Q2 Contribution of genetic and epigenetic mechanisms to Wnt pathway activity in prevalent skeletal disorders

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ABSTRACT

We reported previously that the expression of Wnt-related genes is lower in osteoporotic hip fractures than in osteoarthritis. We aimed to confirm those results by analyzing β-catenin levels and explored potential genetic and epigenetic mechanisms involved.

β-Catenin gene expression and nuclear levels were analyzed by real time PCR and confocal immunofluorescence. Increased nuclear β-catenin was found in osteoblasts isolated from patients with osteoarthritis (99 ± 4 units vs. 76 ± 12, $p = 0.01$, $n = 10$), without differences in gene transcription, which is consistent with a post-translational down-regulation of β-catenin and decreased Wnt pathway activity.

Twenty four single nucleotide polymorphisms (SNPs) of genes showing differential expression between fractures and osteoarthritis (*WNT4*, *WNT10A*, *WNT16* and *SFRP1*) were analyzed in DNA isolated from blood of 853 patients. The genotypic frequencies were similar in both groups of patients, with no significant differences.

Methylation of Wnt pathway genes was analyzed in bone tissue samples (15 with fractures and 15 with osteoarthritis) by interrogating a CpG-based methylation array. Six genes showed significant methylation differences between both groups of patients: *FZD10*, *TBL1X*, *CSNK1E*, *WNT8A*, *CSNK1A1L* and *SFRP4*. The DNA demethylating agent 5-deoxycytidine up-regulated 8 genes, including *FZD10*, in an osteoblast-like cell line, whereas it down-regulated other 16 genes.

In conclusion, Wnt activity is reduced in patients with hip fractures, in comparison with those with osteoarthritis. It does not appear to be related to differences in the allele frequencies of the Wnt genes studied. On the other hand, methylation differences between both groups could contribute to explain the differences in Wnt activity.

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1. Introduction

The Wnt pathway has emerged as an important regulator of skeletal homeostasis. Binding of Wnt ligands to their receptors triggers the activation of a complex signaling pathway. Multiple intracellular mediators are involved, but the best known cascade of Wnt signals constitutes the so-called canonical pathway, which involves the post-transcriptional regulation of β-catenin levels. Wnt ligands induce the disassembly of the GSK3 complex that phosphorylates β-catenin. Since non-phosphorylated β-catenin is less prone to proteasome degradation, this results in increased β-catenin levels and translocation into the nucleus, where it modulates the transcription of target genes (Gaur et al., 2005; Williams and Insogna, 2009), with the collaboration of several co-factors, including members of the T-cell factor/lymphoid enhancing factor family (Gordon and Nusse, 2006; MacDonald et al., 2009). There are 19 different Wnt ligands, some of which, such as

Wnt3a, preferentially activate the canonical pathway, whereas others, such as Wnt4 and Wnt5a, are usually regarded to transmit signals through non-canonical pathways. However, there is no a clear difference between ligands, each one being able to activate preferentially the canonical or non-canonical pathways depending on the target cell and other context-dependent factors (van Amerongen and Nusse, 2009). Furthermore, cross-talks take place between different pathways. Both the canonical and the non-canonical pathways appear to be involved in the regulation of bone homeostasis (Chang et al., 2007; Gaur et al., 2005; Piters et al., 2008). Wnt inhibitors include members of the secreted frizzled related protein family, which are structurally related to the Wnt membrane-bound receptors frizzled and bind directly to Wnt ligands, thereby altering their ability to interact with the Wnt receptor complex at cell membranes (Kawano and Kypta, 2003). One member of this family, sFRP1, has been shown to modulate the activity of cells of the osteoblastic lineage (Bodine et al., 2005; Yao et al., 2010). In line with this, knock-out mice with deletion of the *SFRP1* or other genes encoding secreted frizzled proteins show increased bone mass (Lodewyckx and Lories, 2009).

An association between Wnt-related genes, particularly FRZB, with osteoarthritis was reported in several studies (Loughlin et al., 2004; Valdes et al., 2007), but it could not be confirmed in recent GWAS (Arcogen Consortium, 2012; Panoutsopoulou et al., 2011). On the other hand, variations in genes related to the Wnt pathway, such as the Wnt co-receptors LRP5 and the Wnt inhibitor sclerostin, have been related to osteoporosis in genetic association studies (Ralston, 2010; Riancho et al., 2011; Richards et al., 2009; Styrkarsdottir et al., 2009; Valero et al., 2011). Specifically, Estrada et al. found an association between some polymorphisms of genes encoding Wnt ligands, such as WNT16, and osteoporosis in a large multinational GWAS (Estrada et al., 2012).

Epigenetic mechanisms, and specifically the methylation of CpG sites in gene promoters, are known to play an important role in gene expression regulation during development and in adult organisms (Fraga and Esteller, 2007). The role of DNA methylation in bone homeostasis has not been extensively studied yet, but several lines of evidence point it as a critical regulator of the differentiation of bone cells (Delgado-Calle et al., 2012a,c).

Osteoporosis and osteoarthritis are prevalent skeletal disorders. Whereas bone mass is decreased in osteoporosis, several epidemiological studies suggested that patients with osteoarthritis may show not only periarticular bone formation, but also a generalized trend for higher bone mass (Arokoski et al., 2002; Chaganti et al., 2010; Dequeker et al., 2003). We have previously reported that, in comparison with patients with osteoarthritis, the expression of a number of genes in the Wnt pathway is reduced in bone samples and osteoblast cultures from patients with osteoporotic hip fractures. In line with this, experiments with a reporter vector suggested higher Wnt activity is osteoarthritis, suggesting that differences in Wnt activity may be involved in the opposite changes in bone mass typical of these disorders (Velasco et al., 2010). In the present study we compared nuclear β-catenin levels in primary osteoblasts from patients with hip fractures and hip osteoarthritis, determined β-catenin gene expression, and explored if the differences in Wnt activity were related to genotypic or epigenetic differences between both groups of patients.

2. Materials and methods

2.1. Patients

The study subjects included patients with osteoporotic hip fractures or with severe hip osteoarthritis requiring replacement surgery. Samples from different patients were used for the various experiments. Patients with secondary osteoarthritis or secondary osteoporosis, those taking drugs known to affect bone metabolism, as well as those having fractures related to high-energy trauma, were excluded.

Patients gave informed consent. The study was approved by the institutional review board (Comité de Etica en Investigación Clínica de Cantabria).

2.2. Osteoblast cultures

Bone samples were obtained during hip replacement surgery, in patients with hip fractures (n = 11) or with hip osteoarthritis (n = 9) (mean age 82 ± 5 and 75 ± 7 yr, respectively). Trabecular bone cylinders of the central part of the femoral head (thus avoiding the fractured and the subchondral regions) were obtained with a trephine, cut in small samples, washed extensively in phosphate-buffered saline and used to set up osteoblast cultures by the primary explant technique (Jonsson et al., 1999). 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 appropriate experimental conditions.

2.3. Immunofluorescence microscopy

Primary osteoblasts obtained from 5 patients with fractures and 4 with osteoarthritis were grown on microscope glass coverslips until 70–80% confluence. Then they were fixed with 3.7% paraformaldehyde-PBS and permeabilized with 0.5% Triton 100-X for 30 min at room temperature. After several washes with PBS and 0.05% PBS-Tween, the coverslips were incubated overnight at 4 °C with a rabbit polyclonal anti β-catenin antibody (Abcam, Cambridge, UK), diluted 1/200 in PBS. After washing, cells were incubated with a FITC-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 45 min and mounted with VectaShield (Vector Laboratories, Burlingame, CA, USA). In some experiments, β-catenin immunolabeling was combined with Texas Red-Phalloidin to stain actin filaments.

Confocal microscopy was performed with a laser scanning microscope (LSM 510; Carl Zeiss, Oberkochen, Germany) by using excitation wavelengths of 488 nm (for FITC) and 543 nm (for Texas Red). Each channel was recorded independently, and pseudocolor images were generated and superimposed. TIFF images were transferred to Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA, USA) for presentation.

To measure fluorescence intensities of nuclear and cytoplasmic β-catenin in primary osteoblast cultures from patients with fractures and osteoarthritis (blindly to the origin of the sample), confocal images of at least 130 osteoblasts (without prior selection) of each patient group were captured by using a 63× oil 1.4 (NA) objective. Images were acquired with no saturated pixels, always using the same confocal settings, with eightfold averaging at resolution of 1024 × 1024 pixels and using a pinhole setting of 1. In order to minimize between-day variability bias, in each experiment cells of both patient groups were included. Images were background corrected by reference regions outside the cells and fluorescence intensities were estimated by using the ImageJ software (NIH, Bethesda, Maryland, USA; http://rsb.info.nih.gov/ij/). Fluorescence intensities were measured in four regions of interest of the same area per nucleus, excluding the nucleolus, and in four regions per cytoplasm. Nuclear and cytoplasmic β-catenin average values and the nucleocytoplasmic ratios were computed for each cell analyzed and pooled for each patient. The mean values of each patient group were then compared by two-tailed unpaired t-test.

2.4. β-Catenin expression

Sub-confluent cultures of first-passage primary osteoblasts were used to analyze gene expression by reverse transcription (RT) real-

time PCR. The medium was aspirated and fresh medium with 0.1% bovine serum albumin was added. Forty eight hours later, cells were rinsed with phosphate-buffered saline and the RNA was extracted with Trizol, following manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The purity and integrity of RNA were checked by absorbance and gel electrophoresis.

Aliquots of RNA were reverse-transcribed with the Superscript III kit (Invitrogen), using random hexamers as primers. After RT, the expression of β-catenin was determined by real-time PCR using gene-specific primers and Taqman probes (Applied Biosystems, Foster City, CA, USA) in an ABI7300 apparatus (Applied Biosystems). The amount of PCR product was monitored by fluorescence and the threshold cycle (Ct) for each well was determined. The results were normalized to the expression of the housekeeping gene TATA box protein (TBP) and the specific gene expression was calculated as the $2^{-\Delta Ct}$, where ΔCt is the difference between the gene of interest threshold cycle and the housekeeping threshold cycle.

2.5. Genetic analysis

The genotype analysis included 353 patients with severe hip osteoarthritis requiring replacement surgery (182 men and 171 women, age 69 ± 11 yr) and 500 with hip fractures (92 men and 408 women, age 79 ± 12 yr). DNA was isolated from blood or buccal swabs using a commercial kit (Qiagen, Hilden, Germany) and quantified with Qubit technique (Invitrogen). Several candidate genes of the Wnt pathway were selected on the basis that they showed differential expression in hip osteoarthritis and fractures (Velasco et al., 2010). The gene set included 3 Wnt ligands (WNT4, WNT10A and WNT16) and 1 gene encoding a soluble Wnt-binding protein of the frizzled family (SFRP4). We explored the Hapmap database searching SNPs in those genes with a minimum allelic frequency (MAF) of 10% in the Caucasian population. Tagging SNPs capturing the most common variants of these genes were then selected using the method of Gabriel, implemented in Haploview (Barrett et al., 2005). In addition, we included potentially functional SNPs identified with the PupaSuite web tool (Conde et al., 2006). The SNP set was genotyped using a Sequenom platform, at the Centro Nacional de Genotipado in Santiago de Compostela, Spain. Replicate samples were included for quality control.

2.6. DNA methylation profiling

Bone samples were obtained from femoral heads removed during hip replacement as previously reported (Hernandez et al., 2008). The study population included women aged 61–85 years; with osteoarthritis (age 73 ± 7 yr; n = 15) or with osteoporotic hip fractures (81 ± 3 yr; n = 15). DNA was isolated from bone samples using phenol:chloroform:isoamylalcohol, as previously described (Delgado-Calle et al., 2012c). After bisulphite conversion of DNA using the EZ DNA Methylation kit (Zymo Research, Orange, CA, USA), microarray-based DNA methylation profiling was performed with the Human Methylation Infinium 27 k DNA Analysis BeadChip (Illumina, San Diego, CA, USA), following manufacturer's instructions. This array targets CpG sites located within the proximal promoter regions of transcription start sites of 14,475 consensus coding sequencing (CCDS) in the NCBI Database (Genome Build 36) and 110 miRNA promoters. The assay interrogates the loci using two site-specific probes, one designed for the methylated locus (M bead type) and another for the unmethylated locus (U bead type). The methylation level for the interrogated locus is determined by calculating the ratio of the fluorescent signals from the methylated vs. unmethylated sites as expressed as beta-values, a quantitative measure of DNA methylation levels of specific CpG that ranges from 0 for completely unmethylated to 1 for completely methylated. The analysis included 257 CpG sites present in genes related to the Wnt pathway.

2.7. Gene demethylation and expression

To explore the effects of DNA demethylation on gene expression we treated the osteoblast-like cell line MG-63 with 5-aza-2-deoxy-azacytidine (AzadC) for 4 days. Three independent cultures (each including control and 1 μM AzadC-treated cells) were used for these experiments. Gene expression profiling was performed at the Gene Expression Unit of Genomics Core Facility (SGIKer) of the University of the Basque Country UPV/EHU (Leioa, Spain) using oligonucleotide-based Agilent Whole Human Genome Oligo Microarrays 4×44K G4112F (design ID 014850). Two-color microarray-based gene expression analysis was performed following the Quick Amp Labeling protocol from Agilent Technologies (G4140-90050 v5.7; Agilent Technologies España, Las Rozas, Spain). In brief, Cyanine-3 (Cy3) labeled cRNA was prepared from 500 ng of a pool of control RNA samples, and Cyanine-5 (Cy5) labeled cRNA was prepared from 300 ng of individual AzadC-treated samples. Dye incorporation and cRNA yield were monitored with the NanoDrop ND-1000 Spectrophotometer. Aliquots (825 ng) of each Cy3 and Cy5 labeled cRNA were fragmented, and co-hybridized to microarrays and washed following manufacturer's recommendations. Slides were scanned using an Agilent DNA Microarray Scanner G2565BA and the resulting TIFF images were processed with Agilent Feature Extraction Software v9.5.3. Default parameters for two-color gene expression microarrays were used for image analysis, data extraction, background correction and dye bias correction. Non-uniform outliers or signals not significantly above background intensity in 70% or more of each channel (Cy3 or Cy5) were filtered out.

2.8. Statistical analyses

The statistical significance of the differences in β-catenin levels between samples from patients with hip fractures and osteoarthritis was tested by the Mann–Whitney tests. Genotype and allele frequencies of SNPs were tested for consistency with Hardy–Weinberg equilibrium (HWE) proportions using Plink software (Purcell et al., 2007). The allelic frequency distributions in both groups of patients were compared assuming additive models with Plink, in the whole population and in the male and females subgroups. Power analysis of the genetic analyses was done assuming a log-additive genetic model with Quanto software (available at http://hydra.usc.edu/gxe/).

For methylation, the ratio methylated/unmethylated was estimated as the beta/(1-beta) ratio and log2-transformed. Data were then normalized by the quintile procedure with BRB Array software developed by Dr. Richard Simon and the BRB-ArrayTools Development Team (http://linus.nci.nih.gov/BRB-ArrayTools.html) and between group differences in the methylation of genes related to the Wnt pathway in KEGG (Kyoto Encyclopedia of Genes and Genomes) were analyzed by t-tests. Significance levels were corrected for multiple testing by the method of Benjamini to control the FDR (Benjamini and Yekutieli, 2005).

To assess the AzadC-induced changes in gene expression, we considered as up-regulated those genes consistently increased in the three experiments, with an average fold-change >2; similarly, consistently decreased genes with a fold-change <−2 were regarded as down-regulated.

3. Results

3.1. β-Catenin levels and gene expression

In addition to a weak immunostaining throughout the cytoplasm, β-catenin signal was concentrated at the cell cortex, particularly beneath membrane domains involved in cell–cell interactions. In this localization, β-catenin frequently colocalized with the tip of actin filaments (Fig. 1). Staining was also found at the nuclei of cells of both
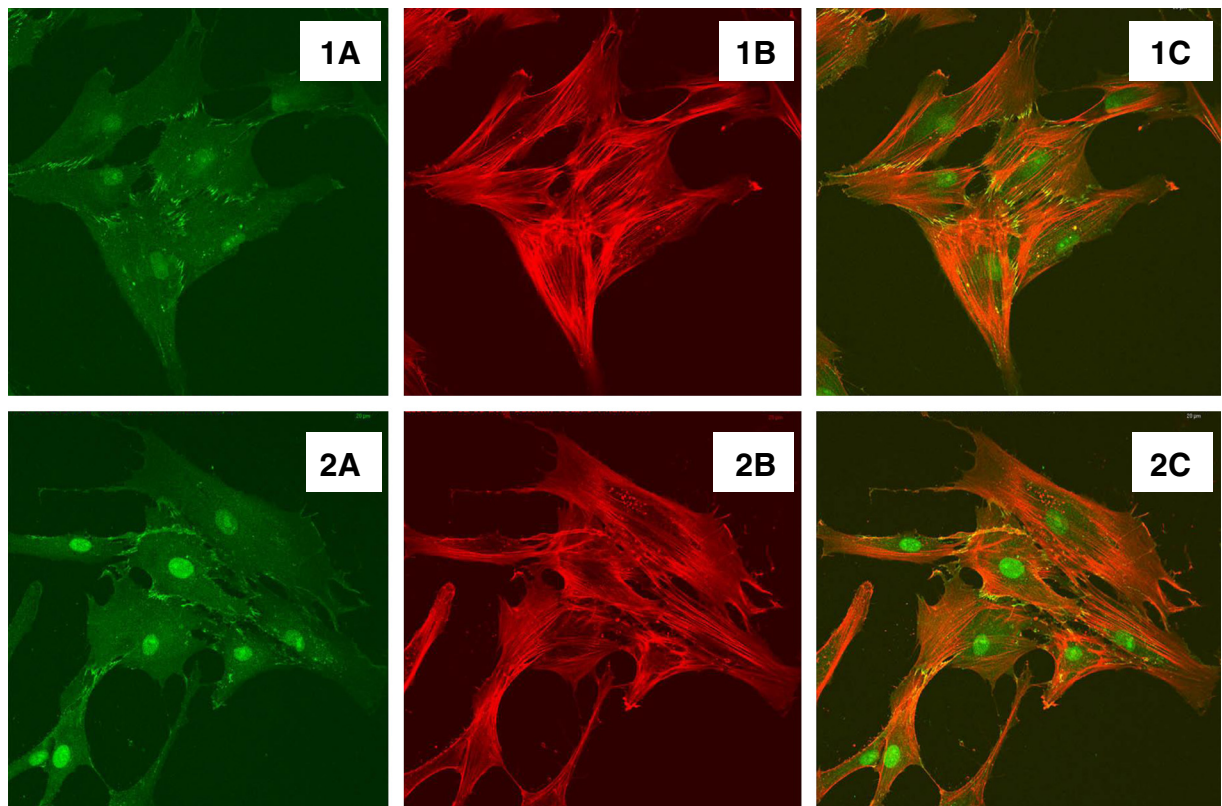
Fig. 1. Immunofluorescence of primary osteoblasts grown from fracture (1) or osteoarthritis samples (2). A, β-Catenin staining; B, phalloidin staining of F-actin; C, merged images.

groups of patients, but the nuclear fluorescence intensity was significantly lower in cultures grown from patients with hip fractures than in patients with osteoarthritis (nucleocytoplasmatic ratios of 2.39 ± 0.38 versus 3.13 ± 0.38, respectively, p = 0.023). To analyze if β-catenin was regulated at transcriptional or post-transcriptional levels, we measured its expression in cell cultures. The abundance of β-catenin mRNA was similar in both groups of patients (Fig. 2).

3.2. Association between gene polymorphisms and disease

The SNPs analyzed and their chromosomal locations are shown in Supplementary Table S1. The genotype distribution did not differ between both groups of individuals (Table 1), neither in the combined analysis nor in the sex-stratified analysis. Similar results were obtained when age was included in the analysis as a covariate.

3.3. DNA methylation and gene expression

The analyzed CpG sites and their statistical significance are listed in Table S2 (Supplementary Online Material). Statistical analysis revealed 6 CpG sites (out of the 257 related to Wnt pathway genes) with differential methylation in samples from patients with fractures and osteoarthritis (FDR < 0.1). They included *FZD10*, *CSNK1E*, *TBL1X*, *WNT8A*, and *SFRP4* genes. P-values and the differences in methylation are shown in Table 2. The heatmap of the probe methylation, shown in Fig. 3, revealed important heterogeneity among patients with fractures, who tended to group into two different classes. Within the age range studied, no gene showed significant age-related differences in methylation.

To explore the potential relationship between DNA methylation and the expression of Wnt-related genes, we treated osteoblast-like MG-63 cells with AzadC, which promotes a global decrease in

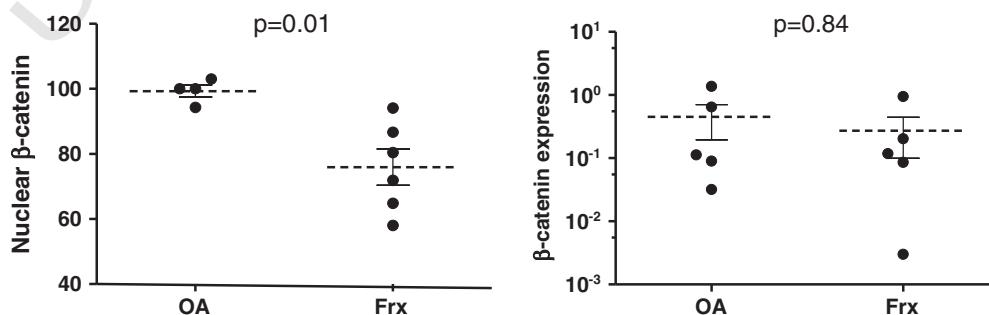**Fig. 2.** β-Catenin nucleocytoplasmatic staining fluorescence intensity (left panel) and expression levels assessed by quantitative real-time PCR (right panel) in primary osteoblast cultures from patients with fractures (Frx) or osteoarthritis (OA). Each point represents the result of an individual patient.

Table 1

Allele and genotype frequencies of patients with hip fractures and hip osteoarthritis (homozygotes for the minor allele/heterozygotes/homozygotes for the major allele), and p-values for the differences between both groups of patients.

SNP	GENE	Fracture (n = 500)		Osteoarthritis (n = 353)		P (all)	P (women)	P (men)
		MAF	Genotypes	MAF	Genotypes			
rs7526484	WNT4	0.23	26/160/284	0.24	18/123/193	0.56	0.46	0.98
rs2235526	WNT4	0.18	17/130/303	0.18	13/93/217	0.92	0.39	0.65
rs10917158	WNT4	0.18	17/133/321	0.17	12/90/234	0.70	0.91	0.51
rs3806557	WNT10A	0.23	30/159/286	0.20	16/108/222	0.18	0.27	0.83
rs10177996	WNT10A	0.21	27/136/280	0.19	8/103/208	0.19	0.15	0.77
rs2385199	WNT10A	0.20	24/149/309	0.17	8/104/231	0.14	0.16	0.89
rs3779381	WNT16	0.26	34/174/258	0.26	22/133/183	0.92	0.88	0.33
rs2908004	WNT16	0.44	87/252/144	0.42	62/168/115	0.46	0.76	0.94
rs2707471	WNT16	0.16	13/122/338	0.15	7/87/247	0.65	0.90	0.91
rs3801385	WNT16	0.07	3/58/412	0.07	4/40/297	0.83	0.65	0.94
rs2707466	WNT16	0.44	87/241/142	0.42	63/153/117	0.37	0.53	0.92
rs17143305	WNT16	0.15	12/120/335	0.14	7/82/244	0.58	0.89	0.90
rs3242	SFRP1	0.38	77/192/183	0.36	46/153/138	0.45	0.88	0.86
rs1127379	SFRP1	0.44	104/229/159	0.44	64/175/106	0.84	0.66	0.95
rs7820647	SFRP1	0.39	76/212/173	0.41	53/163/113	0.58	0.31	0.73
rs11786592	SFRP1	0.35	59/211/206	0.36	44/145/139	0.70	0.54	0.83
rs6651363	SFRP1	0.40	77/225/177	0.42	61/161/114	0.31	0.58	0.33
rs10109536	SFRP1	0.41	83/233/176	0.42	66/159/124	0.64	0.42	0.98
rs17652488	SFRP1	0.51	127/189/121	0.51	94/130/86	0.83	0.62	0.84
rs10958671	SFRP1	0.13	13/98/377	0.13	6/75/259	0.96	0.50	0.86
rs17574424	SFRP1	0.19	22/145/321	0.16	6/99/240	0.09	0.15	0.27
rs7832767	SFRP1	0.05	2/45/425	0.05	1/34/306	0.94	0.63	0.80
rs968427	SFRP1	0.39	77/222/188	0.38	49/165/134	0.74	0.83	0.27
rs921142	SFRP1	0.32	53/205/230	0.31	34/142/168	0.57	0.70	0.98

DNA methylation. In AzadC-treated cells, 8 genes were up-regulated, whereas 16 were down-regulated (Table 3).

4. Discussion

In this study we found increased nuclear β-catenin in osteoblasts from patients with osteoarthritis in comparison with those grown from patients with osteoporotic fractures. β-Catenin participates in cadherin signaling by binding to the cytoplasmic domain of type I cadherins and linking them to the actin cytoskeleton (Mbalaviele et al., 2006; Nelson and Nusse, 2004). On the other hand, it is a major player in the canonical Wnt pathway. The Wnt pathway regulates the differentiation and activity of bone cells, and particularly of the bone-forming cells of the osteoblastic lineage (Williams and Insogna, 2009). On the other hand, Wnt activity has been reported to influence cartilage metabolism and may be involved in the pathogenesis of osteoarthritis (Corr, 2008; Diarra et al., 2007; Lodewyckx and Lories, 2009; Luyten et al., 2009). Although osteoarthritis has been classically understood as a cartilage disorder, important bone changes take place in the vicinity of the joints with osteoarthritis. In fact, several lines of evidence suggest that bone may play more than a merely passive role in the pathogenesis of osteoarthritis (Castaneda et al., 2012). Wnt pathway genes are likely involved in both the bone and cartilage alterations that eventually result in the development of osteoarthritis (Corr, 2008; Kawaguchi, 2009; Lodewyckx and Lories, 2009; Luyten et al., 2009).

We have previously shown a reduced expression of several genes of the Wnt pathway in bone tissue samples and osteoblast cultures from patients with hip fractures, in comparison with samples from patients with osteoarthritis (Velasco et al., 2010). Therefore, it could be speculated that the higher Wnt pathway activity may be involved in the increased bone formation taking place in osteoarthritic joints (causing osteophytes and subchondral bone sclerosis) and perhaps in the differences in bone mass between osteoarthritis and osteoporosis. In the present study we confirmed that nuclear β-catenin is more abundant in primary osteoblast cultures grown from osteoarthritis samples than in samples from patients suffering a hip fracture. This was not accompanied by changes in β-catenin gene transcription, which is consistent with regulation at the posttranscriptional level. Although we did not confirm increased levels of β-catenin by western-blotting, these results are in line with studies using gene expression and reporter vectors that showed higher Wnt activity in osteoblasts from patients with osteoarthritis than in those obtained from fracture cases (Velasco et al., 2010).

Genetic association studies have found Wnt pathway genes, and specifically Wnt ligands such as WNT16, to be associated with bone mineral density and wrist fractures (Estrada et al., 2012; Medina-Gomez et al., 2012; Zheng et al., 2012). Therefore, we hypothesized that the differences in Wnt/β-catenin activity could be related to genetic or epigenetic variants. However, we did not find evidence for genetic differences between both groups of patients regarding three Wnt ligands (WNT4, WNT10A and WNT16) or a Wnt inhibitor (SFRP1). These negative results of the genetic association analysis should be interpreted in the context of the limitations inherent to our study. Most important, the aim of our study was to explore whether genetic differences contributed to explain the differences in Wnt activity between osteoporotic hip

Table 2

Wnt pathway genes showing differential methylation of CpG sites. Mean beta-values (a quantitative measure of DNA methylation levels that ranges from 0 for completely unmethylated to 1 for completely methylated) of samples from patients with osteoarthritis and osteoporotic fractures. The chromosomal location and the distance of the interrogated nucleotide to the transcription start site (TSS) are shown. FDR, false discovery rate.

Gene	CpG location	Distance to TSS	Osteoarthritis (beta)	Fractures (beta)	P-value	FDR
FZD10	47160656	223	0.14	0.08	3.7×10^{-5}	0.0094
CSNK1E	37044362	327	0.29	0.21	0.00036	0.0385
TBL1X	9393597	549	0.19	0.10	0.00045	0.0385
WNT8A	137447943	365	0.54	0.61	0.00079	0.0428
CSNK1A1L	36577573	230	0.64	0.70	0.00083	0.0428
SFRP4	37922543	359	0.15	0.08	0.00176	0.0755
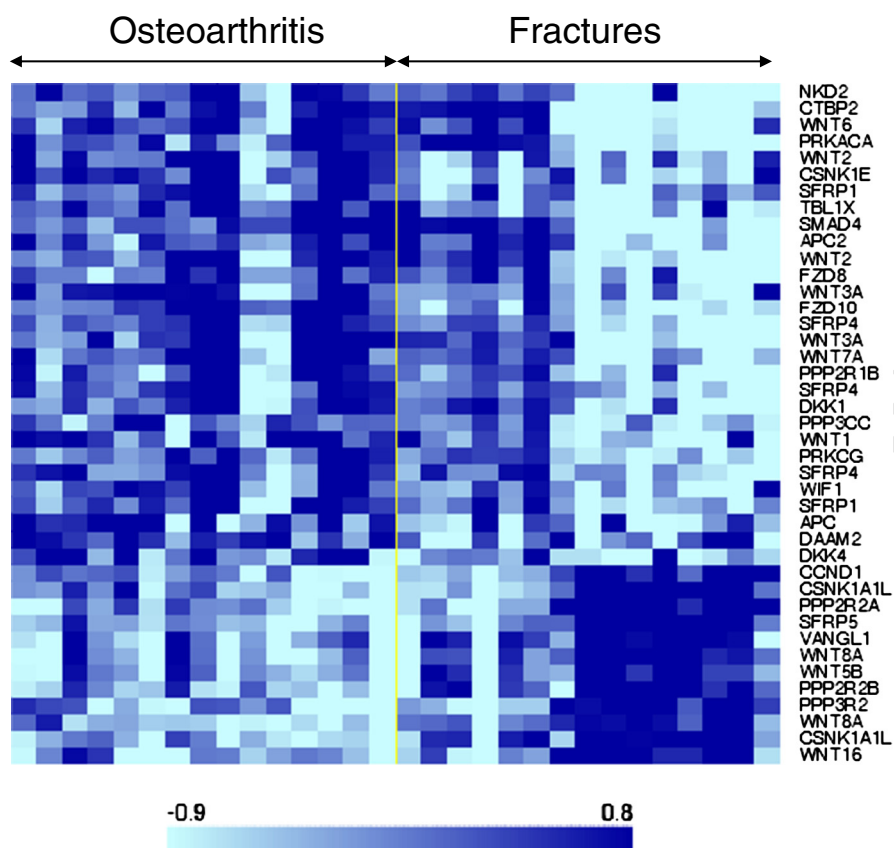
Fig. 3. Heat map representation of the methylation of Wnt-pathway (scaled and centered values). Darker color represents higher methylation. Genes with CpG sites showing a trend for differential methylation between osteoporosis and osteoarthritis (nominal p-values <0.05) are shown.

fractures and hip osteoarthritis rather than discovery of genes associated with these conditions (reason why comparisons with a healthy control group were not done). This was a moderate-sized study, with limited statistical power. With a type I error of 5%, our study had more than 80% power to detect disease-associated polymorphisms with odds ratios of 1.4 and 1.6, when the minor allele frequencies are >0.3 or >0.1, respectively. However, it was underpowered to detect SNPs with smaller odds ratios. For instance, power to detect alleles with 0.3 frequency and odds ratios in the range of 1.1–1.2 would be only 15–41%. In our study sex and age were different in the groups of fractures and osteoarthritis, which reflects the epidemiological differences between these skeletal disorders. A lack of association between the

genotypes and the phenotypes persisted when results were adjusted by age and sex, but the statistical power further decreased under those analysis conditions. We selected genes on the basis of their differential expression, but we cannot exclude the existence of differences in the allelic frequency distributions of other genes in the Wnt pathway. In fact, some investigators reported an association of osteoarthritis with certain polymorphisms of the *FRZB* gene, which encodes secreted frizzled related protein 3, another Wnt inhibitor (Loughlin et al., 2004). However, this has not been replicated in other reports, including some recent genome-wide studies (Panoutsopoulou et al., 2011).

Since genetic differences did not explain the differences in Wnt activity, alternative mechanisms not related to DNA sequence might be involved Thus, we hypothesized that epigenetic marks, and specifically cytosine methylation, might underlie the differences in Wnt activity between osteoporosis and osteoarthritis. The methylation of cytosines of CpG dinucleotides is maintained through cell divisions by DNA methyltransferases. Methylation of CpG-rich sequences of the promoter regions tends to inhibit the transcription of genes known to play important roles in bone formation and bone resorption. On the other hand, the demethylation of those CpG-rich regions is associated with the activation of gene expression (Delgado-Calle et al., 2011, 2012b, 2012c). Little is known about the potential role of CpG methylation in the pathogenesis of bone changes in osteoarthritis and other skeletal disorders. However, promoter methylation has been demonstrated to modulate Wnt pathway activity in other normal and neoplastic tissues (Ekstrom et al., 2011; Kocemba et al., 2012). In line with this, in the present study we identified several Wnt-related genes differentially methylated in osteoporosis and osteoarthritis. WNT8A is a Wnt ligand that may be modulated by estrogen and has been associated with alterations of bone development, such as cleft palate (Chiquet et al., 2008). Proteins of the frizzled family, including FZD10 (frizzled family receptor 10), may act as Wnt co-receptors at the cell membranes. On the other

Table 3
Wnt pathway genes up-regulated and down-regulated by AzadC in MG-63 cells (mean ± of three experiments).

Gene	Fold-increase	Gene	Fold-decrease
RAC2	18.1 ± 3.1	*SFRP1*	7.6 ± 0.9
FZD10	12.1 ± 4.4	*FZD1*	6.2 ± 0.3
WNT11	6.2 ± 1.6	*WNT5B*	5.5 ± 1.2
PLCB2	5.0 ± 1.0	*CTNNBIP1*	4.2 ± 0.7
FZD4	4.9 ± 0.6	*CTNNB1*	4.1 ± 0.8
WNT6	3.7 ± 0.1	*DKK2*	4.0 ± 0.4
MYC	3.2 ± 0.1	*CSNK2A1*	3.2 ± 0.2
NLK	2.5 ± 0.1	*GSK3B*	3.0 ± 0.2
		CAMK2G	2.9 ± 0.2
		PPP2R1A	2.8 ± 0.4
		PLCB3	2.7 ± 0.3
		CTBP1	2.7 ± 0.2
		CACYBP	2.3 ± 0.1
		RHOA	2.3 ± 0.1
		CSNK1A1	2.1 ± 0.1
		FOSL1	2.1 ± 0.1

hand, soluble frizzled-related proteins, including SFRP4 (soluble frizzled related protein 4), are secreted and may bind Wnt ligands, thus preventing their interaction with cell membrane receptors (Kawano and Kypta, 2003; Nakanishi et al., 2007; Wang et al., 2006). *TBL1X* (transducin (beta)-like 1X-linked) encodes a regulatory protein that appears to contribute to the regulation of Wnt target genes (Li and Wang, 2008). Casein kinases, including CSNK1E (casein kinase 1, epsilon), participate in the regulation of a variety of cell functions, and contribute to the signaling cascade initiated by the interaction of Wnt ligands with their receptors (Valle-Perez et al., 2011). The biological role of *CSNK1A1L* (casein kinase 1, alpha 1-like) gene is unknown.

In theory, those methylation differences could influence Wnt pathway activity, but further studies are needed to confirm this hypothesis, including detailed analysis of methylation at different nucleotides by other procedures such as pyrosequencing. The methylation signatures of Wnt-related genes revealed some rather different patterns, suggesting that the osteoarthritis and fracture groups may be heterogeneous and include patients with somewhat different pathogenetic mechanisms, at least regarding the gene methylation pattern. This is in line with the views of other investigators that used a clinico-epidemiological approach (Herrero-Beaumont et al., 2009). In general, DNA methylation tends to inhibit gene expression, but this is not a universal phenomenon (Hantusch et al., 2007). In fact, we found that AzadC upregulated some Wnt-related genes, but downregulated others. We have previously shown that several genes respond similarly to AZadC treatment in primary osteoblasts and in osteoblastic cell lines (Delgado-Calle et al., 2011, 2012b). Thus, although in the present study we used an osteoblastic cell line to assess the response to AzadC, the results are likely similar in nontransformed osteoblasts. Thus, these experiments support the concept that DNA methylation-dependent mechanisms influence the expression of Wnt pathway genes. However, they do not allow establishing to what extent those changes are the direct consequence of the demethylation of the promoters of those genes, or the result of changes in other regulatory genes upstream in the pathway. For instance, the expression of *FZD10* was increased by AzadC, even though its promoter is largely unmethylated in bone samples, with beta-values between 0.08 and 0.14 (see Table 2), and it is even less methylated in cultured osteoblastic cells (unpublished results). Thus, the stimulatory effect of AzadC was likely due to its effect on another regulatory molecule which in turn stimulated FZD10 transcription.

5. Conclusion

In conclusion, nuclear β-catenin levels are higher in osteoblasts form hip osteoarthritis than in osteoblasts from hip fractures. This is in line with previous reports showing higher Wnt pathway activity in osteoarthritis and may be related to the opposite changes in bone mass and bone formation typical of these disorders. However, the difference in Wnt activity is not explained by the allele distribution of common polymorphisms of various Wnt-related genes. On the other hand, despite some heterogeneous patterns, several genes in the Wnt pathway presented differences in methylation. Further studies are needed to elucidate to what extent those epigenetic differences are involved in the differences in Wnt activity.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2013.09.080.

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