RESEARCH PAPER

Epigenetics 7:1, 83-91; January 2012; © 2012 Landes Bioscience

Role of DNA methylation in the regulation of the RANKL-OPG system in human bone

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Key words: DNA methylation, osteoporosis, bone remodeling, osteoblasts

Osteoblasts are specialized cells that form new bone and also indirectly influence bone resorption by producing factors that modulate osteoclast differentiation. Although the methylation of CpG islands plays an important role in the regulation of gene expression, there is still scanty information about its role in human bone. The aim of this study was to investigate the influence of CpG methylation on the transcriptional levels of two osteoblast-derived critical factors in the regulation of osteoclastogenesis: the receptor activator of nuclear factor NFκB ligand (RANKL) and its soluble decoy receptor osteoprotegerin (OPG). Quantitative methylation specific PCR (qMSP) and pyrosequencing analysis in various cell types showed that the methylation of regulatory regions of these genes, in the vicinity of the transcription start sites, repressed gene transcription, whereas an active transcription was associated with low levels of methylation. In addition, treatment with the DNA demethylating agent 5-azadeoxycitidine promoted a 170-fold induction of RANKL and a 20-fold induction of OPG mRNA expression in HEK-293 cells, which showed hypermethylation of the CpG islands and barely expressed RANKL and OPG transcripts at baseline. Transcriptional levels of both genes were also explored in bone tissue samples from patients with hip fractures and hip osteoarthritis. Although RANKL transcript abundance and the RANKL:OPG transcript ratio were significantly higher in patients with fractures than in those with osteoarthritis (RANKL: 0.76 ± 0.23 vs. 0.24 ± 0.08, $p = 0.012$; RANKL:OPG: 7.66 ± 2.49 vs. 0.92 ± 0.21, $p = 0.002$), there was no evidence for differential methylation across patient groups. In conclusion, the association between DNA methylation and the repression of RANKL and OPG expression strongly suggests that methylation-dependent mechanisms influence the transcription of these genes, which play a critical role in osteoclastogenesis. However, other mechanisms appear to be involved in the increased RANKL/OPG ratio of patients with osteoporotic fractures.

Introduction

Bone is a dynamic tissue that is under continuous remodeling. This process involves the synthesis of bone matrix by osteoblasts and its resorption by osteoclasts. Bone remodeling is tightly regulated by the RANKL-RANK-OPG system.[1] RANKL (Receptor activator of NFκB ligand, encoded by the *TNFSF11* gene) binds to its receptor, RANK, present in cells of the osteoclast lineage and stimulates osteoclast formation, activation and survival.[2,3] On the other hand, OPG (Osteoprotegerin, the product of the *TNFRSF11B* gene) protects bone from excessive resorption by binding to RANKL and thus avoiding its interaction with RANK.[4,5] In addition, recent findings suggest that OPG may function as a traffic regulator of RANKL, modulating its ability to reach the cell membrane.[6] Thus, the relative concentrations of RANKL and OPG in bone are regarded as important determinants of bone mass and strength, and the inhibition of RANKL/RANK signaling has become a therapeutic target in osteoporosis and other disorders characterized by an increased bone resorption.[7,8]

Identifying the transcriptional mechanisms that drive RANKL and OPG expression is of great scientific interest and may help to identify new targets for bone therapies. Various cell types, including stromal cells and osteoblasts produce RANKL and OPG in the bone microenvironment.[9,10] Recent studies also suggest that osteocytes, as well as hypertrophic chondrocytes, are other important sources of RANKL in the adult skeleton.[11,12] PTH, $1,25(OH)_2D_3$, glucocorticoids and IL 6-type cytokines stimulate RANKL expression.[13-15] OPG expression is also regulated by different cytokines, hormones and growth factors, as well as by the Wnt/β-catenin pathway.[16,17]

Epigenetic mechanisms and particularly DNA methylation are known to contribute to gene transcription in many tissues. DNA methylation tends to block gene expression by incompletely known mechanisms, including the interference of the binding of transcription factors to the regulatory sites in DNA.[18] Although there are some indications for a role of DNA methylation of the *RANKL* and *OPG* genes in murine models and cancer cells,[19,20] it is still unknown whether DNA methylation regulates the expression of these genes in human bone. Therefore, the aim

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Submitted: 09/14/11; Revised: 10/28/11; Accepted: 11/13/11
http://dx.doi.org/10.4161/epi.7.1.18753

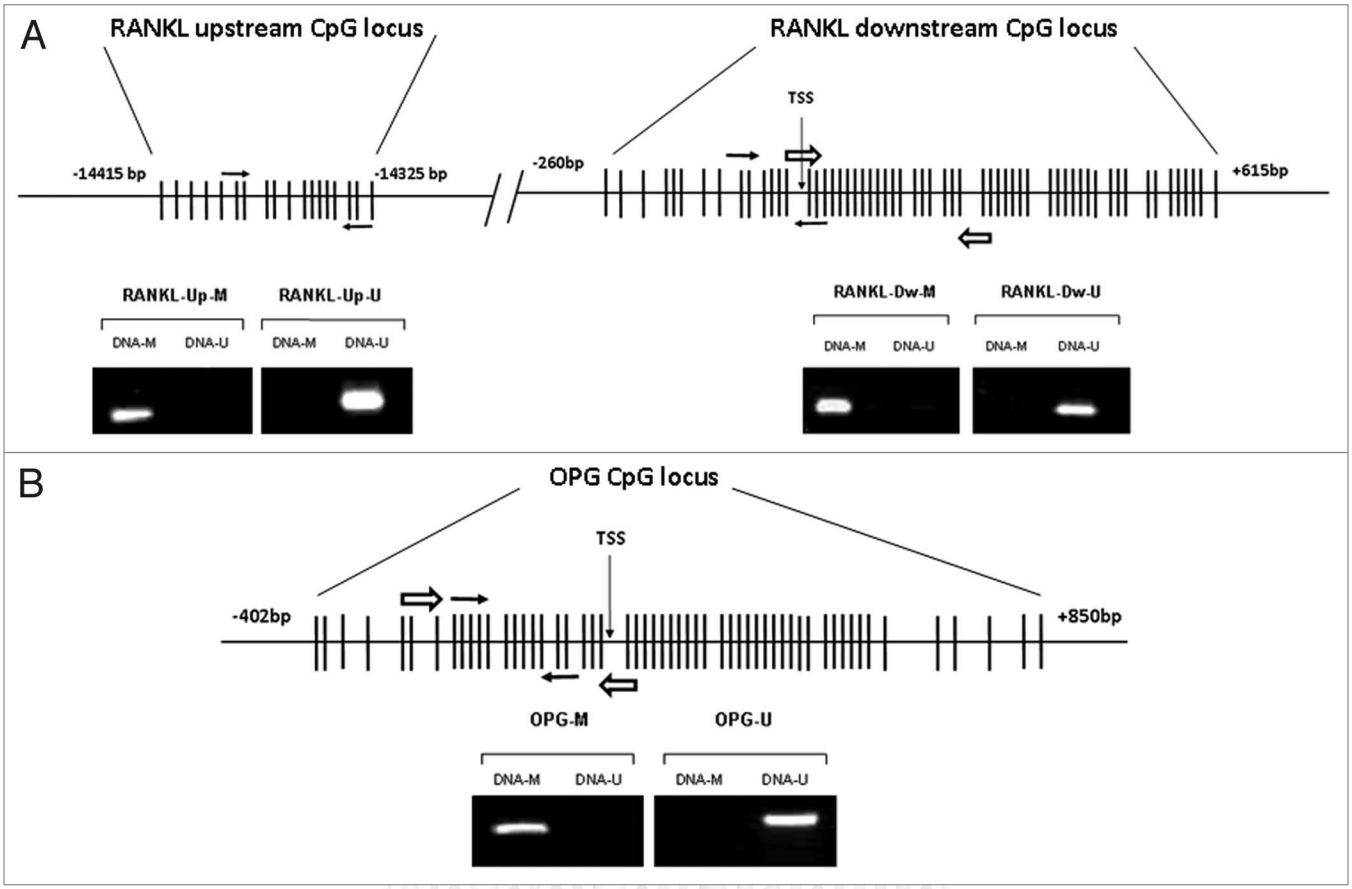

Figure 1. Locations of the *RANKL* and *OPG* CpG islands, qMSP and pyrosequencing amplicons. (A) Non-scaled representation of the *RANKL* gene. Two CpG-rich regions were identified, the upstream one located at -14,415 bp from the TSS of the isoform I and the downstream one, located -260 bp from isoform I TSS. One qMSP amplicon was designed for each region (white arrows). A pyrosequencing amplicon was designed for the downstream CpG region (black arrows). MSP was performed with control fully methylated DNA (DNA-M) or completely unmethylated (DNA-U) to verify the specificity of the MSP primers. (B) Non-scaled representation of the *OPG* gene. One CpG island was found spanning from -402 to +850 bp of the TSS. qMSP and pyrosequencing amplicons were designed within the CpG-rich area (white and black arrows respectively). MSP was performed using DNA-M or DNA-U to verify the specificity of the MSP primers.

of this study was to explore the influence of DNA methylation on *RANKL* and *OPG* expression in human osteoblastic cells and its possible involvement in osteoporotic fractures.

Results

Bioinformatics analysis of the *RANKL* and *OPG* gene sequence. Our bioinformatics analysis revealed two CpG islands in the *RANKL* gene: the upstream one (18 CpG sites), located at -14,415 bp from the transcription start site of isoform I (TSS I), the major *RANKL* transcript; and the downstream one (59 CpG sites) that spans from -260 bp to +615 bp of the TSS I (**Fig. 1A**). One island was found in the *OPG* gene (56 CpG sites), spanning from -402 to +850 bp of the TSS (**Fig. 1B**).

***RANKL* gene expression and methylation.** We studied *RANKL* and *OPG* gene expression in several cell lines and primary cells. *RANKL* mRNA was readily detected in human primary osteoblasts (hOBs) and the osteoblast-like cell line MG-63. However, minimum amounts of *RANKL* mRNA were detected in the osteoblastic cell line HOS-TE85 and in kidney-derived HEK-293 cells, less than 1% of the level of hOBs (**Fig. 2A**).

In parallel experiments we explored the methylation degree of the two CpG-rich regions of the *RANKL* gene by qMSP. MG-63 and hOBs, that expressed higher amounts of RANKL, showed lower methylation in the downstream CpG island than the two other cell types. However, no differences in the methylation of the upstream CpG island were found, which was strongly methylated in all cell types (**Fig. 2B**).

To confirm the differences in DNA methylation we analyzed the downstream CpG-rich region by pyrosequencing (**Fig. 2A**, bottom part). Indeed, hOBs and MG-63 showed smaller degrees of methylation of the overall region than HOS-TE85 and HEK-293 cells (4.1 ± 1.4%; 26.6 ± 0.4%; 63.9 ± 2.2%; and 89.0 ± 5.6%, respectively). The single nucleotide analysis showed a very similar methylation pattern in the 9 cytosines included in the region, with methylation percentages between 1.2 and 5.4% in hOBs, and between 25.1 and 42.3% in MG-63. Much higher methylation percentages were found in the cells with low

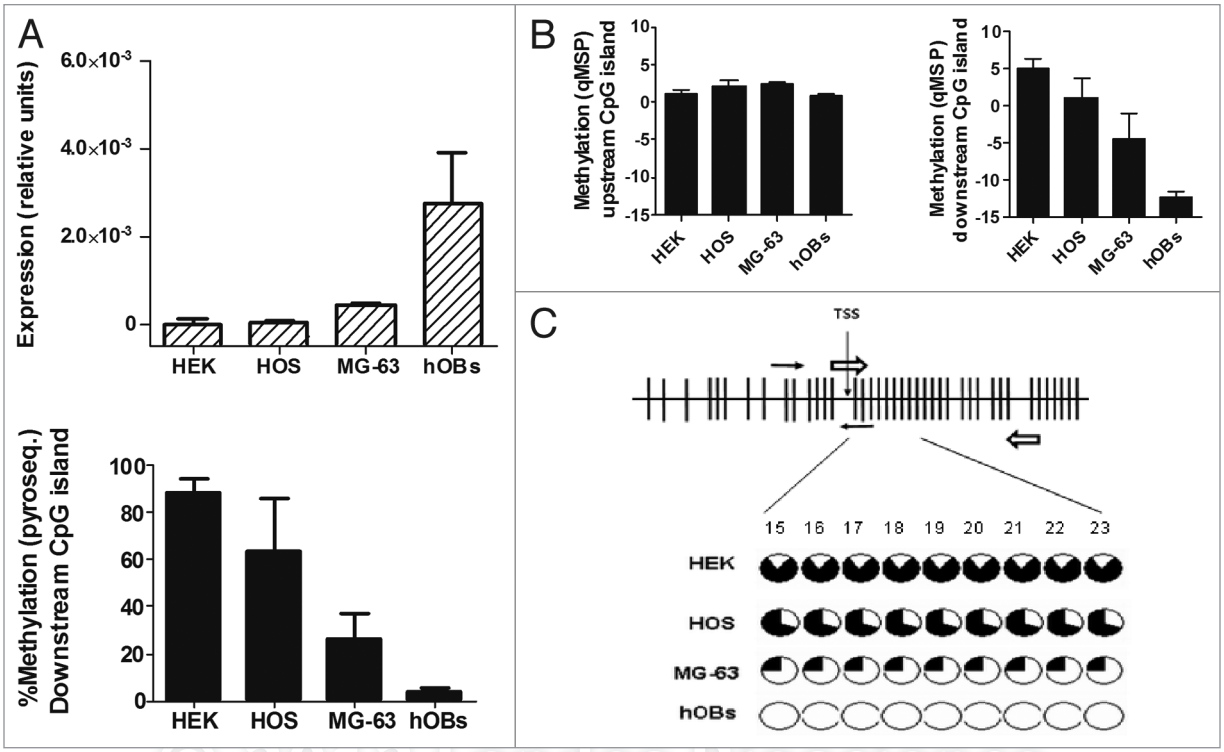

Figure 2. *RANKL* expression and methylation analysis in cell lines and primary osteoblasts. (A) The abundance of *RANKL* transcripts was determined by RT-qPCR. Results are expressed as relative expression to the housekeeping gene TBP. Methylation of the downstream CpG region was studied by pyrosequencing. Bars represent average % methylation of the CpG dinucleotides studied. Mean and SD of three independent experiments for each cell line is represented. (B) Methylation was explored in the upstream (CpGs 5, 6 and 7 in the forward primer; CpGs 17 and 18 in the reverse primer) and downstream CpG islands (CpGs 9 and 10 in the forward primer; 15 and 16 in the reverse primer) by qMSP. Mean and SD of three independent experiments is showed. (C) All individual CpGs studied (CpGs 15 to 23) by pyrosequencing of the *RANKL* downstream island showed a similar methylation status. Black slices represent the percentage of methylation. Note that only CpG 1 to 40 are displayed in (C).

expression of *RANKL*: 68.7–91.2% in HOS-TE85 and 80.0–98.7% in HEK-293 (**Fig. 2C**).

***OPG* gene expression and methylation.** *OPG* mRNA was highly expressed in hOBs and MG-63 cells. HOS-TE85 cells expressed smaller amounts of OPG, whereas *OPG* mRNA was barely detected in the kidney-derived HEK-293 cell line (**Fig. 3A**, upper part).

DNA methylation analysis by qMSP showed that HEK-293 and HOS-TE85 cells, that expressed smaller amounts of OPG, displayed higher methylation in the CpG-rich region of the *OPG* gene than hOBs or MG-63. The differences in methylation were also confirmed by pyrosequencing (**Fig. 3A** and middle and bottom parts), which demonstrated that the *OPG* gene was more methylated in HEK-293 and HOS-TE85 cells (19.5 ± 4.5% and 17.3 ± 5.2%, respectively) than in MG-63 and hOBs (1.2 ± 0.3% and 1.6 ± 0.4%, respectively). The single-base analysis revealed a similar degree of methylation in the 3 cytosines studied (**Fig. 3B**). In hOBs, the methylation of individual cytosines varied between 1.4% and 2.3%; in MG-63, between 0.8% and 1.6%. However, in HOS-TE85, it varied between 14.9% and 21.3%; and in HEK-293 between 12.3% and 21.5%.

5-Aza-2'-deoxycytidine induces *RANKL* and *OPG* expression in HEK-293 cells. The inverse relationship observed between methylation and gene expression suggested that DNA methylation was indeed repressing *RANKL* and *OPG* expression. To confirm this hypothesis, we explored the ability of 5-Aza-2'-Deoxycytidine (AzadC) to induce gene expression in HEK-293 cells. AzadC is incorporated into DNA and blocks DNA methyltransferases. We found that AzadC treatment induced a decrease in the methylation of the CpG-rich regions of *OPG* and *RANKL* genes (**Fig. 4A and B**, middle and bottom part). It was associated with a marked increase in gene expression, with a 170-fold induction of *RANKL* and a 20-fold induction of *OPG* mRNA. Interestingly enough, however, when hOBs, which had the *OPG* and *RANKL* regions already hypomethylated, were treated with AzadC, we did not detect any changes in *OPG* or *RANKL* gene expression. On the other hand, osteocalcin (*BGLAP*) expression was not affected by the demethylating treatment in the HEK-293 cells (**Fig. 4C**).

***RANKL* and *OPG* expression and methylation in osteoarthritic and osteoporotic bone samples.** We explored if differences in *RANKL-OPG* expression were involved in the opposite changes in bone mass typical of osteoarthritis (OA) and osteoporotic (OP) fractures. *RANKL* expression was significantly higher in samples from patients with osteoporotic fractures (0.76 ± 0.23 vs. 0.24 ± 0.08, p = 0.012; **Fig. 5A**). No significant differences were observed in *OPG* expression (0.26 ± 0.06 in OP and 0.44 ± 0.11 in OA; **Fig. 5B**), but the RANKL:OPG ratio was much

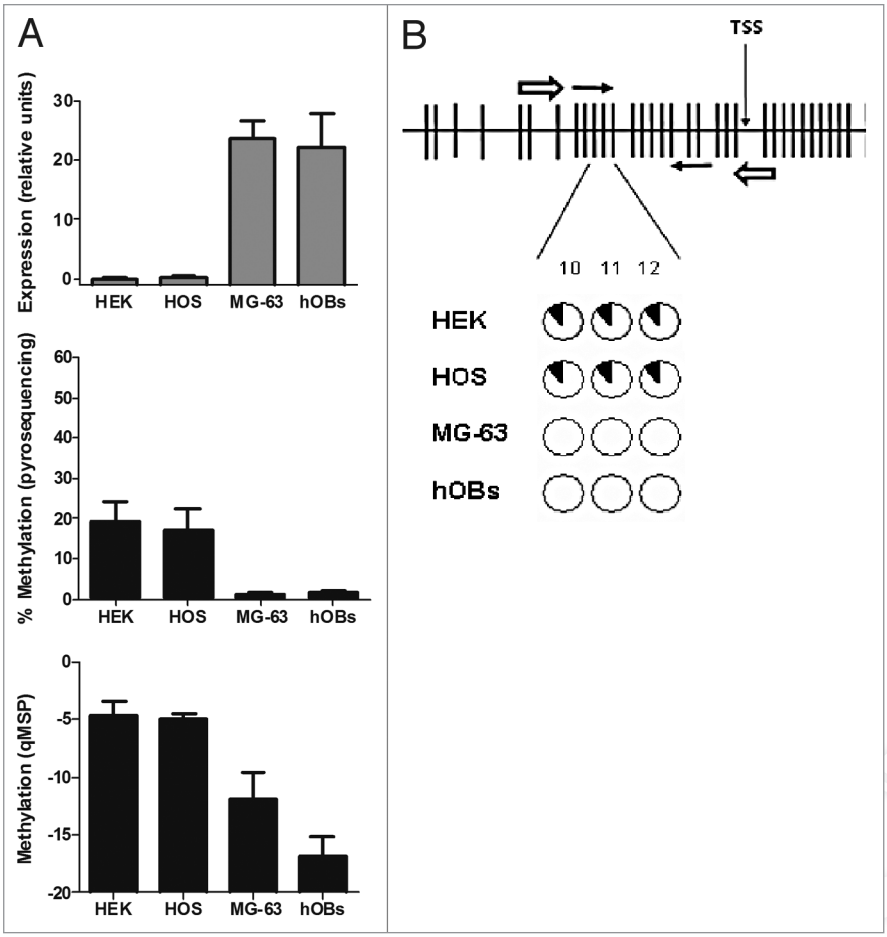

Figure 3. *OPG* expression and methylation analysis in culture cell lines and primary osteoblasts. (A) *OPG* expression was detected by RT-qPCR. Results are expressed as relative expression in relation to the housekeeping gene TBP. Methylation was studied by qMSP (CpGs 10, 11 and 12 in the forward primer; CpGs 18, 19 and 20 in the reverse primer). For pyrosequencing bars represent the average % methylation of the CpG dinucleotides studied. Mean and SD of three independent experiments for each cell line is presented. (B) The CpG sites studied by pyrosequencing (CpGs 10–12) showed a similar degree of methylation. Each circle represents one CpG site. The black slices represent the percentage of methylation. Note that only CpGs 1 to 33 are displayed in (B).

higher in OP bone tissue samples (7.66 ± 0.23 vs. 0.92 ± 0.21, p = 0.002; **Fig. 5C**).

We next investigated if the differences in the *RANKL*:*OPG* expression pattern between the two groups of patients were related to differential methylation. DNA was extracted from femoral head samples from 9 patients with OA and 12 with fractures (age 77 ± 4 and 79 ± 3 y, respectively) and methylation was studied by qMSP. As shown above in cell cultures, the *RANKL* upstream region appeared highly methylated in all bone samples. However, the *RANKL* downstream and the *OPG* CpG islands appeared hypomethylated, without differences between OP and OA samples (**Fig. 5A and B**, bottom parts). The results were confirmed by pyrosequencing (**Fig. 5A and B**, middle parts). The average methylation of the *RANKL* downstream region was 6.6 ± 3.8% in hip fracture samples and 5.9 ± 2.8% in OA samples. The CpG-rich region of the *OPG* gene was also poorly methylated in both samples (1.9 ± 0.4% in OP and 3.0 ± 0.7% in OA).

Discussion

There is increasing experimental evidence showing that the methylation status of CpG islands plays an important role in the regulation of gene expression.[21] This has been extensively studied in neoplastic disorders, including bone tumors.[22,23] However, little is known about the potential role of DNA methylation in normal bone and non-neoplastic bone disorders.

A bioinformatic search revealed two CpG-rich regions in the *RANKL* gene, the upstream CpG island and the downstream one. The human downstream CpG-rich sequence showed greater than 75% homology with the previously reported CpG island in the mouse *RANKL* gene.[19] In fact, there are not only sequence homologies, but also the location of the island is very similar in both species, around the transcription start site of the isoform I (TSS I). We did not find homologies for the upstream CpG-rich region between those species, which was located quite far from the TSS I (-14,415 bp), but relatively close to the isoform II TSS (around -3,000 bp). On the other hand, we found a CpG island in the vicinity of the *OPG* transcription start site, consistent with the location reported by Lu et al.[20] The presence of the CpG islands suggested that these genes might be regulated by cytosine methylation. Indeed, we found an inverse association between DNA methylation and gene expression. Thus, the CpG-rich regions of HEK-293 cells, which hardly expressed *RANKL* and *OPG* transcripts, were hypermethylated, whereas low percentage of methylation was observed in hOBs, which expressed large amounts of both genes. Pyrosequencing confirmed the semiquantitative results of qMSP and allowed us to establish that all individual CpG sites analyzed had a similar degree of methylation within a certain CpG island, a consistent result in all cell types studied. Interestingly enough, the methylation of the *RANKL* downstream CpG island was associated with the repression of transcription, but no differences in the methylation level of the upstream CpG island were found between the cells studied, despite the marked differences in *RANKL* expression. This observation, along the conservation, both in sequence and location, suggests that the methylation at the downstream CpG island, and not at the upstream one, actually modulates *RANKL* transcription. Nevertheless, in this study we analyzed the isoform I, which is the most widely expressed and active form of *RANKL*.[24] Thus, we cannot exclude an influence of the upstream CpG island on the transcription of alternative minor isoforms of RANKL. However, its consistent hypermethylation

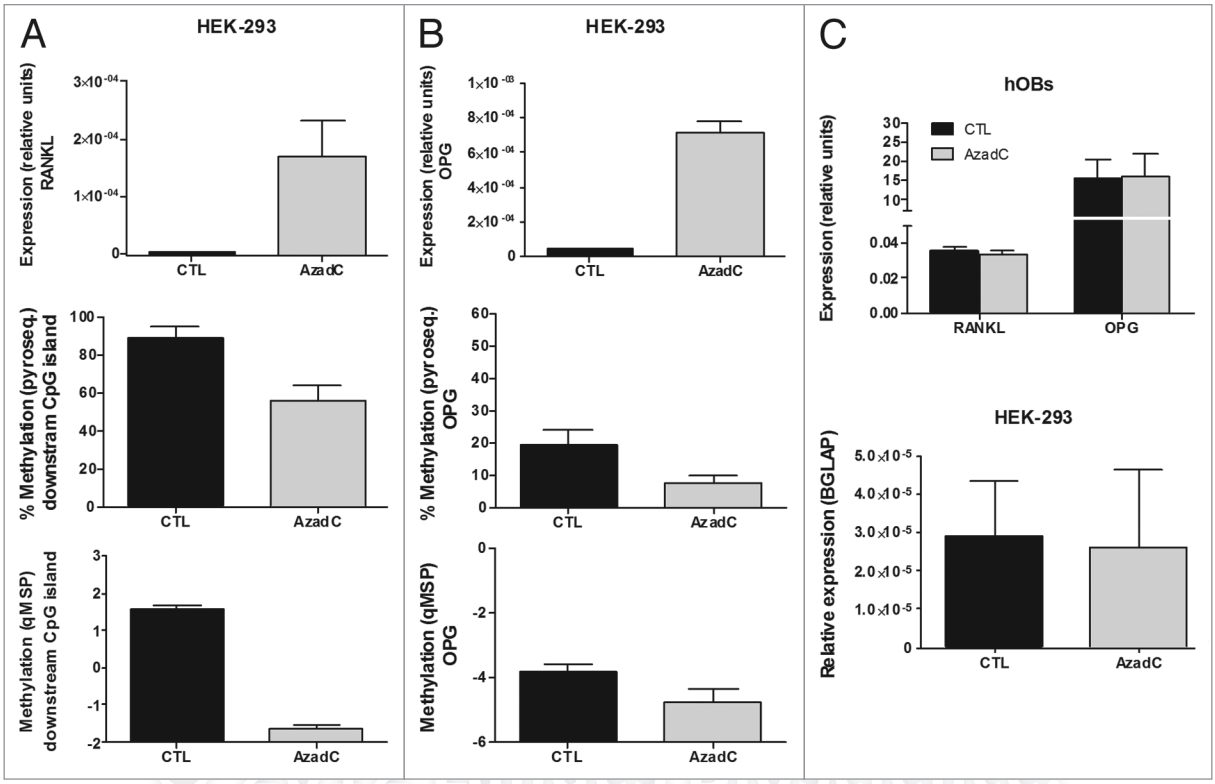

Figure 4. Effect of AzadC on *RANKL* and *OPG* expression in HEK-293 cells and primary osteoblasts. (A) *RANKL* expression was explored by RT-qPCR in HEK-293 cells, either untreated or treated with AzadC 5 μM for 4 d. Graph shows *RANKL* expression relative to the housekeeping gene *TBP*. AzadC effect on DNA methylation of the *RANKL* downstream CpG region was studied by pyrosequencing and qMSP. (B) *OPG* transcriptional levels were explored by RT-qPCR in HEK-293 cells, either untreated or treated with AzadC 5 uM for 4 d. Graph shows *OPG* expression relative to the housekeeping *TBP*. DNA methylation was explored by pyrosequencing and qMSP. (C) *RANKL* and *OPG* expression was assayed in primary osteoblasts treated with 5 μM of AzadC for 4 d (upper plot). Osteocalcin expression (*BGLAP*) was studied in HEK-293, either untreated or treated with AzadC 5 μM for 4 d (lower plot). Graphs show *OPG*, *RANKL* and *BGLAP* expression relative to *TBP*. Mean and SD of three independent experiments for each cell line is showed.

in all the samples studied raises doubts about the potential physiological role of methylation changes in this region, at least in the skeleton.

We have also studied the methylation profile of the *RANKL* downstream CpG-rich region in microdissected mature human osteocytes and lining osteoblasts and found that those cells show a low degree of methylation, similar to hOBs (unpublished observations). This strongly suggests that osteocytes are epigenetically ready to express *RANKL*. Indeed, recent studies suggest that osteocytes may be a major source of RANKL within the bone microenvironment.[11,12] Furthermore, the expression of both genes in human mesenchymal stem cells (MSCs), which differentiate into various types of cells, including osteoblasts, is easily detectable. In line with the results here reported, the CpG islands of *RANKL* and *OPG* genes are also largely demethylated in MSCs (unpublished data). Therefore, the DNA methylation pattern of *RANKL* appears to be early established in osteoblast precursors and does not undergo significant changes during the process of differentiation toward osteocytes, which is consistent with reports demonstrating that *RANKL* is expressed throughout all stages of differentiation of the osteoblastic lineage.[25]

To get further evidence for the role of DNA methylation in the expression of *RANKL* and *OPG* we studied the effect of AzadC on the heavily methylated HEK-293 cells. AzadC is a cytidine analog with a nitrogen atom replacing the carbon at the 5 position of the pyrimidine ring. When AzadC is incorporated into DNA, DNA-methyltransferases cannot methylate the modified cytidine, thus perturbing the methylation pattern in daughter cells.[26] AzadC was actually effective in decreasing gene methylation at the CpG sites studied, as shown by qMSP and pyrosequencing analysis. This hypomethylating effect was accompanied by a marked upregulation of *OPG* and *RANKL* (20-fold and 170-fold induction, respectively), a result consistent with a repressive role of CpG methylation in gene expression. This was not just the consequence of a non-specific increase in gene expression, because other genes typical of an osteoblastic phenotype, such as osteocalcin, remained unaltered. Moreover, when primary osteoblasts, which show a low degree of methylation at the studied CpG sites, were treated with AzadC, no further upregulation of *RANKL* or *OPG* was found.

OP and OA are two common age-related, chronic disorders of the skeleton that tend to show opposite changes in bone mass.[27] Although OA has long been considered to be primarily a cartilage disorder, with secondary changes in the subchondral bone, several studies suggest a more active role of the bone tissue in the pathogenesis of the disease.[28,29] As expected, *RANKL* and

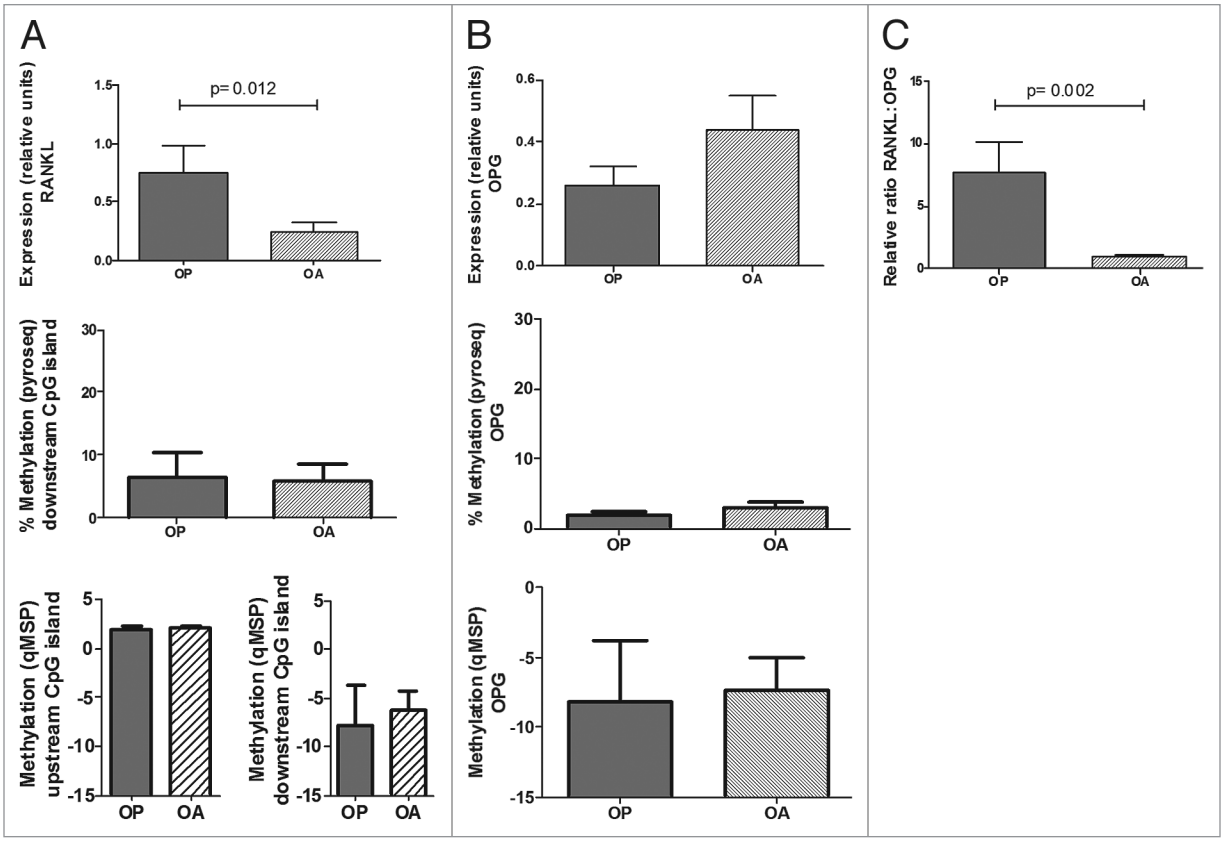

Figure 5. Expression and methylation of *RANKL* and *OPG* genes in bone tissue from osteoporotic (OP) and osteoarthritic (OA) patients. (A) *RANKL* expression was studied in bone tissue samples. Bars represent *RANKL* expression relative to *TBP*. DNA methylation in the downstream CpG island was studied by pyrosequencing and qMSP, whereas methylation in the upstream region was studied by qMSP. (B) *OPG* studies were performed in the same samples. (C) *RANKL*:*OPG* transcript ratio. Each bar represents mean and SE values for each group.

OPG transcripts were readily detected in bone tissue samples from both groups. No significant differences in *OPG* expression were observed. However we found that *RANKL* expression and the *RANKL*:*OPG* ratio were significantly higher in samples from patients with hip fractures than in those with osteoarthritis, which is consistent with enhanced osteoclastogenesis in the former group of patients. These results confirm previous reports by Logar et al.[30] and a recent report by D'Amelio[31] and are consistent with the hypothesis that an increased signaling of the RANK pathway and the resultant stimulus of osteoclastogenesis may contribute to osteoporotic fractures.

Emerging evidence suggests that epigenetic mechanisms may be involved in age related diseases and bone biology[32,33] and a few genes have been reported as epigenetically regulated in bone.[34,35] Since we found an epigenetic modulation of *RANKL* and *OPG* gene expression levels, we explored if differential levels of DNA methylation could explain the differences in gene transcription between the OP and OA groups. In bone tissue samples, low DNA methylation was found in the downstream *RANKL* island and in the *OPG* island. This is in line with the results in cell cultures, showing an association between active expression and low methylation levels. However, despite the marked differences in gene expression, we found no differences in the methylation of *RANKL* between both groups of patients at the bone tissue level. These results would suggest that mechanisms other than DNA methylation are responsible for the differences in *RANKL* expression between patients with OA and OP. We can speculate that DNA methylation represents an on/off regulator of *RANKL-OPG* gene expression, whereas other factors, such as hormones and cytokines in the bone microenvironment, are responsible for the fine tuning of expression in cells with hypomethylated islands. In fact, PTH and estrogens, may be appealing candidates because they have been shown to modulate RANKL expression in several experimental system,[11,15] in some cases by interacting with distant regulatory regions.[36] Besides these humoral factors, we cannot exclude the involvement of other epigenetic mechanisms, such as the post-translational modifications of histones or miRNA production, but their role, if any, remains speculative at the moment. Nevertheless, it is important to note that bone is formed by different cell types, many of which express *RANKL*. Since we analyzed whole bone tissue samples, we cannot rule out that DNA methylation differences in specific cell types, such as osteocytes or osteoblasts, could underlie the dysregulation of *RANKL* in osteoporotic patients.

In summary, we have shown that DNA methylation represses *RANKL* and *OPG* transcription. This was the case for the *OPG* island and the downstream *RANKL* CpG island, but not for the upstream *RANKL* island, suggesting that the latter does not

play an important role in the regulation of gene expression. The silencing role of DNA methylation was further supported by the fact that treatment with a demethylating agent induced a marked upregulation of *RANKL* and *OPG* transcripts in cells that hardly expressed these genes at baseline. Together, these data suggest that changes in DNA methylation contribute to regulate the expression of these genes, which are critical for bone homeostasis. However, other mechanisms independent of DNA methylation appear to be involved in the increased RANKL/OPG ratio of patients with osteoporotic fractures.

Materials and Methods

Cell culture. The human osteoblastic cell line HOS-TE85 [37] was maintained in culture with Eagle Minimum Essential Medium (MEM, Sigma-Aldrich, M0643) supplemented with 10% fetal bovine serum (FBS) and antibiotics. The human osteoblastic cell line MG-63 [38] and the human embryonic kidney cell line HEK-293,[39] were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, 12800-116) supplemented as described above.

Bone tissue samples were obtained during arthroplasty, from patients with osteoporotic hip fractures (24 women, 2 men; age 80 ± 4 y) and with hip osteoarthritis (19 women, 5 men; age 75 ± 6 y), after obtaining informed consent. The study was approved by the Cantabria Clinical Research Ethics Committee and all participants gave informed written consent. Patients with secondary osteoporosis, fractures due to high-energy trauma or secondary osteoarthritis were excluded. Trabecular bone cylinders of the central part of the femoral head (thus avoiding the fractured and the subchondral regions) were obtained with a trephine, washed extensively in phosphate-buffered saline, snap-frozen in liquid nitrogen and stored at -80°C or used to generate primary human osteoblasts (hOBs) by the explant technique.[40] The osteoblastic phenotype was confirmed by the ability to express osteocalcin when stimulated with vitamin D and to form a mineralized matrix. hOBs were cultured in DMEM supplemented with 10% FBS and antibiotics. 5-Aza-2'-Deoxycytidine (AzadC) was purchased from Sigma-Aldrich (A2385). For demethylation experiments cells were plated at 5,000 cells/cm^2 and treated for 4 d in the presence/absence of AzadC, 5 μM, prior to the analysis of gene expression and DNA methylation.

Gene expression. *RANKL* and *OPG* expression was determined by reverse transcription followed by real time quantitative polymerase chain reaction (RT-qPCR). RNA was isolated from cell cultures or bone tissue samples with Trizol following the manufacturer's protocol (Invitrogen, 15596-018). Then cDNA was synthesized from 1 μg total RNA, using random hexamers as primers, and the Superscript III First Strand kit (Invitrogen, 18080-051). Gene expression was measured using Taqman probes following the manufacturer's protocol (Applied Biosystems). The following Taqman Gene Expression Assays were used: *TNFSF11* (isoform I, *RANKL*): Hs00243519_m1; *TNFRSF11B* (*OPG*): Hs00900360_m1; and *BGLAP* (osteocalcin): Hs01587814_g1. Results are shown as the relative gene expression, using the expression of the TATA Box Binding Protein (*TBP*) housekeeping gene as a reference. The cycle thresholds of each gene were estimated and gene expression was computed as $2^{-\Delta Ct}$, where ΔCt is the difference between the gene of interest threshold cycle and the threshold cycle of *TBP*.

DNA bioinformatic search and methylation analysis by qMSP. We performed a bioinformatics analysis to identify potential CpG islands in the genomic sequences of *RANKL* and *OPG* genes. The USCS Human Genome Browser public database (genome.ucsc.edu), the Primer Express software (available from Applied Biosystems), and CpG Island Explorer software (cpgie.sourceforge.net) were used to explore the nucleotide sequences surrounding de transcription start site (TSS) of *RANKL* isoforms I and II (NG_008990.1 Ref Seq Gene), and *OPG* (NG_012202.1 Ref Seq Gene). A (C + G)/total bases ratio >0.5 and CpG observed/CpG expected ratio >0.6 were used as criteria. Quantitative methylation-specific PCR (qMSP) is based on the amplification of bisulfite converted DNA.[41] Sodium bisulfite converts unmethylated cytosines to uracils, whereas methylated cytosines are unaffected. Thus, it allows distinguishing between methylated and unmethylated DNA. Primers, which targeted CpG-rich regions within the *RANKL* and *OPG* regions, were designed with Methyl Primer Express software (available from Applied Biosystems). For each region, a pair of primers was selected; one specifically recognized the bisulfite-treated methylated DNA and other recognized only unmethylated bisulfite-treated DNA (**Table 1**).

Genomic DNA from cells and bone tissue samples was isolated with phenol:chloroform: isoamyl alcohol (Invitrogen, 15593031). For optimized bisulfite conversion, we used 1,000 ng of genomic DNA and the Zymo EZ DNA Methylation-Gold kit (Zymo Research, D5005). After bisulfite conversion, the DNA was bound to a Zymo spin column and desulfonated. The bisulfite converted DNA was eluted from the column in 10 μl of elution buffer. In the qMSP reaction, duplicate aliquots of 1 μl of the eluted bisulfite-treated genomic DNA were amplified with AmpliTaq Gold DNA Polymerase buffer (Applied Biosystems, N808-0240) with 2 mM $MgCl_2$, 0.4 pmol primers, 400 μM dNTP mix, 8 μl of Sybr Green and 1 U of AmpliTaq Gold DNA polymerase in a total volume of 25 μl. Amplification conditions were: 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 30 s, with a final extension of 72°C for 5 min. Epitec methylated and unmethylated human control DNA, bisulfite converted, as well as unmodified human DNA (Qiagen, 59695) were used as controls to check primer specificity (**Fig. 1**). The degree of DNA methylation was estimated as the unmethylated primer cycle threshold minus the methylated primer cycle threshold for each amplicon. Thus, smaller values are associated with smaller proportions of methylated DNA in the sample. The mean and SD was calculated from series of three experiments for each amplicon and cell line.

Pyrosequencing. The results obtained by qMSP were confirmed by pyrosequencing.[42,43] Sodium bisulphite modification of 0.5 μg genomic DNA was performed as described above. Bisulphite-treated DNA was eluted in 15 μl, using 2 μl for each PCR. The set of primers for PCR amplification and sequencing were designed using the PyroMark assay design software,

Table 1. Primer sequences and amplicon sizes for the quantitative methylation-specific PCR (qMSP) of the *RANKL* and *OPG* gene

Name	Sequence (5'-3')	Amplicon (bp)
MET-OPG-Fwd	TTC GGA TTT TGG TTG GAT C	148
MET-OPG-Rev	GCA AAC TCT AAA ATT TCC CG	148
UNMET-OPG-Fwd	GTT TGG ATT TTG GTT GGA TT	148
UNMET-OPG-Rev	CAC AAA ACT CTA AAA TTT CCC A	148
MET-RANKL-upstream -Fwd	TCG GTG TTT GTC GGT TAGC	142
MET-RANKL-upstream-Rev	CGA CGA AAA ATT AAA CAC AAC A	142
UNMET-RANKL-upstream-Fwd	GTT TGG TGT TTG TTG GTT AGT	142
UNMET-RANKL-upstream-Rev	CAA CAA AAA ATT AAA CAC AAC AAC	142
MET-RANKL-downstream-Fwd	AGG AGA TGG GCG TTT TTA GTC	134
MET-RANKL-downstream-Rev	CCG CTC CAT ATT CGT AAC C	134
UNMET-RANKL-downstream-Fwd	GGA AGG AGA TGG GTG TTT TTA GTT	134
UNMET-RANKL-downstream-Rev	CCC ACT CCA TAT TCA TAA CCC T	134

bp, base pairs; Fwd, forward; Rev, reverse.

version 2.0.01.15. Primer sequences were designed to hybridize with CpG-free sites to ensure methylation-independent amplification. The primers were located flanking the qMSP amplicons (see **Fig. S1**). Primer sequences were:

Pyro-RANKL-F: 5'-TTT TGG GAA GGT GGT TAT TTA T-3';

Pyro-RANKL-R[Btn]: 5'-CCA ACA AAA ACT ACA CCA AAT AC-3';

Pyro-RANKL-Seq: 5'-GTT TTA GTT TTA GGA GGG TTA-3';

Pyro-OPG-F: 5'-GGG TTT TGT AAT TTG AGG TTT TAG AA-3';

Pyro-OPG-R[Btn]: 5'-ACT TAT ATC TCC TCC ACC CTA AA-3';

Pyro-OPG-Seq: 5'-GAT AAA GGT TTG GGA TAT ATT-3'.

PCR was performed with primers biotinylated to convert the PCR product to single-stranded DNA templates, using the Vacuum Prep Tool (Biotage), according to manufacturer's instructions. Pyrosequencing reactions and methylation quantification were performed in a PyroMark Q24 System version 2.0.6 (Qiagen). Series of three independent experiments were performed for each cell line and condition.

Statistical analysis. The statistical significance of the differences between osteoporotic and osteoarthritic patients was tested by Mann-Whitney tests. All reported p values are two-tailed and 0.05 was the significance threshold.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported in part by grants from Instituto de Salud Carlos III-Fondo de Investigaciones Sanitarias (Spanish Ministry of Health) PI09/539, PI06/1267, PS09/02454, the Spanish National Research Council (CSIC 200820I172 to M.F.F.) and the Community of Asturias (FICYT IB09-106). The IUOPA is supported by the Obra Social Cajastur, Spain. J.D.C. is recipient of a grant from IFIMAV. A.F.F. is supported by the IUOPA. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Note

Supplemental materials can be found at:
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