Production of Wnt Inhibitors by Myeloma Cells: Potential Effects on Canonical Wnt Pathway in the Bone Microenvironment

Nicola Giuliani,[1] Francesca Morandi,[1] Sara Tagliaferri,[1] Mirca Lazzaretti,[2] Gaetano Donofrio,[3] Sabrina Bonomini,[1] Roberto Sala,[4] Marcellina Mangoni,[1] and Vittorio Rizzoli[1]

[1]Hematology and Bone Marrow Transplantation Center, Department of Internal Medicine and Biomedical Science, [2]Pathology, [3]"Dipartimento di Salute Animale, Sezione di Malattie Infettive degli Animali," and [4]Department of Experimental Medicine, University of Parma, Parma, Italy

Abstract

Osteoblast impairment occurs within multiple myeloma cell infiltration into the bone marrow. Canonical Wnt signaling activation in osteoprogenitor cells is involved in osteoblast formation through the stabilization of dephosphorylated β-catenin and its nuclear translocation. The effects of multiple myeloma cells on Wnt signaling in human mesenchymal/osteoprogenitor cells are unclear. In 60 multiple myeloma patients checked, we found that among the Wnt inhibitors, Dickkopf-1 and secreted frizzled-related protein-3 were produced by multiple myeloma cells. However, although multiple myeloma cells or multiple myeloma bone marrow plasma affected expression of genes in the canonical Wnt signaling and inhibited β-catenin stabilization in murine osteoprogenitor cells, they failed to block the canonical Wnt pathway in human mesenchymal or osteoprogenitor cells. Consistently, Wnt3a stimulation in human osteoprogenitor cells did not blunt the inhibitory effect of multiple myeloma cells on osteoblast formation. Consequently, despite the higher Wnt antagonist bone marrow levels in osteolytic multiple myeloma patients compared with nonosteolytic ones, β-catenin immunostaining was not significantly different. Our results support the link between the production of Wnt antagonists by multiple myeloma cells and the presence of bone lesions in multiple myeloma patients but show that myeloma cells do not inhibit canonical Wnt signaling in human bone microenvironment. [Cancer Res 2007;67(16):7665–74]

Introduction

Multiple myeloma is a plasma cell malignancy characterized by a high capacity to induce osteolytic bone lesions (1). Histomorphometric studies have shown that in multiple myeloma patients with bone lesions, there is uncoupled or imbalanced bone remodeling with increased bone resorption and decreased or absent bone formation due to osteoblast impairment that occurs with multiple myeloma cell infiltration into the bone marrow (2–4). The mechanisms by which myeloma cells affect osteoblast formation and function are currently under investigation (4). Recently, we have shown that multiple myeloma cells inhibit osteoblastogenesis by blocking the activity of the critical osteoblast transcription factor Runx2 in osteoprogenitor cells (5). However, other mechanisms could be involved in multiple myeloma–induced osteoblast impairment.

Growing evidence suggests that Wnt signaling has a critical role in the regulation of osteoblast formation (6). Canonical Wnt signaling pathway is activated by Wnt1/3a that triggers the glycogen synthase kinase 3 (GSK3)/Axin complex leading to the stabilization and nuclear translocation of the active dephosphorylated β-catenin, which in turn activates the LEF-1/TCF transcription system (6, 7). It has been shown in murine systems that activation of canonical Wnt signaling in osteoblast progenitors induces osteogenic differentiation (6, 8). In particular, bone morphogenic protein (BMP)-2 or other osteogenic molecules induce osteoblastic differentiation of murine mesenchymal stem cells by stimulating Wnt signaling through the modulation of Wnt stimulators and/or inhibitors (6, 8). Several molecules negatively regulate canonical Wnt signaling inducing phosphorylation and subsequent degradation of β-catenin. Dickkopfs (DKK), including DKK-1, the secreted frizzled-related proteins (sFRP), such as sFRP-1, sFRP-2, sFRP-3, and sFRP-4, and Wnt inhibitory factor (Wif-1) are the major soluble Wnt inhibitors present in murine osteoblasts, which block early osteoblast formation and induce the death of immature cells (6, 9, 10).

Recently, it has been reported that multiple myeloma cells produce the Wnt inhibitor, DKK-1, and a correlation between its expression by microarray analysis and the presence of bone lesions in multiple myeloma patients has been shown (11). However, the potential effect of multiple myeloma cells on Wnt signaling cascade in osteoblasts and osteoblast progenitors has not yet been extensively investigated, and the mechanism by which DKK-1 production by multiple myeloma cells is related to bone destruction is still unclear. For example, a neutralizing anti-DKK-1 antibody could block the inhibitory effect of bone marrow plasma from multiple myeloma patients on BMP-2–induced alkaline phosphatase expression and osteoblast formation by a murine mesenchymal cell line (11) but failed to block the inhibitory effects of multiple myeloma cells on human bone marrow osteoblast formation (5). In this study, we evaluate the production of Wnt inhibitors by human myeloma cells and their potential effect on Wnt signaling in osteoblast progenitors in human and murine systems.

Materials and Methods

Reagents and Cytokines

Recombinant human (rh) interleukin-6, rhBMP-2, and murine BMP-2 were obtained from Endogen. rhDKK-1, Wnt3a, and sFRP-3 were purchased from R&D Systems. Dexamethasone, ascorbic acid, and β-glycerophosphate were obtained from Sigma-Aldrich. RPMI 1640, DMEM, and α-MEM culture media as well as glutamine, penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Invitrogen Life Technologies.

Requests for reprints: Nicola Giuliani, Hematology and Bone Marrow Transplantation Center, Department of Internal Medicine and Biomedical Science, University of Parma, via Gramsci 14, 43100 Parma, Italy. Phone: 00390521903299; Fax: 00390521903264; E-mail: Nicola.Giuliani@unipr.it or N_giuliani@yahoo.com.

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Patients

We studied 60 patients with newly diagnosed multiple myeloma in stages I to III. In addition, 16 subjects with monoclonal gammopathy of uncertain significance were included in the study. All patients underwent total body X-rays to identify the presence of osteolytic bone lesions and or diffuse osteoporosis. Patients negative by standard X-rays underwent magnetic resonance imaging scans. Bone disease was defined based on the presence of one or more lesions on X-rays or magnetic resonance imaging scan. Bone marrow aspirates and bone biopsies were obtained from the iliac crest of all multiple myeloma patients at diagnosis after obtaining informed consent according to the Declaration of Helsinki and our local ethics committee. The approval of the study was obtained from the University of Parma Institutional Review Board. Bone marrow plasma was obtained after centrifugation from bone marrow aspirates treated with EDTA to prevent clotting.

Cells and Cell Culture Conditions

Cell lines. Human myeloma cell lines (HMCL) XG-6, XG-1, and JJN3 were obtained from Dr. Bataille (INSERM UMR 601, Nantes, France). U266 was obtained from the American Type Culture Collection. OPM2 and RPMI-8226 were purchased from DSM. HMCLs were cultivated as described previously (12). Human trabecular SV40-transfected osteoblasts (HOBIT) were a generous gift from Dr. Riggs (Mayo Clinic, Rochester, MN). An immortalized mesenchymal/stromal cell line was a kind gift from Dr. Giuseppe Gaipa (Clinica Pediatrica Universitá, Milano-Bicocca, Monza, Italy). Murine cell lines C2C12 and MC3T3 were obtained from DSM.

Primary human multiple myeloma cells, mesenchymal cells, and osteoprogenitor cells (PreOB). Primary multiple myeloma cells were isolated from bone marrow mononuclear cells (BMMNC) of multiple myeloma patients at diagnosis by immunomagnetic beads using anti-CD138 monoclonal antibody (mAb)–coated microbeads (MACS, Miltenyi Biotec). Only samples with purity >90%, checked by flow cytometry, were used immediately after purification in the study. In selected experiments, primary multiple myeloma cells or HMCLs (5×10^5) were incubated in the presence or absence of rhDKK-1 (20–700 ng/mL) or blocking anti-DKK-1 mAb (1–5 μg/mL; ref. R&D Systems) for 24 to 48 h.

Human bone marrow mesenchymal cells (HMSC) and human osteoprogenitor cells (PreOB) were obtained from primary bone marrow adherent cells after an attachment period and incubated in the absence or presence of osteogenic differentiation medium [α-MEM with 15% FCS, 2 mmol/L glutamine, ascorbic acid (50 μg/mL), and dexamethasone at 10^{-8} mol/L] as described previously (12).

Semiconfluent HMSC or human PreOB or osteoblastic cells (HOBIT) or murine MC3T3 and C2C12 cells (2×10^6) were incubated with or without BMP-2 (50 ng/mL; Endogen) in the presence or absence of rhDKK-1 or sFRP-3 (20–700 ng/mL) or Wnt3a (50–500 ng/mL) for 12 to 48 h.

Cocultures. A series of coculture experiments were done with HMCLs or fresh purified multiple myeloma $CD138^+$ cells (10×10^6 cells) and confluent HMSC or immortalized stromal cell lines or osteoprogenitor cells (PreOB) or HOBIT cells (2×10^6) or the murine osteoprogenitor cell lines MC3T3 or C2C12. Myeloma cells were added directly to the cultures or placed in a Transwell insert in RPMI 1640 with 10% FCS or bone marrow plasma (10%) from multiple myeloma patients for 12 to 48 h in the presence or absence of Wnt3a (50–500 ng/mL) or BMP-2 (50 ng/mL). At the end of culture period, cocultures were depleted of myeloma cells using a negative immunoselection with anti-CD138 mAb (MACS).

Alkaline phosphatase and bone nodule assays. Osteogenic differentiation was assessed in cocultures in the presence of a Transwell insert using either human BMMNC cells with HMCLs as described previously (5) or murine osteoprogenitor cells MC3T3 with HMCLs in RPMI 1640 at 15% of FBS in the presence of ascorbic acid (50 μg/mL) and BMP-2 (50 ng/mL). Colony-forming unit fibroblasts (CFU-F) positive for alkaline phosphatase expression were checked after 15 days in human BMMNC by a semiquantitative histochemical kit (Sigma-Aldrich) followed by 1D Image Analysis Software analysis (Kodak Digital Science). Similarly, alkaline phosphatase expression was determined after 7 days in MC3T3 cells. Nodule formation was evaluated in human and murine cell cultures after 21 and 12 days, respectively, by 2% of Alizarin red staining (Sigma-Aldrich) and microscopic quantification as described previously (5).

Reverse Transcription-PCR Amplification

For reverse transcription-PCR (RT-PCR), 1 μg of RNA was reverse transcribed with 400 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer's protocol. cDNAs were amplified by PCR using specific primer pairs: DKK-1, 5′-GGTATTCCAGAAGAACCACC-3′ (forward) and 5′-GAGAGCCTTTT-CTCCTATGC-3′ (reverse); sFRP-1, 5′-TCTACACCAAGCCACCTCAG-3′ (forward) and 5′-CAGTCACCCCATTCTTCAGG-3′ (reverse); sFRP-2, 5′-AGTT-CCTGTGCTCGCTCTTC-3′ (forward) and 5′-AATGGTCTTGCTCTT-GGTCTC-3′ (reverse); sFRP-3, 5′-ACATGACTAAGATGCCCAACCAC-3′ (forward) and 5′-GAGTGCATCCCTCACACTTCTCAG-3′ (reverse); sFRP-4, 5′-AACATCACGCGGATGCCCAACCA-3′ (forward) and 5′-GATTACTACGA-CTGGTGCGCCCG-3′ (reverse); and β_2-microglobulin, 5′-CTCGCGCTAC-TCTCTTCTCTTTCTGG-3′ (forward) and 5′-GCTTACATGTCTCGATCC-CACTTAA-3′ (reverse). The annealing temperatures (T_a) are as follows: 58°C for DKK-1, 60°C for sFRP-1, 57°C for sFRP-2, 60°C for sFRP-3, 69°C for sFRP-4, and 63°C for β_2-microglobulin. The product sizes are the following: 185 bp for DKK-1, 616 bp for sFRP-1, 386 bp for sFRP-2, 701 bp for sFRP-3, 825 bp for sFRP-4, and 334 bp for β_2-microglobulin. PCRs were done in a thermal cycler (MiniCycler MyResearch) for 30 cycles. The following positive controls have been used for the PCRs: sFRP-1, sFRP-2, sFRP-3, and sFRP-4 (fetal and adult human cardiomyocytes) and DKK-1 (HOBIT).

Microarray Expression Analysis

Total cellular RNA was extracted from cells using RNeasy Mini kits (Qiagen SpA). RNA was quantified by Nanodrop ND-1000 (Celbio SpA). Using the True-Labeling AMP Linear RNA amplification kit (SuperArray, DBI), the mRNA was reversely transcribed to obtain cDNA and converted into biotin-labeled cRNA using biotin-16-UTP (Roche) by *in vitro* transcription. Before hybridization, the cRNA probes were purified with an ArrayGrade cRNA cleanup kit (SuperArray). The purified cRNA probes were then hybridized to the pretreated Oligo GEArray Human Wnt Signaling Pathway arrays (OHS-043, SuperArray), which cover 114 Wnt-related genes plus controls or to Oligo GEArray Murine Wnt Signaling Pathway arrays (OMM-043, SuperArray). After washing steps, array spots binding cRNA were detected by the chemiluminescence method according to the manufacturer's procedure. Spots were then analyzed and converted into numerical data by using the Personal Densitometer and ImageQuant software (Molecular Dynamics).

Real-time PCR

Total cellular RNA was extracted from cells using RNeasy Mini kits. RNA was quantified by Nanodrop ND-1000. Using Reaction Ready First-Strand cDNA Synthesis kit (SuperArray), 1 μg total RNA was reverse transcribed and then real-time PCR was done by adding cDNA directly to PCR Master Mix containing SYBR Green and References Dyes (SuperArray). The mixtures were then aliquoted into 96-well PCR array plates, which profile the expression of 84 Wnt signaling-specific genes plus controls for both the human (APH-043) and mouse (APM-043) Wnt signaling pathways. Thermal cycling was done according to the manufacturer's protocol (T_a, 55°C; total cycles, 40). Finally, we determined the fold changes in expression by $\Delta\Delta C_t$ as described previously (13).

Western Blot Analysis

Nuclear and cytosolic extracts were obtained using a commercial kit (Active Motif). One hundred micrograms of cytosolic proteins and 20 μg of nuclear proteins were evaluated, respectively. β-Catenin protein expression was checked using as primary antibody either anti-β-catenin mAb for total β-catenin (Chemicon International) or anti-β-catenin mAb recognizing the active dephosphorylated nuclear form of human and mouse origin (amino acids 27–37; Alexis Biochemicals) or anti-β-catenin mAb recognizing the inactive phosphorylated form ($pSer^{45}$; Sigma). The following primary antibodies were also used in the study: polyclonal anti-sFRP-3, anti-TCF4, anti-LEF-1, anti-Axin, and anti-GSK3β (Santa Cruz Biotechnology, Inc., DBI) and anti-DKK-1 (R&D Systems). Anti-β-actin (Sigma) and anti–histone H1 mAbs were used as internal controls (Upstate) for cytosol and nuclear lysates, respectively. Immunoblot procedures were done as described previously (5).

TOP/FOP-FLASH Wnt Reporter

Both human (PreOB) and murine osteoprogenitor cells (C2C12) and human osteoblasts (HOBIT) were electroporated with TOP-FLASH or FOP-FLASH Wnt reporter plasmids (Millipore Corp.) containing wild-type (WT) or mutant TCF DNA binding sites by using the apparatus Opti Plus (Equibio). Cells were also cotransfected with the RL-TK *Renilla* plasmid (Promega). Following transfection, cells were plated at 5×10^4 per well in a 24-well plate and exposed to Wnt3a (50–500 ng/mL) or bone marrow plasma from multiple myeloma either positive or negative for DKK-1 expression for 12 h before luciferase assay. Reporter activity was assayed by using the Dual-Luciferase Reporter System (Promega). Results were normalized to *Renilla* values of each sample. The reporter assay results represent the average of two independent experiments.

ELISA and Enzyme Immunometric Assay

The amount of DKK-1 and sFRP-3 in myeloma cell conditioned media and in bone marrow plasma was evaluated by ELISA assay in 96-well immunoplates previously coated overnight with 100 μL anti-rhDKK-1 or anti-sFRP-3 mAb (R&D System) at 4 μg/mL according to the manufacturer's protocol. The range of sensitivity for the ELISA test was 4 to 0.125 ng/mL for DKK-1 and 40 to 0.312 ng/mL for sFRP-3 with a coefficient of variation (CV) intra-assay of 0.4% and 1% and a CV interassay of 10% and 8%, respectively.

The total levels of β-catenin were evaluated in cell lysates (nuclear and cytosol) using a specific enzyme immunometric assay according to the manufacturer's procedures (Assay Design).

Immunohistochemistry

For immunohistochemical staining, human PreOB (3×10^4) or HOBIT cells were incubated in tissue culture chambers on glass slides (Falcon, BD) with $CD138^+$ multiple myeloma cells or HMCLs placed in a Transwell insert for 48 to 72 h. At the end of culture period, glass slides were fixed in cold acetone or 4% paraformaldehyde and after washing incubated with anti-β-catenin (1:1250; Chemicon International MAB2081) at 4°C. Slides were then incubated with fluorescinate goat anti-mouse secondary antibody (goat anti-mouse IgG-FITC, Sigma F0257) for 1 h at 37°C and counterstained with 4′,6-diamidino-2-phenylindole.

β-Catenin immunostaining was also done on bone marrow biopsies obtained from a subgroup of 20 multiple myeloma patients (13 osteolytic and 7 nonosteolytic ones). Samples were fixed in B5-formalin mixture, decalcified by EDTA, and embedded in paraffin. Serial sections of 3 μm thick were processed for immunohistochemical staining with anti-β-catenin (working dilution, 1:1250) using the immunoperoxidase technique as described previously (12). After the acquisition of bioptic images by FOTOVIX (Tamron), the total trabecular area of each section was measured using a software analyzer (ImagePro Plus 4.5) and osteoblastic cells were identified, as described previously (12).

Results

Production of Wnt inhibitors by human multiple myeloma cells and their bone marrow levels in multiple myeloma patients. We determined the potential expression and production of the Wnt inhibitors by HMCLs and fresh purified $CD138^+$ multiple myeloma cells and found that DKK-1 mRNA was expressed in XG-1 and JJN3 and weakly expressed in XG-6 of the six HMCLs tested (Fig. 1*A*). sFRP-3 mRNA was present in XG-1 and XG-6 and negative in the other HMCLs (Fig. 1*A*). Weak expression of sFRP-2 was observed in U266 and JJN3 cells, whereas sFRP-1 and sFRP-4 were not detected in all HMCLs tested (data not shown).

We tested 60 $CD138^+$ multiple myeloma cells by RT-PCR and found that 74% of multiple myeloma patients expressed DKK-1 mRNA at diagnosis, whereas 60% expressed sFRP-3 mRNA. All the patients tested were negative for sFRP-1, sFRP-2, and sFRP-4. DKK-1 and sFRP-3 expression was further analyzed in relation to the bone status of the multiple myeloma patients. Although DKK-1 and sFRP-3 expression was positive in 81% and 73% of osteolytic patients and 50% and 37% of nonosteolytic ones, respectively, these values do not reach statistical significance (two-tailed $P = 0.2$ and 0.07, χ^2). On the other hand, comparing multiple myeloma patients positive for DKK-1 and sFRP-3 with those negative for both molecules, we found a significant statistical correlation with the presence of bone lesions ($P = 0.03$). DKK-1 and sFRP-3 expression was analyzed at protein level in HMCLs and fresh purified multiple myeloma cell lysates and conditioned media by Western blot analysis (Fig. 1*B*) and ELISA assay (Fig. 1*C*), respectively. We found that a lower number of multiple myeloma patients expressed the Wnt inhibitors at the protein level compared with mRNA. DKK-1 and sFRP-3 were detectable in bone marrow plasma of 90% and 76% of multiple myeloma patients, respectively. Significant higher DKK-1 and sFRP-3 levels were detected in multiple myeloma patients (median DKK-1 levels, 2.84 ng/mL; range, 0.55–91.55 ng/mL; median sFRP-3 levels, 1.53 ng/mL; range, 0–27 ng/mL) compared with monoclonal gammopathy of uncertain significance subjects (DKK-1, 1.37 ng/mL; range, 0–4.12 ng/mL; sFRP-3, 0.53 ng/mL; range, 0–6.82 ng/mL; $P = 0.05$ and 0.02, respectively, nonparametric two-tailed test; Fig. 1*D*). The median bone marrow plasma levels of DKK-1 were 7.70 ng/mL (range, 1.55–91.55) in $DKK\text{-}1^+$ patients compared with 1.19 ng/mL (0–1.47) observed in negative patients ($P < 0.01$). Median levels of sFRP-3 in bone marrow were 2.05 ng/mL in $sFRP\text{-}3^+$ multiple myeloma patients and undetectable in $sFRP\text{-}3^-$ ones (Fig. 1*D*). Finally, considering the bone status of multiple myeloma patients, we found that osteolytic patients showed significantly higher bone marrow DKK-1 levels compared with nonosteolytic ones (median, 6.85 versus 1.19 ng/m; $P = 0.03$), whereas those of sFRP-3 did not reach a statistical significance [3.3 versus 1.12 ng/mL; $P = 0.088$ not significant (NS); Fig. 1*D*].

Effect of multiple myeloma cells on Wnt signaling gene expression by osteoprogenitor cells in coculture. Wnt signaling gene expression was evaluated in PreOB after 24 h of coculture with purified $CD138^+$ multiple myeloma cells positive for DKK-1 in the presence of a Transwell insert. No significant effect was observed on the expression of the negative regulators of Wnt (fold change, <1) DKK-1; sFRP-1, sFRP-2, sFRP-3, and sFRP-4; or the Wnt activator ligands Wnt1, Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt6, Wnt8a, and Wnt10a or CTNNB1 (β-catenin) or on the transcription factors LEF-1 and TCF7L1. A significant inhibitory effect was observed on the expression of the Frizzled-1 signaling pathway molecules AXIN1 (fold change, −4.73), frizzled homologue (FZD) 4 (fold change, −2.23), and FZD5 (fold change, −3.48). Similar results were obtained with the HMCLs XG-1 in coculture (data not shown). To compare the effect of multiple myeloma cells on Wnt signaling in human and murine osteoprogenitor cells, we did microarrays and real-time PCR for Wnt signaling in murine osteoprogenitor cells MC3T3 incubated in the presence of BMP-2 and cocultured with $CD138^+$ multiple myeloma cells positive for DKK-1. Similarly to human PreOB, no significant effect was observed in MC3T3 on the expression of the negative regulators of Wnt (fold change, <1) DKK-1 and sFRP-1, sFRP-3, and sFRP-4, on the Wnt activator ligands Wnt1, Wnt3, Wnt3a, Wnt4, Wnt5a, and Wnt8a, and on CTNNB1, whereas a significant inhibition of AXIN1 (fold change, −5.05), FZD2 (fold change, −1.93), and FZD4 (fold change, −3.73) expression was detected. Interestingly, we found a significant down-regulation of the Wnt activator ligands Wnt6 (fold change, −4.77) and Wnt10a (fold change, −4.28) together with an up-regulation of the Wnt antagonist sFRP-2 (fold change, 1.47) and the Wnt receptor LRP5 (fold change, 5.14) and LRP6 (fold change, 6.88).

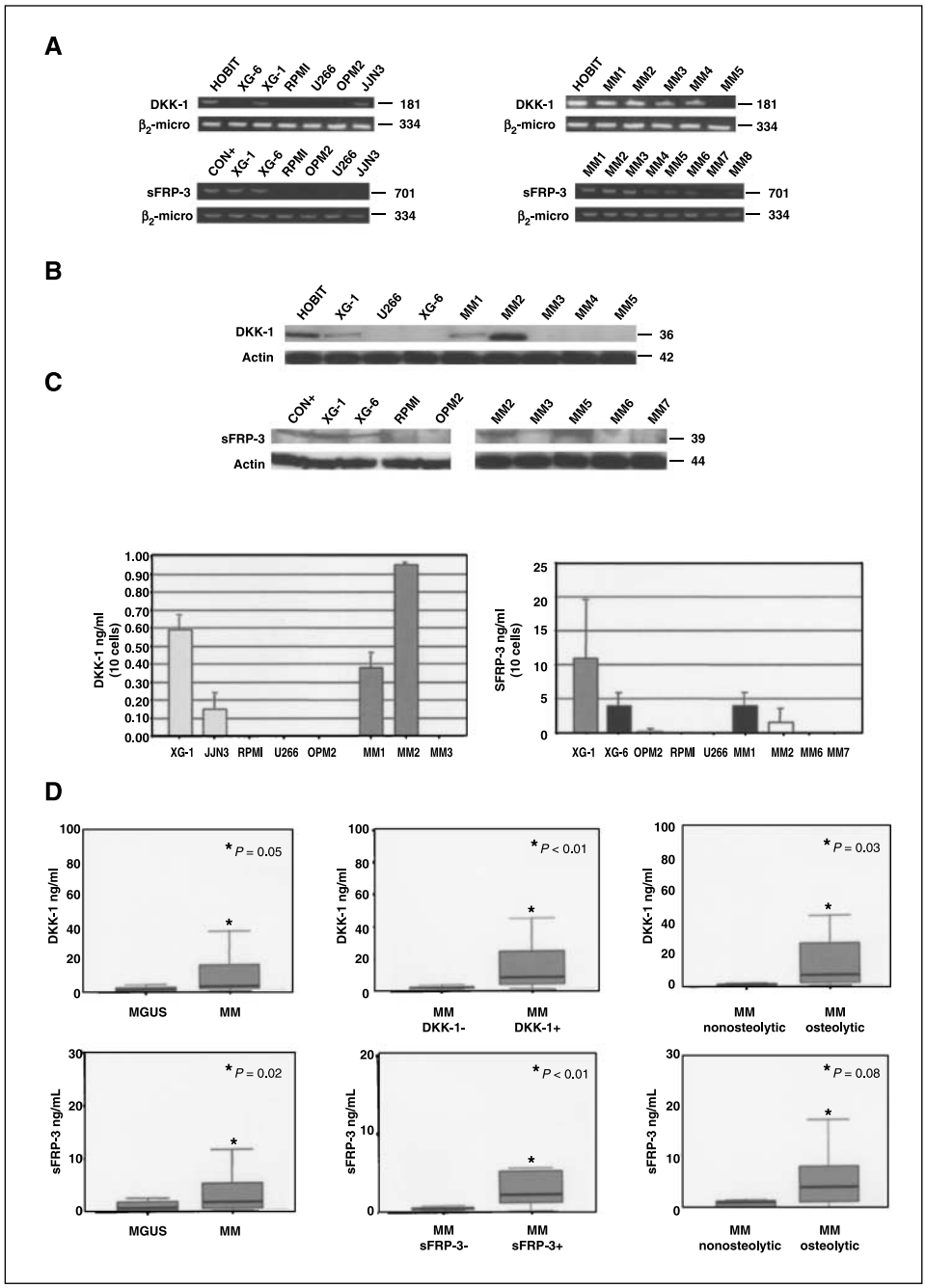


Figure 1. Production of DKK-1 and sFRP-3 by multiple myeloma cells and their bone marrow levels in multiple myeloma patients: relationship with bone lesions. mRNA was extracted from HMCLs [XG-6, XG-1, RPMI-8226 (*RPMI*), U266, OPM2, and JJN3] and purified CD138$^+$ multiple myeloma (*MM*) cells from multiple myeloma patients. The expression of DKK-1 and sFRP-3 was evaluated by RT-PCR using specific primer pairs. The following positive control (*CON+*) was used: sFRP-3 (human cardiomyocytes) and DKK-1 (HOBIT; *A*). DKK-1 and sFRP-3 protein expression was evaluated by Western blot analysis in cell lysates from HMCLs and purified CD138$^+$ multiple myeloma cells (*B*). Aliquots of HMCLs and purified multiple myeloma cell conditioned media were assessed for DDK-1 and sFRP-3 levels by ELISA as described in Materials and Methods. *Columns,* mean levels of three repeated samples; *bars,* SD (*C*). DKK-1 and sFRP-3 levels were measured by ELISA assay in the bone marrow plasma of monoclonal gammopathy of uncertain significance (n = 16) and multiple myeloma patients (n = 50) and compared between multiple myeloma patients positive and negative in DKK-1 and sFRP-3, respectively, and between osteolytic and nonosteolytic ones. Box plots represent the median of DKK-1 and sFRP-3 bone marrow levels with the 25th to 75th percentiles (*D*).

Effect of multiple myeloma cells on canonical Wnt signaling in mesenchymal/osteoprogenitor cells: β-catenin accumulation and stabilization. To investigate whether multiple myeloma cells may affect canonical Wnt signaling, we have evaluated the accumulation and stabilization of β-catenin as the hallmark of the activation of canonical Wnt signaling. First, to validate our assay system, we tested the expression of β-catenin in osteoprogenitor cells. The presence of dephosphorylated β-catenin was shown in bone marrow adherent cells and a slight increase of both cytosolic and nuclear β-catenin levels was observed after 2 weeks of osteogenic differentiation compared with control. In contrast, we found that HMCLs did not express dephosphorylated β-catenin with the exception of JJN3 but that the majority of purified CD138$^+$ multiple myeloma cells expressed dephosphorylated β-catenin (Fig. 2*A*).

We then did cocultures with or without a Transwell insert to analyze the expression of β-catenin in PreOB and undifferentiated HMSC. No significant inhibitory effect on total β-catenin was observed at both cytosolic and nuclear level after coculture in both the conditions using either XG-1 (positive for DKK-1 and sFRP-3) or OPM2 (negative for both the Wnt inhibitors; Fig. 2*B*). Consistently, active dephosphorylated β-catenin was not modified at both nuclear and cytosolic level and LEF-1 and TCF4 nuclear levels were not changed (Fig. 2*B*). Similarly, HMCLs did not affect cytosolic or nuclear β-catenin expression in osteoblastic HOBIT cells (data not shown). The lack of effect of HMCLs on β-catenin accumulation in PreOB was also confirmed by ELISA assays by measuring β-catenin levels in both cytoplasm and nucleus and by immunofluorescence (Fig. 2*C*).

These observations were expanded by testing either purified $CD138^+$ multiple myeloma cells or bone marrow plasma from multiple myeloma patients, which were both positive and negative for DKK-1 expression. As shown in Fig. 2*D*, no significant inhibitory effect was observed on cytosolic or nuclear expression of total and active dephosphorylated β-catenin. Similar results were obtained when cocultures were done in the presence of BMP-2 or Wnt3a (Fig. 2*D*).

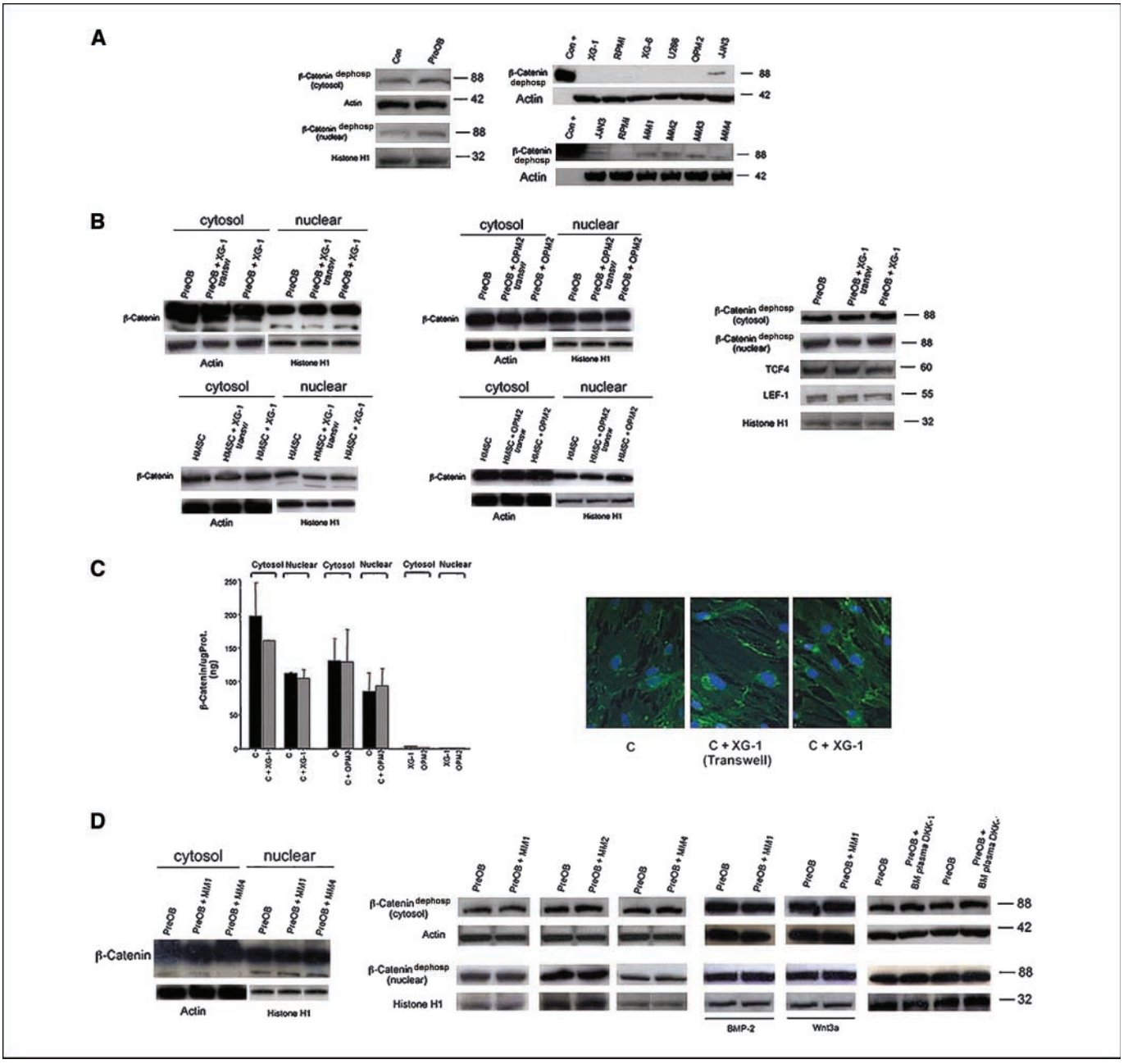


Figure 2. Effects of multiple myeloma cells on β-catenin accumulation and levels in human osteoprogenitor cells. The expression of active dephosphorylated (*dephosp*) β-catenin was evaluated by Western blot at the cytosolic and nuclear level in human bone marrow adherent cells incubated for 2 wks in α-MEM with 15% FBS (Con) or in the presence of osteogenic differentiation medium (PreOB). Several HMCLs and purified multiple myeloma cells were tested for the presence of dephosphorylated β-catenin by Western blot (Con+ = positive control = dephosphorylated purified β-catenin; *A*). A series of cocultures was done between human mesenchymal (HMSC) or PreOB cells and XG-1 (positive for DKK-1 and sFRP-3) or OPM2 (negative for both the Wnt inhibitors) in the presence or absence of a Transwell (*transw*) insert. After 48 h, total β-catenin was evaluated in both nuclear and cytosolic extracts running the samples side by side. Actin and histone H1 were used as appropriate internal controls, respectively. Active dephosphorylated β-catenin was evaluated in both cytosolic and nuclear extracts of PreOB cocultured with HMCLs as well as the transcription factor TCF4 and LEF-1 protein levels were evaluated in the nuclear samples. Actin and histonic protein H1 were used as internal controls (*B*). β-Catenin protein levels were detected at the cytosolic and nuclear level from PreOB cocultured with XG-1 or OPM2 by ELISA assay. *C,* PreOB. *Columns,* mean β-catenin protein level of three independent experiments; *bars,* SD. Human PreOB cells (3×10^4) were incubated in tissue culture chambers on glass slides in the presence or absence of XG-1 placed in a Transwell insert for 48 h. At the end of culture period, glass slides were fixed in cold acetone, and β-catenin was stained for immunofluorescence as described in Materials and Methods. Original magnification, ×400 (*C*). Human PreOB cells were cocultured with purified $CD138^+$ multiple myeloma cells placed in a Transwell insert in the presence or absence of BMP-2 or Wnt3a or with bone marrow plasma (*BM*; 10%) from DKK-1^+ and DKK-1^- multiple myeloma patients. Total β-catenin was detected by Western blot analysis at cytosolic and nuclear level in PreOB running samples side by side and active dephosphorylated β-catenin was shown at both cytosolic and nuclear level. Actin and histonic protein H1 were used as internal controls (*D*).

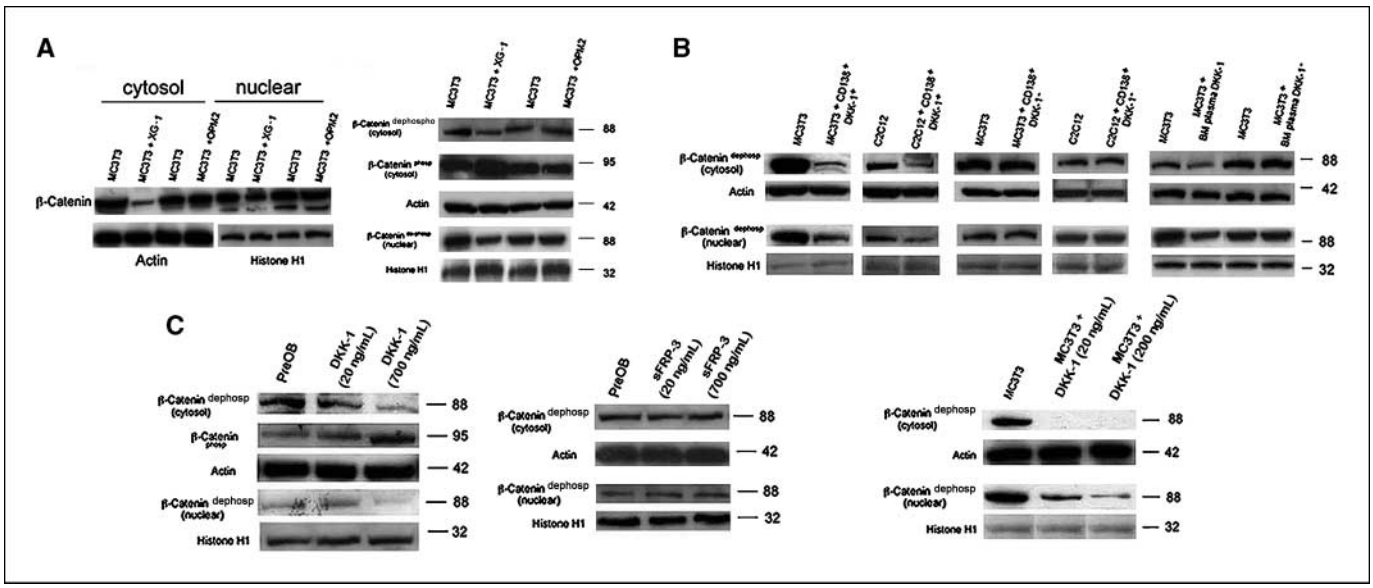


Figure 3. Effects of multiple myeloma cells on β-catenin accumulation and levels in murine osteoprogenitor cells. Murine osteoprogenitor cells, MC3T3 and C2C12, were cultured in appropriate media in the presence of BMP-2 (100 ng/mL) and either cocultured with XG-1 and OPM2 cells placed in a Transwell (*A*) or incubated with fresh purified CD138$^+$ multiple myeloma cells and bone marrow plasma (10%) from both DKK-1$^+$ and DKK-1$^-$ multiple myeloma patients (*B*). After 48 h, total β-catenin was detected by Western blot analysis at cytosolic and nuclear level in PreOB running samples side by side and active dephosphorylated β-catenin at both cytosolic and nuclear level was shown. Actin and histone H1 were used as internal controls. Human PreOB or murine osteoprogenitor cells (MC3T3 and C2C12) were incubated in the presence or absence of DKK-1 (20–700 ng/mL) or sFRP-3 (20–700 ng/mL) for 48 h. The expression of both dephosphorylated and phosphorylated (*phosp*) β-catenin was evaluated by Western blot at cytosolic and nuclear level (*C*).

In contrast, we found that XG-1 but not OPM2 reduced the expression of total β-catenin and the dephosphorylated β-catenin, inducing accumulation of the inactive phosphorylated form in murine MC3T3 after coculture (Fig. 3*A*). In line with this observation, purified CD138$^+$ multiple myeloma cells, which expressed DKK-1 but not those negative for DKK-1, inhibited both cytosolic and nuclear levels of dephosphorylated β-catenin in the murine osteoprogenitor cell lines MC3T3 and C2C12. A similar effect was induced by bone marrow plasma from DKK-1$^+$ and DKK-1$^-$ multiple myeloma patients, respectively (Fig. 3*B*).

The different behavior of human and murine osteoprogenitor cells in response to multiple myeloma cells was further investigated. First, we checked the effect of Wnt inhibitors on β-catenin stabilization in both human PreOB and murine osteoprogenitor cells. Consistent with the lack of effect of myeloma cells on β-catenin stabilization in PreOB, we found that rhDKK-1 suppressed nuclear and cytosol dephosphorylated β-catenin inducing accumulation of phosphorylated inactive β-catenin in PreOB only at concentration ≥700 ng/mL. sFRP-3 did not show any significant inhibitory effect on dephosphorylated β-catenin levels at concentration ranging between 20 to 700 ng/mL (Fig. 2*C*).

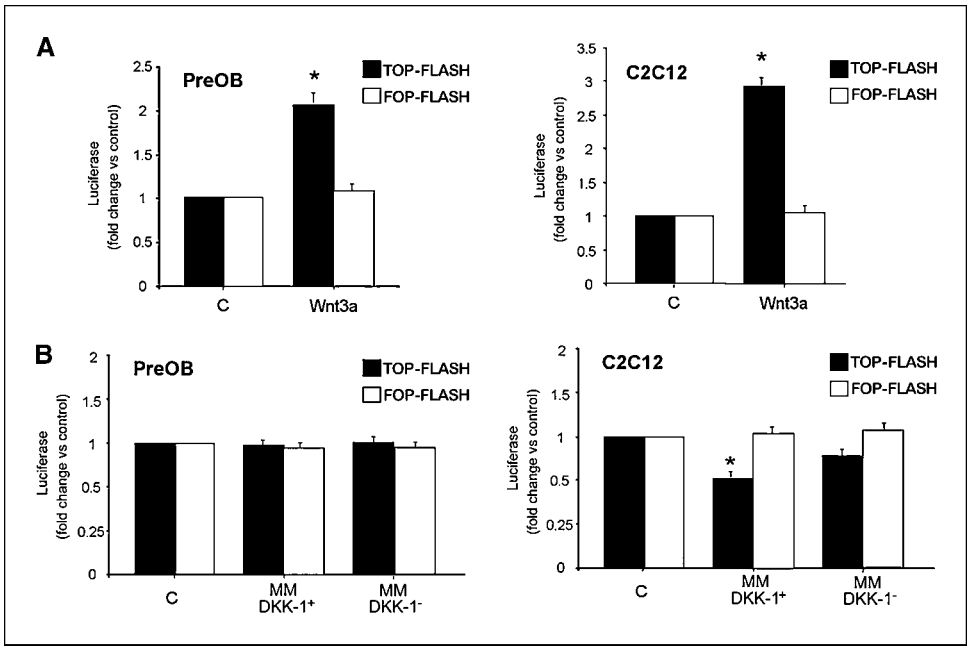


Figure 4. Modulation of TCF transcriptional activity in human and murine osteoprogenitor cells. Human PreOB or murine C2C12 cells were transfected with TOP-FLASH or FOP-FLASH Wnt reporter plasmids containing WT or mutant TCF DNA binding sites. Following transfection, cells were plated at 5 × 10^4 per well in a 24-well plate and exposed to Wnt3a 500 ng/mL (*A*) or bone marrow plasma from multiple myeloma patients either positive or negative for DKK-1 expression (*B*) for 12 h before luciferase assay. Reporter activity was assayed by using the Dual-Luciferase Reporter System. Results were normalized to *Renilla* values of each sample. *Columns,* mean of the fold change versus control of the luciferase activity of two independent experiments done twice; *bars,* SD. *, $P = 0.01$.

However, we found that DKK-1 significantly reduced dephosphorylated β-catenin expression at both the nuclear and cytosol level in MC3T3 in a dose-dependent manner, at concentrations ranging between 20 to 200 ng/mL (Fig. 3*C*). Similar results were obtained with sFRP-3 (data not shown).

Modulation of TCF transcriptional activity in human and murine osteoprogenitor cells. To confirm our observations on the different behavior of human and murine osteoprogenitor cells in response to multiple myeloma cells, we use a molecular approach evaluating the TCF transcriptional activity using the TOP/FOP reporter assay.

First, we show that Wnt3a stimulation increased the luciferase activity in both PreOB and murine C2C12 (Fig. 4*A*) as well as in osteoblastic cells HOBIT (data not shown), indicating that murine Wnt3a is able to activate canonical Wnt signaling in either human or murine osteoprogenitor cells.

Thereafter, we check the effect of multiple myeloma cells on TCF transcriptional activity in human (PreOB) and murine (C2C12) osteoprogenitor cells testing bone marrow plasma of multiple myeloma patients. In human PreOB, accordingly with the lack of effect on β-catenin nuclear accumulation, any significant inhibitory effect was not observed on luciferase activity by bone marrow plasma either positive or negative for DKK-1 (Fig. 4*B*). Similar results were obtained with osteoblastic cells HOBIT (data not shown). On the other hand, a significant inhibitory effect on luciferase activity was observed in murine C2C12 osteoprogenitor cells treated with bone marrow plasma of DKK-1$^+$ multiple myeloma patients but not with DKK-1$^-$ ones (Fig. 4*B*).

Potential involvement of canonical Wnt signaling in the inhibitory effect of multiple myeloma cells on osteoblastogenesis. To investigate whether the effects induced by multiple myeloma cells on Wnt signaling in human and murine osteoprogenitor cells could be involved in osteoblastogenesis, we evaluated the capacity of Wnt signaling stimulation to block multiple myeloma–induced suppression of osteoblast formation *in vitro*. In human bone marrow cultures, we found that Wnt3a did not blunt the inhibitory effect on CFU-F and bone nodule formation induced by XG-1 (Fig. 5*A*). Similar results were previously obtained using anti-DKK-1 in the same experimental conditions (5). In contrast, we found that Wnt3a blocked or partially reduced the inhibitory effect of XG-1 on alkaline phosphatase expression and bone nodules formation in murine MC3T3 cocultures (Fig. 5*B*).

β-Catenin immunostaining in multiple myeloma patients. Finally, the potential role of Wnt signaling in the osteoblast deficiency induced by multiple myeloma cells and the occurrence of bone lesions in multiple myeloma patients was evaluated by doing β-catenin immunostaining in bone biopsies of multiple myeloma patients. We found that β-catenin was expressed in endothelial cells, multiple myeloma cells, and osteoblastic cells (Fig. 6). In multiple myeloma cells, we found that both nuclear and

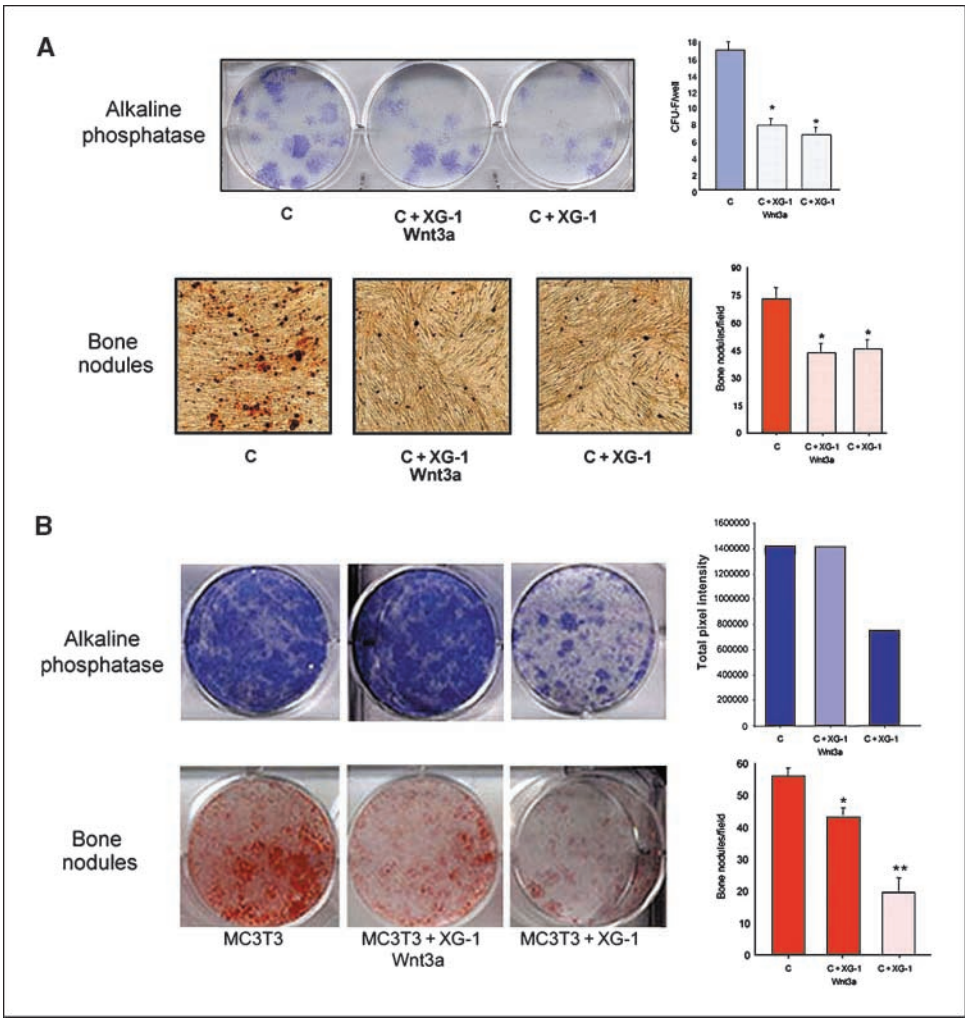
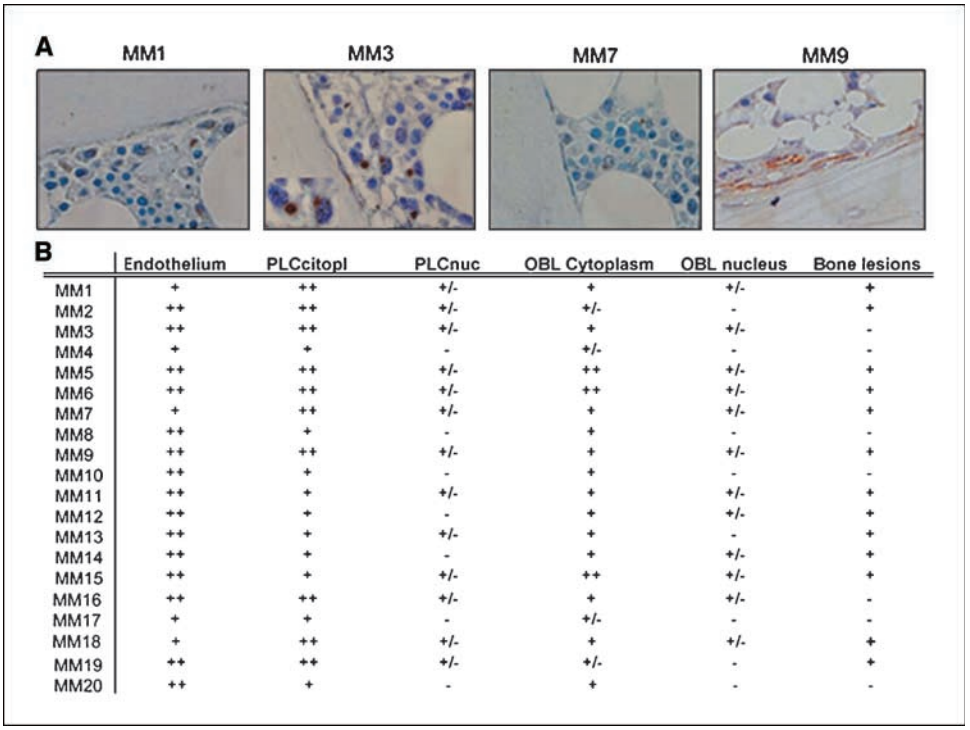


Figure 5. Effect of Wnt3a on multiple myeloma–induced inhibition of osteoblastogenesis in human and murine osteoprogenitor cells. Human BMMNCs were cocultured in the appropriate medium with XG-1 cells placed in a Transwell insert in the presence or absence of Wnt3a. CFU-F formation was evaluated after 14 d by alkaline phosphatase expression. Bone nodules were stained after 21 d with Alizarin red. Original magnification, ×5. *C,* control. *Columns,* mean number of CFU-F per well and bone nodules per field of six independent experiments done in triplicate; *bars,* SD. *, $P < 0.01$ (*A*). Murine osteoprogenitor MC3T3 cells were incubated with BMP-2 (100 ng/mL) in appropriate medium and cocultured with XG-1 cells in the presence or absence of Wnt3a. After 7 and 12 d, respectively, alkaline phosphatase and Alizarin red staining was done. *Columns,* the total pixel intensity and the number of bone nodules per well from representative experiments done in triplicate; *bars,* SD. *, $P = 0.05$; **, $P = 0.001$ (*B*).



B

	Endothelium	PLCcitopl	PLCnuc	OBL Cytoplasm	OBL nucleus	Bone lesions
MM1	+	++	+/-	+	+/-	+
MM2	++	++	+/-	+/-	-	+
MM3	++	++	+/-	+	+/-	-
MM4	+	+	-	+/-	-	-
MM5	++	++	+/-	++	+/-	+
MM6	++	++	+/-	++	+/-	+
MM7	+	++	+/-	+	+/-	+
MM8	++	+	-	+	-	-
MM9	++	++	+/-	+	+/-	+
MM10	++	+	-	+	-	-
MM11	++	+	+/-	+	+/-	+
MM12	++	+	-	+	+/-	+
MM13	++	+	+/-	+	-	+
MM14	++	+	-	+	+/-	+
MM15	++	+	+/-	++	+/-	+
MM16	++	++	+/-	+	+/-	-
MM17	+	+	-	+/-	-	-
MM18	+	++	+/-	+	+/-	+
MM19	++	++	+/-	+/-	-	+
MM20	++	+	-	+	-	-

Figure 6. β-Catenin immunostaining in marrow biopsies from multiple myeloma patients. β-Catenin immunostaining was done on bone marrow biopsies from multiple myeloma patients either with bone lesions or without skeletal involvement as shown for four representative patients (original magnification, ×100) with an insert at higher magnification (×1,000) for the patient MM3 (*A*). Data about the β-catenin immunostaining intensity observed in bone biopsies on multiple myeloma patients in relationship with the presence of bone lesions (*B*).

cytoplasmic β-catenin are present; however, nuclear staining shows a lower intensity with a punctiform/granular pattern compared with the cytoplasm.

As expected, a significant reduction of the number of osteoblastic cells per millimeter square was observed in multiple myeloma patients with osteolysis compared with ones without bone lesions (mean ± SE, 90.4 ± 4.1 versus 130 ± 2.8; $P < 0.05$) and a reduction of the osteoid surface was also observed in osteolytic versus nonosteolytic multiple myeloma patients (mean, 25 ± 0.4% versus 15 ± 0.5%; $P < 0.05$). β-Catenin expression was not correlated with the number of osteoblastic cells and the osteoid surface ($P = 0.3$). In addition, no significant difference was detected in β-catenin expression by osteoblastic cells of multiple myeloma patients with or without bone lesions.

Discussion

In this study, we have evaluated for the first time the effects of myeloma cells on Wnt signaling in osteoprogenitor and osteoblastic cells to investigate the potential role of the Wnt system in multiple myeloma–induced bone destruction. Previous data (11) found that primary $CD138^+$ multiple myeloma cells overexpress the Wnt inhibitor DKK-1 compared with plasma cells from monoclonal gammopathy of uncertain significance patients and normal plasma cells. Further, using gene expression profiling, they showed a tight relationship between DKK-1 expression by multiple myeloma cells and the occurrence of focal lytic bone lesions in multiple myeloma patients (11). In this study, we confirmed that fresh purified multiple myeloma cells secrete DKK-1 and we show that the Wnt inhibitor sFRP-3 is also produced but sFRP-2 was not detected in contrast to previous reports (14). A significant correlation was found between the presence of osteolytic bone lesions in multiple myeloma patients and DKK-1 bone marrow plasma levels but not DKK-1 expression by $CD138^+$ multiple myeloma cells. In contrast, the difference between sFRP-3 bone marrow plasma levels in osteolytic and nonosteolytic multiple myeloma patients did not reach statistical significance, suggesting a weaker link between sFRP-3 and the presence of bone lesions.

The presence of active dephosphorylated β-catenin was also shown in purified multiple myeloma cells as reported previously (15) and in the HMCL JJN3. The lack of active β-catenin detection in other HMCLs as XG-1 and OPM2 in contrast to those reported by others (15, 16) could be due to the different sensitivity of either the anti-β-catenin antibody used or the Western blot procedures.

Thereafter, the presence of dephosphorylated β-catenin was detected in bone marrow stromal cells and at higher level in osteoprogenitor cells, suggesting that Wnt signaling is activated in these cells. Nevertheless, we failed to find an inhibitory effect on β-catenin accumulation and translocation in human osteoprogenitor and osteoblastic cells by either HMCLs, purified multiple myeloma cells, or bone marrow plasma of $DKK\text{-}1^+$ multiple myeloma patients, suggesting that multiple myeloma cells did not block canonical Wnt signaling in these cells. DKK-1 inhibited dephosphorylated β-catenin only at high concentrations that are not reached either in conditioned media from multiple myeloma cells or in bone marrow plasma of multiple myeloma patients. In line with our observations, others have observed recently that DKK-1 can block osteoblast differentiation in human bone marrow stromal cells only at high concentration (17). We consequently found that activation of Wnt signaling in osteoprogenitor cells did not blunt multiple myeloma–induced inhibition of osteoblast formation *in vitro*. A molecular approach has been used to support our evidence. Using the TOP/FOP reporter assay, we confirm that human osteoprogenitor and osteoblastic cells respond to the activation of the canonical Wnt signaling by Wnt3a and that multiple myeloma cells or bone marrow plasma from multiple myeloma patients did not inhibit TCF reporter activity. Finally,

our *in vitro* observations have been confirmed *in vivo* by examining the expression of active β-catenin in bone biopsies of multiple myeloma patients.

On the other hand, using murine osteoprogenitor cells in coculture with multiple myeloma cells, we found a different result compared with human osteoprogenitor cells. In both murine MC3T3 and C2C12 cells, we found that multiple myeloma cells and bone marrow plasma of DKK-1$^+$ patients blocked Wnt signaling in the presence of BMP-2. Data obtained by quantitative PCR and by the TOP/FOP reporter assay further supported the different effects observed on β-catenin accumulation between human and murine osteoprogenitor cells.

Multiple myeloma cells induced a significant up-regulation of the Wnt antagonist sFRP-2 and Wnt receptors LRP5 and LRP6 in murine but not human osteoprogenitor cells. This could explain the capacity of multiple myeloma cells to block Wnt signaling in murine but not in human osteoprogenitor cells and their differential sensitivity to DKK-1. The different response of murine and human mesenchymal cells could be also due to the lack of production of activators of canonical Wnt signaling as Wnt3a that permits the antagonist effect of DKK-1 by HMSCs as reported recently (18).

However, the different results found with murine and human system are not completely surprising. Consistently with our findings, Tian et al. (11) reported that neutralizing anti-DKK-1 antibody blocked the inhibitory effect of bone marrow plasma from multiple myeloma patients on BMP-2–induced alkaline phosphatase expression and osteoblast formation by the murine mesenchymal cell line C2C12. In contrast, we showed previously that anti-DKK-1 failed to block the inhibitory effects of multiple myeloma cells on human bone marrow osteoblast formation (5).

Most data on the role of canonical Wnt signaling in the regulation of osteoblast differentiation have been obtained in murine cells stimulated by BMP-2 (6–8). *In vivo* mouse models also support the role of Wnt signaling in the regulation of bone mass (19). Transgenic mice overexpressing DKK-1 develop osteopenia, whereas mice expressing a mutant *LRP5* gene with low affinity for DKK-1 develop increased bone mass (20, 21). In humans, inactivating mutations of the LRP5 Wnt coreceptor cause osteoporosis (22), indicating that Wnt activation may have a critical role in human bone formation. However, other authors have shown that Wnt activation in human bone marrow cells suppresses osteogenic differentiation (23, 24).

Alternative mechanisms to block the canonical Wnt signaling could be involved in DKK-1–mediated bone destruction. It has been shown that multiple myeloma cell migration and invasion (25) as well as adhesion (26) are not mediated by Wnt canonical β-catenin pathway. Expression of Wnt inhibitors, such as DKK-1, is triggered by cell contact and modulates adhesion of leukemia cells to osteoblasts (27). Consistently with this hypothesis, increasing data indicate a potential Wnt-independent activity of Wnt antagonists in cancer (28), as reported in both melanoma (29, 30) and choriocarcinoma tumors (28, 30), where DKK-1 is able to antagonize Wnt signaling independent of β-catenin. A correlation between DKK-1 expression by multiple myeloma cells, the presence of specific genetic abnormalities, and the occurrence of focal bone lesions has also been shown (31), suggesting that the relationship between DKK-1 and the presence of bone lesions in multiple myeloma patients could be a genetic signature linked to specific chromosomal abnormalities.

Finally, growing *in vivo* data indicate that Wnt signaling pathway may be a target for the treatment of bone disease. In ovariectomized rats, GSK3α and GSK3β inhibitors increase markers of bone formation and bone mass (32). Recent studies in the severe combined immunodeficient-hu mice model of multiple myeloma reported that anti-DKK-1 increases bone mineral density and the number of osteocalcin-positive osteoblasts compared with control mice (33). Interestingly, the authors observed a reduction of the number of osteoclastic cells, suggesting that DKK-1 could be also involved in the regulation of bone resorption.

In conclusion, our data support a link between Wnt antagonists and the presence of bone lesions in multiple myeloma even if we show that multiple myeloma cells failed to block the canonical Wnt/β-catenin pathway in human mesenchymal and osteoprogenitor cells.

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Production of Wnt Inhibitors by Myeloma Cells: Potential Effects on Canonical Wnt Pathway in the Bone Microenvironment

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