ARTHRITIS & RHEUMATISM
Vol. 65, No. 1, January 2013, pp 197–205
DOI 10.1002/art.37753

Genome-Wide Profiling of Bone Reveals Differentially Methylated Regions in Osteoporosis and Osteoarthritis

Jesús Delgado-Calle,[1] Agustín F. Fernández,[2] Jesús Sainz,[3] María T. Zarrabeitia,[4] Carolina Sañudo,[1] Raúl García-Renedo,[5] María I. Pérez-Núñez,[5] Carmen García-Ibarbia,[1] Mario F. Fraga,[2] and José A. Riancho[1]

Objective. **To determine genome-wide methylation profiles of bone from patients with hip osteoarthritis (OA) and those with osteoporotic (OP) hip fractures.**

Methods. **Trabecular bone pieces were obtained from the central part of the femoral head of 27 patients with hip fractures and 26 patients with hip OA. DNA was isolated, and methylation was explored with Illumina methylation arrays. RNA was extracted, pooled, and deep-sequenced to obtain the whole transcriptome. Differentially methylated regions were identified, and connections between genes with differentially methylated regions were explored by pathway and text-mining analyses.**

Results. **After quality control, methylation of 23,367 CpG sites (13,463 genes) was analyzed. There was a genome-wide inverse relationship between methylation and gene expression in both patient groups. Comparison of OP and OA bones revealed 241 CpG sites, located in 228 genes, with significant differences in methylation (false discovery rate <0.05). Of them, 217 were less methylated in OP than in OA. The absolute methylation differences were >5% in 128 CpG sites and >10% in 45 CpG sites. The differentially methylated genes were enriched for association with bone traits in the genome-wide association study catalog. Pathway analysis and text-mining analysis with Gene Relationships Across Implicated Loci software revealed enrichment in genes participating in glycoprotein metabolism or cell differentiation, and particularly in the homeobox superfamily of transcription factors.**

Conclusion. **Genome-wide methylation profiling of bone samples revealed differentially methylated regions in OP and OA. These regions were enriched in genes associated with cell differentiation and skeletal embryogenesis, such as those in the homeobox superfamily, suggesting the existence of a developmental component in the predisposition to these disorders.**

Bone increases in size during the growth period by a modeling process driven by the formation of new bone. Thereafter, it is constantly remodeled by the concerted action of bone-resorbing osteoclasts and bone-forming osteoblasts, originating from hematopoietic and mesenchymal precursors, respectively. When remodeling is to start at a certain site, osteoclast precursors are attracted and committed to differentiate into mature osteoclasts. When the resorption phase ends, surrounding osteoblast precursors proliferate and differentiate into mature osteoblasts that synthesize bone matrix that eventually mineralizes and replaces the old bone resorbed by osteoclasts. Thus, bone remodeling requires the cyclic and sequential proliferation and differentiation of osteoclast and osteoblast precursors. Any disturbance of this process will result in abnormal bone mass. This is the case in osteoporosis (OP), which is characterized by a decrease in bone mass, due to high bone resorption and/or low bone formation, and consequently propensity to fracture. In contrast to OP, in osteoarthritis (OA) bone formation is increased in the

Supported by a grant from the Instituto de Salud Carlos III (PI 09/539). Dr. Delgado-Calle is recipient of a fellowship from the Instituto de Formación e Investigación Marqués de Valdecilla.

[1]Jesús Delgado-Calle, PhD, Carolina Sañudo, Carmen García-Ibarbia, MD, PhD, José A. Riancho, MD, PhD: Hospital U. M. Valdecilla–Instituto de Formación e Investigación Marqués de Valdecilla, University of Cantabria, and RETICEF, Santander, Spain; [2]Agustín F. Fernández, PhD, Mario F. Fraga, PhD: Instituto Universitario de Oncología del Principado de Asturias, HUCA, and University of Oviedo, Oviedo, Spain; [3]Jesús Sainz, PhD: Institute of Biomedicine and Biotechnology of Cantabria, and Spanish Research Council, Santander, Spain; [4]María T. Zarrabeitia, MD, PhD: University of Cantabria, Santander, Spain; [5]Raúl García-Renedo, MD, María I. Pérez-Núñez, MD, PhD: Hospital U. M. Valdecilla, Santander, Spain.

Address correspondence to José A. Riancho, Departamento de Medicina Interna, Hospital U. M. Valdecilla, Avenida Valdecilla s/n, 39011 Santander, Spain. E-mail: rianchoj@unican.es.

Submitted for publication May 9, 2012; accepted in revised form October 9, 2012.

affected joints, characteristically resulting in subchondral bone sclerosis and osteophyte formation (1). Several epidemiologic studies have shown a tendency toward a generalized increase in bone mineral density in patients with OA (2,3).

Although the methylation level of cytosines in CpG-rich regions influences gene transcription and cell differentiation in many tissues, little is known about its role in skeletal homeostasis. Nevertheless, we have recently reported that DNA methylation is involved in the differentiation of cells in the osteoblastic lineage and the osteoblast–osteocyte transition (4). Therefore, we speculated that differences in DNA methylation could be involved in the pathogenesis of bone mass alterations. To test this hypothesis, we performed genome-wide methylation profiling of bone tissue in patients with OP and those with OA and explored the relationship between methylation and gene expression.

MATERIALS AND METHODS

DNA methylation profiling. Bone samples were obtained from the femoral heads of patients undergoing hip replacement. Trabecular bone cylinders were obtained from the central part of the head with a trephine, thus avoiding subchondral and fractured regions. Bones were cut into small pieces, extensively washed with phosphate buffered saline, snap-frozen in liquid nitrogen, and stored at −70°C until assayed.

The study population included 53 women ages 59–85 years; 26 had OA (mean ± SD age 75 ± 6 years), and 27 had OP fractures (mean ± SD age 80 ± 3 years). Patients with fractures due to high-energy trauma, or with disorders causing secondary OP or OA were not included. Informed consent was obtained from all subjects, and the study was approved by the institutional review board (Comité de Etica en Investigación Clínica de Cantabria).

DNA was isolated from bone samples using phenol–chloroform–isoamyl alcohol, as previously described (5). Microarray-based DNA methylation profiling was performed using an Infinium HumanMethylation27 DNA Analysis BeadChip (Illumina), which targets CpG sites located within the proximal promoter regions of transcription start sites of 14,475 CCDS coding sequences in the National Center for Biotechnology Information Database (genome build 36) and 110 microRNA promoters. It accomplishes this high multiplexing by combining bisulfite conversion of genomic DNA and whole-genome amplification sample preparation with direct, array-based capture and enzymatic scoring of the CpG loci. Bisulfite conversion of DNA was performed using an EZ DNA Methylation Kit according to the recommendations of the manufacturer (Zymo Research), with the modifications described in the Infinium Methylation Assay Protocol Guide. Processed DNA samples were hybridized to the BeadChip. The assay interrogates the chemically differentiated loci using two site-specific probes, one designed for the methylated locus (M bead type) and another for the unmethylated locus (U bead type). Single-base extension of the probes incorporates a labeled ddNTP, which is subsequently stained with a fluorescent reagent. The methylation level for the interrogated locus is determined by calculating the ratio of the fluorescent signals from the methylated versus unmethylated sites. The ratio of fluorescent signals was then computed from the two alleles according to the following formula:

$$\text{Beta} = \frac{\text{Max}(M,0)}{\text{Max}(U,0) + \text{Max}(M,0) + 100}$$

A constant value of 100 is added as a pseudocount to prevent division by 0 for background subtraction. This beta value is a quantitative measure of DNA methylation levels of specific CpG sites, and ranges from 0 for completely unmethylated to 1 for completely methylated. Before analyzing the methylation data, we excluded possible sources of technical biases that could alter the results. Every beta value in the Infinium platform is accompanied by a detection *P* value. We based the filtering criteria on these *P* values reported by the assay. All probes with detection *P* values greater than 0.01 in 5% or more of the samples were removed.

Pyrosequencing. The methylation status of selected genes was confirmed by pyrosequencing. Sodium bisulfite modification of 0.5 μg genomic DNA was carried out as described above. Bisulfite-treated DNA was eluted in 15 μl, using 2 μl for each polymerase chain reaction (PCR). The sets of primers for PCR amplification and sequencing were designed using PyroMark assay design software, version 2.0.01.15. Primer sequences were designed to hybridize with CpG-free sites to ensure methylation-independent amplification (sequences are available from the corresponding author upon request).

PCR was performed with primers biotinylated to convert the PCR product to single-stranded DNA templates, using a Vacuum Prep Tool, according to the recommendations of the manufacturer (Biotage). Pyrosequencing reactions and methylation quantification were performed in a PyroMark Q24 System version 2.0.6 (Qiagen).

Determination of gene expression by RNA sequencing and real-time PCR. Bone samples were obtained as described above from patients with hip fractures or hip OA. Total RNA was isolated with Trizol reagent (Invitrogen), and quality was estimated using an Agilent 2100 Bioanalyzer. Only samples with an RNA integrity number ≥8.0 were selected for sequencing in a next-generation sequence platform (Genome Analyzer IIx; Illumina). Samples from patients with hip fractures (n = 11) and those with hip OA (n = 11) were pooled independently and sequenced to obtain 35-nucleotide reads. Libraries for sequencing were constructed from the RNA pools using reagents and protocols from the manufacturer (Illumina). Alignments of the reads to the reference human genome, counts of reads per gene, and estimates of reads per kilobase per million reads (RPKM) (6), used to normalize the expression data, were obtained using the Cassava pipeline from Genome Analyzer System software. Bone specimens used for this analysis were distinct from those used for the methylation analyses. Since the samples were pooled, a single comparison was done between gene expression in pooled OA samples versus pooled OP samples.

The expression of some selected genes was studied in

individual samples by quantitative real-time PCR. Aliquots of RNA extracted from femoral heads as described above were reverse-transcribed with a Superscript III kit (Invitrogen), using random hexamers. The abundance of gene transcripts was determined using TaqMan assays (Applied Biosystems) and normalized to the expression of the housekeeping gene TATA box binding protein (7).

Statistical analysis. After probe filtering, 23,367 CpG sites were analyzed, corresponding to 13,463 genes. To determine differentially methylated regions between OA and OP, two approaches were used. First, we used a Bayesian analysis of variance (ANOVA) for microarrays approach, which takes the multiple test problem into account by controlling both the false discovery rate (FDR) and the false nondiscovery rate, thus trying to optimize the number of well-classified targets, minimizing the number of false positives and false negatives, with BAMarray software. This approach may have increased power in comparison to other methods that only try to control the FDR (8). Since the assumptions of the calculations include a continuous variable, and beta values are restricted to the 0–1 range, we computed the function arcsin$\sqrt{}$beta (arcsine of the square root of beta) prior to inputting data for the analysis, and assumed unequal group variances.

Second, a more stringent analysis was done by a standard 2-group comparison by *t*-tests with multiple test correction. For this analysis, the ratio methylated:unmethylated was estimated as the beta:(1−beta) ratio and $\log_2$-transformed. Data were then normalized by the quintile procedure. Between-group comparisons were performed with linear models with blocking by array batch using BRB ArrayTools developed by Dr. Richard Simon and the BRB ArrayTools Development Team (http://linus.nci.nih.gov/BRB-ArrayTools.html). Significance levels were corrected for multiple testing by the Benjamini method to control the FDR (9). In some analyses, age was included as a covariate (categorized according to the median) using a multiway ANOVA. The existence of pathways or functional groups enriched in genes showing differentially methylated regions was explored with the DAVID version 6.7 web tool, and *P* values were corrected for multiple testing (10).

The representation of genes with differentially methylated regions among those related to bone disorders was also tested by searching the Gene Reference into Function (GeneRIF) database (www.ncbi.nlm.nih.gov/gene/about-generif) with the strings "osteo," "fracture," "cartilage," "bone," and "arthritis," and relative enrichment was assessed by chi-square test. We also explored the presence of differentially methylated genes among those associated with bone phenotypes in the genome-wide association studies (GWAS) catalog (www.genome.gov/gwastudies/), interrogated with the terms "osteoporosis," "fractures," "bone mineral density," "bone size," "hip geometry," "height," and "calcium levels." Networks of genes showing significant functional relationships were generated through the use of Ingenuity Pathways Analysis (Ingenuity Systems; www.ingenuity.com). Genes showing differentially methylated regions with an FDR of <0.05 were uploaded into the application and overlaid onto a global molecular network developed from information contained in Ingenuity's Knowledge Base. Gene networks were then algorithmically generated based on their connectivity and scored with Fisher's exact test (the network score is the negative logarithm of the *P* value).

Relationships between genes were also explored by a text-mining procedure using the literature-based annotation with Gene Relationships Across Implicated Loci (GRAIL) software with correction for multiple testing (11). This analysis is based upon the assumption that genomic changes are more likely to be biologically relevant when they involve several genes within a certain pathway. The relationship between methylation and gene expression was assessed by Spearman's correlation coefficient. Also, genes were separated in quartiles according to expression level, measured as RPKM (as described above). Then, methylation levels across quartiles of gene expression were compared using an ANOVA test. For genes with more than one CpG site in the methylation array, each CpG site was considered independently.

RESULTS

Global methylation and gene expression. Most loci showed low methylation values (Figure 1). The overall methylation was similar in patients with OP fractures and those with OA (mean beta values 0.273 and 0.276, respectively). In fact, there was a strong correlation between samples from patients with OP and those from patients with OA with regard to average methylation at each CpG locus ($r = 0.989$). Gene methylation and expression levels were inversely correlated in both groups of patients. Thus, when genes were separated into quartiles of expression, a marked difference in methylation was observed ($P < 10^{-36}$) (Figure 2).

Differentially methylated regions. BAM software identified 201 hypermethylated and 627 hypomethylated CpG sites in OP samples (in comparison with OA samples). The comparison of the methylation of individual loci by conventional tests using BRB ArrayTools revealed 241 CpG sites with differences between OA and OP (FDR < 0.05). They were mapped to 228 genes;

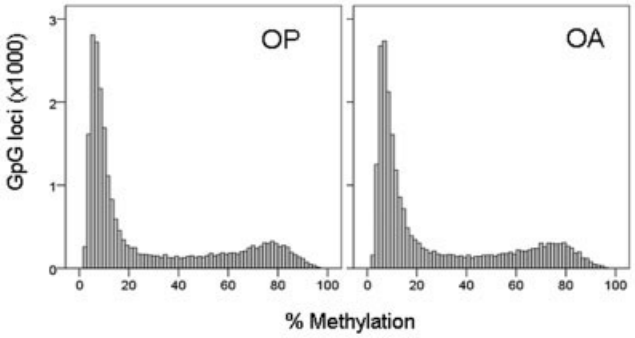

Figure 1. Average distribution of methylation in the group of patients with osteoporotic (OP) hip fractures and the group of patients with hip osteoarthritis (OA).

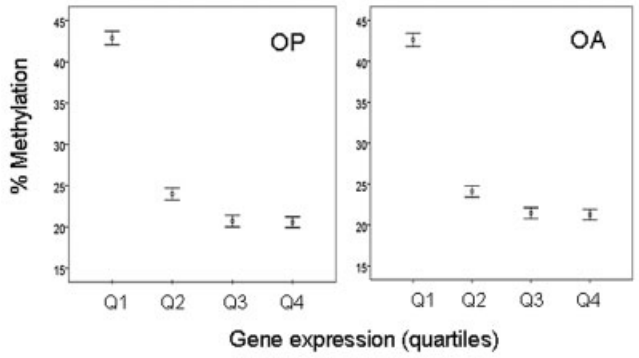

Figure 2. Methylation of CpG sites across quartiles of gene expression in samples from patients with osteoporosis (OP) and patients with osteoarthritis (OA). Quartile 1 (Q1) consists of genes with <0.9 reads per kilobase per million reads (RPKM); Q2 of genes with 1–5.4 RPKM, Q3 of genes with 5.5–15.9 RPKM, and Q4 of genes with >16 RPKM. Values are the mean and 95% confidence interval.

most of them (199) overlapped with those identified in the Bayesian analysis. Among the CpG sites, 217 were more methylated in OA and 24 were more methylated in OP (see Supplementary Table 1, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131). In 128 CpG sites the differences in beta values were >0.05, and in 45 CpG sites the differences were >0.1 (see Supplementary Table 2, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131). Since patients with OP were somewhat older than those with OA, we repeated the analysis after adjusting for age. Overall, similar results were obtained (see Supplementary Tables 1 and 2). In contrast, we did not find any CpG locus with significant differences in methylation across age strata.

Most loci showing differential methylation were located in regions that are considered to be true CpG islands in the vicinity of the gene translation start sites. In fact, 229 of the differentially methylated regions were in a true CpG island located within 600 bp of the translation start sites, whereas the global analysis showed 241 differentially methylated regions among 23,667 CpG sites (1.5% versus 1%; $P = 2 \times 10^{-8}$).

Pathway and phenotype analyses. Pathway analysis showed that differentially methylated regions were overrepresented in genes participating in the glycoprotein, neuronal differentiation, adherence, homeobox, and cell proliferation pathways (Table 1). Genes with differentially methylated regions were also overrepresented among those with skeleton-related annotations in the GeneRIF database. Whereas 1,225 (9.2%) of 13,262 genes included in the database had skeleton-related annotations, 40 (17.6%) of 228 genes with differentially methylated regions had such annotations ($P = 0.00002$). Likewise, genes with differentially methylated regions were overrepresented among those associated with bone phenotypes in the GWAS catalog (4.8% versus 1.6%; $P = 0.0003$). Molecular network analysis with Ingenuity software resulted in a very likely network (score 40), that revealed several "hidden hubs" consisting of molecules involved in skeletal homeostasis, such as type I collagen, alkaline phosphatase, and NF-κB (Figure 3). The network was enriched in genes associated with the development of the appendicular skeleton ($P = 0.00006$), limb morphogenesis ($P = 0.0014$), and osteoblast differentiation ($P = 0.0001$).

We also explored associations between genes by a text-mining analysis using GRAIL software. The 45 genes with differentially methylated regions and beta value differences of >0.1 between OP samples and OA samples were included. This analysis revealed significant

Table 1. Functional annotation analysis of terms overrepresented among genes showing differentially methylated loci*

Category	Term	Fold enrichment	*P*	*P* after Bonferroni correction
INTERPRO	Iroquois-class homeodomain protein	58.1	2.8×10^{-5}	1.0×10^{-2}
INTERPRO	Cadherin prodomain like	49.8	4.9×10^{-5}	2.0×10^{-2}
GOTERM_BP_FAT	Regulation of epithelial cell proliferation	9.1	2.6×10^{-5}	4.0×10^{-2}
UP_SEQ_FEATURE	Cadherin 5	8	6.2×10^{-5}	4.0×10^{-2}
INTERPRO	Homeobox	5.2	3.0×10^{-6}	1.0×10^{-3}
GOTERM_BP_FAT	Sensory organ development	4.9	5.2×10^{-6}	8.0×10^{-3}
GOTERM_BP_FAT	Neuron differentiation	4.8	1.5×10^{-10}	2.4×10^{-7}
GOTERM_BP_FAT	Neuron development	4.5	2.0×10^{-7}	3.2×10^{-4}
GOTERM_BP_FAT	Cell adhesion	3.1	4.4×10^{-7}	7.1×10^{-4}
GOTERM_BP_FAT	Regulation of cell proliferation	2.8	3.9×10^{-6}	6.0×10^{-3}
SP_PIR_KEYWORDS	Glycoprotein	2.1	1.3×10^{-14}	4.0×10^{-12}

* Functional annotation analysis was conducted using the DAVID web tool. Loci with a *P* value of <0.05 after Bonferroni correction were considered differentially methylated.

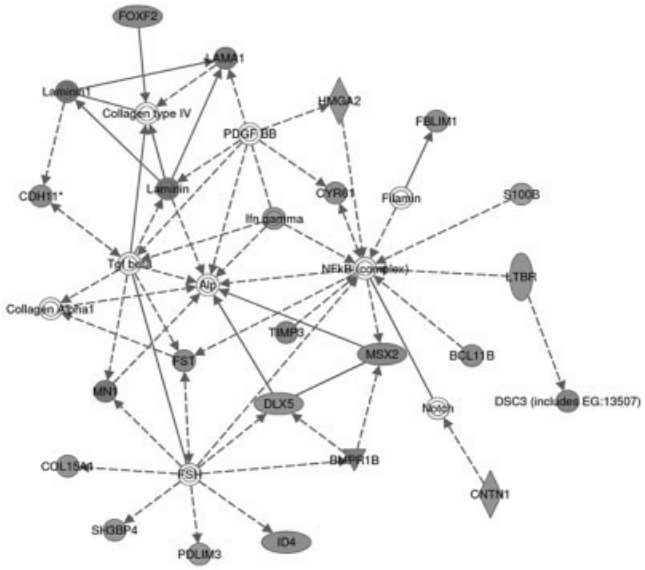

Figure 3. Molecular function network. Gray symbols correspond to genes with differentially methylated regions. Broken lines represent indirect interactions. PDGF-BB = platelet-derived growth factor BB; TGFβ = transforming growth factor β; Alp = alkaline phosphatase; IFNγ = interferon-γ; FSH = follicle-stimulating hormone.

underlying connections between 28 genes at $P < 0.05$ and between 17 genes at $P < 0.001$ (threshold for significance after adjustment for multiple testing) (Table 2)

Table 2. Genes showing significant connections after adjustment of the significance threshold for multiple testing*

Gene symbol	Gene name	P
IRX2	Iroquois homeobox 2	1.1×10^{-9}
TLX3	T cell leukemia homeobox 3	1.5×10^{-9}
GSX1	GS homeobox 1	2.6×10^{-9}
FOXI2	Forkhead box I2	8.9×10^{-9}
IRX4	Iroquois homeobox 4	1.2×10^{-8}
NEUROG1	Neurogenin 1	1.2×10^{-8}
LHX1	LIM homeobox 1	1.6×10^{-8}
MSX2	Msh homeobox 2	2.8×10^{-8}
NKX2-8	NK2 homeobox 8	3.8×10^{-8}
MAFB	v-*maf* musculoaponeurotic fibrosarcoma oncogene homolog B	4.0×10^{-8}
FGF4	Fibroblast growth factor 4	3.8×10^{-7}
HOXA9	Homeobox A9	7.7×10^{-7}
SOX1	SRY (sex-determining region Y) box 1	1.3×10^{-6}
SALL3	Sal-like 3	6.7×10^{-5}
LAMA1	Laminin, α1	2.4×10^{-4}
MN1	Meningioma	5.8×10^{-4}
LTBR	Lymphotoxin β receptor (tumor necrosis factor receptor superfamily, member 3)	9.3×10^{-4}

* Genes were analyzed with Gene Relationships Across Implicated Loci software. The significance level after adjustment for multiple testing was $P = 0.001$.

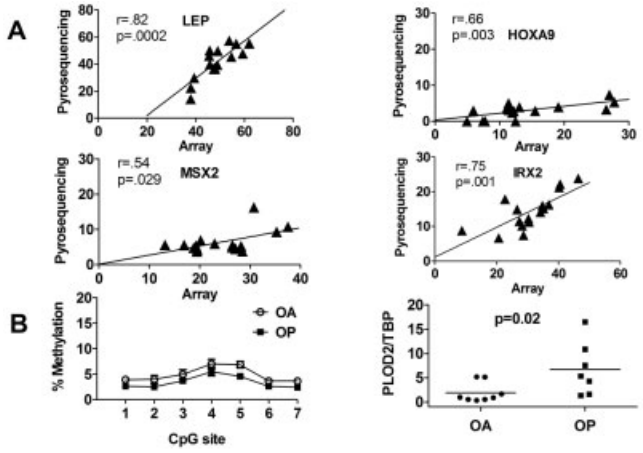

Figure 4. A, Correlation of the methylation of several genes as assessed by arrays and the methylation of the same genes as assessed by pyrosequencing. Each data point represents the methylation level of the same individual CpG site analyzed with both procedures. **B,** Methylation of individual CpG sites of the *PLOD2* gene (left) and *PLOD2* gene expression (right) in samples from patients with osteoarthritis (OA) and those from patients with osteoporosis (OP). Values in the left panel are the mean ± SEM. In the right panel, symbols represent individual patients; horizontal lines represent the median. TBP = TATA box binding protein.

(also see Supplementary Figure 1A, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131). The most common connecting terms were "development," "homeobox," "differentiation," "leptin," "laminin," "lymphotoxin," and "neuronal."

We selected the following 5 genes with differentially methylated regions to validate the array results by pyrosequencing: *LEP* (encoding leptin), *HOXA9* (homeobox A9), *PLOD2* (procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2), *IRX2* (iroquois homeobox 2), and *MSX2* (msh homeobox 2). The analysis was performed in 8 samples from patients with fractures and 8 samples from patients with OA, all of which had previously been analyzed with methylation arrays. There was a tight correlation between the methylation values obtained using the arrays and the values obtained by pyrosequencing (global r = 0.91; $P < 0.00001$) (Figure 4A). Pyrosequencing demonstrated that individual CpG sites within a certain CpG island had similar levels of methylation (Figure 4B) (additional data for other genes are available from the corresponding author upon request). This confirmed the informativeness of the single CpG sites explored in the methylation arrays.

We next explored the relationship between gene expression and methylation levels. Transcripts of the leptin gene tended to be inversely correlated with the methylation level in both groups of patients (r = −0.76;

$P = 0.0009$). Similarly, expression and methylation of the *PLOD2* gene tended to show an inverse correlation, particularly in OA ($r = -0.62$; $P = 0.0099$). However, there was no evidence for correlation between individual expression levels and methylation for the genes *HOXA9*, *IRX2,* or *MSX2*.

For comparison purposes we also pyrosequenced 4 samples obtained at autopsy from individuals without known bone disorders (ages 69–85). The methylation levels of these genes tended to be lower in bone samples from controls than in those from the patient groups (see Supplementary Figure 1B, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).

DISCUSSION

Complex disorders such as OP and OA are the final result of genetic and acquired factors. In fact, candidate gene studies and, more recently, GWAS, have identified several genes involved in these disorders. However, surprisingly, they still explain only a minor proportion of the disease risk (12,13). The explanation for the missing heritability is disputed, but may include epigenetic mechanisms, such as DNA methylation. The methylation of cytosines of CpG dinucleotides is maintained through cell divisions by DNA methyltransferases and influences DNA stability, chromatin structure, and the regulation of gene transcription. DNA methylation is believed to play an important role in the differentiation of various cell types, both in embryonic and adult tissues. We have recently shown that the methylation of CpG-rich sequences of the promoter regions represses genes known to play important roles in bone formation and bone resorption (4,5,14). Epigenetic mechanisms, and specifically DNA methylation, modulate the expression of proteases and other cartilage constituents (15–17), suggesting that these mechanisms may be involved in OA (recently reviewed by Reynard [18] and Barter [19]). However, little is known about the potential role of CpG methylation in the pathogenesis of bone changes in OA and other skeletal disorders.

In this study, we aimed to compare the genome-wide methylation profiles of bone from patients with OA and bone from patients with OP, two conditions characterized by changes in bone mass in opposite directions (2,3). The comparative analysis identified several genes showing differential methylation levels in both conditions. Although they were significantly enriched for bone trait–associated genes (as determined by GWAS), supporting the hypothesis that the genes found to have differentially methylated regions are good candidates for bone disease, they were not classic bone gene candidates, such as those known to play major roles in osteoblast and osteoclast differentiation (RANKL, *RUNX2*, etc.), or in bone formation and resorption (alkaline phosphatase, collagen, cathepsin K, etc.). Some differentially methylated regions were related to lymphotoxin and neuronal pathways. Although important connections between the immune system and bone, as well as between the nervous system and bone, are known to exist (20,21), the actual significance of those differentially methylated regions is unknown at present.

Most differentially methylated regions tended to be located in genes generally associated with the fate of less differentiated cells and with cell–matrix interactions. The analysis of relationships between genes by several complementary methods, such as standard pathway analysis and a text-mining procedure, revealed underlying connections between several genes showing differentially methylated regions, and enrichment in gene sets associated with skeletal development, which further supports the biologic relevance of these findings and points to the homeobox group of genes as particularly overrepresented among those with differentially methylated regions. Most of them were more methylated in OA than in OP. Data suggested that in both conditions the methylation level of several genes tended to be higher than in control bones, but this result should be interpreted cautiously due to the small number of control samples and limited clinical information available for them.

The homeobox superfamily includes genes that direct the formation of many body structures during early embryonic development. They encode transcription factors with a conserved group of 60 amino acids known as the homeodomain. Genes in the homeobox family are involved in a wide range of critical activities during embryonic development, including the position and shape of the skeletal rudiments of the limbs (22,23).

These results would suggest that a developmental factor (either genetic, environmental, or epigenetic), acting either during growth or in later years, is involved in the pathogenesis of these disorders, a concept that has been proposed previously (24,25). Consistent with this hypothesis, epidemiologic data show that patients with OP and those with OA have different anthropometric characteristics, such as height, weight, and bone size and shape. For instance, a long femoral neck axis is associated with an increased risk of fractures, whereas a wider femoral neck is associated with the osteophytic and composite hip OA phenotypes (26). Likewise, the shape

of the femoral head may influence the risk of OA (27). Genetic association studies have identified gene variants that are associated with OA, including some *FRZB* polymorphisms. *FRZB* encodes the secreted Frizzled-related protein 3, a soluble Wnt inhibitor. The Wnt pathway is up-regulated in patients with OA, in comparison with those with OP (28), and animal models suggest that it is involved in joint damage in OA (29,30). Interestingly, some *FRZB* alleles associated with OA appear to influence the shape of the proximal femur (31). In the present study, *SFRP1*, which encodes the secreted Frizzled-related protein 1, a Wnt-related inhibitor, was included in the set of genes that were differentially methylated.

Obesity is associated with OA, whereas low body weight is a well-recognized risk factor for OP and fractures (27,32,33). The pathophysiologic links between body weight and skeletal disorders have not been elucidated, but may include both physical factors (i.e., mechanical loads on the skeleton) and humoral factors, such as estrogens, leptin, and other cytokines synthesized in the adipose tissue (34). Alternatively, skeletal and adipose phenotypes may share genetic, environmental, or epigenetic factors (35). Interestingly enough, it has been reported that high-fat diets, which tend to be associated with obesity, may induce the hypermethylation of homeobox genes in cynomolgus monkeys (36). Given the lack of detailed information about nutrient intake, we cannot establish if the higher methylation of homeobox genes in the patients with OA in the present study was the result of differences in fat intake. Consistent with the emerging role of leptin in bone homeostasis (37), the differences in leptin methylation between samples from OP patients and those from OA patients found in this study may represent another link between energy metabolism and bone cells that merits further investigation.

Homeodomain transcription factors not only play a major role in the specification of skeletal structures during embryogenesis, but also influence the differentiation and activity of adult skeletal cells, specifically osteoblasts (38–42), which may also be relevant for OP and OA. In fact, the role of undifferentiated precursors in the pathogenesis of skeletal disorders is suggested by studies showing a decreased ability of mesenchymal stem cells from patients with OP to differentiate into bone matrix–synthesizing osteoblasts (32,43). Epigenetic mechanisms, and specifically DNA methylation, may be involved, since demethylating agents and other epigenetic modifiers modulate the differentiation of osteoblast and chondrocyte precursors in vitro (44–46). Consistent with this, although genes directly related to osteoblast and osteoclast activity did not show differentially methylated regions, network analysis revealed that several well-known bone genes (collagen, alkaline phosphatase, and NF-κB, a critical factor in osteoclastogenesis) appeared as "hubs" receiving inputs from genes with differentially methylated regions. This suggests that epigenetic changes in upstream regulatory genes may have downstream influences on bone cell activity.

To our knowledge, this study represents the first attempt to characterize the genome-wide methylation of bone in OP and OA. However, it has some limitations. Although hip fractures are frequently considered to be a single entity, there may be clinical and genetic differences between cervical and trochanteric fractures (47). Only cervical fractures were included in this study. We did not separate different hip OA phenotypes, such as the so-called atrophic and hypertrophic phenotypes, which may also have different pathogenetic mechanisms (26). Most important, DNA was isolated from bone tissue samples, which include a variety of cell types from bone and some contaminating hemopoietic cells. Therefore, the methylation profile represents the contributions of all of them. Of course, not all cells in bone or any other tissue have the same epigenome. In fact, we have previously reported marked differences in promoter methylation of some genes across cells of the osteoblastic lineage (4). However, current technologies do not allow obtaining genome-wide methylation in individual cells.

Unlike the genome, epigenetic markers may be transmitted unmodified through cell divisions or change with the cell environment. Our results represent a picture at a single point in time, and the bone epigenome might change throughout the lifetime of an individual. Of course, exploring the bone epigenome at different time points represents major practical and ethical difficulties. Age-related changes in DNA methylation have been reported, with a general trend toward a decrease in methylation level with aging (48). Although the age range was similar in patients with OP and those with OA, the mean age was somewhat higher in those with OP. However, age did not seem to have an important influence on the results. Within the age range of the patients included in this study, we did not find evidence for statistically significant differences in gene methylation across age strata; very similar results were obtained when age was included as a covariate. Epigenetic studies in humans often raise the question of whether

changes are the cause or the consequence of the disease. Our study is not an exception. We obtained bone samples apart from the fracture site and the subchondral bone, thus trying to minimize the direct influence of fracture or joint degeneration. However, we cannot completely exclude the possibility that changes in methylation are not causally related to disease, but are rather the consequence of prolonged disease processes. Since ethical and practical reasons preclude sampling bone specimens repeatedly, experimental approaches with animal models will be required to further clarify this issue.

The differences in methylation between OP and OA found in this study were <15%. Although small, they still may be biologically relevant. A small change in methylation in a whole bone sample may reflect a much more important change in the methylation level of a particular cell type, which is obscured by other cells present in the tissue. Moreover, we and others have shown that decreasing methylation by only 10–20% markedly induces gene expression in osteoblast cultures (4,5). Transcript analysis of RNA samples allowed us to demonstrate an overall inverse relationship between DNA methylation and gene expression on a genome-wide basis, thus confirming previous findings in samples from other tissues. However, there was an inconsistent relationship between methylation and gene expression at the level of the individual gene. In fact, we found evidence for such a relationship in only 2 of the 5 genes studied. This is not a unique result, and other investigators have reported similar observations (49,50). DNA methylation is only one of the many factors influencing gene expression. Other epigenetic, genetic, and microenvironmental factors may also have major influences in determining gene expression at a given point in time. Therefore, the absence of correlation between methylation and expression at the individual level in a given tissue is not unexpected. As suggested by Deaton et al, who recently found a rather poor correlation between gene expression and methylation in immune cells, differences in DNA methylation may be more important at the early stages of lineage differentiation (50).

In conclusion, genome-wide methylation profiling of bone samples reveals differentially methylated regions in OP and OA. These differentially methylated regions are enriched in genes associated with cell differentiation and osteogenesis, such as those in the homeobox family, suggesting that these disorders involve a developmental component. Further studies are needed to elucidate the mechanisms involved.

ACKNOWLEDGMENT

We thank Dr. Sunil Rao for advice regarding the use of BAMarray software.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Riancho had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Delgado-Calle, Fraga, Riancho.

Acquisition of data. Zarrabeitia, Sañudo, García-Renedo, Pérez-Núñez, García-Ibarbia.

Analysis and interpretation of data. Delgado-Calle, Fernández, Sainz, Fraga, Riancho.

REFERENCES

1. Goldring SR. The role of bone in osteoarthritis pathogenesis. Rheum Dis Clin North Am 2008;34:561–71.
2. Dequeker J, Aerssens J, Luyten FP. Osteoarthritis and osteoporosis: clinical and research evidence of inverse relationship. Aging Clin Exp Res 2003;15:426–39.
3. Chaganti RK, Parimi N, Lang T, Orwoll E, Stefanick ML, Nevitt M, et al. Bone mineral density and prevalent osteoarthritis of the hip in older men for the Osteoporotic Fractures in Men (MrOS) Study Group. Osteoporos Int 2010;21:1307–16.
4. Delgado-Calle J, Sanudo C, Bolado A, Fernandez AF, Arozamena J, Pascual-Carra MA, et al. DNA methylation contributes to the regulation of sclerostin expression in human osteocytes. J Bone Miner Res 2012;27:926–37.
5. Delgado-Calle J, Sanudo C, Fernandez AF, Garcia-Renedo R, Fraga MF, Riancho JA. Role of DNA methylation in the regulation of the RANKL-OPG system in human bone. Epigenetics 2012;7:83–91.
6. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Methods 2008;5:621–8.
7. Hernandez JL, Garcés CM, Sumillera M, Fernandez-Aldasoro EV, Garcia-Ibarbia C, Ortiz-Gomez JA, et al. Aromatase expression in osteoarthritic and osteoporotic bone. Arthritis Rheum 2008; 58:1696–1700.
8. Ishwaran H, Rao JS, Kogalur UB. BAM: Java software for Bayesian analysis of variance for microarray data. BMC Bioinformatics 2006;7:59.
9. Benjamini Y, Yekutieli D. Quantitative trait loci analysis using the false discovery rate. Genetics 2005;171:783–90.
10. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009;4:44–57.
11. Raychaudhuri S, Plenge RM, Rossin EJ, Ng AC, Purcell SM, Sklar P, et al. Identifying relationships among genomic disease regions: predicting genes at pathogenic SNP associations and rare deletions. PLoS Genet 2009;5:e1000534.
12. Panoutsopoulou K, Southam L, Elliott KS, Wrayner N, Zhai G, Beazley C, et al. Insights into the genetic architecture of osteoarthritis from stage 1 of the arcOGEN study. Ann Rheum Dis 2011;70:864–7.
13. Estrada K, Styrkarsdottir U, Evangelou E, Hsu YH, Duncan EL, Ntzani EE, et al. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. Nat Genet 2012;44:491–501.
14. Delgado-Calle J, Sanudo C, Sanchez-Verde L, Garcia-Renedo RJ, Arozamena J, Riancho JA. Epigenetic regulation of alkaline

phosphatase in human cells of the osteoblastic lineage. Bone 2011;49:830–8.
15. Cheung KS, Hashimoto K, Yamada N, Roach HI. Expression of ADAMTS-4 by chondrocytes in the surface zone of human osteoarthritic cartilage is regulated by epigenetic DNA demethylation. Rheumatol Int 2009;29:525–34.
16. Roach HI, Yamada N, Cheung KS, Tilley S, Clarke NM, Oreffo RO, et al. 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 2005;52:3110–24.
17. Roach HI, Aigner T. DNA methylation in osteoarthritic chondrocytes: a new molecular target. Osteoarthritis Cartilage 2007;15: 128–37.
18. Reynard LN, Loughlin J. Genetics and epigenetics of osteoarthritis. Maturitas 2012;71:200–4.
19. Barter MJ, Bui C, Young DA. Epigenetic mechanisms in cartilage and osteoarthritis: DNA methylation, histone modifications and microRNAs. Osteoarthritis Cartilage 2012;20:339–49.
20. He JY, Jiang LS, Dai LY. The roles of the sympathetic nervous system in osteoporotic diseases: a review of experimental and clinical studies. Ageing Res Rev 2011;10:253–63.
21. Geusens P, Lems WF. Osteoimmunology and osteoporosis. Arthritis Res Ther 2011;13:242.
22. Cillo C, Cantile M, Faiella A, Boncinelli E. Homeobox genes in normal and malignant cells. J Cell Physiol 2001;188:161–9.
23. Franceschi RT. The developmental control of osteoblast-specific gene expression: role of specific transcription factors and the extracellular matrix environment. Crit Rev Oral Biol Med 1999; 10:40–57.
24. Aspden RM, Scheven BA, Hutchison JD. Osteoarthritis as a systemic disorder including stromal cell differentiation and lipid metabolism. Lancet 2001;357:1118–20.
25. Holroyd C, Harvey N, Dennison E, Cooper C. Epigenetic influences in the developmental origins of osteoporosis. Osteoporos Int 2011;22:1277–88.
26. Javaid MK, Lane NE, Mackey DC, Lui LY, Arden NK, Beck TJ, et al. Changes in proximal femoral mineral geometry precede the onset of radiographic hip osteoarthritis: the study of osteoporotic fractures. Arthritis Rheum 2009;60:2028–36.
27. Aspden RM. Osteoarthritis: a problem of growth not decay? Rheumatology (Oxford) 2008;47:1452–60.
28. Velasco J, Zarrabeitia MT, Prieto JR, Perez-Castrillon JL, Perez-Aguilar MD, Perez-Nunez MI, et al. Wnt pathway genes in osteoporosis and osteoarthritis: differential expression and genetic association study. Osteoporos Int 2010;21:109–18.
29. Luyten FP, Tylzanowski P, Lories RJ. Wnt signaling and osteoarthritis. Bone 2009;44:522–7.
30. Corr M. Wnt-beta-catenin signaling in the pathogenesis of osteoarthritis. Nat Clin Pract Rheumatol 2008;4:550–6.
31. Baker-Lepain JC, Lynch JA, Parimi N, McCulloch CE, Nevitt MC, Corr M, et al. Variant alleles of the WNT antagonist FRZB are determinants of hip shape and modify the relationship between hip shape and osteoarthritis. Arthritis Rheum 2012;64:1457–65.
32. Jiang LS, Zhang ZM, Jiang SD, Chen WH, Dai LY. Differential bone metabolism between postmenopausal women with osteoarthritis and osteoporosis. J Bone Miner Res 2008;23:475–483.
33. Kanis JA, Borgstrom F, De Laet C, Johansson H, Johnell O, Jonsson B, et al. Assessment of fracture risk. Osteoporos Int 2005;16:581–9.
34. Gomez R, Lago F, Gomez-Reino J, Dieguez C, Gualillo O. Adipokines in the skeleton: influence on cartilage function and joint degenerative diseases. J Mol Endocrinol 2009;43:11–8.
35. Tang ZH, Xiao P, Lei SF, Deng FY, Zhao LJ, Deng HY, et al. A bivariate whole-genome linkage scan suggests several shared genomic regions for obesity and osteoporosis. J Clin Endocrinol Metab 2007;92:2751–7.
36. Howard TD, Ho SM, Zhang L, Chen J, Cui W, Slager R, et al. Epigenetic changes with dietary soy in cynomolgus monkeys. PLoS One 2011;6:e26791.
37. Grethen E, Hill KM, Jones R, Cacucci BM, Gupta CE, Acton A, et al. Serum leptin, parathyroid hormone, 1,25-dihydroxyvitamin D, fibroblast growth factor 23, bone alkaline phosphatase, and sclerostin relationships in obesity. J Clin Endocrinol Metab 2012; 97:1655–62.
38. Gotoh M, Notoya K, Ienaga Y, Kawase M, Makino H. Enhancement of osteogenesis in vitro by a novel osteoblast differentiation-promoting compound, TAK-778, partly through the expression of Msx2. Eur J Pharmacol 2002;451:19–25.
39. Cheng SL, Shao JS, Cai J, Sierra OL, Towler DA. Msx2 exerts bone anabolism via canonical Wnt signaling. J Biol Chem 2008; 283:20505–22.
40. Sierra OL, Towler DA. Runx2 trans-activation mediated by the MSX2-interacting nuclear target requires homeodomain interacting protein kinase-3. Mol Endocrinol 2010;24:1478–97.
41. Sierra OL, Cheng SL, Loewy AP, Charlton-Kachigian N, Towler DA. MINT, the Msx2 interacting nuclear matrix target, enhances Runx2-dependent activation of the osteocalcin fibroblast growth factor response element. J Biol Chem 2004;279:32913–23.
42. Gordon JA, Hassan MQ, Koss M, Montecino M, Selleri L, van Wijnen AJ, et al. Epigenetic regulation of early osteogenesis and mineralized tissue formation by a HOXA10-PBX1-associated complex. Cells Tissues Organs 2011;194:146–50.
43. Jin WJ, Jiang SD, Jiang LS, Dai LY. Differential responsiveness to 17β-estradiol of mesenchymal stem cells from postmenopausal women between osteoporosis and osteoarthritis. Osteoporos Int 2012;23:2469–78.
44. El Serafi AT, Oreffo RO, Roach HI. Epigenetic modifiers influence lineage commitment of human bone marrow stromal cells: Differential effects of 5-aza-deoxycytidine and trichostatin A. Differentiation 2011;81:35–41.
45. Vaes BL, Lute C, van der Woning SP, Piek E, Vermeer J, Blom HJ, et al. Inhibition of methylation decreases osteoblast differentiation via a non-DNA-dependent methylation mechanism. Bone 2010;46:514–23.
46. Dansranjavin T, Krehl S, Mueller T, Mueller LP, Schmoll HJ, Dammann RH. The role of promoter CpG methylation in the epigenetic control of stem cell related genes during differentiation. Cell Cycle 2009;8:916–24.
47. Riancho J, Garcia-Ibarbia C, Perez-Nunez MI, Alonso MA, Diaz T, Perez-Castrillon JL, et al. Genetic polymorphisms of the Wnt receptor LRP5 are differentially associated with trochanteric and cervical hip fractures. Calcif Tissue Int 2012;90:137–43.
48. Fraga MF, Esteller M. Epigenetics and aging: the targets and the marks. Trends Genet 2007;23:413–8.
49. Kaneko KJ, Rein T, Guo ZS, Latham K, DePamphilis ML. DNA methylation may restrict but does not determine differential gene expression at the Sgy/Tead2 locus during mouse development. Mol Cell Biol 2004;24:1968–82.
50. Deaton AM, Webb S, Kerr AR, Illingworth RS, Guy J, Andrews R, et al. Cell type-specific DNA methylation at intragenic CpG islands in the immune system. Genome Res 2011;21:1074–86.