comparisons between the three groups (nominal uncorrected *p*-values and false discovery rate values assuming dependence—FDRD). Genes with FDRD values above 0.05 are not shown

used to compute the significance thresholds after adjustment for multiple comparisons [25]. The procedure takes into account the linkage disequilibrium of the SNPs analyzed in each gene and, when applied independently to each of the four genes studied, resulted in adjusted significance thresholds of 0.003, 0.006, 0.005, and 0.008, for the SNPs of LRP5, LRP6, FRZB, and SOST, respectively.

Results

Gene expression

After excluding some samples of suboptimal RNA quality, we analyzed the following bone tissue samples: 15 from hip fracture patients, with a mean age of 80±9 years (14 women, one man); 13 with hip OA, mean age 72±7 years (11 women, two men); and 11 with knee OA, mean age 74±5 years (eight women, three men). Cell cultures were grown from five women with hip fractures (mean age 76±9 years), six patients with hip OA (three women and three men; 75±5 years), and six patients with knee OA (four women, two men; 76±5 years).

When expression data in bone tissue samples were compared by ANOVA, 55 genes showed statistical significant differences between the study groups (hip fractures, hip OA, knee OA) at the 0.05% FDRD cut-off level (Table 1). They included Wnt ligands (such as *WNT2B*, *4*, *6*, *10A*, and *11*), Wnt receptors/co-receptors (such as *LRP5*, and *frizzleds 3*, *4*, *5*, *6*, *7*, and *8*), other Wnt binding proteins (such as *SFRP1*, *SFRP3 (FRZB)*, *SFRP4*, and *WIF1*), cytoplasmic proteins in the downstream signaling pathways (such as casein kinases, *DVL2*, *GSK3B*, and β-catenin), nuclear transcription factors and co-factors (including *BCL9*, *BTRC*, *TCF7L1*, *TLEs*, *EP300*) and Wnt

target genes (such as cyclins, *FOSL1/FRA1*, and *FZD7*). In general, the highest levels were found in knee OA samples, and the lowest in fracture samples. Thus, pairwise comparisons showed significant differences between fracture and knee samples for all those genes. For 17 genes we also found evidence for differential expression between hip and knee OA and for ten genes between hip fracture and hip OA. Very similar results were obtained when only female samples were included in the analysis. We found no evidence for age-related differences in Wnt expression, nor evidence for interaction between age and the underlying condition.

The analysis of gene expression in cell cultures by using a two-way ANOVA procedure did not reveal evidence for interaction between patient group and culture conditions (i. e., serum-free or serum-containing). Therefore, data from both culture conditions were combined together. Fourteen genes showed evidence for differential expression in cell cultures, with FDRD values below the 0.05 cut-off (Table 2). Thus, seven genes (*BCL9*, *FZD5*, *DVL2*, *EP300*, *FRZB*, *LRP5*, and *TCF7L1*) were differentially expressed in both bone tissue samples and osteoblast cultures (Fig. 1).

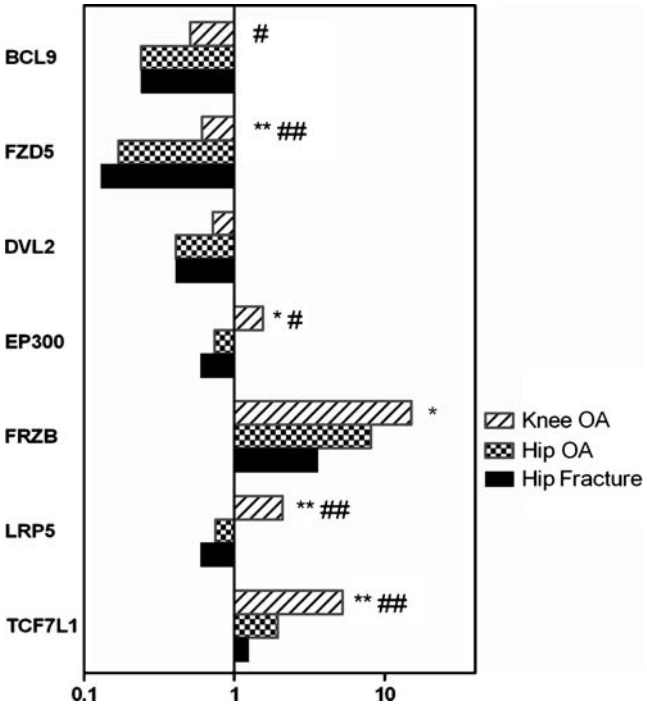

Fig. 1 Wnt-related genes showing significant differential expression, consistently both in cell cultures and in bone samples. *Bars* represent the abundance of gene transcripts in cell cultures, expressed as relative units in comparison with reference RNA. *Symbols* represent the statistical significance of pairwise comparisons. Knee osteoarthritis vs. hip fractures: *False discovery rate (FDRD) <0.05, ** FDRD <0.005. Knee osteoarthritis vs. hip osteoarthritis: # FDRD <0.05, ## FDRD <0.005

Wnt activity

The expression of *AXIN2* was measured as an index of the overall Wnt activity. As shown in Fig. 2, it was significantly higher in samples from patients with knee OA, in comparison with samples from fractured patients. On the other hand, we assayed Wnt activity in primary osteoblast cultures by using a luciferase reporter system. In basal medium, activity was somewhat higher in cultures derived from OA patients (p=0.07) and the difference increased markedly when the cells were cultured in the presence of LiCl, which inhibits glycogen synthase kinase and therefore tends to stabilize β-catenin. As expected, luciferase activity was increased in the presence of LiCl in both fracture and OA cultures, but the effect was more marked in the latter (Fig. 2).

Table 2 Genes showing differential expression in primary osteoblast cultures

Gene	Hip OA	Knee OA	nominal p-value	FDRD
FRD5	1.35	4.72	5.14E−08	2.17E−05
TCF7L1	1.58	4.24	1.43E−06	2.01E−04
LRP5	1.25	3.50	1.26E−06	2.65E−04
WNT5A	0.76	3.90	4.29E−06	4.51E−04
EP300	1.22	2.58	8.03E−06	5.64E−04
GSK3A	1.23	3.52	7.15E−06	6.02E−04
WNT16	7.88	0.64	3.40E−05	2.05E−03
FRZB	2.25	4.20	5.74E−05	3.02E−03
CCND3	1.26	2.42	8.48E−05	3.97E−03
CSNK1A1	1.32	2.56	1.15E−04	4.84E−03
BCL9	0.97	2.09	1.99E−04	7.63E−03
FRAT1	1.03	2.17	2.27E−04	7.97E−03
CTBP2	1.71	2.79	4.00E−04	1.30E−02
DVL2	1.02	1.76	1.13E−03	3.40E−02

The fold-changes in comparison with values in cultures from patients with hip fractures (which were given a value of 1) are shown. Statistical significance in ANOVA global comparisons between the three groups (nominal uncorrected p-values and false discovery rate values assuming dependence—FDRD). Genes with FDRD values above 0.05 are not shown

Genetic association study

We chose a set of four genes, two Wnt co-receptors (*LRP5* and *6*) and two Wnt inhibitors (*FRZB* and *SOST*), to be studied looking for allelic variants that could explain the differences in gene expression and activity reported in the previous section (see Table S1 in online supplemental material). The study power to detect an effect size of 0.12 was higher than 90% (this is roughly equivalent to a 10% difference in the proportion of the most common homozygotes, for a minor allele frequency of 20%).

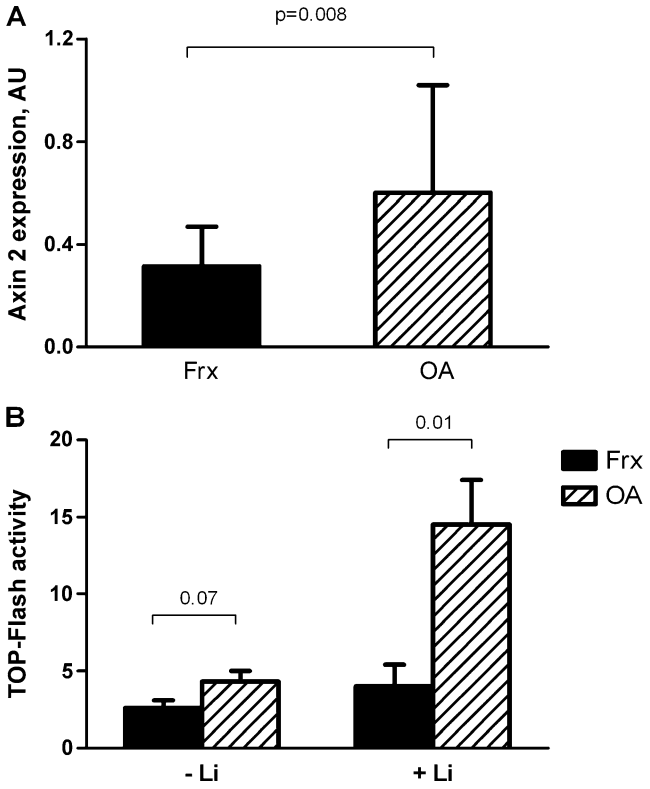

Fig. 2 Analysis of Wnt activity. **a** Expression of axin 2, a Wnt target gene, in bone samples from patients with hip fracture or knee osteoarthritis (arbitrary units, mean and SEM). **b** TCF/LEF-dependent reporter activity in cultures of osteoblasts grown from patients with hip fractures or knee osteoarthritis, in the presence or the absence of lithium chloride (relative luciferase to galactosidase activity, mean and SEM)

Three SNPs of the *LRP5* gene (rs4988330, rs4988331, and rs312786) and one of the *LRP6* gene (rs1181334) showed differences in the allele frequency distributions in the study groups (Fig. 3), with nominal *p*-values between 0.008 and 0.03. However, none remained significant after adjustment for multiple comparisons. The genotype frequencies are shown in Fig. 4.The allele frequencies of the SNPs of the FRZB and SOST genes were similarly distributed in the three groups. In the sex-stratified analysis, some other SNPs showed marginally significant differences in allelic frequency distribution, but they were inconsistent in men and women and did not remain significant after multiple-test adjustment (not shown).

Discussion

In this study, we found evidence for disease- and site-dependent differences in the expression of genes related to the Wnt pathway in bone tissue and bone-derived osteoblast cultures. A number of studies suggest that the Wnt pathway is involved in the degradation of the osteoarthritic cartilage (reviewed in [7, 26]). Nakamura found an upregulation of *WNT7B* in OA cartilage, with conflicting results about SFRPs [6, 9]. Others reported an upregulation of *WNT5B* in bone samples of patients undergoing hip arthroplasty [27]. We did not find differences in the expression of *WNT5B* or *WNT7B* between bone samples of hip fractures and hip OA. We found a trend to reduced expression of *WNT5B* and *WNT7B* in bone samples of

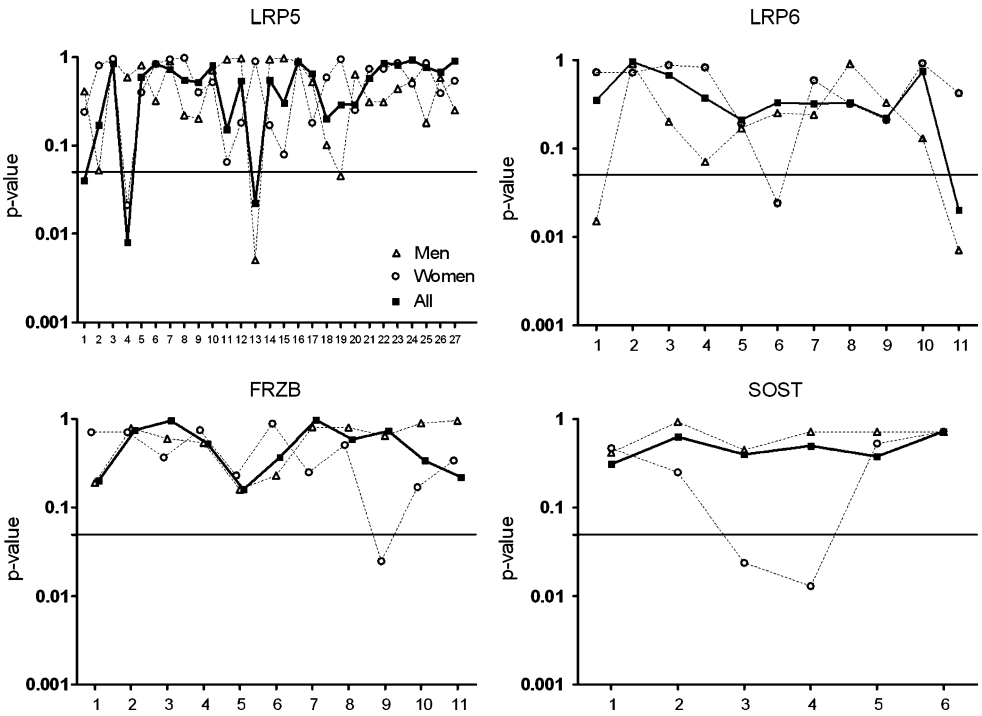

Fig. 3 Differences in the genotypic frequencies of polymorphisms of the LRP5, LRP6, FRZB, and SOST genes across patient groups. *p*-values for the frequency distribution in patients with hip OA, knee OA, and hip fractures are shown, in the whole group as well as in the male and female subgroups. The *horizontal line* marks the conventional *p*-value of 0.05

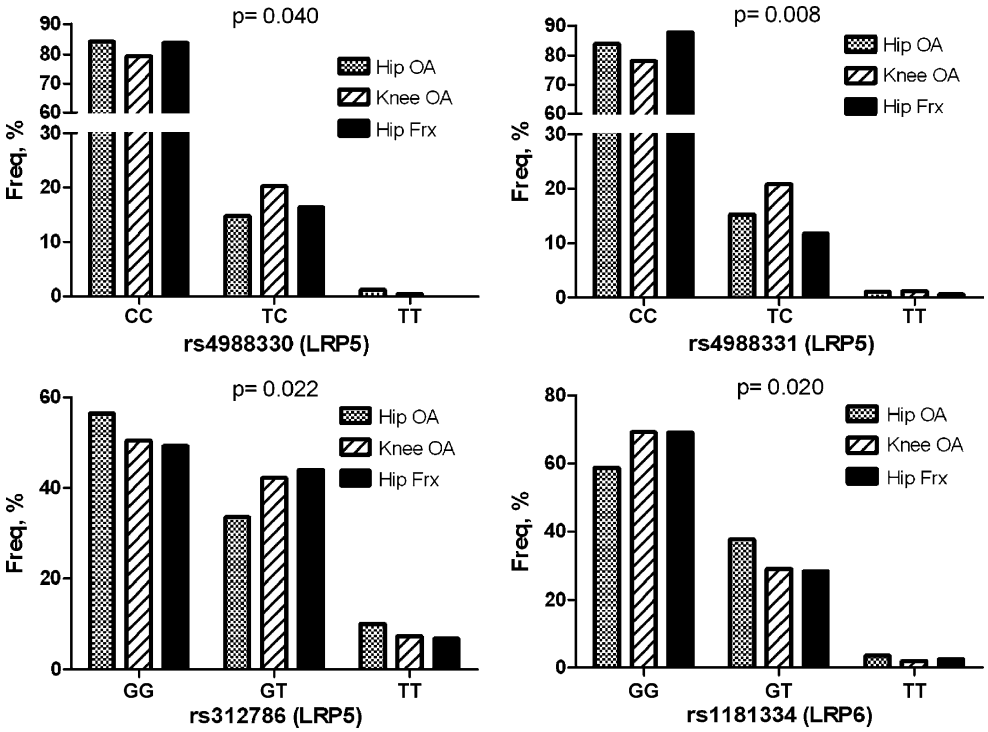

Fig. 4 Genotype frequency distributions of the four SNPs showing nominal *p*-values <0.05 in the whole group analysis

patients with hip fractures in comparison with those undergoing knee arthroplasty, but the differences were not statistically significant after multiple-test adjustment. Likewise, although it has been suggested that the high circulating levels of DKK1, a Wnt inhibitor, may be associated with a slower progression of hip OA, we did not find significant differences in *DKK1* expression at the tissue level [28]. Also in favor of the Wnt involvement in OA are the results of a recent study by Blom, who found an increased expression of the Wnt-induced signaling protein 1 in an experimental model of OA and in human OA cartilage [29]. However, our knowledge about the actual role of Wnt in this disorders is still far from complete, as exemplified by the finding that both ß-catenin induction and ß-catenin activation results in OA-like changes in genetically engineered mice [30, 31].

In the present study, the comparison of gene expression in the trabecular bone of the femoral head revealed significant differences between patients with hip fractures and hip OA. Thus, ten genes were upregulated in OA, in comparison with fracture samples. They included some nuclear transcription factors and co-regulators (*BTRC*, *EP300*), Wnt ligands (*WNT4*, *6* and *10A*), and the secreted proteins *SFRP1* and *3*, among others (see Table 1). On the other hand, when samples from patients with hip and knee OA were compared, an upregulation of several genes was found in knee samples (see Table 1). The expression of *AXIN2* was also increased, thus reflecting the enhanced Wnt activity in the bones of patients with OA. *AXIN2* is a Wnt target gene commonly used a monitor the overall activity of the pathway [32]. The upregulation of the Wnt pathway may be deleterious for the joint cartilage, for Wnt signals induce the expression of matrix metalloproteases that promote cartilage catabolism and degradation [8]. On the other hand, the Wnt pathway upregulation may have a stimulatory effect on bone formation, in part because it is involved in mechanotransduction [33, 34]. At the local level, it may be related with the bone sclerosis and osteophyte formation seen in the affected joints. On the other hand, if this was a generalized phenomenon, it could contribute to the increased bone mineral density found in patients with hip or knee OA [2, 35].

Cells of multiple lineages are present in bone tissue. Therefore, to get a better knowledge of gene expression in osteoblasts, we studied it in primary cultures. In general, similar results were found in bone samples and in primary cultures, with upregulation of several genes in OA. However, two interesting differences existed. First, fewer genes showed significant upregulation; second, significant differences existed between cells derived from knee OA and either hip OA or hip fracture, but there were no significant differences between the primary cultures of hip OA and hip fracture.

Seven genes showed the most consistent evidence for differential expression, both in bone samples and in osteoblast primary cultures. They included two membrane-associated Wnt receptor molecules, *LRP5* and *FZD5*; a soluble molecule which also binds Wnt ligands, *FRZB* (SFRP3); a protein needed for Wnt action downstream of its receptors, *DVL2*; and three nuclear factors (*BCL9*, *EP300*, and *TCF7L1*).

LRP5 forms, with frizzled proteins, the receptor complex for Wnt ligands. Its role in bone is emphasized by the skeletal consequences of natural and experimental mutations [36, 37]. Thus, upregulation of LRP5 could theoretically increase Wnt signaling and favor osteoblastic bone formation. The family of human frizzled comprises at least ten genes with a high level of homology [38]. In the present study, we found upregulation of the *FZD5* gene in OA. FZD5 binds WNT7A and WNT5B and may have a more important role in the non-canonical than in the canonical pathway [39, 40]. The secreted frizzled-related proteins (SFRPs) bind Wnt ligands. Although first considered as inhibitors of Wnt function, they may have a more complex role, including the transport of Wnts to cellular sites that have a high concentration of receptors, where they can be released as active ligands [41]. Indeed, Cho et al. recently demonstrated that SFRPs may enhance the Wnt-induced differentiation of osteoblastic precursors in mice [40]. According to those results, the increased expression of *FRZB* (SFRP3) found in OA bone might theoretically have a bone anabolic influence. However, SFRP3 has been shown to inhibit Wnt signaling in other experimental systems and it has been recently reported that the targeted disruption of the *FRZB* gene promotes cartilage degradation in mice [10]. On the other hand, some coding polymorphisms of the *FRZB* gene, which appear to reduce its binding affinity for Wnt, have been associated with knee OA [14, 16]. Therefore, further studies are needed to elucidate the actual role of FRZB in the physiology of human bone and cartilage and in the pathogenesis of skeletal disorders.

According to a recently proposed model, the binding of Wnt molecules to their LRP5/6-frizzled receptors recruits dishevelled molecules (DVL) to frizzled. Three genes encoding isoforms of Dishevelled (*DVL1*, *DVL2*, and *DVL3*) appear to function cooperatively as well as uniquely with respect to mediation of WNT3A-stimulated canonical signaling [42]. Therefore, it is possible to speculate that increased transcription of *DVL2* in knee OA may result in increased Wnt-like signals.

Canonical Wnt signaling results in the accumulation of non-phosphorilated ß-catenin, which translocates into the nucleus. There it regulates gene transcription by binding to proteins of the T-cell factor/lymphoid enhanced factor (TCF/LEF) family. Thus, the upregulation of these genes probably results in increased Wnt signaling. BCL9 also binds ß-catenin and thus takes part in the formation of multimeric complexes of ß-catenin, TCFs, and different co-activators and adaptors which finally bind to conserved Wnt response elements in DNA [43]. We also found increased expression of the transcriptional coactivator E1A binding protein p300 (EP300, also known as P300). EP300 participates in the transduction of signals elicited by a number of regulatory factors, including TGFß and Wnt [44].

The upregulation of several genes in the Wnt pathway observed in OA in the present study was accompanied by a parallel increase in Wnt activity, as revealed by higher expression of the Wnt target gene *AXIN2*, as well as increased activity of a luciferase reporter sensitive to TCF/LEF. Dell'Accio et al. have recently reported an upregulation of the Wnt pathway in the articular cartilage of patients with OA, which may be directly related to the mechanical injury [45]. Our results extend those findings and suggest that Wnt activation is not only present in the cartilage, but also in the bone tissue. However, some differences exist, as *FRZB* was found to be downregulated in the cartilage [45], whereas we observed an upregulation in bone and osteoblast cultures. On the other hand, we observed some differences between samples from patients with hip and knee OA, suggesting that, besides the disease-related differences, there are site-dependent differences in the behavior of bone cells.

Our study has several limitations. We do not have data about gene expression in regions far apart from the affected joints. Therefore, we do not know to which extent the differences in gene expression are just a local phenomenon or a generalized one. There were some differences in age between patients with hip fractures and OA, which might raise some concerns about age-dependent differences in gene expression. However, we did not find evidence for a significant influence of age in the results. Also, there might be relevant differences regarding co-morbidities and drug therapy. Most patients with OA were taking analgesics or nonsteroidal anti-inflammatory drugs (NSAIDs). Nevertheless, it is an unlikely explanation for the differences observed in our study, as NSAIDs, including aspirin and COX-2 inhibitors, tend to decrease Wnt signaling, at least in cancer cells [46, 47]. Although we were careful to obtain samples apart from the fracture focus itself, the possibility of some regional changes in gene expression cannot be completely excluded. Since we could not obtain hip and knee samples from normal individuals, we cannot compare our results with those in a completely normal skeleton. Hence, it could be difficult to establish with certainty if Wnt was upregulated in OA or downregulated in fractures. However, since no differences in gene expression were found between osteoblast cultures in hip OA and hip fractures, it seems likely that the higher levels found in knee OA actually represented an upregulation in the latter condition.

We studied a series of polymorphisms of two Wnt co-receptors and two inhibitors, looking for allelic differences that could explain the differences in Wnt activity. However, although we found some SNPs with marginally significant differences in the genotype distribution across the patients'

groups, they were small and did not resist the multiple-test corrections. Moreover, they were not consistent in men and women. The FRZB polymorphisms have been studied in relation with OA by several investigators. Although they were found to be associated with OA of the lower limbs in some studies [14, 16], the results could not be replicated recently in two population-based cohorts [48]. Our analysis was limited by the moderate size of the study population, particularly for subgroup analysis. It had an adequate power to detect moderate-size effects, but the study does not allow to exclude small effects, particularly under recessive genetic models (for example, it had a 30% power to detect a 2% difference in the genotype frequency distribution for a recessive allele with a frequency of 20%). Also, the average age of the groups of patients with OA and fractures was different. This reflects the different epidemiology of those diseases. Nevertheless, when the analysis was restricted to individuals of less advanced age, similar results were found (not shown). Therefore, the marked differences in Wnt gene expression are unlikely to be accounted for those small differences in allelic frequencies. Polymorphisms of other Wnt-related genes not analyzed in the present study could be involved and those showing evidence for differential expression might be particularly interesting candidates. Alternatively, epigenetic changes, which can be transmitted through the mitosis of osteoblasts in culture, including the DNA methylation pattern and modifications of nuclear proteins, could be a more likely explanation. This remains to be confirmed experimentally, but differential DNA methylation has been suggested to have a role in OA, in particular in the induction of matrix-degrading proteases in the joint cartilage [49].

In summary, we have shown that several genes in the Wnt pathway are differentially expressed in bone samples of patients with hip fractures and hip/knee OA. The upregulation of the Wnt pathway may be deleterious for the joint cartilage, because it induces the release of metalloproteases that promote cartilage catabolism and degradation. On the other hand, Wnt signals have a stimulatory effect on bone formation. At the local level, it may be involved in the subchondral sclerosis and osteophyte formation seen in the affected joints. On the other hand, if this was a generalized phenomenon, it would contribute to the increased bone mineral density found in patients with hip or knee OA. Differences in the allelic distribution of common polymorphisms of the LRP5/6 co-receptors and the Wnt inhibitors FRZB and SOST do not seem to account for the differences in Wnt activity.

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Conflicts of interest None.

References

1. Sanchez C, Deberg MA, Bellahcene A et al (2008) Phenotypic characterization of osteoblasts from the sclerotic zones of osteoarthritic subchondral bone. Arthritis Rheum 58:442–455
2. Dequeker J, Aerssens J, Luyten FP (2003) Osteoarthritis and osteoporosis: clinical and research evidence of inverse relationship. Aging Clin Exp Res 15:426–439
3. Little RD, Carulli JP, Del Mastro RG et al (2002) A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone-mass trait. Am J Hum Genet 70:11–19
4. Goldring SR, Goldring MB (2007) Eating bone or adding it: the Wnt pathway decides. Nat Med 13:133–134
5. Baron R, Rawadi G (2007) Wnt signaling and the regulation of bone mass. Curr Osteoporos Rep 5:73–80
6. Nakamura Y, Nawata M, Wakitani S (2005) Expression profiles and functional analyses of Wnt-related genes in human joint disorders. Am J Pathol 167:97–105
7. Corr M, Lane NE (2007) FRZB: a bone and joint connection. Arthritis Rheum 56:3881–3883
8. Yuasa T, Otani T, Koike T et al (2008) Wnt/beta-catenin signaling stimulates matrix catabolic genes and activity in articular chondrocytes: its possible role in joint degeneration. Lab Invest 88:264–274
9. Imai K, Morikawa M, D'Armiento J et al (2006) Differential expression of WNTs and FRPs in the synovium of rheumatoid arthritis and osteoarthritis. Biochem Biophys Res Commun 345:1615–1620
10. Lories RJ, Peeters J, Bakker A et al (2007) Articular cartilage and biomechanical properties of the long bones in Frzb-knockout mice. Arthritis Rheum 56:4095–4103
11. Lane NE, Lian K, Nevitt MC et al (2006) Frizzled-related protein variants are risk factors for hip osteoarthritis. Arthritis Rheum 54:1246–1254
12. Urano T, Narusawa K, Shiraki M et al (2007) Association of a single nucleotide polymorphism in the WISP1 gene with spinal osteoarthritis in postmenopausal Japanese women. J Bone Miner Metab 25:253–258
13. Min JL, Meulenbelt I, Riyazi N et al (2005) Association of the Frizzled-related protein gene with symptomatic osteoarthritis at multiple sites. Arthritis Rheum 52:1077–1080
14. Loughlin J, Dowling B, Chapman K et al (2004) Functional variants within the secreted frizzled-related protein 3 gene are associated with hip osteoarthritis in females. Proc Natl Acad Sci USA 101:9757–9762
15. Smith AJ, Gidley J, Sandy JR et al (2005) Haplotypes of the low-density lipoprotein receptor-related protein 5 (LRP5) gene: are they a risk factor in osteoarthritis? Osteoarthritis Cartilage 13:608–613
16. Lories RJ, Boonen S, Peeters J et al (2006) Evidence for a differential association of the Arg200Trp single-nucleotide polymorphism in FRZB with hip osteoarthritis and osteoporosis. Rheumatology (Oxford) 45:113–114
17. Jonsson KB, Frost A, Nilsson O et al (1999) Three isolation techniques for primary culture of human osteoblast-like cells: a comparison. Acta Orthop Scand 70:365–373

18. Barrett JC, Fry B, Maller J et al (2005) Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21:263–265
19. Conde L, Vaquerizas JM, Dopazo H et al (2006) PupaSuite: finding functional single nucleotide polymorphisms for large-scale genotyping purposes. Nucleic Acids Res 34:W621–W625
20. Trivedi P, Edwards JW, Wang J et al (2005) HDBStat!: a platform-independent software suite for statistical analysis of high dimensional biology data. BMC Bioinformatics 6:86
21. Benjamini Y, Drai D, Elmer G et al (2001) Controlling the false discovery rate in behavior genetics research. Behav Brain Res 125:279–284
22. Benjamini Y, Yekutieli D (2005) Quantitative trait loci analysis using the false discovery rate. Genetics 171:783–790
23. Hernandez JL, Garcés CM, Sumillera M et al (2008) Aromatase expression in osteoarthritic and osteoporotic bone. Arthritis Rheum 58:1696–1700
24. Faul F, Erdfelder E, Lang AG et al (2007) G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods 39:175–191
25. Nyholt DR (2004) A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. Am J Hum Genet 74:765–769
26. Luyten FP, Tylzanowski P, Lories RJ (2009) Wnt signaling and osteoarthritis. Bone 44:522–527
27. Hopwood B, Tsykin A, Findlay DM et al (2007) Microarray gene expression profiling of human osteoarthritic bone suggests altered bone remodelling, WNT and TGF beta/BMP signalling. Arthritis Res Ther 9:R100
28. Lane NE, Nevitt MC, Lui LY et al (2007) Wnt signaling antagonists are potential prognostic biomarkers for the progression of radiographic hip osteoarthritis in elderly Caucasian women. Arthritis Rheum 56:3319–3325
29. Blom AB, Brockbank SM, van Lent PL et al (2009) Involvement of the Wnt signaling pathway in experimental and human osteoarthritis: prominent role of Wnt-induced signaling protein 1. Arthritis Rheum 60:501–512
30. Kawaguchi H (2009) Regulation of osteoarthritis development by Wnt-beta-catenin signaling through the endochondral ossification process. J Bone Miner Res 24:8–11
31. Zhu M, Tang D, Wu Q et al (2009) Activation of beta-catenin signaling in articular chondrocytes leads to osteoarthritis-like phenotype in adult beta-catenin conditional activation mice. J Bone Miner Res 24:12–21
32. Leung JY, Kolligs FT, Wu R et al (2002) Activation of AXIN2 expression by beta-catenin-T cell factor. A feedback repressor pathway regulating Wnt signaling. J Biol Chem 277:21657–21665
33. Bonewald LF, Johnson ML (2008) Osteocytes, mechanosensing and Wnt signaling. Bone 42:606–615
34. Robinson JA, Chatterjee-Kishore M, Yaworsky PJ et al (2006) Wnt/beta-catenin signaling is a normal physiological response to mechanical loading in bone. J Biol Chem 281:31720–31728
35. Bergink AP, Uitterlinden AG, van Leeuwen JP et al (2005) Bone mineral density and vertebral fracture history are associated with incident and progressive radiographic knee osteoarthritis in elderly men and women: the Rotterdam Study. Bone 37:446–456
36. Gong Y, Slee RB, Fukai N et al (2001) LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. Cell 107:513–523
37. van Amerongen R, Berns A (2006) Knockout mouse models to study Wnt signal transduction. Trends Genet 22:678–689
38. Wang HY, Liu T, Malbon CC (2006) Structure-function analysis of Frizzleds. Cell Signal 18:934–941
39. Carmon KS, Loose DS (2008) Wnt7a interaction with Fzd5 and detection of signaling activation using a split eGFP. Biochem Biophys Res Commun 368:285–291
40. Cho SW, Her SJ, Sun HJ et al (2008) Differential effects of secreted frizzled-related proteins (sFRPs) on osteoblastic differentiation of mouse mesenchymal cells and apoptosis of osteoblasts. Biochem Biophys Res Commun 367:399–405
41. Kawano Y, Kypta R (2003) Secreted antagonists of the Wnt signalling pathway. J Cell Sci 116:2627–2634
42. Lee YN, Gao Y, Wang HY (2008) Differential mediation of the Wnt canonical pathway by mammalian Dishevelleds-1, -2, and -3. Cell Signal 20:443–452
43. Hoppler S, Kavanagh CL (2007) Wnt signalling: variety at the core. J Cell Sci 120:385–393
44. Li J, Sutter C, Parker DS et al (2007) CBP/p300 are bimodal regulators of Wnt signaling. EMBO J 26:2284–2294
45. Dell'accio F, De Bari C, Eltawil NM et al (2008) Identification of the molecular response of articular cartilage to injury, by microarray screening: Wnt-16 expression and signaling after injury and in osteoarthritis. Arthritis Rheum 58:1410–1421
46. Tuynman JB, Vermeulen L, Boon EM et al (2008) Cyclooxygenase-2 inhibition inhibits c-Met kinase activity and Wnt activity in colon cancer. Cancer Res 68:1213–1220
47. Bos CL, Kodach LL, van den Brink GR et al (2006) Effect of aspirin on the Wnt/beta-catenin pathway is mediated via protein phosphatase 2A. Oncogene 25:6447–6456
48. Kerkhof JM, Uitterlinden AG, Valdes AM et al (2008) Radiographic osteoarthritis at three joint sites and FRZB, LRP5, and LRP6 polymorphisms in two population-based cohorts. Osteoarthritis Cartilage 16:1141–1149
49. Roach HI, Yamada N, Cheung KS et al (2005) Association between the abnormal expression of matrix-degrading enzymes by human osteoarthritic chondrocytes and demethylation of specific CpG sites in the promoter regions. Arthritis Rheum 52:3110–3124