

The Role of the Wnt-Signaling Antagonist DKK1 in the Development of Osteolytic Lesions in Multiple Myeloma

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ABSTRACT

BACKGROUND

Myeloma cells may secrete factors that affect the function of osteoblasts, osteoclasts, or both.

METHODS

We subjected purified plasma cells from the bone marrow of patients with newly diagnosed multiple myeloma and control subjects to oligonucleotide microarray profiling and biochemical and immunohistochemical analyses to identify molecular determinants of osteolytic lesions.

RESULTS

We studied 45 control subjects, 36 patients with multiple myeloma in whom focal lesions of bone could not be detected by magnetic resonance imaging (MRI), and 137 patients in whom MRI detected such lesions. Different patterns of expression of 57 of approximately 10,000 genes from purified myeloma cells could be used to distinguish the two groups of patients ($P < 0.001$). Permutation analysis, which adjusts the significance level to account for multiple comparisons in the data sets, showed that 4 of these 57 genes were significantly overexpressed by plasma cells from patients with focal lesions. One of these genes, *dickkopf1* (DKK1), and its corresponding protein (DKK1) were studied in detail because DKK1 is a secreted factor that has been linked to the function of osteoblasts. Immunohistochemical analysis of bone marrow–biopsy specimens showed that only myeloma cells contained detectable DKK1. Elevated DKK1 levels in bone marrow plasma and peripheral blood from patients with multiple myeloma correlated with the gene-expression patterns of DKK1 and were associated with the presence of focal bone lesions. Recombinant human DKK1 or bone marrow serum containing an elevated level of DKK1 inhibited the differentiation of osteoblast precursor cells in vitro.

CONCLUSIONS

The production of DKK1, an inhibitor of osteoblast differentiation, by myeloma cells is associated with the presence of lytic bone lesions in patients with multiple myeloma.

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LUNG, BREAST, AND PROSTATE CANCER and multiple myeloma have an affinity for bone, where they cause osteoblastic lesions (prostate cancer) or osteolytic lesions (lung and breast cancer and multiple myeloma).¹ Research on the mechanisms by which multiple myeloma cells induce osteolysis has focused on the osteoclast's role in shifting the normal balance between bone formation and bone resorption in favor of resorption.² Bone resorption is blocked by bisphosphonates,³ but the inability of these compounds to repair lytic lesions indicates that a functional defect of osteoblasts is also important in the lytic process. Indeed, the number and function of osteoblasts are decreased in myeloma with osteolytic lesions.⁴⁻⁷

The Wnt signaling pathway is important for the growth and differentiation of osteoblasts and acts in several developmental processes.⁸ Disabling mutations in the gene for the Wnt coreceptor low-density lipoprotein receptor–related protein 5 (LRP5) cause the osteoporosis–pseudoglioma syndrome,⁹ and *Lrp5*-deficient mice have osteopenia with diminished osteoblast proliferation.¹⁰ In the syndrome of hereditary high bone density,^{11,12} mutations in the LRP5 gene prevent binding of dickkopf1 (DKK1), a soluble inhibitor of Wnt, to LRP5.¹²⁻¹⁶ The importance of DKK1 in skeletal development has been further demonstrated by the extra digits in *dkk1* null mice¹³ and by the loss of bony structures in chicken and mouse embryos after exposure to elevated levels of DKK1.^{13,14} In vitro, short-term exposure to low levels of DKK1 induces moderate proliferation of mesenchymal stem cells, whereas long-term exposure to high levels of DKK1 causes a loss of cell viability.¹⁵

In this study, we compared patterns of gene expression in myeloma cells, as has been done previously in the study of myeloma,¹⁶⁻¹⁸ to determine whether they were related to the presence or absence of bone lesions in myeloma.

METHODS

PATIENTS

We studied 173 patients with newly diagnosed multiple myeloma, 16 patients with monoclonal gammopathy of undetermined significance, 9 patients with Waldenström's macroglobulinemia, and 45 control subjects. The institutional review board of the University of Arkansas for Medical Sciences approved the research studies, and all subjects provided written informed consent. Table 1 shows the

characteristics of the patients with multiple myeloma.

BONE IMAGING

Images were reviewed by one of the investigators, who had no prior knowledge of the gene-expression data, using a Canon Picture Archiving and Cataloging System. Magnetic resonance imaging (MRI) scans were performed on 1.5-Tesla Signa scanners (General Electric). The radiographs were digitized from film in accordance with American College of Radiology standards. MRI scans and radiographs were transferred to the Canon Picture Archiving and Cataloging System with the use of the American College of Radiology's Digital Imaging and Communications in Medicine standard. Imaging was performed in accordance with the manufacturers' specifications. MRI images were created with T₁-weighting both before and after the administration of gadolinium, with fat suppression, and with short-tau inversion recovery (STIR) weighting.

PLASMA-CELL ISOLATION AND GENE-EXPRESSION PROFILING

After Ficoll–Hypaque gradient centrifugation, plasma cells obtained from the bone marrow were isolated from the mononuclear-cell fraction by immunomagnetic bead selection with the use of a monoclonal mouse antihuman CD138 antibody (Miltenyi-Biotec). More than 90 percent of the cells used for gene-expression profiling were plasma cells, as shown by two-color flow cytometry with the use of CD138+/CD45– and CD38+/CD45– markers, the presence of cytoplasmic immunoglobulin light chains on immunocytochemical analysis, and morphologic features as determined with the use of Wright–Giemsa staining. Total RNA was isolated with an RNeasy Mini Kit (Qiagen). Preparation of labeled complementary RNA and hybridization to U95Av2 microarrays containing approximately 10,000 genes (Affymetrix) were performed as previously described.^{16,17} RNA amplification was not required. The results of gene-expression profiling were deposited in Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under the accession number GSE755.

IMMUNOHISTOCHEMISTRY

An antibody from a goat that had been immunized against the entire human DKK1 protein (R&D Systems) was diluted 1:200 in TRIS buffer and added to formalin-fixed, paraffin-embedded bone mar-

Table 1. Relation of the Characteristics of 173 Patients with Multiple Myeloma to the Presence or Absence of Bone Lesions on Magnetic Resonance Imaging (MRI).

| Characteristic | No. of Patients/ Total No. (%) | ≥1 Lesions on MRI (N=137) | 0 Lesions on MRI (N=36) | P Value |
|--|-----------------------------------|------------------------------|----------------------------|---------|
| | | no./total no. (%) | | |
| Age ≥65 yr | 26/173 (15) | 20/173 (15) | 6/36 (17) | 0.76* |
| White race | 149/172 (87) | 115/136 (85) | 33/36 (92) | 0.32* |
| Female sex | 69/173 (40) | 56/137 (41) | 13/36 (36) | 0.60† |
| Kappa light chains | 104/164 (63) | 79/128 (62) | 24/36 (67) | 0.59† |
| Lambda light chains | 61/164 (37) | 49/128 (38) | 12/36 (33) | 0.59† |
| IgA subtype | 39/168 (23) | 25/132 (19) | 14/36 (39) | 0.012† |
| Beta ₂ -microglobulin ≥4 mg/liter | 60/168 (36) | 47/132 (36) | 13/36 (36) | 0.96† |
| C-reactive protein ≥4 mg/liter | 12/165 (7) | 11/129 (8) | 1/36 (3) | 0.47* |
| Creatinine ≥2 mg/dl (177 μmol/liter) | 19/168 (11) | 16/132 (12) | 3/36 (8) | 0.77* |
| Lactate dehydrogenase ≥190 IU/liter | 52/168 (31) | 44/132 (33) | 8/36 (22) | 0.20† |
| Albumin <3.5 g/dl | 23/168 (14) | 19/132 (14) | 4/36 (11) | 0.79* |
| Hemoglobin <10 g/dl | 39/168 (23) | 31/132 (23) | 8/36 (22) | 0.87† |
| Plasma-cell labeling index ≥1% | 22/149 (15) | 18/119 (15) | 4/30 (13) | 1.00* |
| Bone marrow–aspirate plasma cells ≥33% | 108/165 (65) | 82/129 (64) | 26/36 (72) | 0.33† |
| Bone marrow–biopsy plasma cells ≥33% | 103/165 (62) | 79/129 (61) | 24/36 (67) | 0.55† |
| Cytogenetic abnormalities | 57/151 (38) | 50/117 (43) | 7/34 (21) | 0.02 |
| Chromosome 13 deletion or hypodiploidy | 37/57 (63) | 34/50 (68) | 3/7 (43) | 0.23† |
| Other cytogenetic abnormalities | 20/57 (35) | 16/50 (32) | 4/7 (57) | 0.23† |
| Interphase FISH13‡ | 72/137 (53) | 56/106 (53) | 16/31 (52) | 0.90† |
| Osteopenia | 131/172 (76) | 103/136 (76) | 28/36 (78) | 0.80 |
| ≥1 Lesions on MRI | 137/173 (79) | | | |
| ≥3 Lesions on MRI | 108/173 (62) | | | |
| ≥1 Lesions on radiography | 104/173 (60) | | | |
| ≥3 Lesions on radiography | 69/173 (40) | | | |

* Fisher's exact test was used.

† The chi-square test was used.

‡ FISH13 denotes fluorescence in situ hybridization deletion of chromosome 13.

row–biopsy sections, which were then incubated for two hours at room temperature. Adjacent sections were stained with hematoxylin and eosin. Antigen–antibody reactions were developed with diaminobenzidine tetrahydrochloride (DAB) (after staining with biotinylated anti-goat antibody [Vector Laboratories], at 1:400 dilution, and streptavidin–horseradish peroxidase [Dako]) and counterstained with hematoxylin-2.

ENZYME-LINKED IMMUNOSORBENT ASSAY

Microtiter plates (Nunc-Immuno MaxiSorp surface) were coated with 50 μl of anti-DKK1 antibody at a concentration of 1 μg per milliliter in phosphate-

buffered saline, pH 7.2, and incubated at 4°C overnight, and the reaction was blocked with 4 percent bovine serum albumin. Bone marrow plasma was diluted 1:50 in dilution buffer (1× phosphate-buffered saline plus 0.1 percent Tween-20 and 1 percent bovine serum albumin). A total of 50 μl was loaded per well and incubated overnight at 4°C, washed, and incubated with biotinylated goat antihuman DKK1 IgG (R&D Systems) diluted to a concentration of 0.2 μg per milliliter in dilution buffer, followed by the addition of 50 μl of a 1:10,000 dilution of streptavidin–horseradish peroxidase (Vector Laboratories), all according to the manufacturers' rec-

ommendations. Color development was achieved with the OPD substrate system (Dako), used according to the manufacturer's instructions. Serial dilutions of recombinant human DKK1 (R&D Systems) were used to establish a standard curve. The cell line T293, which does not express endogenous DKK1, and T293 with stably transfected DKK1¹⁹ were used to validate the enzyme-linked immunosorbent assay.

OSTEOBLAST-DIFFERENTIATION ASSAYS

C2C12 mesenchymal precursor cells (American Type Tissue Culture) were cultured in Dulbecco's minimal essential medium (Invitrogen) supplemented with 10 percent heat-inactivated fetal-calf serum. Alkaline phosphatase activity in C2C12 cells was measured as described previously.^{20,21} Cell lysates were analyzed for protein content with the use of the micro-BCA assay kit (Pierce). Each experiment was done in triplicate.

STATISTICAL ANALYSIS

Logistic regression was used to model bone disease in multiple myeloma. The independent variables considered were gene-expression intensity values (referred to as signals) from approximately 10,000 genes (12,625 probe sets), measured with the use of MAS software, version 5.01 (Affymetrix), from 173 patients with newly diagnosed multiple myeloma. The signal, a quantitative measure of gene expression, for each probe set was log transformed on a base-2 scale before it was entered into the logistic-regression model and subjected to permutation analysis, which adjusts the significance level to account for multiple comparisons in data sets with high dimensionality.

There was no prior hypothesis with regard to genes that might be associated with bone disease in myeloma. As a result, we used a univariate model of bone disease for each of the 12,625 probe sets. Candidate genes were refined with the use of t-tests with permutation-adjusted significance levels.²² Westfall and Young analysis was used to adjust for the multiple univariate-hypothesis tests. Group differences in DKK1 signal and DKK1 protein levels were tested with the use of the Wilcoxon rank-sum test.

Significant differences in patients' characteristics according to their bone-disease status were evaluated with the use of either Fisher's exact test or the chi-square test. The expression intensities of genes identified by logistic regression were classified with the use of Clusterview.²³ Spearman's correlation co-

efficient was used to measure the correlation between the level of gene expression and protein levels. Significant differences in osteoblast differentiation between the control and each experimental condition were tested with the use of the Wilcoxon rank-sum test; separate comparisons were made for each unique C2C12 experiment. Two-sided P values of less than 0.05 were considered to indicate statistical significance, and two-sided P values of less than 0.10 were considered to indicate marginal statistical significance.

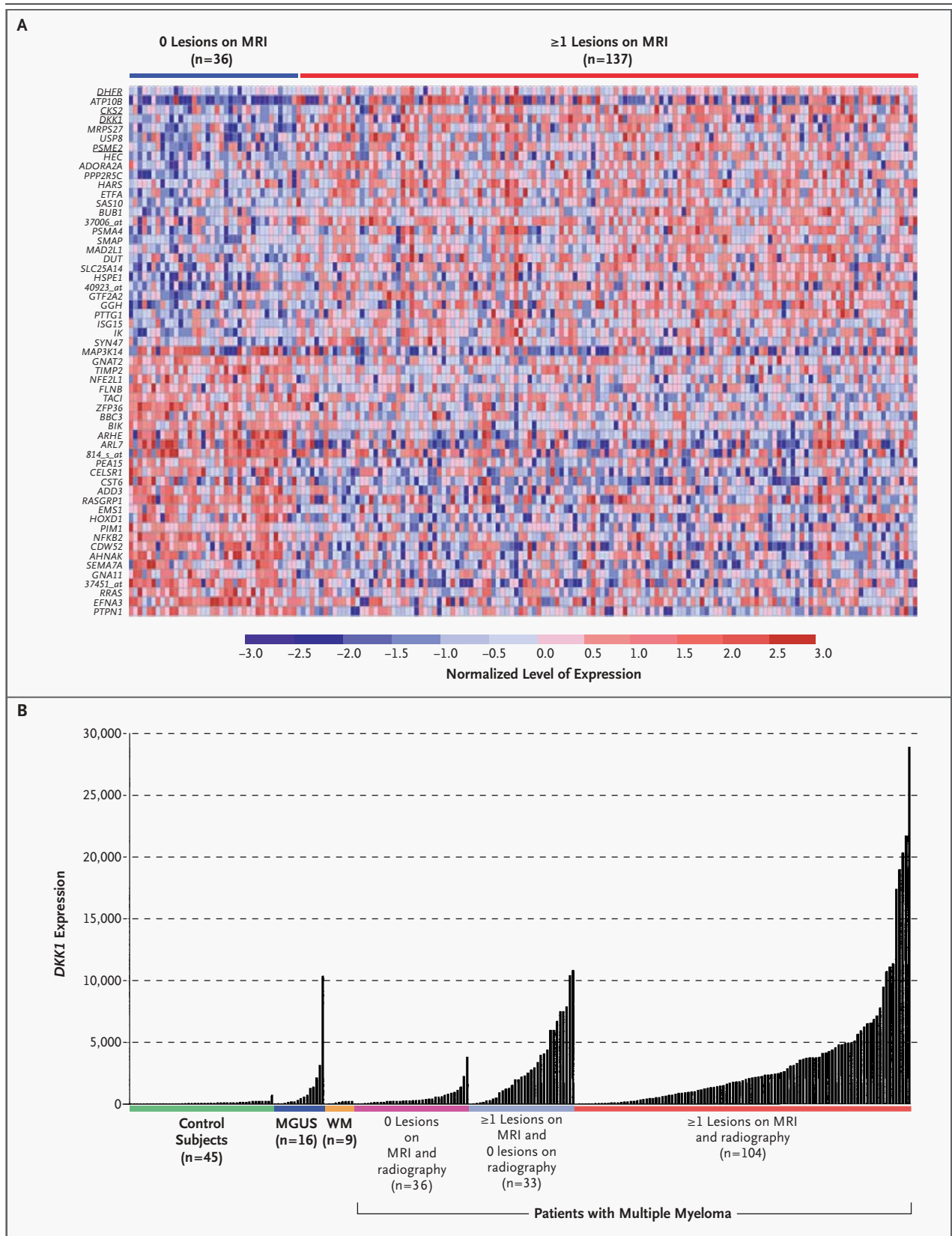
RESULTS

GENE-EXPRESSION PROFILING OF MYELOMA CELLS

We sought to identify genes that were overexpressed and associated with the presence of bone lesions in patients with myeloma by comparing microarray data for patients who had bone lesions with data for those who did not have bone lesions. Since focal lesions of bone can be seen on MRI before lytic lesions can be identified radiologically, we used T₁-weighted and STIR-weighted imaging to detect bone le-

Figure 1 (facing page). Differences in Global Patterns of Gene Expression between 137 Patients with Myeloma and One or More Bone Lesions on MRI and 36 Patients with Myeloma without Bone Lesions on MRI (Panel A) and DKK1 Gene Expression in Plasma Cells from 45 Control Subjects with Normal Bone Marrow, 16 Patients with Monoclonal Gammopathy of Undetermined Significance (MGUS), 9 with Waldenström's Macroglobulinemia (WM), and the 173 with Multiple Myeloma (Panel B).

Panel A shows normalized expression levels of 57 genes identified by logistic-regression analysis as being expressed significantly differentially in malignant plasma cells from patients with no focal lesions on MRI and patients with one or more focal lesions on MRI ($P < 0.001$). The 28 genes with elevated levels of expression in plasma cells from patients with one or more lesions on MRI are rank ordered from top to bottom on the basis of significance. Likewise, the 29 genes with significantly elevated levels of expression in patients with no lesions on MRI are rank ordered from bottom to top on the basis of significance. The genes (or Affymetrix probe-set identifiers in cases in which the gene is unnamed) are listed on the left. The four genes that remained significantly correlated with the presence of bone lesions after permutation adjustment are underlined. In Panel B, the Affymetrix signal, a quantitative measure of gene expression, is indicated on the y axis. The level of expression of DKK1 in each sample is indicated by the height of the bar. Samples are ordered from the lowest to highest level of expression of DKK1 gene from left to right on the x axis.



sions. The patterns of expression of approximately 10,000 genes in purified plasma cells from the marrow of 36 patients with no detectable bone lesions and 137 with one or more focal lesions on MRI were modeled by logistic-regression analysis. The model identified 57 genes that were expressed differently ($P < 0.001$) in the two groups of patients (Fig. 1A).

These 57 genes were further analyzed by t-tests after adjustment for permutation.²² These statistical tests showed that 4 of the 57 genes were overexpressed in patients with one or more lesions on MRI: the genes for dihydrofolate reductase (DHFR), proteasome activator subunit (PSME2), CDC28 protein kinase 2 (CKS2), and DKK1. Given that the gene for the Wnt-signaling antagonist DKK1 is the only one of the four that codes for a secreted factor and that Wnt signaling is implicated in bone formation, we carried out further tests on DKK1.

An analysis of the results from the 173 patients with myeloma showed that the DKK1 plasma-cell signal for patients who had one or more lesions on MRI and no radiographic evidence of lesions differed significantly from the signal for patients who had no lesions detectable on MRI or radiography (the median signals were 2220 and 285, respectively; $P < 0.001$) (Table 2). The DKK1 plasma-cell signals for patients who had one or more lesions on MRI and no radiographic evidence of lesions did not differ significantly from those for patients with one or more lesions on MRI and radiography (the median signals were 2220 and 1865, respectively; $P = 0.63$) (Fig. 1B and Table 2).

Monoclonal gammopathy of undetermined sig-

nificance is a plasma-cell dyscrasia that does not cause lytic bone lesions and that can precede multiple myeloma. In 15 of 16 patients with monoclonal gammopathy of undetermined significance, DKK1 was expressed by bone marrow plasma cells at levels similar to those in patients with multiple myeloma who had no lesions of bone on MRI or radiology (Fig. 1B). DKK1 was essentially undetectable in plasma cells from 45 control subjects and 9 patients with Waldenström's macroglobulinemia, a plasma-cell cancer of the bone that does not cause bone lesions (Fig. 1B).

SYNTHESIS OF DKK1 BY PLASMA CELLS

Serial sections of bone marrow–biopsy specimens from 65 patients with multiple myeloma were stained for the presence of DKK1 (Fig. 2). The levels of DKK1 in plasma cells from these patients were consistent with the level of expression of DKK1 (data not shown). Similar experiments with biopsy specimens from five control subjects failed to identify DKK1 in any cell. Forty-two of 45 DKK1-positive myelomas had low-grade morphologic findings (abundant cytoplasm without apparent nucleoli) with an interstitial pattern of growth; staining was greatest in plasma cells adjacent to bone. Nineteen of 20 DKK1-negative myelomas had high-grade, plasmablastic morphologic findings (enlarged nuclei and prominent nucleoli) with a nodular or obliterative pattern of growth. In biopsy specimens with an interstitial pattern of growth, DKK1 was either present in various percentages of cells or absent. In contrast, DKK1 was uniformly absent from myelomas with the more aggressive nodular growth pattern. In the three patients with both interstitial and nodular growth, the interstitial cells were positive for DKK1, and the nodular cells were negative.

DKK1 IN BONE MARROW PLASMA

An enzyme-linked immunosorbent assay showed that the mean (\pm SD) level of DKK1 protein in the bone marrow plasma from 107 of the 173 patients with newly diagnosed multiple myeloma for whom gene-expression data were also available was 24.0 ± 49.6 ng per milliliter. In contrast, the DKK1 level was 8.9 ± 4.2 ng per milliliter in bone marrow plasma from 14 control subjects, 7.5 ± 4.5 ng per milliliter in 14 patients with monoclonal gammopathy of undetermined significance, and 5.5 ± 2.4 ng per milliliter in 9 patients with Waldenström's macroglobulinemia. The level of DKK1 expression and the level of DKK1 in bone marrow plasma were pos-

Table 2. Levels of Expression of the DKK1 Gene and DKK1 Protein in Patients with Multiple Myeloma, According to the Presence or Absence of Bone Lesions on MRI and Radiography.

| Variable | 0 Lesions on MRI and Radiography | ≥ 1 Lesions on MRI, 0 Lesions on Radiography | ≥ 1 Lesions on MRI and Radiography |
|-----------------------|----------------------------------|---|---|
| DKK1 | | | |
| No. of patients | 36 | 33 | 104 |
| Mean \pm SD | 536.1 \pm 720.7 | 3146.5 \pm 3079.9 | 3415.1 \pm 4870.8 |
| Minimum | 19 | 16 | 9 |
| Median | 285 | 2,220 | 1,865 |
| Maximum | 3810 | 10,828 | 28,859 |
| DKK1 | | | |
| No. of patients | 18 | 9 | 41 |
| Mean \pm SD (ng/ml) | 9.0 \pm 4.7 | 24.0 \pm 17.7 | 34.3 \pm 75.3 |
| Minimum (ng/ml) | 2 | 7 | 2 |
| Median (ng/ml) | 9 | 20 | 14 |
| Maximum (ng/ml) | 20 | 62 | 476 |

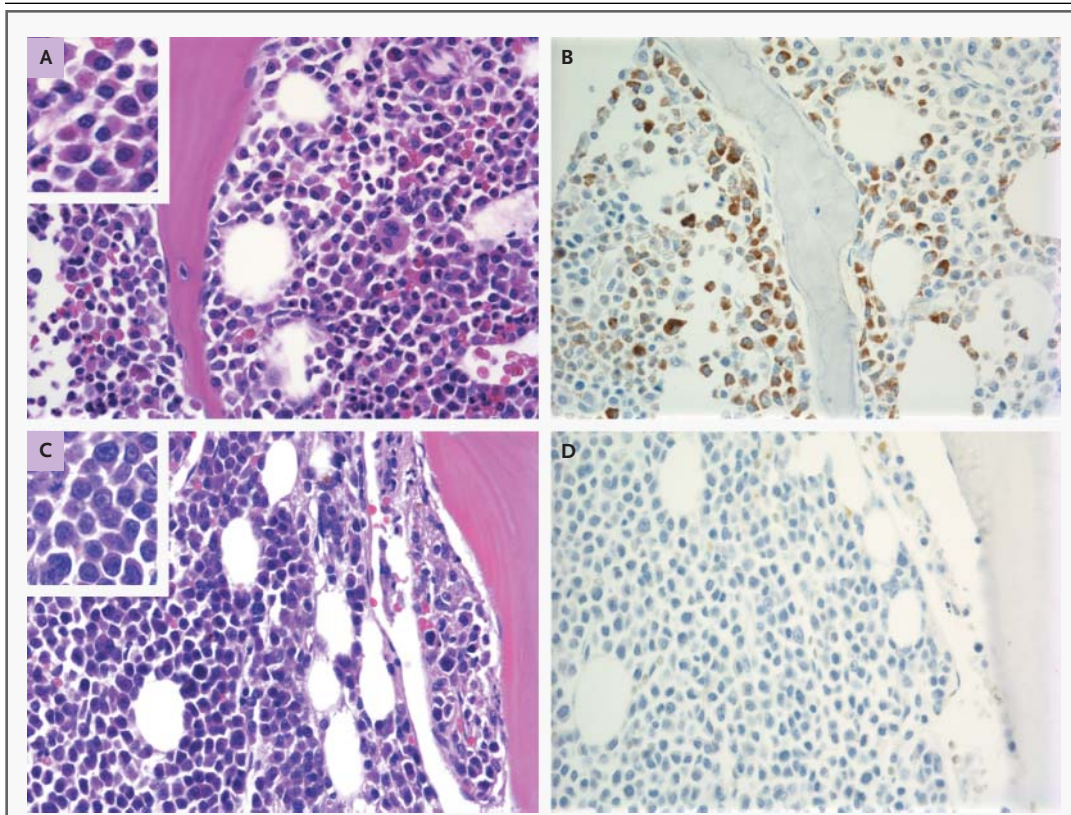


Figure 2. Overexpression of DKK1 in Low-Grade Myeloma, with the Loss of Expression with Disease Progression.

The level of expression of DKK1 was examined immunohistochemically in bone marrow–biopsy specimens from patients with myeloma. Panels A and B show specimens with high levels of DKK1 expression, and Panels C and D show specimens with low levels of DKK1 expression ($\times 550$). Slides are stained with hematoxylin and eosin (Panels A and C) or anti-DKK1 antibody and secondary antibody (Panels B and D). Use of secondary antibody alone failed to stain cells (data not shown). The insets in Panels A and C show a higher magnification ($\times 1200$). Panel A shows a myeloma with an interstitial pattern of involvement, in which plasma cells have low-grade morphologic features with abundant cytoplasm and no apparent nucleoli. Panel B shows staining of plasma cells with anti-DKK1 antibody in an interstitial pattern that was greatest adjacent to bone. Panel C shows a myeloma with a nodular or obliterative pattern of growth, in which plasma cells have high-grade morphologic features with enlarged nuclei and prominent nucleoli. Panel D shows no staining of plasma cells with anti-DKK1 antibody.

itively correlated ($r=0.65$, $P<0.001$) in the 107 patients with myeloma (Fig. 3A). There was also a strong correlation between DKK1 protein levels in bone marrow plasma and peripheral-blood plasma in 41 patients with myeloma from whom both samples were obtained simultaneously ($r=0.57$, $P<0.001$). In 68 patients in whom both DKK1 levels in the bone marrow plasma and the presence of bone lesions were determined, DKK1 levels in patients with one or more lesions on MRI and no radiographic evidence of lesions differed significantly from those in patients with no lesions on MRI or radiography (median level, 20 ng per milliliter and

9 ng per milliliter, respectively; $P=0.002$), but not from those in patients with one or more lesions on both MRI and radiography (median level, 14 ng per milliliter; $P=0.36$) (Fig. 3B and Table 2).

EFFECT OF BONE MARROW PLASMA ON OSTEOBLAST DIFFERENTIATION IN VITRO

Bone morphogenetic protein type 2 (BMP-2) can induce differentiation of the uncommitted mesenchymal progenitor-cell line C2C12²⁴ into osteoblasts through a mechanism that involves Wnt/ β -catenin signaling.^{25,26} Only small amounts of alkaline phosphatase, a specific marker of osteoblast differen-

tiation, were detectable in C2C12 cells grown in 5 percent fetal-calf serum for five days (Fig. 4A). Treatment of C2C12 cells with 50 ng of BMP-2 per milliliter for five days induced them to produce alkaline phosphatase, whereas alkaline phosphatase was inhibited in C2C12 cells that were concomitantly cultured with BMP-2 and 50 ng of recombinant human DKK1 per milliliter. This in vitro effect on alkaline phosphatase production was neutralized by a polyclonal anti-DKK1 antibody but not by a nonspecific polyclonal goat IgG. Bone marrow plasma with a DKK1 level of more than 12 ng per milliliter, obtained from five patients with myeloma, inhibited the production of alkaline phosphatase by C2C12 cells treated with BMP-2, and this effect was reversed by the anti-DKK1 antibody but not by nonspecific IgG (Fig. 4B). By contrast, C2C12 cells treated with 50 ng of BMP-2 per milliliter and 10 percent plasma from the bone marrow of a control subject induced the production of alkaline phosphatase by the cells (Fig. 4B).

DISCUSSION

Using oligonucleotide microarrays, we examined the expression of approximately 10,000 genes by purified plasma cells from the marrow of 137 patients with and 36 without detectable bone lesions at the time of diagnosis of multiple myeloma. After logistic-regression analysis and permutation adjustment, we found significant overexpression of four genes by plasma cells from patients with bone lesions. Only one of these four, DKK1, codes for a secreted protein with a demonstrated role in bone formation. We also detected DKK1 protein in myeloma cells from patients with bone lesions but not in normal plasma cells or in plasma cells from patients who had myeloma without bone lesions, and we found that marrow plasma and blood plasma from patients with myeloma and bone lesions contained higher amounts of the protein. Moreover, an elevated level of DKK1 was associated not only with the presence of bone lesions, but also with increased levels of DKK1 transcripts in myeloma cells. We also demonstrated that plasma from the marrow of some patients with myeloma can block osteoblast differentiation in vitro and that this effect was neutralized by an anti-DKK1 antibody.

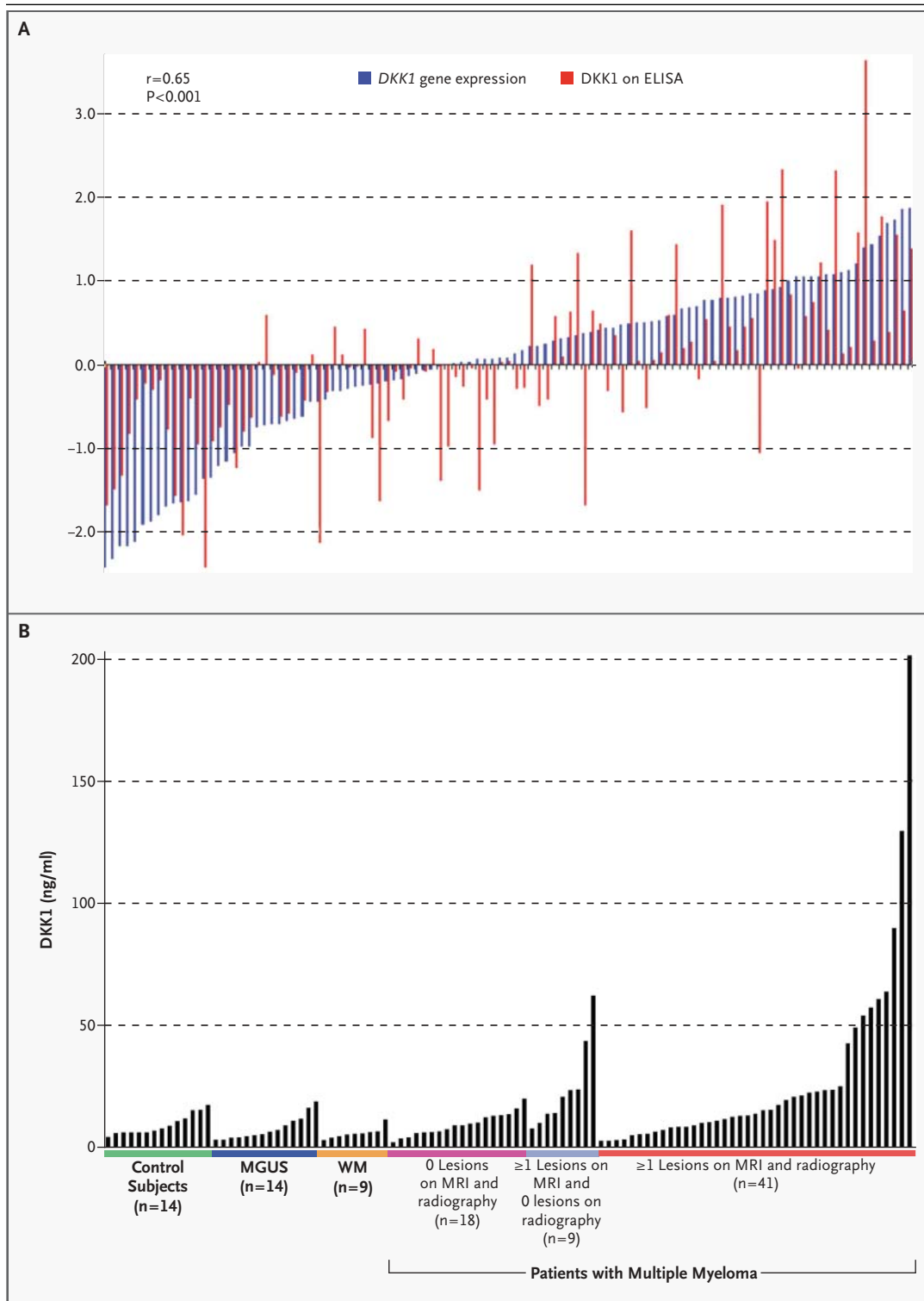
The function of osteoblasts is dramatically reduced when the proportion of myeloma cells in the marrow exceeds 50 percent,^{5,7,27,28} a finding that suggests a link between elevated DKK1 levels in the

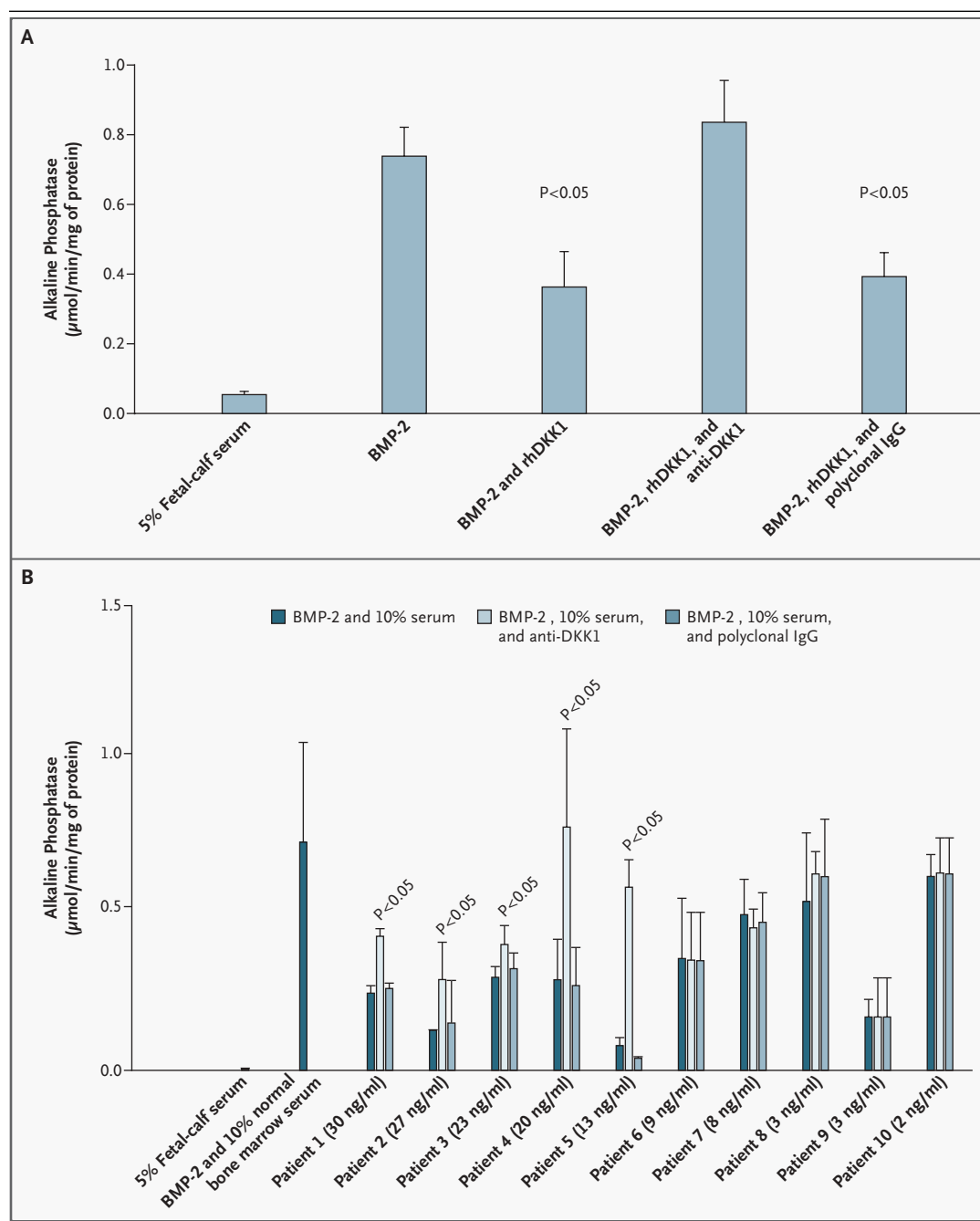
Figure 3 (facing page). Correlation between Levels of DKK1 in Bone Marrow Plasma and Level of DKK1 Expression in Plasma Cells (Panel A) and Correlation of Levels of DKK1 in Bone Marrow Plasma and the Presence or Absence of Bone Lesions (Panel B).

In Panel A, the level of expression of DKK1 messenger RNA (mRNA) was quantitated by microarray analysis and DKK1 protein was determined by enzyme-linked immunosorbent assay (ELISA) in 107 patients with newly diagnosed myeloma. Results of both assays were log transformed with the use of a base-2 scale and normalized to give a mean of 0 and variance of 1. Each bar indicates the relative relation of DKK1 expression and DKK1 expression in a single sample. There was a significant correlation between the level of DKK1 mRNA expressed in myeloma plasma cells and the level of DKK1 protein in bone marrow plasma ($r=0.65$, $P<0.001$). Panel B shows DKK1 protein levels in bone marrow plasma from 14 control subjects, 14 patients with monoclonal gammopathy of undetermined significance (MGUS), 9 patients with Waldenström's macroglobulinemia (WM), and 68 patients with multiple myeloma. To make possible comparisons of DKK1 levels in the lower ranges, a value of 200 ng per milliliter was made the maximum. This resulted in the truncation of a single sample with a DKK1 level of 476 ng per milliliter. The DKK1 level in each sample is indicated by the height of the bar. Samples are ordered from the lowest to highest DKK1 levels from left to right on the x axis.

circulating blood and the diffuse osteopenia that can occur in multiple myeloma. Recent in vitro studies have shown that short-term exposure of mesenchymal stem cells (osteoblast precursors) to low levels of recombinant DKK1 caused them to proliferate, whereas long-term exposure to high levels caused a loss of viability.¹⁵ Thus, in addition to blocking the terminal differentiation of osteoblasts, the sustained high levels of DKK1 in the bone marrow of patients with multiple myeloma may also cause a loss in viability of osteoblast stem cells.

Immunosuppression and anemia represent serious complications in patients with multiple myeloma and are thought to be caused by a cancer-related defect in hematopoiesis. Given that hematopoietic stem-cell proliferation is dependent on a bone marrow niche that is created by osteoblasts^{29,30} and that canonical Wnt signals can directly regulate the capacity of hematopoietic stem cells for self-renewal,^{31,32} elevated levels of DKK1 may also have a role in causing immunosuppression and anemia. We hypothesize that DKK1 could block proliferation of hematopoietic stem cells directly by blocking canonical Wnt signaling on such cells or indirectly by





inhibition of osteoblast differentiation — hence the establishment of the bone marrow microenvironmental niche that hematopoietic stem cells require for proliferation. Elevated levels of DKK1 may also negatively influence several important steps in the mobilization, engraftment, and proliferation of hematopoietic stem cells during the course of autologous transplantation, which is now considered the

therapy of choice in the treatment of multiple myeloma.³³

Not all patients with newly diagnosed multiple myeloma who had lytic bone lesions had elevated levels of expression of the DKK1 gene or protein. DKK1 is rarely detected in plasma cells from patients with monoclonal gammopathy of undetermined significance or patients with myeloma who have

Figure 4 (facing page). Effect of Recombinant DKK1 (rhDKK1) and Bone Marrow Plasma from Patients with Multiple Myeloma on Alkaline Phosphatase Production in C2C12 Cells Treated with Bone Morphogenetic Protein Type 2 (BMP-2).

In Panel A, alkaline phosphatase levels, a marker of osteoblast differentiation, were measured in C2C12 cells after five days of culture in the presence of 5 percent fetal-calf serum alone or with 50 ng of BMP-2 per milliliter; BMP-2 and 50 ng of rhDKK1 per milliliter; BMP-2, rhDKK1, and anti-DKK1 antibody; or BMP-2, rhDKK1, and polyclonal IgG. Each bar represents the mean (\pm SE) of triplicate experiments. The activity of alkaline phosphatase increased in the presence of BMP-2 and was significantly reduced by coinubation with rhDKK1. Anti-DKK1 antibody, but not polyclonal IgG, blocked the suppressive activity of rhDKK1. P values are for the comparison with BMP2 alone. In Panel B, alkaline phosphatase levels were measured in C2C12 cells after culturing these cells for five days in 5 percent fetal-calf serum; 50 ng of BMP-2 per milliliter and 10 percent normal bone marrow plasma; BMP-2 and 10 percent bone marrow plasma from 10 patients with newly diagnosed myeloma; BMP-2, 10 percent bone marrow plasma from the patients, and anti-DKK1 antibody; or BMP-2, 10 percent bone marrow plasma from the patients, and goat polyclonal IgG. Each bar represents the mean (\pm SE) of triplicate experiments. The DKK1 level in each bone marrow plasma sample (shown in parentheses below the graph) was determined by enzyme-linked immunosorbent assay, and final levels in culture after 1:10 dilution are indicated. Samples with more than 12 ng of DKK1 per milliliter had an effect on alkaline phosphatase production that could be significantly inhibited by anti-DKK1 antibody. P values are for the comparison between the alkaline phosphatase levels in response to BMP-2 plus 10 percent serum and in response to BMP-2, 10 percent serum, and anti-DKK1 in all 10 patients.

nuclear factor- κ B (RANK) ligand and RANK, or osteoprotegerin, has a dominant role in the activation and survival of osteoclasts,^{34,35} and elevated serum levels of RANK ligand are associated with markers of bone resorption, osteolytic lesions, and reduced survival in multiple myeloma.³⁶⁻³⁸ Moreover, immature, but not mature, osteoblasts are rich sources of RANK ligand.³⁹ For these reasons, it is plausible that the DKK1-mediated block in osteoblast differentiation may stimulate osteoclasts, because osteoblast precursors produce relatively large amounts of RANK ligand. We have recently shown that in vitro coculture of DKK1-positive myeloma cells with osteoclasts results in significant down-regulation of the expression of DKK1 in plasma cells.⁴⁰ Thus, the findings in the subgroup of patients who have myeloma with lytic lesions who do not express DKK1 may be representative of more advanced disease, in which the down-regulation of DKK1 is mediated by an increase in the numbers of osteoclasts.

We propose that the DKK1 produced by myeloma cells blocks the differentiation of osteoblasts and promotes the early proliferation and, subsequently, the reduced viability of mesenchymal stem cells. During progression of the disease, these events would shift the balance between osteoblasts and osteoclasts in favor of osteoclasts, thereby diminishing bone formation and enhancing bone resorption and, hence, the development of lytic lesions.

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end-stage disease or secondary plasma-cell leukemia (unpublished data), indicating that increased DKK1 expression is restricted to a specific stage of the disease.

An interaction between the receptor activator of

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