

## Negative Regulation of the Osteoblast Function in Multiple Myeloma through the Repressor Gene E4BP4 Activated by Malignant Plasma Cells

Franco Silvestris, Paola Cafforio, Monica De Matteo, Nicola Calvani, Maria A. Frassanito, and Franco Dammacco

**Abstract** **Purpose:** To explore the pathogenetic mechanisms that suppress the osteoblast function in multiple myeloma because osteogenesis results in defective new bone formation and repair. **Experimental Design:** Microarray gene analysis revealed the overexpression of E4BP4, a transcriptional repressor gene, in normal osteoblasts cocultured with myeloma cells that were releasing the parathyroid hormone-related protein (PTHrP). Thus, the effect of E4BP4 was assessed in PTHrP-stimulated osteoblasts by measuring the RNA levels of both Runx2 and Osterix as major osteoblast transcriptional activators. Because E4BP4 is a negative regulator of the cyclooxygenase-2 (COX-2) pathway that drives the expression of both Runx2 and Osterix, these factors were investigated after prostaglandin E<sub>2</sub> treatment to overcome the COX-2 defect as well as in E4BP4-silenced osteoblasts. Finally, E4BP4, PTHrP, Osterix, and osteocalcin levels were measured *in vivo* in patients with bone disease together with the E4BP4 protein in bone biopsies. **Results:** E4BP4 was specifically induced by PTHrP and inhibited both Runx2 and Osterix, whereas E4BP4-silenced osteoblasts expressed functional levels of both factors. The prostaglandin E<sub>2</sub> treatment of E4BP4-up-regulated osteoblasts promptly restored Runx2 and Osterix activities, suggesting that integrity of COX-2 pathway is essential for their transcription. Down-regulation of Osterix by E4BP4 was confirmed *in vivo* by its inverse levels in osteoblasts from myeloma patients with increased serum PTHrP, whose bone biopsies expressed the E4BP4 protein. **Conclusions:** Our data support the role of E4BP4 as osteoblast transcriptional repressor in inhibiting both Runx2 and Osterix in myeloma bone disease and correlate its effect with the increased PTHrP activity.

Myeloma bone disease (MBD) is the hallmark of multiple myeloma. It is primarily associated with hyperactive osteoclastogenesis promoted by malignant plasma cells and the bone destruction is not accompanied by compensatory remodeling (1). Minimal bone formation occurs early within the initial bone erosions (2), whereas osteoblast activity is suppressed as the disease progresses and is reflected by low serum levels of osteogenic markers as osteocalcin, type I collagen, alkaline phosphatase, and osteoprotegerin (3).

Different mechanisms concurrently lead to osteoblast suppression. The canonical Wnt signaling pathway, which regulates pre-osteoblast differentiation by mesenchymal stem cells, is deranged by inhibitors produced by myeloma cells. They mainly include dickkopf1, whose RNA levels correlate with both serum protein and skeletal involvement (4), and the soluble Frizzled related protein-2 (5). Serum levels of other osteoblast-inhibiting factors, as noggin, gremlin, interleukin (IL)-3, IL-7, IL-11, and insulin growth factor binding protein 4, are also enhanced in MBD (6, 7).

Osteogenic differentiation from mesenchymal cells is critically regulated by Runx2/Cbfa1 (8), a transcription factor that, through the downstream inducer Osterix/Sp7 (9), promotes the expression of bone matrix proteins as collagen 1a1, bone sialoprotein, osteopontin, and osteocalcin in maturing osteoblasts (10). Down-regulation of its activity in MBD has been shown in osteoprogenitor cells whose coculture with human myelomas resulted in general suppression of the osteoblast lineage cells in terms of inefficient differentiation, short survival, and weak expression of osteocalcin and other bone proteins (11). Moreover, histomorphometric analyses of bone biopsies documented the decrease of Runx2-positive osteoblasts in patients with active MBD compared with those with no evidence of bone lesions (12).

**Authors' Affiliation:** Department of Internal Medicine and Clinical Oncology, University of Bari Medical School, Bari, Italy

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**Requests for reprints:** Franco Silvestris, Department of Internal Medicine and Clinical Oncology, University of Bari Medical School, P.za Giulio Cesare, 11, 70124 Bari, Italy. Phone: 39-80-5478771; Fax: 39-80-5478831; E-mail: f.silvestris@dim.uniba.it.

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Impairment of Runx2 in MBD has been attributed to molecular interactions between myeloma cells and osteoblast progenitors. One involves the VLA-4/VCAM-1 pathway (12), because inactivation of VLA-4 on myeloma cells by a neutralizing monoclonal antibody prevents the defect of Runx2 activity in cocultured pre-osteoblasts (13). Other cell-to-cell interactions by NCAM molecules expressed by both myeloma and cells of the osteoblast lineage may negatively influence the production of bone morphogenetic proteins, although their role in Runx2 deregulation is unclear (14). Soluble factors may also suppress Runx2 in MBD. IL-7 inhibits Runx2 promoter activity and results in feeble osteoblast maturation, whereas IL-7-blocking antibodies reduce suppression of osteoblast differentiation by myelomas (11). Thus, physical cell-to-cell interactions and increased IL-7 activity by myeloma cells evidently affect Runx2 activity, although their intracellular inhibitory pathways are still undefined.

This study also focused on osteoblast inhibition in MBD. Microarray analysis of normal pre-osteoblasts stimulated by myeloma cells showed that they modify a broad number of osteoblast genes. E4BP4, a repressor gene induced in osteoblasts through the type 1 parathyroid hormone receptor (PTH-1-R; ref. 15), by IL-3 in murine pro-B cells (16) and by glucocorticoids in human T lymphoblasts (17), was over-expressed. Hyperactivity of E4BP4 in myeloma-conditioned osteoblasts definitely inhibited the cyclooxygenase-2 (COX-2) pathway that regulates the expression of both Runx2 and Osterix (18). Involvement of E4BP4 in the defective osteoblast function in multiple myeloma was also shown *in vivo* in myeloma osteoblasts as both defect of Runx2 and Osterix and concurrent accumulation of E4BP4 protein.

## Materials and Methods

**Pre-osteoblasts and myeloma cocultures.** Osteoblast progenitors were derived from a 23-year-old donor undergoing orthopedic surgery and cultured as described (19). Their maturation to osteoblasts was assessed by alkaline phosphatase detection (Molecular Probes) within 4 weeks. Pre-osteoblasts at the third week were thus used for functional assays. Osteoblasts and sera were also obtained from 11 patients: 5 with symptomatic multiple myeloma, hypercalcemia, and multiple osteolytic lesions (20); 4 with monoclonal gammopathy of undetermined significance (MGUS), and 2 with non-Hodgkin's lymphoma (NHL). All subjects gave their informed consent to the study, which was approved by the local ethical committee.

Osteoblasts from the normal donor were cocultured in  $\alpha$ -MEM up to 48 h with U-266 myeloma by transwell insert (Becton Dickinson Labware; pore size, 1  $\mu$ m) to avoid cell-to-cell contacts and, in parallel, with four plasma cell lines (BUT-1, GRA-2, MCC-2, and PAS-2; ref. 21). Each coculture included approximately  $1 \times 10^5$  osteoblasts and  $2.5 \times 10^5$  myeloma cells in 1 mL medium. Raji and CEM lymphoblasts served as controls. Osteoblasts were recovered after removing the inserts containing myeloma cells.

**Osteoblast microarray analysis.** Total RNA (10  $\mu$ g) was purified by TRIzol from osteoblasts cocultured with U-266 and transcribed in cDNA (ThermoScript, Invitrogen). RNA from unconditioned osteoblasts provided the basic gene profile of normal osteoblasts before the myeloma coculture. Thus, cDNA from U-266-conditioned osteoblasts was conjugated with Cy5 red fluorescent dye and that from unconditioned osteoblasts with Cy3 green fluorescent dye. Hybridization was done on a microarray chip containing 50-mer oligo probes for 20,000 genes. Spots of fluorescence intensity were read by dual laser scanner (BioDiscovery) and values were processed with dedicated

software (MAVI Pro-2.6.0, MWG Biotech), calculated by background subtraction, normalized to a number of housekeeping genes, and compared with unconditioned osteoblasts. Therefore, the Cy5/Cy3 ratio for each gene indicated its expression. Ratio values higher than 2.0 or lower than 0.5 were defined as significant by the software. To include only highly deregulated genes, we arbitrarily extended this range and ultimately evaluated those with a spot intensity of at least one channel greater than 0.1, as detected by relative  $\log_2$  values from the intensities in the scatter plot. Thus, we designated as highly deregulated genes those with Cy5/Cy3 ratio above 3.0 or below 0.33.

**Real-time PCR analysis.** Osteoblast RNA (1  $\mu$ g) was transcribed to cDNA (Applied Biosystems) and E4BP4 was measured by real-time PCR (ABI Prism 7000 Sequence Detector by Assay-On-Demand product Hs00356605\_g1) in parallel with glyceraldehyde 3-phosphate dehydrogenase (TaqMan assay). AmpliTaq Gold amplification used 40 cycles at 95°C (15 s) and 60°C (1 min). Each RNA was tested in triplicate and the mean threshold cycle (Ct) value of glyceraldehyde 3-phosphate dehydrogenase was subtracted from E4BP4 gene to yield the  $\Delta$ Ct. E4BP4 gene expression was then calculated as  $2^{-\Delta\Delta Ct}$  absolute value, where  $\Delta\Delta Ct$  was the difference of treated less untreated osteoblast  $\Delta$ Ct (22). Thus, basic  $2^{-\Delta\Delta Ct}$  was considered as 1.0, whereas values higher or lower than 0.5 unit were the significance limits regarded as indicative of gene up-regulation or down-regulation, respectively.

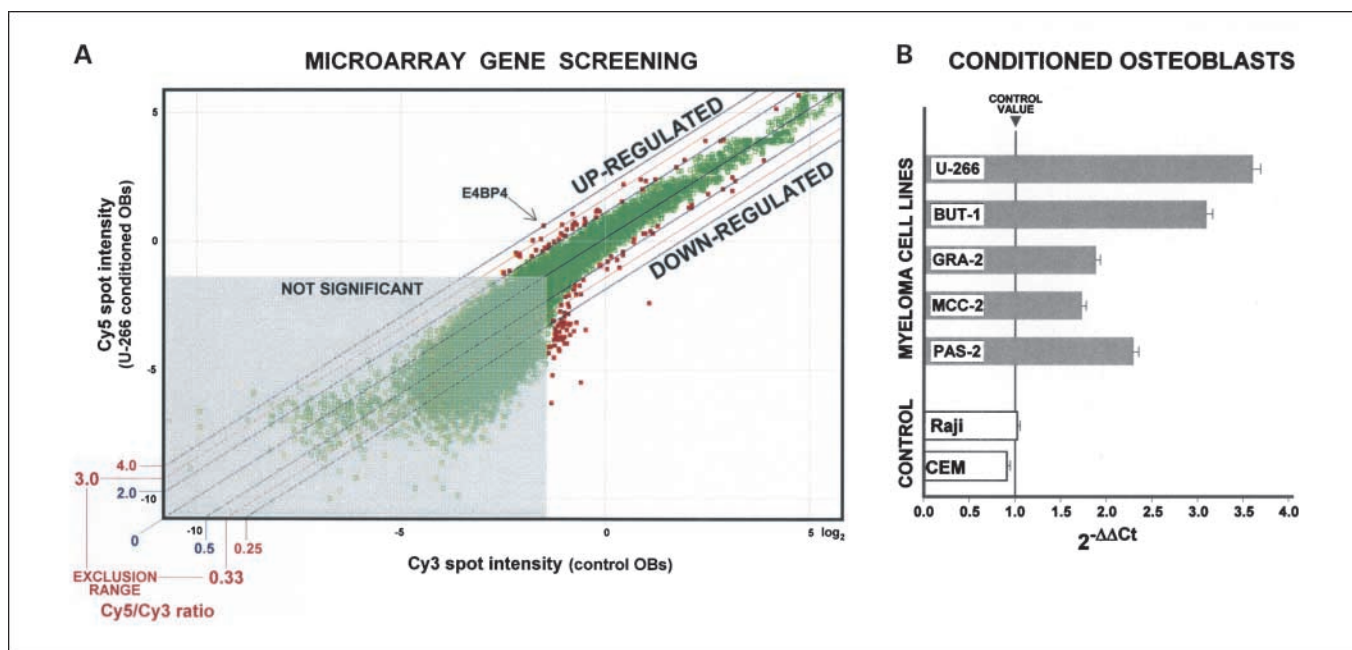
Runx2 and Osterix RNAs were also measured in other osteoblast preparations by real-time PCR (Runx2: Hs00231692\_m1; Osterix: Hs00541729\_m1).

**In vitro osteoblast stimulation and PCR molecular analysis.** Since E4BP4 is inducible in osteoblasts by PTH (15), we investigated the parathyroid hormone-related protein (PTHrP) release by myeloma cells in cocultures. Thus, we treated unconditioned osteoblasts with recombinant PTHrP (PeproTech) at 10 nmol/L (19) for 48 h in parallel with IL-3 at 100 pg/mL (23), dexamethasone at  $10^{-6}$  mol/L and recombinant dickkopf1 (R&D Systems) at 50 ng/mL (24) to assess their potential on E4BP4 induction.

PTHrP expression by myelomas was evaluated by PCR using MCF7 as positive control. The reaction used 5'-TCTTCCTTTCACCATCTGATCG-3' and 5'-TGTCCCTTGAAGGTCTCTGC-3' as forward and reverse primers in 35 cycles at 94°C (15 s), 58°C (15 s), and 72°C (30 s) to obtain the PTHrP product (249 bp; ref. 19). Semiquantitative analysis on agarose gel was assessed as trace quantity value for each determination by the Fluor-S gel analyzer (Bio-Rad). Graded cDNA amounts were amplified to provide reference curves.

The expression of BMP-2, Runx2, and Osterix by PTHrP-, IL-3-, and dexamethasone-stimulated osteoblasts was also explored by PCR in relation to the unequal E4BP4 induction. BMP-2 (230-bp PCR product) was amplified by 35 cycles at 95°C (1 min), 50°C (1 min), and 72°C (2 min) with 5'-CCTGAGCGAGTTCGAGTTG-3' and 5'-CACTCGTTTCTGGTTCTGGTAGITC-3' as forward and reverse primers, respectively (25), whereas to obtain Runx2 (270 bp) the amplification was extended to 40 cycles at 94°C (1 min), 64°C (1 min), and 72°C (1 min) using 5'-CCCCACGACAACCGCACCAT-3' and 5'-CACTCCGGCCCCAAATC-3' as forward and reverse primers (8). The 162-bp product of the short form of Osterix was obtained with 35 cycles at 94°C (30 s), 60°C (1 min), and 72°C (1 min) using 5'-ACCGGTTGCTGCCTCTC-3' as forward primer and 5'-AGTCCCGCAGAGGGCTAGAG-3' as reverse primer (26).

**PTHrP dosage.** PTHrP was measured by dedicated ELISA (Phoenix Pharm.). Briefly,  $1 \times 10^6$  cells from each myeloma and from MCF7 (27) were grown up to 96 h and 0.5 mL supernatant was processed for PTHrP detection in parallel with transforming growth factor- $\beta$ -stimulated cultures as control. PTHrP was also measured in serum from multiple myeloma, MGUS and NHL patients providing bone biopsies for osteoblast cultures with respect to reference values (28, 29) and to control sera from healthy donors. Serum osteocalcin was detected by ELISA (Diagnostic Systems Labs). Specificity of PTHrP in inducing E4BP4 was verified by removing the protein from



**Fig. 1.** Microarray gene screening of myeloma-conditioned osteoblasts (OBs) and induction of the E4BP4 transcriptional repressor. *A*, osteoblasts from a healthy donor were incubated for 48 h with U-266 and the relative cDNA was screened on a microarray chip containing probes relative to 20,000 gene sequences. Spot intensities of gene expression in U-266-conditioned osteoblasts (Cy5) were related to their basal levels in unconditioned osteoblasts (Cy3) and the Cy5/Cy3 ratio provided the relative variation value (deregulated genes in red). To identify only highly deregulated genes, the exclusion range was increased to comprise 0.33 to 3.0 Cy5/Cy3 ratio values. The test thus selected the 38 genes (7 up-regulated and 31 down-regulated) listed in Table 1 in relation to their role in bone metabolism and identified E4BP4 as the highest up-regulated gene. Values were calculated by dedicated software (MWG Biotech), which defined the values in the shaded area as not significant. *B*, changes in E4BP4 RNA transcription as  $2^{-\Delta\Delta Ct}$  absolute value induced by different myeloma cell lines compared with control B cell lines, including CEM and Raji. In all instances, the myeloma cell lines produced a significant increase ( $2^{-\Delta\Delta Ct} > 1.5$ ) of the basal transcription value, whereas no variation was induced by the control lines.

supernatants before osteoblast conditioning by immunoprecipitation with goat anti-PTHrP IgG (Santa Cruz Biotechnology) coupled to protein A-activated beads. Thus, centrifugation at 13,000 rpm depleted the beads including PTHrP-containing immune complexes and PTHrP-depleted supernatants were tested for their residual capacity to induce E4BP4 in osteoblasts.

**Molecular pathways regulated by E4BP4.** The 81-kDa E4BP4 protein acts as a negative regulator of several genes by the consensus sequence "(A/G)T(G/T)A(T/C)GTAA(T/C)" expressed by their promoter (30). In accordance with previous studies (31, 32), we investigated their structure with PatSearch program (33) and found this sequence expressed by the COX-2 promoter (15), whereas unrelated sequences were expressed by both promoters of Runx2 and Osterix (30). Therefore, because COX-2 promotes the transcription of both Runx2 and Osterix through prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) activation (18), we investigated the COX-2 pathway with respect to the E4BP4-negative regulation. Thus, RNA of both Runx2 and Osterix from PTHrP-treated osteoblasts incubated overnight with either PGE<sub>2</sub> at 10<sup>-7</sup> mol/L (Sigma-Aldrich) or the COX-2 inhibitor NS-398 (Sigma) at the same concentration or both were measured and compared with control PTHrP-conditioned or unconditioned osteoblasts.

**E4BP4 gene silencing.** E4BP4 was silenced by small interfering RNA (Santa Cruz Biotechnology). Briefly, 3 × 10<sup>5</sup> untreated osteoblasts were incubated with three target-specific 20- to 25-nucleotide small interfering RNA and Lipofectamine 2000 (Invitrogen) for 2 h in serum-free α-MEM and then maintained for 12 h in complete α-MEM to induce transient silencing of the gene. Efficiency of transfection was evaluated by using siGLO (Dharmacon) at 1 μmol/L, whereas the positive and negative controls were small interfering RNA for glyceraldehyde 3-phosphate dehydrogenase and scramble small interfering RNA (Dharmacon), respectively. E4BP4 was thus measured by real-time PCR within 24 h after silencing.

**Detection of E4BP4.** E4BP4 protein was revealed by both immunoblotting and immunohistochemistry. Briefly, 100 μg nuclear protein

extract from osteoblasts was run by SDS-PAGE and transferred on nitrocellulose membrane (Bio-Rad), incubated with goat anti-E4BP4 IgG (Santa Cruz Biotechnology), and developed with peroxidase-conjugated anti-goat. Finally, E4BP4 protein was revealed by ECL Plus (Amersham) using the ChemiDoc gel equipment (Bio-Rad).

Bone biopsies from multiple myeloma, MGUS, or NHL patients were investigated for E4BP4 protein. Slices (5 μm thick) were first treated with xylene and ethanol, then with EDTA to expose antigens, permeabilized with Triton X-100, and lastly with anti-E4BP4 antiserum. Parallel specimens were treated with anti-λ-chain antiserum to detect plasma cells, or with goat anti-osteopontin antiserum (Chemicon).

**Statistical analysis.** Differences were evaluated by Student's *t* test and nonparametric Mann-Whitney test (significance cutoff: *P* < 0.05).

## Results

**Gene profile of U-266-stimulated osteoblasts and E4BP4 induction.** Figure 1A illustrates the microarray results of normal osteoblasts conditioned by the U-266 myeloma (Cy5 fluorochrome) with respect to unconditioned osteoblasts (Cy3) from the same donor. Gene expression values are distributed in relation to each Cy5/Cy3 ratio and normalized to housekeeping genes. The test revealed 38 highly deregulated genes among the 20,000 genes of the chip. Seven were definitely up-regulated (Cy5/Cy3 ≥ 3.0), whereas 31 were down-regulated (Cy5/Cy3 ≤ 0.33). These genes are listed in relation to their classified functions (National Center for Biotechnology Information, Entrez Gene)<sup>1</sup> in Table 1A for those involved in bone metabolism and in Table 1B for other cell functions. Known

<sup>1</sup> <http://www.ncbi.nlm.nih.gov>

**Table 1.****(A) Major bone metabolism genes deregulated by U-266 cells in normal osteoblasts**

Gene	Accession reference	Gene product classification	Ratio
Up-regulated gene			
E4 promoter-binding protein (nuclear factor IL-3 regulated); E4BP4 (NFIL3)	NM_005384_1	Transcription factor	4.0169
Down-regulated genes			
$\alpha$ 1 type I collagen (col1a1)	M20789_1	Matrix bone protein	0.3275
Bone morphogenetic protein 8 (osteogenic protein 2; Bmp-8)	AL033527_1	Growth factor	0.3203
Runt-related transcription factor 2 (Runx2)	NM_004348	Transcription factor	0.2371
COX-2	M90100_1	Prostaglandin synthase	0.2264
Bone morphogenetic protein 4 (Bmp-4)	BC020546	Growth factor	0.2196
Bone $\gamma$ -carboxyglutamate (gla) protein osteocalcin (Bglap)	NM_199173_1	Matrix bone protein	0.2119
Bone morphogenetic protein 2 (Bmp-2)	AF040249_1	Growth factor	0.1967
Sp7 transcription factor (Osterix)	NM_152860_1	Transcription factor	0.1826

**(B) List of genes by cell functions, deregulated by U-266 cells in normal osteoblasts**

Up-regulated genes			
Cell migration and adhesion			
Unc-5 homologue c (UNC5C)	NM_003728_1	Transmembrane receptor	3.3594
Vascular cell adhesion molecule 1 (VCAM1)	M30257_1	Adhesion molecule	3.1212
Extracellular matrix organization			
Similar to tissue plasminogen activator	BC013968_1	Enzyme	3.2794
UDP-glucose pyrophosphorylase 2 (UGP2)	NM_006759_1	Enzyme	3.1043
Follistatin precursor	M19481_1	Secreted protein	3.025
Miscellaneous			
Protein kinase cAMP-dependent regulatory type II $\alpha$ (PRKAR2A)	NM_004157_1	Enzyme	3.010
Down-regulated genes			
Cell cycle			
Postmeiotic segregation increased 2-like 9 (PMS2L9)	NM_005395_1	DNA-binding protein	0.0816
Dynein light intermediate chain 2 (LIC2)	AF035812_1	Cytoplasmic protein	0.1322
Patched (Drosophila) homologue (PTCH)	NM_000264_1	Membrane receptor	0.2397
SART-1	AB006198_1	Nuclear protein	0.2973
Cytoskeletal organization			
Coronin	BC026335_1	Actin-associated protein	0.1657
Similar to calmodulin 1 (phosphorylase kinase $\delta$ )	XM_062854_1	Enzyme	0.2142
Caldesmon	M83216_1	Cytoplasmic protein	0.2308
CDC42 GAP-related protein	U62794_1	Enzyme	0.2912
Cellular stress response			
Cytochrome P450 subfamily IIIA polypeptide 43 isoform 3 (CYP3A43)	NG_000004_5	Enzyme	0.1803
Similar to ferritin heavy chain (ferritin H subunit)	XM_165439_1	Enzyme	0.2658
Na/K-ATPase $\gamma$ -subunit	U50743_1	Membrane protein	0.3206
Extracellular matrix organization			
Type XVIII collagen	AF184060	Extracellular matrix	0.1338
Hyaluronidase 1 variant 2 (HYAL1)	AF502905_1	Enzyme	0.212
Fibronectin (FN1)	U42404_1	Extracellular matrix	0.3205
Miscellaneous			
Similar to serine/threonine kinase 4	BC005231_1	Enzyme	0.0961
Methionine synthase reductase (MTRR)	AF025794_1	Enzyme	0.111
NRIF3	AF175306_1	Nuclear receptor coactivator	0.1169
Similar to protease (prosome, macropain) 26S subunit, ATPase I	XM_115995_1	Enzyme	0.1364
Diacylglycerol kinase, $\beta$ isoform 2 (DGKB)	NM_145695_1	Enzyme	0.1376
Nuclear VCP-like protein NVLP.2 (NVL.2)	U68140_1	Enzyme	0.1461
Na/K-ATPase $\beta$ -3 subunit	AF005898_1	Membrane protein	0.1531
Galactokinase 2	BC005141_1	Enzyme	0.1793
Similar to acetyl-coenzyme A transporter	BC014416_1	Enzyme	0.1815

NOTE: Gene expression levels are shown as ratio with respect to nonconditioned osteoblasts. Only genes with three-fold deregulated expression (ratio  $\geq 3.0$  or  $\leq 3.0$ ) are listed.

genes of bone metabolism including BMPs, Runx2, osteocalcin, COX-2, and Osterix were variably down-regulated, whereas E4BP4 was the up-regulated gene with the highest ratio (Cy5/Cy3 = 4.0169). We therefore investigated its induction by the other myelomas.

Figure 1B shows the levels of E4BP4 induction in normal osteoblasts by all myeloma cell lines. As depicted, a significant

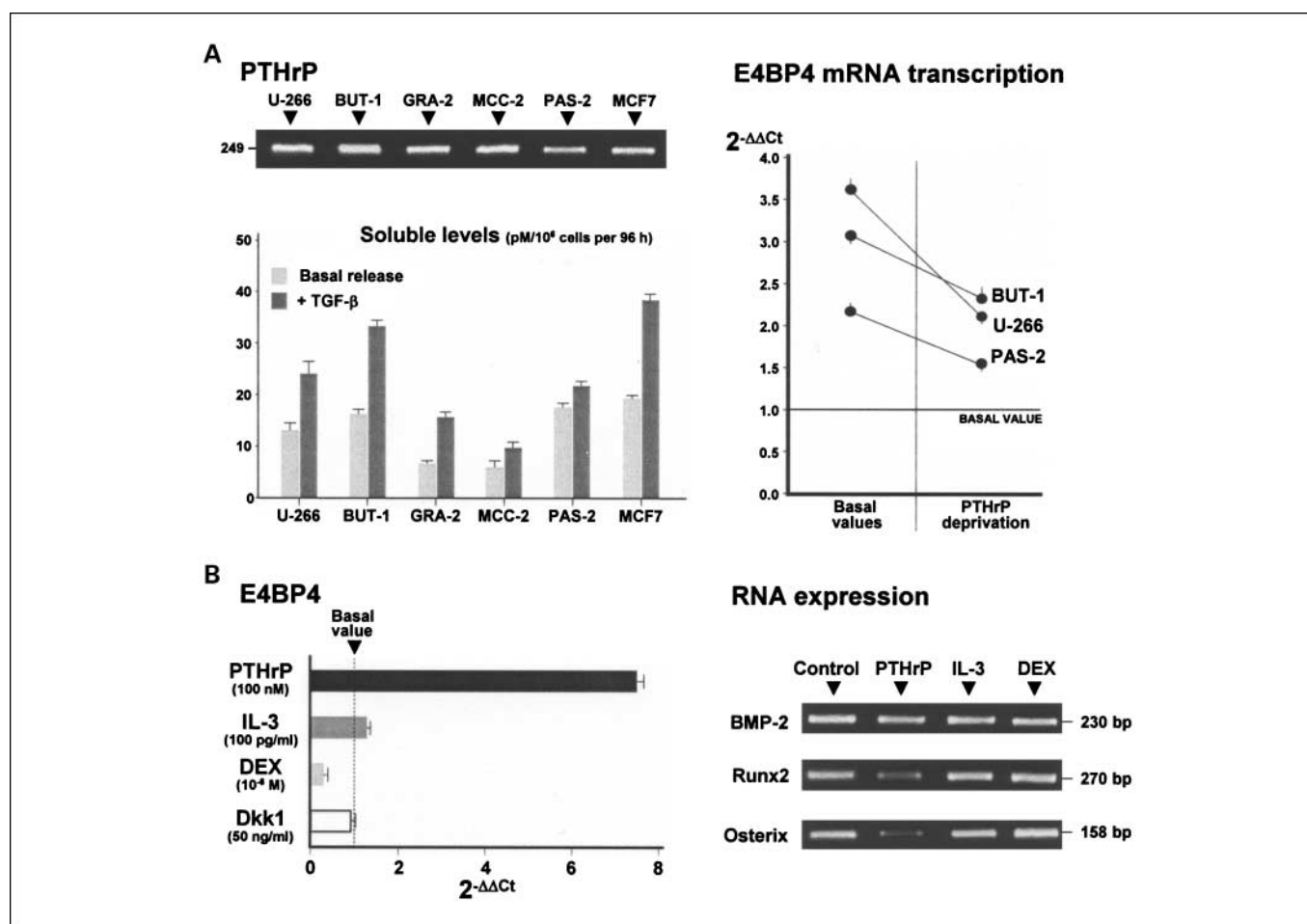
increase ( $2^{-\Delta\Delta Ct} > 1.5$ ) of E4BP4 RNA transcription, normalized to glyceraldehyde 3-phosphate dehydrogenase, was recorded in all instances compared with the control value ( $2^{-\Delta\Delta Ct} = 1.0$ ) unconditioned osteoblasts. The highest  $2^{-\Delta\Delta Ct}$  value occurred in U-266-conditioned osteoblasts ( $3.781 \pm 0.2$ ), yet lower although still significant levels of RNA transcription were induced by the other myelomas ( $P < 0.05$ ). By contrast,

osteoblasts stimulated by both Raji and CEM cells displayed little change in their basal E4BP4 RNA ( $2^{-\Delta\Delta Ct} = 1.0 \pm 0.5$  and  $0.82 \pm 0.5$ , respectively).

**PTHrP expression by myelomas and osteoblast stimulation.** Figure 2A shows that PTHrP was variably expressed as 249-bp PCR product by all myeloma cell lines and by MCF7, the PTHrP-positive control cell (*left*), whereas no band was detected by using RNA of human CEM lymphoblasts as negative control (data not shown). Soluble levels of PTHrP were also unequal because the ELISA revealed higher production by U-266, BUT-1, and PAS-2 with basic levels almost similar to MCF7 (up to 19 pmol/L). In parallel, increased PTHrP levels were always detected by transforming growth factor- $\beta$ , in keeping with plasma cell sensitivity to this cytokine (28). To support the specificity of PTHrP in inducing E4BP4, the PTHrP deprivation from supernatants of U-266, BUT-1, and PAS-2 resulted in variable although significant reductions of E4BP4 RNA ( $P < 0.05$  in all instances) in osteoblasts (*right*).

Figure 2B illustrates the real-time PCR of E4BP4 in osteoblasts treated with PTHrP, IL-3, or dexamethasone (*left*). A dramatic increase in PTHrP-treated osteoblasts gave a  $2^{-\Delta\Delta Ct}$  value of 7.621, whereas IL-3 was ineffective ( $2^{-\Delta\Delta Ct} = 1.293$ ). E4BP4 was inhibited by dexamethasone because its  $2^{-\Delta\Delta Ct}$  value was the lowest (0.185). These results corroborated the PTHrP specificity in inducing E4BP4, whereas its suppression by dexamethasone was contrary to previous data in T lymphoblasts (17). The defective E4BP4 induction by dickkopf1 ( $2^{-\Delta\Delta Ct} = 0.890$ ) is in line with the independence of this osteoblast inhibitor from the PTH-1-R pathway.

PTHrP suppressed both Runx2 and Osterix in osteoblasts (*right*). As shown, the PCR products of BMP-2, Runx2, and Osterix were quantitatively different in response to PTHrP, IL-3, or dexamethasone. Measurement of relative PCR bands by Fluor-S confirmed the decrease of Runx2 and Osterix detected by real-time PCR in PTHrP-treated osteoblasts, with respect to control or to IL-3- or dexamethasone-treated osteoblasts,



**Fig. 2.** PTHrP production by myeloma cells and *in vitro* stimulation of osteoblasts. *A, left*, PCR analysis of PTHrP expression by myeloma cell lines compared with control breast cancer cells (MCF7). The 249-bp PCR product was variably amplified by the cDNA of all myelomas (*left*) that released different amounts of soluble PTHrP molecules. Measurement of PTHrP in pmol/L was completed on 96 h cultures by dedicated ELISA and confirmed the release of PTHrP by all myelomas with lower release in both GRA-2 and MCC-2 cell cultures and by MCF7. Parallel stimulation with transforming growth factor- $\beta$  at 10 ng/mL increased the secretion of PTHrP in all instances. *Right*, PTHrP was removed by immunoprecipitation in U-266, BUT-1, and PAS-2 supernatants whose residual E4BP4 activation in normal osteoblasts was measured by real-time PCR. These PTHrP-deprived supernatants induced lower E4BP4 RNA transcription ( $P < 0.1$ ) in line with the specificity of the protein on E4BP4 gene expression. *B, left*, stimulation of osteoblasts by PTHrP, IL-3, and dexamethasone and measurement of E4BP4 (*left*) and BMP-2, Runx2, and Osterix (*right*). Significant real-time PCR values ( $2^{-\Delta\Delta Ct} > 1.5$ ) of E4BP4 transcription were detected in PTHrP-stimulated osteoblasts, whereas no effect was observed by IL-3. By contrast, dexamethasone significantly inhibited ( $2^{-\Delta\Delta Ct} < 0.5$ ) E4BP4 RNA transcription. The PCR expression of bone morphogenetic factors calculated with the Quantity One software revealed a substantial absence of any effect of PTHrP, IL-3 and dexamethasone on the expression of BMP-2, whereas Runx2 and particularly Osterix were significantly inhibited by PTHrP ( $P < 0.05$  in both instances). By contrast, both morphogenetic factors were definitely up-regulated in dexamethasone-treated osteoblasts ( $P < 0.05$ ), whereas no effect was induced by IL-3.

whereas BMP-2 was minimally affected. Linearity of semiquantitative PCR was assessed by using graded (0, 0.1, 1, 2, and 4  $\mu$ g) amounts of cDNA (data not shown). The Fluor-S trace quantity values in PTHrP-treated osteoblasts were 0.881 and 0.227 for Runx2 and Osterix, respectively, compared with 1.304 and 1.511 in untreated osteoblasts. Differences in both factors after PTHrP treatment were significant ( $P < 0.03$  in each instance), whereas IL-3 was substantially inert. By contrast, in dexamethasone-treated osteoblasts, Runx2 and Osterix PCR products increased to 3.630 and 2.296, respectively, whereas BMP-2 was again unchanged. PTHrP thus produced a clear-cut increase of E4BP4 and a concomitant inhibition of Runx2 and particularly Osterix, whereas an equivalent inverse effect was attributable to dexamethasone.

**E4BP4-silenced osteoblasts and COX-2 pathway inhibition.** E4BP4 was silenced to assess its regulatory effect in the transcription of morphogenetic factors. Figure 3A shows the efficiency of E4BP4 small interfering RNA (*left*). E4BP4 RNA transcription was decreased and clearly retarded both with ( $2^{-\Delta\Delta C_t} = 0.004$ ) and without ( $2^{-\Delta\Delta C_t} = 0.0006$ ) PTHrP stimulation compared with control nonsilenced osteoblasts. In E4BP4-silenced osteoblasts, both Runx2 and Osterix RNA  $2^{-\Delta\Delta C_t}$  values were virtually unchanged and within their basal levels after PTHrP, whereas those from nonsilenced osteoblasts reflected a significant RNA suppression (Runx2:  $0.390 \pm 0.03$ ; Osterix:  $0.19 \pm 0.002$ ). This was evidence of the negative regulation of both transcription factors by E4BP4 because its silencing kept their basal RNA levels stable after PTHrP.

We also measured both Runx2 and Osterix by investigating the COX-2 pathway in relation to E4BP4 interference. Figure 3B shows the real-time PCR levels of both morphogenetic factors in osteoblasts treated with NS-398 as COX-2 inhibitor, PGE<sub>2</sub>, or both. In osteoblasts with E4BP4 up-regulated by PTHrP, basal transcription of both factors was lower than in control osteoblasts and was unaffected by the COX-2 inhibitor, suggesting intrinsic inhibition of COX-2. Conversely, by supplementation with PGE<sub>2</sub>, Runx2 and Osterix transcription was restored to levels higher than control osteoblasts ( $P < 0.003$ ), whereas NS-398 and PGE<sub>2</sub> resulted in a minimal reduction of Runx2 and Osterix RNA probably in relation to the predominant effect of PGE<sub>2</sub>. However, employment of E4BP4-silenced osteoblasts corroborated the inhibitory effect of the gene. The basal production of both RNAs was higher than in control osteoblasts as  $2^{-\Delta\Delta C_t}$  values ( $P < 0.02$  in both instances). Its significant suppression in the presence of NS-398 is evidence of the role of COX-2. PGE<sub>2</sub> further increased the expression of both factors, particularly Osterix, whereas NS-398 more weakly inhibited PGE<sub>2</sub>-regulated RNA expression. Thus, restoration of Runx2 and Osterix by PGE<sub>2</sub> in E4BP4-up-regulated osteoblasts provided definitive evidence of the negative regulation of both factors by E4BP4 through COX-2. To support the role of this osteoblast repressor, Fig. 3C shows the alkaline phosphatase expression in PTHrP-treated as well as in E4BP4-silenced and control osteoblasts after 4 weeks of culture. Cytoplasmic accumulation of brown granules including the enzyme was apparently defective in E4BP4-up-regulated osteoblasts in response to the PTHrP treatment compared with both E4BP4-silenced and untreated osteoblasts.

**E4BP4 protein expression.** Figure 4A shows E4BP4 by Western blot analysis in osteoblasts under different conditions. Control untreated osteoblasts, as normally regulated

in E4BP4, displayed no E4BP4 in their nuclear extract, whereas those treated with U-266 or PTHrP were remarkably positive compared with CEM-conditioned and E4BP4-silenced osteoblasts.

Figure 4 also depicts both E4BP4 and Osterix RNAs in osteoblasts from patients (Fig. 4B) with respect to relative serum levels of osteocalcin and PTHrP (Fig. 4C). In all instances, the E4BP4 RNA transcription of osteoblasts from multiple myeloma patients was increased and higher than in control osteoblasts from MGUS or NHL patients. Moreover, E4BP4 was inversely related to Osterix. This was apparently inhibited because each  $2^{-\Delta\Delta C_t}$  value dropped to minimal levels of transcription ( $\leq 0.384$  in all samples). Conversely, Osterix was normally regulated in MGUS and NHL patients. With the exception of MGUS patient 3 with a minor  $2^{-\Delta\Delta C_t}$  value (0.815), all osteoblasts expressed normal or higher levels of Osterix RNA transcription ( $\geq 1.0$ ), thus emphasizing that the Osterix defect in multiple myeloma was correlated with intrinsic E4BP4 up-regulation.

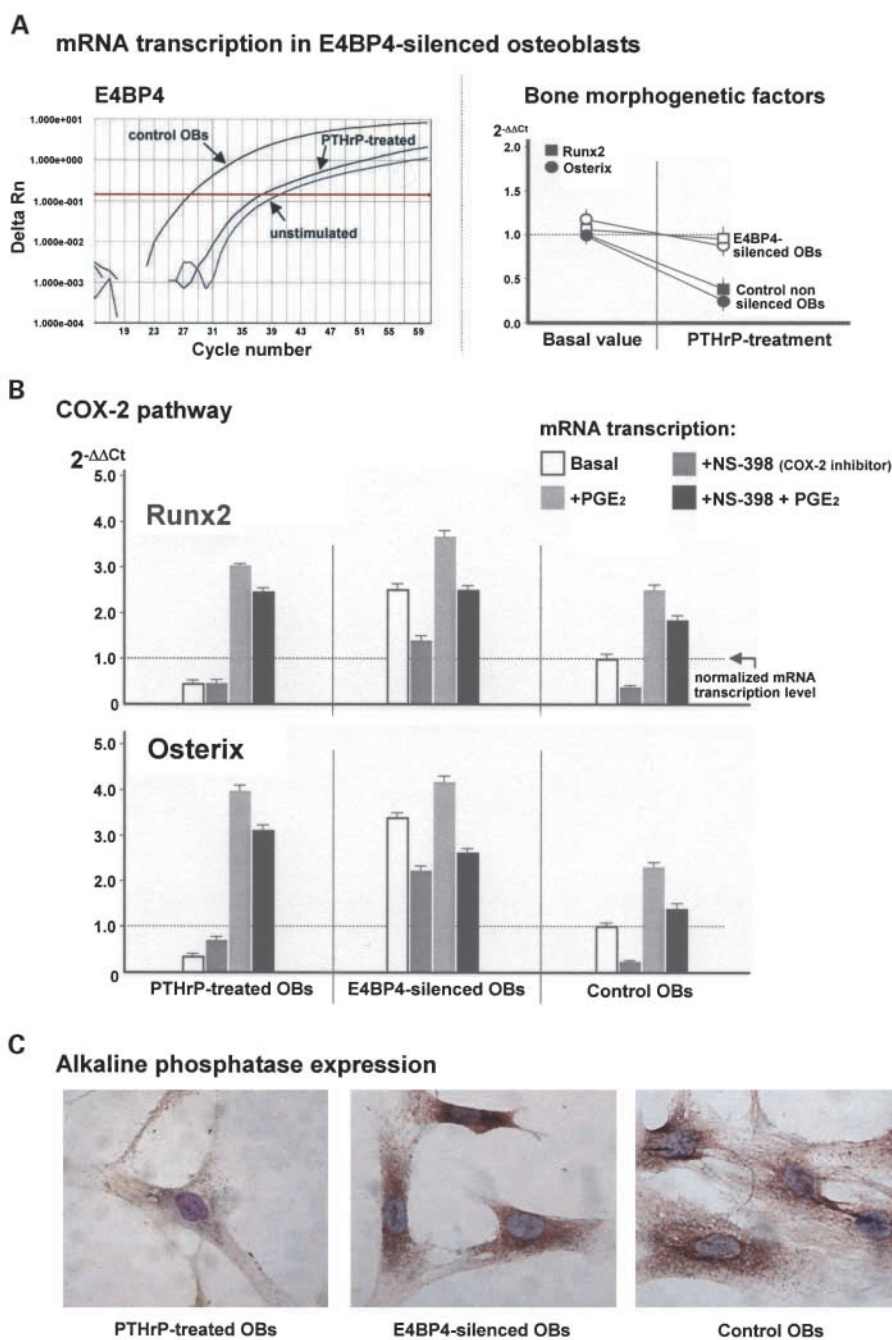
The Osterix defect in myeloma osteoblasts was paralleled by minor osteocalcin levels and elevations of serum PTHrP (Fig. 4C). All multiple myeloma patients expressed variable levels of PTHrP, ranging from 120 to 430 pmol/L with a decrease of osteocalcin in three of them. The NHL and MGUS controls uniformly expressed normal osteocalcin levels in the presence of lower concentrations of PTHrP. However, in both NHL patients and the MGUS patient 3, the PTHrP was slightly higher than the control reference limit. The quantitative difference with the multiple myeloma group of sera was approximately  $1/4.57$  ( $P < 0.05$ ). The parallel increase of PTHrP serum levels and E4BP4 RNA in multiple myeloma patients was inversely related to the concurrent reduction of both Osterix transcription and serum osteocalcin.

**E4BP4 protein detection in bone biopsies.** Figure 5A shows E4BP4 in myeloma osteoblasts (*top*), located within the trabecular bone (*left*) and facing the cortical bone (*right*). These cells are osteoblasts (*bottom*) for the expression of osteopontin (*left*), whereas the myeloma bone specimen included malignant plasma cells (*right*) identified by intracytoplasmic  $\lambda$ -chain. By contrast, no evidence of E4BP4 was revealed in control biopsies from patients with MGUS or NHL (data not shown). Detection of E4BP4 as protein product in osteoblasts from multiple myeloma patients emphasized the role *in vivo* of E4BP4 as a transcriptional repressor of osteoblast activity in MBD. Figure 5B summarizes the sequential events that may occur in bone marrow and are responsible for the functional osteoblast exhaustion.

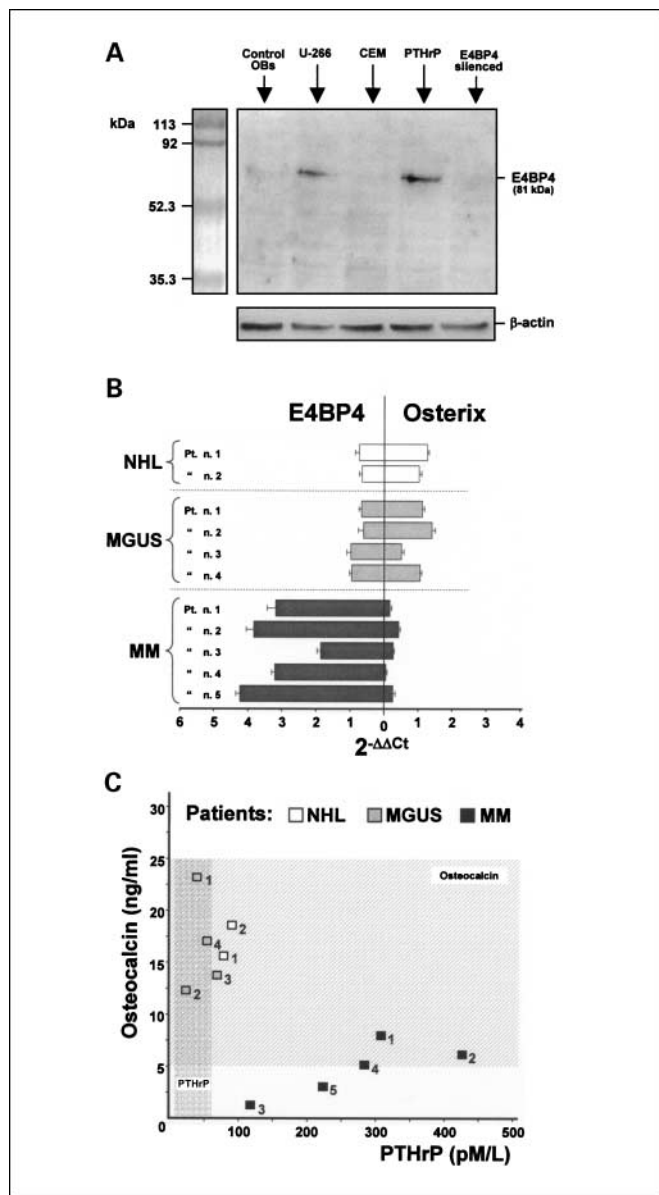
## Discussion

The pathophysiology of MBD includes the inefficacy of bone repair within typical osteolytic lesions. This study describes a novel inhibitory pathway of the osteoblast function activated by the PTHrP released by malignant plasma cells, which induces in osteoblasts E4BP4, a repressor of the bone morphogenetic factors Runx2 and Osterix.

Osteoblasts from a healthy donor were strongly conditioned by U-266 and other myelomas to overexpress E4BP4, in contrast with inhibition of most genes involved in transcription of bone factors. E4BP4 expression was closely dependent on PTHrP stimulation, and E4BP4-up-regulated osteoblasts were



**Fig. 3.** Effect of E4BP4 silencing in normal osteoblasts and COX-2 pathway activity. *A, left*, measurement of E4BP4 by real-time PCR in E4BP4-silenced osteoblasts showed a retarded and minimal RNA transcription in both unstimulated ( $2^{-\Delta\Delta C_t} = 0.0006$ ) and PTHrP-stimulated ( $2^{-\Delta\Delta C_t} = 0.004$ ) cells, whereas control nonsilenced osteoblasts produced efficient transcription of the repressor gene. *Right*, real-time PCR measurement of both Runx2 and Osterix transcription in E4BP4-silenced compared with control nonsilenced osteoblasts. Basal levels of both Runx2 and Osterix RNAs were virtually mostly unchanged after PTHrP treatment in E4BP4-silenced osteoblasts with respect to control cells. *B*, real-time PCR measurement of Runx2 and Osterix transcription through the COX-2 pathway. Levels of both Runx2 and Osterix, as major bone morphogenetic factors, were measured by real-time PCR in both PTHrP-treated and E4BP4-silenced osteoblasts with respect to control osteoblasts. Parallel cultures in each group of osteoblasts explored the effect of NS-398 as COX-2 inhibitor, PGE<sub>2</sub>, or both. In PTHrP-treated osteoblasts, the  $2^{-\Delta\Delta C_t}$  values of both factors were constitutively down-regulated compared with control osteoblasts and supplementation with NS-398 failed to produce evident variation of relative RNAs in relation to the functional expression of E4BP4. However, by adding PGE<sub>2</sub> at  $10^{-7}$  mol/L, a dramatic increase of both Runx2 and Osterix was detected compared with basal levels ( $P < 0.05$  in both instances), whereas the concurrent presence of NS-398 induced a slight down-regulation of the PGE<sub>2</sub>-restored transcription. By contrast, E4BP4-silenced osteoblasts showed high constitutive activity of both Runx2 and Osterix, whose enhanced levels were inhibited by NS-398 and slightly increased by PGE<sub>2</sub> in response to the inhibitor or activator of the COX-2 pathway, respectively. These effects were associated to the overexpression and vice versa, with the absence of E4BP4 in PTHrP-treated or E4BP4-silenced osteoblasts, respectively, and were confirmed in control osteoblasts sensitive to the inhibition of COX-2 by NS-398 or its activation by PGE<sub>2</sub>. These results supported the hypothesis that the expression of both Runx2 and Osterix in osteoblasts occurred through the integrity of COX-2 and that the E4BP4-mediated suppression of both morphogenetic factors occurred through the COX-2 inhibition. *C*, differential expression of granular cytoplasmic alkaline phosphatase in E4BP4-up-regulated osteoblasts by PTHrP compared with E4BP4-silenced and control unstimulated osteoblasts. As shown, the overexpression of the transcriptional repressor induced a maturation arrest revealed by the defective expression of the enzyme (magnification,  $\times 100$ ).



**Fig. 4.** Detection of E4BP4 protein product in osteoblasts and measurement of osteogenic factors. **A**, unstimulated (control) osteoblasts, U-266-conditioned and CEM-conditioned osteoblasts, PTHrP-treated, and E4BP4-silenced osteoblasts were screened by immunoblotting in their expression of E4BP4 protein using a polyclonal goat antiserum. The 81-kDa E4BP4 protein was expressed, although at different intensity, by osteoblasts cocultured with the myeloma cell line U-266 and by osteoblasts stimulated by PTHrP. By contrast, no evidence of E4BP4 was shown in osteoblasts stimulated by the CEM cell line. The E4BP4-silenced osteoblasts provided the negative control. The secondary antibody was revealed by ECL Plus reagent (Amersham).  $\beta$ -Actin was detected as loading control for each nuclear extract. **B**, comparison by real-time PCR quantification of E4BP4 and Osterix transcription in cultured osteoblasts. A discrepant expression of E4BP4 was detected in osteoblasts from bone biopsies of patients with multiple myeloma compared with those from control patients with NHL or MGUS. In contrast with significantly higher transcription of E4BP4 ( $2^{-\Delta\Delta Ct} > 1.5$ ) in osteoblasts from multiple myeloma patients with respect to those from NHL and MGUS patients, all five osteoblast preparations from myeloma bone biopsies showed dramatic Osterix deficiency ( $2^{-\Delta\Delta Ct} < 0.5$ ) compared with the normal reference range ( $2^{-\Delta\Delta Ct} = 1.0 \pm 0.5$ ) in the controls. This analysis supported the inversely correlated expression of E4BP4 and Osterix existing *in vivo* in osteoblasts from multiple myeloma bone biopsies. **C**, scatter plot correlating serum PTHrP with osteocalcin levels. All multiple myeloma patients displayed variable increases of PTHrP, three with a concurrent reduction of osteocalcin. By contrast, patients with NHL and MGUS expressed normal serum levels of osteocalcin and differential (sometimes increased) PTHrP concentrations. However, the mean PTHrP value in multiple myeloma patients was 4.57 higher than in the controls ( $P < 0.05$ ). Numbers refer to patients in each group; shaded areas include the corresponding normal ranges.

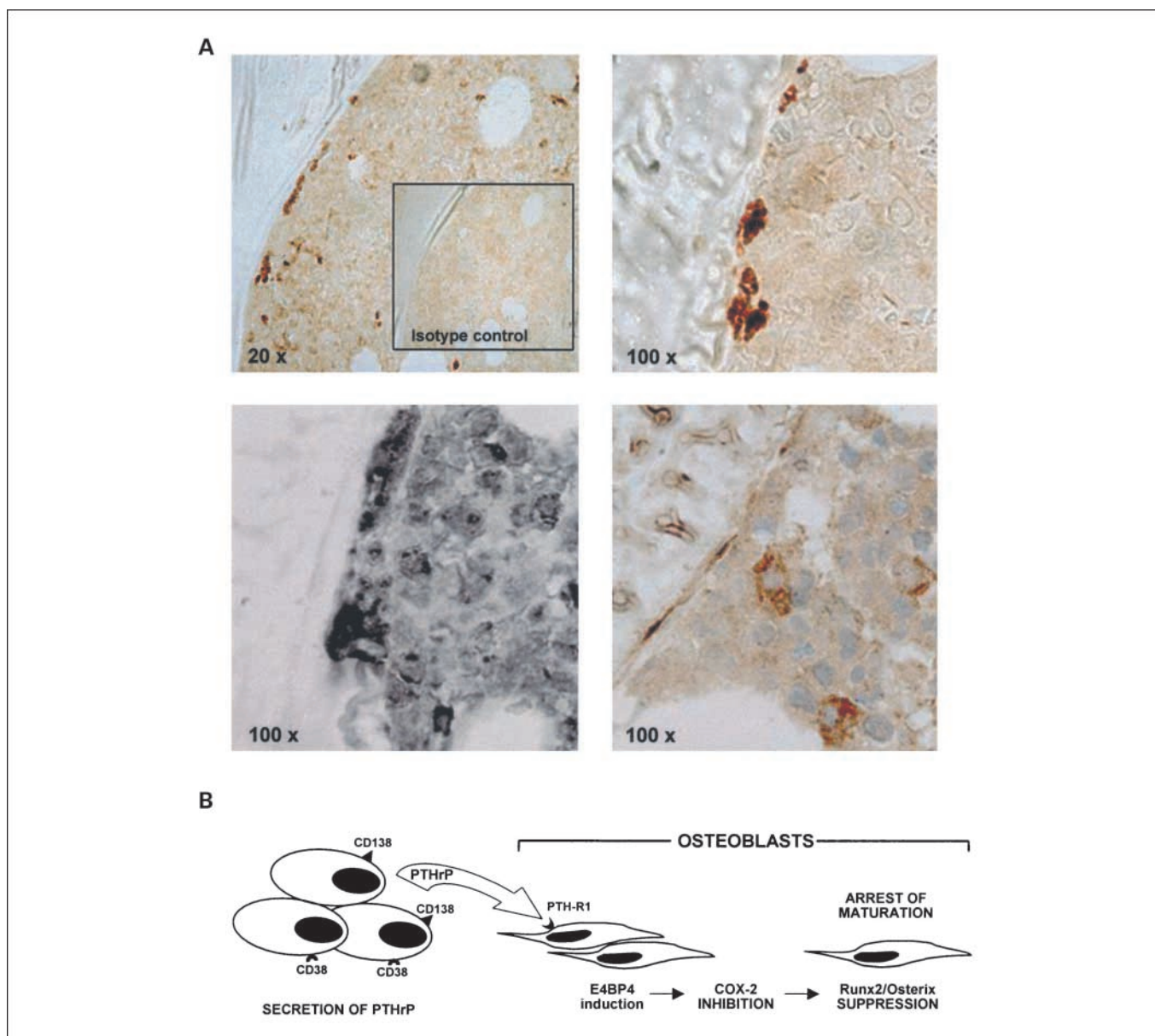
restrained in Runx2 and Osterix transcription, whereas E4BP4-silenced osteoblasts were not affected by PTHrP. Negative regulation of the COX-2 pathway by E4BP4 was shown by supplementing PTHrP-treated osteoblasts with PGE<sub>2</sub>, which promptly reestablished the transcription of both factors and overcame the defect of COX-2. That E4BP4 is active in MBD is also indicated by its high levels in osteoblasts from multiple myeloma patients showing serum elevations of PTHrP and, conversely, lower osteocalcin levels in parallel with minimal Osterix transcription in their osteoblasts. In addition, E4BP4 as protein product was detectable in osteoblasts in myeloma biopsies. These data emphasize the role of E4BP4 in the inhibitory pathway of both Runx2 and Osterix.

E4BP4, a mammalian basic leucine zipper transcription factor, was initially reported as repressor of viral promoter sequences (34), thus as effector of cell survival versus apoptosis (16, 35) in the anti-inflammatory response (36), as antagonist of proline and acidic amino acid-rich transcription factors in mammalian circadian mechanisms (37), as well as negative regulator of COX-2-mediated signals induced by the PTH in osteoblasts (30). Such a diversity of functions is related to the regulatory pathways interacting with E4BP4 in different cell types including Ras through IL-3 in pro-B cells, the glucocorticoid receptor in human T lymphoblasts, as well as the COX-2-regulated induction of both Runx2 and Osterix in osteoblasts. E4BP4 RNA is inducible in osteoblasts by the PTH binding to PTH-1-R, a G protein-coupled heptahelical molecule, which primes the transcription of primary response genes driving the suppression of osteogenesis (38). Both PTH and PTHrP bind the PTH-1-R by competitive link to regulate the calcium homeostasis, and malignant tumors as myeloma (28) release both in marrow and bloodstream variable amounts of PTHrP that promote PTH-like effects, such as inhibition of osteoblast growth by cell cycle arrest in G<sub>1</sub>, hypercalcemia, and osteoclast activation (39). By stimulating normal osteoblasts with PTHrP, we induced E4BP4 and postulated that the sequential events, including increased PTHrP secretion, induction of E4BP4 in osteoblasts, inhibition of COX-2, and suppression of both Runx2 and Osterix, may occur *in vivo* because serum elevations of PTHrP were associated to concurrent decrease of osteocalcin and defective Osterix transcription in patients with severe MBD.

E4BP4 is also inducible by IL-3 and glucocorticoids in B and T cells, respectively (16, 17). With the purpose of exploring their effect on osteoblasts, we stimulated them with IL-3 and dexamethasone and measured the expression of E4BP4 and morphogenetic factors. Interestingly, IL-3 is also thought to be detrimental to osteoblast survival in MBD, although its release by myeloma cells was first postulated (40) and then denied (41). In our study, IL-3 was unable to induce E4BP4 in osteoblasts and displayed no regulatory effect on BMP-2, Runx2, and Osterix. On the other hand, dexamethasone-treated osteoblasts underwent a concurrent down-regulation of E4BP4 and increased expression of both Runx2 and Osterix. Although E4BP4 is induced by dexamethasone in osteoblasts (21), this up-regulation in response to dexamethasone is in line with other studies (42), and correlates with its concomitant inhibition.

The E4BP4 repression domain is located within the amino acid stretch 298 to 363 and confers transcriptional inhibition through the consensus sequence expressed by the COX-2





**Fig. 5.** Expression of E4BP4 protein in myeloma bone biopsies. *A*, immunohistochemistry assays revealed the protein in osteoblasts (*top*) located within the spongy bone (*left*) and facing the cortical bone (*right*), which were positive to both osteopontin (*left*) as osteoblast marker and to the presence of myeloma cells (*right*) identified by the detection of intracytoplasmic lambda chain (*bottom*). *B*, sequential events driving the osteoblast functional exhaustion through E4BP4. In bone marrow, malignant plasma cells secrete PTHrP that induces E4BP4 in osteoblasts through the PTH-1-R. The transcriptional repressor inhibits COX-2, which in turn leads to the suppression of both Runx2 and Osterix. The final effect is the arrest of osteoblast maturation.

promoter (15), whereas both promoters of Runx2 and Osterix display E4BP4-unrelated sequences (33). The COX-2 promoter regulates, through PGE<sub>2</sub>, the differentiation of mesenchymal cells to functional osteoblasts by transcription of Runx2, Osterix, and other morphogenetic factors (34, 43). Thus, suppression of the COX-2 promoter by E4BP4 blocks this transcription. Because supplementation with PGE<sub>2</sub> restored the activity of both Runx2 and Osterix, it appeared that E4BP4 provoked the negative regulation of COX-2 in osteoblasts. However, the COX-2 constitutive defect in osteoblasts during MBD has not been investigated, although the increased expression by malignant plasma cells is apparently associated to poor prognosis in multiple myeloma (44).

Recent pathogenetic studies of MBD emphasize the defective transcription of Runx2 in pre-osteoblasts as a result of the inhibitory effect induced by myeloma cells through cell-to-cell contact or by soluble factors released in the marrow environment (1, 9). This inhibition may partially explain the sluggishness of pre-osteoblasts to progress in their maturation and the defective repair of osteolytic lesions. Dickkopf1, a critical osteoblast inhibitor, has been reported to correlate with MBD severity (4), although it is variably secreted by malignant plasma cells (5). Its inhibitory pathway, however, does not affect Runx2 (45) and is inactive in our system because U-266 cells are unable to produce functional amounts of dickkopf1 as protein product (5, 46). Here, we show that the PTHrP released

by myelomas acts as a soluble factor that inhibits Runx2 through the negative regulation of COX-2. Because bone marrow stromal cells express the PTH-1-R (47), they may also be inhibited by PTHrP in multiple myeloma.

Osterix is a three-zinc-finger transcription factor independently regulated by both Runx2 (9) and COX-2 (18) and is essential for the differentiation of Runx2-induced pre-osteoblasts to immature osteoblasts (10). Its pivotal role in osteogenesis has been shown by the inability of *Osx*<sup>-/-</sup> mesenchymal cells to differentiate to immature osteoblasts as well as the lack of osteoblastogenesis in *Osx*<sup>-/-</sup> mice (48). Osterix expression has also been associated with decreased osteolytic lesions in experimental models of murine osteosarcoma (49). In this study, we revealed by real-time PCR a severe constitutive defect of Osterix in osteoblasts from multiple myeloma patients, which was absent in those from patients with either MGUS or NHL. Moreover, RNA levels of Osterix were inversely related to E4BP4 overexpressed in patients with PTHrP serum elevations, and E4BP4 protein was detected *in vivo* in myeloma bone biopsies. That Osterix was inhibited through E4BP4 transcription was proved by the intact Osterix expression in E4BP4-silenced osteoblasts refractory to PTHrP. This provides further evidence that PTHrP stimulation of PTH-1-R critically induces E4BP4.

That E4BP4 may be involved in the defective osteogenesis of myeloma is suggested by the accumulation of its protein product in osteoblasts from patients with active MBD. Detection of E4BP4 as either RNA in cultured osteoblasts or protein in bone biopsies is in line with the concurrent demonstration of both Runx2 and Osterix deficiency and with the increased activity of PTHrP released by myeloma cells and detectable in sera. Previous studies have shown either at mRNA level or by immunohistochemistry the production of PTHrP by myeloma cells (28, 50), although an extended screening of myeloma sera by ELISA could better correlate its role with the severity of MBD.

In conclusion, based on previous studies on PTHrP in osteoblast biology (39), we have explored its role in MBD and identified a new molecular defect in myeloma osteoblastogenesis. This defect involves the deregulated transcription of Runx2 through a transcriptional repressor of the osteoblast function directly elicited by myeloma cells that release PTHrP. Moreover, Osterix is also suppressed through the same inhibitory pathway and contributes to the pathogenesis of MBD.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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