The bone marrow niche in the pathogenesis of multiple myeloma: A role for Wnt signaling and adrenomedullin
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N-cadherin-mediated interaction with multiple myeloma cells inhibits osteoblast differentiation

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ABSTRACT

BACKGROUND
Multiple myeloma is a hematologic malignancy characterized by a clonal expansion of malignant plasma cells in the bone marrow, which is accompanied by the development of osteolytic lesions and/or diffuse osteopenia. The intricate bidirectional interaction with the bone marrow microenvironment plays a critical role in sustaining the growth and survival of myeloma cells during tumor progression. Identification and functional analysis of the (adhesion) molecules involved in this interaction will provide important insights into the pathogenesis of multiple myeloma.

DESIGN AND METHODS
Multiple myeloma cell lines and patients’ samples were analyzed for expression of the adhesion molecule N-cadherin by immunoblotting, flow cytometry, immunofluorescence microscopy, immunohistochemistry and expression microarray. In addition, by means of blocking antibodies and inducible RNA interference we studied the functional consequence of N-cadherin expression for the myeloma cells, by analysis of adhesion, migration and growth, and for the bone marrow microenvironment, by analysis of osteogenic differentiation.

RESULTS
The malignant plasma cells in approximately half of the multiple myeloma patients, belonging to specific genetic subgroups, aberrantly expressed the homophilic adhesion molecule N-cad-herin. N-cadherin-mediated cell-substrate or homotypic cell-cell adhesion did not contribute to myeloma cell growth in vitro. However, N-cadherin directly mediated the bone marrow localization/retention
of myeloma cells *in vivo*, and facilitated a close interaction between myeloma cells and N-cadherin-positive osteoblasts. Furthermore, this N-cadherin-mediated interaction contributed to the ability of myeloma cells to inhibit osteoblastogenesis.

**CONCLUSIONS**

Taken together, our data show that myeloma cells frequently display aberrant expression of N-cadherin and that N-cadherin mediates the interaction of myeloma cells with the bone marrow microenvironment, in particular the osteoblasts. This N-cadherin-mediated interaction inhibits osteoblast differentiation and may play an important role in the pathogenesis of myeloma bone disease.
INTRODUCTION

Multiple myeloma (MM) is a neoplasm characterized by clonal expansion of malignant plasma cells. The transition of a normal plasma cell to a fully transformed, aggressive myeloma is considered to be a multistep process, which requires the acquisition of chromosomal translocations and mutations in multiple genes.\textsuperscript{1,2} Most of this evolution takes place in the bone marrow (BM), indicating that the interaction with the BM microenvironment plays a critical role in the pathogenesis of MM.

As in normal plasma cell homing, a major player in the recruitment to and retention of MM plasma cells in the BM is the CXCL12/CXCR4 axis.\textsuperscript{3,4} The chemokine CXCL12 promotes transendothelial migration and induces α4β1 mediated adhesion to VCAM-1 and fibronectin.\textsuperscript{5} Malignant plasma cells express several cell-surface molecules which mediate either homotypic cell adhesion, e.g. N-CAM, or adhesion with the extracellular matrix or other cells in the BM microenvironment, e.g. the integrins α4β1 and α5β1 and mucin1.\textsuperscript{1,6} These interactions control long-term survival and proliferation,\textsuperscript{7} and may also render the MM cells resistant to the pro-apoptotic effects of conventional chemotherapies,\textsuperscript{6} a process known as cell adhesion-mediated drug resistance (CAM-DR). In addition, these interactions may also affect the BM microenvironment.

Under physiological conditions bone remodeling is a continuous process in which osteoclasts mediate resorption of “old” bone tissue, followed by new bone formation by the osteoblasts. These two processes are tightly regulated by signals involving the RANKL-RANK axis, the Wnt signaling pathway, and N-cadherin engagement,\textsuperscript{8–10} with the last interaction serving an important role in osteoblast function and differentiation. One of the characteristic features of MM is osteolytic bone destruction, resulting from the activation of osteoclasts and inhibition of osteoblast function. Notably, the actions of several factors produced by
MM cells and involved in the deregulation of osteoblast differentiation, such as DKK1,11 sFRP-2,12 and HGF,13 were shown to be partially dependent on adhesion of myeloma cells to the osteoblasts via integrin α4β1.14

We have previously shown that malignant plasma cells from MM patients overexpress β-catenin, including the non-phosphorylated form, leading to active β-catenin/TCF-mediated transcription and MM cell proliferation.15 Subsequent studies confirmed the presence of Wnt pathway activation in MM and its importance for MM cell proliferation and survival.16,17 Interestingly, our immunohistochemical studies revealed that β-catenin is localized in the nucleus as well as at the plasma membrane, at the site of cell-cell contact. Indeed, in addition to its role as a transcriptional regulator in the Wnt signaling pathway, β-catenin is also a key regulator of cadherin-mediated adhesion.18 Cadherins comprise a family of transmembrane adhesion molecules that mediate calcium-dependent cell-cell adhesion through homophilic interactions. In the “classical” cad-herins, the conserved cytoplasmic domain forms a complex with the catenins p120catenin, β-catenin and α-catenin, which are possible regulators of cadherin function and link it to the cytoskeleton.19 In this study, we show that malignant plasma cells of a subset of MM patients express N-cadherin. We explored the function of N-cadherin expression in MM pathogenesis, focusing on its potential role in bone marrow localization of malignant cells and myeloma bone disease.

DESIGN AND METHODS

CELL LINES AND ANTIBODIES
MM cell lines were cultured as described previously.15 Further details on the cell lines, culture conditions, and antibodies are provided in the Supplementary Design and Methods.

A doxycycline-inducible NCI-H929 MM cell line was generated as described previously,20 using the T-REx™ System (Invitrogen Life Technologies), containing the TET repressor only (H929 TR), or combined with a specific shRNA against CDH2, 5′-GAGCCT-GAAGCCAACCTTA-3′ (H929 shCDH2). N-cadherin knockdown was obtained by incubating NCI-H929 shCDH2 cells for 5 days with 0.2 μg/mL doxycycline.

CELL GROWTH ASSESSMENT
Cells were plated (5 × 10³) in a 96-well plate coated with recombinant N-cadherin/Fc chimera (1 μg/mL; R&D Systems, Abingdon, UK), or BSA as control. Further
details on the cell growth assessment assays are provided in the **Supplementary Design and Methods**.

**IMMUNOFLUORESCENT MICROSCOPY, IMMUNOHISTOCHEMISTRY AND FLOW CYTOMETRY**

N-cadherin (mouse mAb clone 32) and β-catenin (rabbit pAb H-102) expression in MM cell lines was analyzed by immunofluorescence microscopy on paraformaldehyde-fixed cytopsins. Expression was detected, using Alexa488-conjugated goat anti-mouse and Alexa568-conjugated goat anti-rabbit as secondary antibodies, and analyzed by confocal laser scan microscopy (CLSM).

Immunohistochemical staining was performed on formalin-fixed, plastic-embedded tissue sections as described elsewhere. Further details are provided in the **Supplementary Design and Methods**.

Flow cytometry analysis of N-cadherin expression was determined by incubation of cells with an anti-N-cadherin monoclonal antibody (clone GC-4, Sigma-Aldrich) followed by biotinylated goat anti-mouse IgG1 (Southern Biotechnology, Birmingham, AL, USA) and subsequently RPE-conjugated streptavidin (DAKO). Analysis was carried out on a FACScalibur flow cytometer (BD Biosciences) with CellQuestTM software (BD Biosciences).

**IMMUNOPRECIPITATION, IMMUNOBLOT ANALYSIS, CELL ADHESION AND MIGRATION ASSAYS**

Immunoprecipitation, western blotting, adhesion and migration assays were performed essentially as described previously. Further details on these assays are provided in the **Supplementary Design and Methods**.

**SAMPLE PREPARATION AND MICROARRAY HYBRIDIZATION AND ANALYSIS**

Isolation of plasma cells and profiling of RNA were performed as described previously. Further details are provided in the **Supplementary Design and Methods**.

**HOMING ASSAY**

H929 shCDH2 cells were incubated for 5 days with (KD) or without (WT) 0.2 μg/mL doxycycline. Cells were labeled with either 0.25 μM Cell tracker Green (CMFDA; Invitrogen) or 2 μM Cell tracker Orange (CMTMR; Invitrogen), mixed (1:1), and 15×10⁶ cells were injected intravenously into RAG2⁻/‒γc⁻/‒ mice. Each WT/KD combination was analyzed by adoptive transfer of eight recipient mice, and included a dye-swap. After 24 h, blood and bone marrow were collected and
analyzed by FACS to quantify dye-labeled cells. The percentage of homing cells was corrected for the input ratio. All animal experiments were conducted according to the Institutional Guidelines of the University Medical Center Utrecht, after acquiring permission from the local Ethical Committee for Animal Experimentation and in accordance with current Dutch laws on animal experiments.

OSTEOBLAST DIFFERENTIATION
KS483 cells and primary human mesenchymal stromal cells were cultured and differentiated essentially as described elsewhere,\textsuperscript{24,25} in the absence or presence of MM cells. Further details are provided in the \textit{Supplementary Design and Methods}.

REAL-TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION
RNA isolation and cDNA synthesis were performed as described previously.\textsuperscript{15} The quantitative reverse transcription-polymerase chain reaction (qRT-PCR) runs were performed on a Roche LightCycler 1.5 using FastStart DNA Master SYBR Green I kit (Roche, Basel, Switzerland). Results were analyzed using LinReg PCR analysis software (version 7.5\textsuperscript{26}). Expression was normalized over β-2-microglobulin expression. Further details are provided in the \textit{Supplementary Design and Methods} and Supplementary Tables 1 and 2.

STATISTICAL ANALYSIS
The unpaired two-tailed Student’s \( t \)-test was used to determine the statistical significance of differences between means, unless otherwise stated. *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \); ns: not significant.

RESULTS
MULTIPLE MYELOMA CELLS EXPRESS A FUNCTIONAL β-CATENIN/N-CADHERIN COMPLEX AT THE PLASMA MEMBRANE
We previously reported that MM plasma cells over-express β-catenin. Stimulation of Wnt signaling with either the glycogen synthase kinase-3β (GSK3β) inhibitor LiCl or Wnt3a led to further accumulation and nuclear localization of β-catenin, resulting in enhanced TCF-mediated transcription and increased cell proliferation.\textsuperscript{15} Interestingly, however, β-catenin was not only localized in the nucleus of the MM cells, but was also frequently observed at the plasma membrane, at the
cell-cell contact sites between adjacent MM cells (Figure 1A). This observation suggested that β-catenin could also be involved in intercellular adhesion, presumably through interaction with a classical cadherin. To explore this hypothesis, we screened a panel of MM cell lines for expression of cadherins. Western blot analysis revealed that, while none of the myeloma cell lines tested expressed E-cadherin, four of seven were N-cadherin positive (Figure 1B). This expression was confirmed by FACS analysis (Supplementary Figure S1). Notably, the cell lines with most prevalent expression of N-cadherin also displayed the highest levels of β-catenin (Figure 1B). Furthermore, confocal laser scan microscopy revealed co-localization of N-cadherin and β-catenin at the cell-cell junctions between adjacent MM cells, suggesting a physical interaction between the two proteins (Figure 1C). Indeed, co-precipitation of N-cadherin and β-catenin confirmed the existence of an association between these molecules at the MM plasma membrane, with the majority of N-cadherin attached to β-catenin (Figure 1D).

Figure 1. Multiple myeloma cells express N-cadherin
(A) β-catenin is expressed in MM cells at the cell-cell junctions. The MM cell line OPM-1 is stained with a pAb H-102 against β-catenin and detected with Alexa568-conjugated goat anti-rabbit by confocal laser scan microscopy (CLSM). (B) Cadherin expression in MM cell lines. Cell lysates were immunoblotted using monoclonal antibodies against E-cadherin (HECD-1), N-cadherin (clone 32) and β-catenin (clone 14). The breast carcinoma cell line T47D and the malignant glioma cell line U251 MG were used as positive controls for E-cadherin and N-cadherin, respectively. β-actin was used as the loading control. (C) N-cadherin co-localizes with β-catenin at the cell-cell contacts. OPM-1 cells were double-stained with an antibody against N-cadherin (clone 32) and β-catenin (H-102), followed by secondary antibodies Alexa488-conjugated goat anti-mouse and Alexa568-conjugated goat anti-rabbit. Expression of N-cadherin (green; left panel) and β-catenin (red; middle panel) and co-localization (orange; right panel) was detected by CLSM. (D) Co-immunoprecipitation of N-cadherin with β-catenin. Cell
lysates of the MM cell line OPM-1 were immunoprecipitated (IP) with monoclonal antibody clone 32 (N-cadherin), clone 14 (β-catenin), or control IgG1 antibody, pre-coupled to Protein G-Sepharose beads. Western blots (WB) were stained with anti-N-cadherin (clone 32) and anti-β-catenin (clone 14). As a specificity control, mock incubations without lysates, were immunoblotted and stained. Total lysates (TL) were immunoblotted and stained with anti-β-catenin (clone 14), as an input control.

Classical cadherins, including N-cadherin, mediate cell-cell adhesion via homophilic interaction. To assess the functional integrity of the N-cadherin/β-catenin complexes on the MM cells we tested the ability of N-cadherin-expressing MM cells to adhere to an N-cadherin-coated surface. As shown in Figure 2A, the N-cadherin-positive MM cell lines OPM-1 and NCI-H929 specifically adhered to recombinant N-cadherin, an effect which could be prevented completely by the N-cadherin blocking antibody (GC-4). Notably, the N-cadherin-negative MM cell lines L363 and UM3 did not show specific binding to N-cadherin (data not shown). CD138-positive MACS-purified primary MM cells, expressing N-cadherin, also showed specific adhesion to coated N-cadherin (Figure 2B), whereas primary MM cells without N-cadherin did not adhere (data not shown).

![Figure 2. N-cadherin-mediated adhesion of MM cells](image)

(A) Adhesion of MM cells to recombinant N-cadherin. OPM-1 (left panel) and NCI-H929 (right panel) cells adhere to a surface coated with recombinant N-cadherin (1 μg/mL), compared to BSA-coating as a negative control. This adhesion could be prevented by an N-cadherin blocking antibody, GC-4 (10 μg/mL). (B) Adhesion of primary patients’ material to recombinant N-cadherin. Primary myeloma cells (pMM) were MACS purified using CD138-coated beads (> 95% pure) and were allowed to adhere to a surface coated with recombinant N-cadherin (1 μg/mL), compared to BSA-coating as a negative control.

**EXPRESSION OF N-CADHERIN IN PRIMARY MULTIPLE MYELOMAS**

To assess the prevalence of N-cadherin expression in primary MM, in purified plasma cells from a panel of 559 MM patients, using Affymetrix oligonucleotide microarrays we analyzed N-cadherin expression in relation to characteristic recurrent chromosomal translocations and expression of cyclin D1 and D2 (TC groups). As depicted, CDH2, the gene encoding N-cadherin, was highly expressed (> 2x) in 83% of the MM samples bearing the t(4;14)(p16;q32) translo-
cation involving MMSET. Furthermore, the subgroups characterized by high expression of cyclin D1, either alone (D1) or together with high expression of cyclin D2 (D1 + D2), consisted of two distinct populations: one with high and one with low expression of CDH2. Expression of CDH2 was less prevalent in samples with the translocations involving 11q13, 6p21, or MAF, and in the subgroup characterized by low cyclin D1 but high cyclin D2 expression (D2) (Figure 3A, left panel). Although the 4p16 translocation is known to correlate with poor prognosis, no independent prognostic value could be found for N-cadherin expression (data not shown). Importantly, analysis of the freely accessible microarray data of the Lambert Laboratory revealed that expression of N-cadherin is absent/low in normal bone marrow plasma cells from healthy donors, whereas expression is weakly elevated in MGUS (Figure 3A, right panel). In agreement with our data, analysis of CDH2 expression in this data set, in which the major myeloma subtypes are defined by gene expression profile-derived classification, revealed high expression of CDH2 in over 90% of the MMSET expression subgroup (MS) characterized by the 4p16 MMSET translocation, and low expression in the MAF expression subgroup (MF) characterized by MAF/MAFB translocations. Furthermore, the hyperdiploid subgroup (HY) revealed distinct populations with either high or low CDH2 expression, which is in line with the high concordance of the HY subgroup with our D1 subgroup.

Consistent with the mRNA expression data, immunohistochemical study of bone marrow biopsies of MM patients (n = 43) demonstrated N-cadherin protein expression in the malignant cells of approximately 50% of the patients (Figure 3B). Besides membrane expression, several of these tumors displayed strong cytoplasmic N-cadherin staining. As in the MM cell lines (Figure 1C), N-cadherin and β-catenin in the primary MM often localized at the cell-cell junctions between adjacent MM cells (Figure 3B), and between MM cells and the bone-lining cells (Figure 3C). Our observations identify N-cadherin as a myeloma-associated protein displaying deregulated expression in a subset of MM.

N-CADHERIN-MEDIATED ADHESION DOES NOT AFFECT MULTIPLE MYELOMA GROWTH
Since N-cadherin expression has been described to promote survival and to suppress cell proliferation in other cell types, we examined the role of both heterotypic as well as homotypic N-cadherin-mediated adhesion in MM growth. The direct effect of heterotypic adhesion was mimicked by seeding MM cell lines, with different levels of N-cadherin expression (Figure 1B), on recombinant N-cadherin and monitoring the growth for 4 days. Although the cells of the N-cadherin-
expressing cell lines essentially grew as single cells on the N-cadherin coating as compared to the formation of cell aggregates on the BSA coating (Supplementary Figure S2A), no differences in growth rate were observed (Supplementary Figure S2B). Similarly, no difference in cell proliferation was observed, as determined by $^3$H-thymidine incorporation (data not shown). In addition, the survival of MM cells in a single cell growth assay was not altered by culturing on a recombinant N-cadherin-coated surface (data not shown).

Figure 3. Expression of N-cadherin in primary MM

(A) Affymetrix expression profiles of N-cadherin in MM. Gene expression of 559 newly diagnosed MM patients was measured by U133 Plus2.0 Affymetrix oligonucleotide microarray probeset 203440_at, summarized with MAS5, median normalized and plotted against chromosomal translocations/cyclin D expression (left panel); and publicly available genetic MM data of 345 MM patients from the total therapy 2 (TT2) patient set were plotted against disease progression and genetic profiles (right panel). The expression of N-cadherin by plasma cells of patients in the MMSET expression (MS) and the hyperdiploid (HY) subgroups was statistically higher than that by normal bone marrow plasma cells (BMPC) from healthy donors (P < 0.001 by the Kruskal-Wallis test). (B) Expression of N-cadherin in primary MM. Consecutive sections of plastic-embedded BM-biopsies of MM patients were immunohistochemically stained with antibodies against CD138 (B-B4; left panels), N-cadherin (clone 32; middle panels), and β-catenin (clone 14; right panels). Original magnifications x64. (C) Immunohistochemical stainings of N-cadherin and β-catenin expression on MM cells adjacent to N-cadherin positive bone lining osteoblasts. N-cadherin is often localized between MM cells and the bone-lining cells (arrowhead). Plastic-embedded sections from a MM patient stained with antibodies against β-catenin (clone 14; upper panel) and N-cadherin (clone 32; lower panel) are shown. Original magnification x100.
To examine the contribution of the homotypic adhesion to the growth of MM cells, we cultured MM cells in the presence of an N-cadherin blocking antibody. However, addition of antibodies to the culture did not result in altered proliferation (*data not shown*). To determine the direct effect of N-cadherin expression on MM growth, we generated NCI-H929 cells stably transfected with a doxycycline-inducible shRNA against *CDH2* (H929 shCDH2). As shown in Supplementary Figure S2C, doxycycline treatment of the cells for 5 days resulted in a 70% reduction of N-cadherin expression. Assessment of the growth of the H929 shCDH2 cells in comparison to the negative control H929 TR cells, containing the TET repressor but not the CDH2 shRNA, showed that the doxycycline-induced knockdown of N-cadherin did not result in an aberrant growth pattern (Supplementary Figure S2D). Performing similar experiments on a N-cadherin coating also did not result in a difference in the growth rate of the cells (Supplementary Figure S2E). Collectively, these data show that neither N-cadherin expression as such, nor N-cadherin-mediated cell-substrate or homotypic cell-cell adhesion, directly affects MM cell growth in vitro.

**N-CADHERIN PLAYS A ROLE IN THE RETENTION OF MULTIPLE MYELOMA CELLS IN THE BONE MARROW**

Since N-cadherin has been implicated in migration and tumor metastasis,19,30,31 we investigated whether N-cadherin plays a role in MM cell homing. H929 shCDH2 cells were incubated with or without doxycycline for 5 days, and subsequently fluorescently labeled with either CMFDA or CMTMR. Cells were mixed (1:1), and injected intravenously into Rag-2−/−γc−/− mice. Analysis of blood and bone marrow of these mice (*n* = 4) revealed that reduced N-cadherin expression resulted in higher levels of circulating cells and reduced homing to the bone marrow (Figure 4A), with a dye swap (*n* = 4) resulting in similar results (*data not shown*). In support of these results, both basal motility (Figure 4B) and SDF-1-induced migration (Figure 4C) of the N-cadherin-expressing OPM-1 cells was potentiated in transwell migration assays when the transwells were coated with recombinant N-cadherin (from 9% to 15%, and from 45% to 80%, respectively), whereas migration of the N-cadherin-negative L363 cells was not affected. Since endothelial cells express N-cadherin, we further explored a possible role for N-cadherin in MM cell homing by transendothelial migration assays using HUVEC cells. However, in contrast to the strong inhibition observed with an α4-integrin blocking antibody, neither blocking nor knockdown of N-cadherin affected transendothelial migration towards SDF-1 (Figure 4D). Taken together, our results establish the importance of N-cadherin in localization of MM cells.
in the bone marrow, most likely reflecting a role for N-cadherin in bone marrow retention rather than in active homing of MM cells.

Figure 4. N-cadherin-mediated retention of MM cells in the bone marrow

(A) H929 shCDH2 cells were incubated with or without doxycycline for 5 days, and subsequently labeled with either CMFDA or CMTMR. Cells were mixed (1:1), and injected intravenously into Rag-2−/− γc−/− mice. Each CMFDA/CMTMR combination was analyzed by adoptive transfer of eight recipient mice, which included a dye-swap. After 24 h, blood and bone marrow were collected and FACS analyzed to quantify dye-labeled cells. The percentage of homing cells was corrected for the input ratio. The bars represent the means ± SD for four mice. (B) MM cells were allowed to migrate for 4 h in the absence of SDF-1 in transwells coated with recombinant N-cadherin (1 μg/mL), compared to BSA-coating as a negative control. (C) MM cells were allowed to migrate for 4 h towards 100 ng/mL SDF-1 in transwells coated with recombinant N-cadherin (1 μg/mL), or sVCAM-1 (1 μg/mL) compared to BSA-coating as a negative control. (D) H929 shCDH2 cells were allowed to migrate for 5 h towards 100 ng/mL SDF-1 over an endothelial monolayer. H929 shCDH2 cells were cultured in the presence (+dox) or absence (-dox) of doxycycline for 5 days before migration (left panel). In addition to the doxycycline-treatment, MM cells were coated with blocking antibodies against N-cadherin (GC-4) or α4-integrin (HP2/1) before migration (right panel).

**N-CADHERIN-MEDIATED INTERACTION WITH MULTIPLE MYELOMA CELLS SUPPRESSES OSTEOBLAST DIFFERENTIATION**

MM-related osteolytic bone disease is caused by an imbalance between osteoblast and osteoclast activity. MM cells have been shown to suppress osteoblast differentiation and activity via at least two different mechanisms, *i.e.*, by secreting soluble factors11–13 and by direct cell-cell contact with osteoblasts.14 This,
combined with the observations that N-cadherin-positive MM cells reside in close proximity to the osteoblasts in the bone marrow of MM patients (Figure 3C) and that N-cadherin-mediated adhesion plays an important role in osteoblast maturation,\textsuperscript{9,32,33} prompted us to explore the possible contribution of N-cadherin-mediated adhesion to the contact-dependent suppression of osteoblast differentiation by MM cells.

After confirming the high N-cadherin expression by pre-osteoblastic cells (Figure 5A), we determined whether MM cells could adhere to these cells in an N-cadherin-dependent manner. To avoid integrin-mediated adhesion, experiments were performed in the presence of calcium as the only divalent cation. Indeed, more than 60% of the N-cadherin-positive MM cells adhered to the osteoblasts (Supplementary Figure S3A, left panels), and, moreover, this adhesion could be blocked by pre-incubation of the MM cells with an antibody that blocked N-cadherin function (Supplementary Figure S3A, right panels and Figure 5B). This heterotypic cell-cell interaction was further investigated using doxycycline-inducible H929 shCDH2 cells, which upon doxycycline-treatment displayed an approximately 70% reduction of N-cadherin expression (Supplementary Figure S2C). In line with the blocking antibody results (Supplementary Figure S3A and Figure 5B), these cells showed diminished adhesion to osteoblasts upon silencing of N-cadherin expression, whereas no difference in adhesion was observed with the control H929 TR cells (Supplementary Figure S3B and Figure 5C).

To investigate the effect of N-cadherin-mediated MM adhesion on osteoblast differentiation, the doxycycline-inducible cells were co-cultured with murine KS483 pre-osteoblastic cells which, upon reaching confluence and the addition of ascorbic acid, differentiate into mature osteoblasts expressing alkaline phosphatase (ALP). Co-cultures of KS483 cells with either H929 shCDH2 cells or H929 TR cells resulted in a strong inhibition of ALP activity (Figure 5D). Interestingly, doxycycline-induced knockdown of N-cadherin markedly attenuated the ability of H929 shCDH2 cells to inhibit osteoblast differentiation, whereas doxycycline treatment of the control H929 TR cells had no effect (Figure 5D). The inhibitory effect of this N-cadherin-mediated interaction on osteoblast differentiation was further substantiated by measuring the mRNA levels of the early osteogenic markers \textit{Akp2} and \textit{Col1a1} and the late marker \textit{Bglap} (Figure 5E), encoding alkaline phosphatase, collagen type I, alpha1 and osteocalcin, respectively. As for ALP activity, the ability of MM cells to inhibit the expression of \textit{Akp2}, \textit{Col1a1} and \textit{Bglap} was significantly diminished upon N-cadherin knockdown (Figure 5E), whereas no significant change was observed in the expression of
the (pre-)osteogenic transcription factors Osx and Runx2 (Supplementary Figure S4A). Notably, co-culture of the H929 MM cells did not affect the expression of the osteoclastogenic factors Cdhl, Vcam1, Tnfsf11/Rankl, or Il6 by the KS483 cells (Supplementary Figure S4A-B). In addition, co-cultures of primary human mesenchymal stromal cells with H929 shCDH2 cells or H929 TR cells also revealed a diminished inhibition of the late osteogenic marker BGLAP upon N-cadherin knockdown, while there was no change in expression of the early markers ALPL or COL1A1 (Figure 5F). Furthermore, in the co-cultures with either KS483 or these primary mesenchymal stromal cells, N-cadherin silencing did not reduce MM growth. Thus, N-cadherin-mediated adhesion does not control production of MM-growth supportive cytokines by the osteoblasts, and the observed impaired inhibition of osteoblast differentiation upon N-cadherin silencing is not due to reduced MM cell numbers (Supplementary Figure S4C). Taken together, these observations show that N-cadherin plays an important role in the interaction of MM cells with osteoblasts, and establish an important role for this N-cadherin-mediated interaction in the inhibition of osteoblastogenesis.

Figure 5. N-cadherin mediates inhibition of osteoblast differentiation by MM cells
(A) N-cadherin expression in osteoblastic cell lines. Cell lysates were immunoblotted using a monoclonal antibody against N-cadherin (clone 32), and β-actin was used as a loading control. (B) N-cadherin-mediated adhesion of MM cells to osteoblasts. MM cell lines were allowed to adhere to C3H10T1/2

![Image](image.jpg)
cells in the presence of an N-cadherin blocking antibody (GC-4) or isotype control antibodies. (C) N-cadherin knockdown abolishes N-cadherin-mediated adhesion of MM cells to osteoblasts. H29 TR and H929 shCDH2 cells were incubated with or without doxycycline for 5 days, and subsequently allowed to adhere to C3H10T1/2 cells. (D) N-cadherin mediates MM cell-controlled inhibition of osteoblast differentiation. Murine (pre-)osteoblastic KS483 cells were, upon confluence, further differentiated in the presence or absence of MM cells, either treated with (black bars) or without doxycycline (white bars), and subsequently stained for alkaline phosphatase (ALP) expression and quantified. (E) N-cadherin represses alkaline phosphatase (Akp2), collagen type I, alpha1 (Col1a1), and osteocalcin (Bglap) gene expression. Murine KS483 cells were differentiated in the presence or absence of H929 TR or H929 shCDH2 cells with (black bars) or without doxycycline (white bars), and subsequently analyzed by qRT-PCR. (F) N-cadherin represses the late human osteogenic differentiation. Human primary MSC were differentiated in the presence or absence of H929-TR or H929-shCDH2 cells with (black bars) or without doxycycline (white bars), and subsequently analyzed by qRT-PCR for the expression of the early osteogenic markers alkaline phosphatase (ALPL), and collagen type I, alpha1 (COL1A1), and the late marker osteocalcin (BGLAP).

DISCUSSION

Although MM cell growth is driven by genetic alterations like translocation and mutations, these cells still remain dependent upon the BM microenvironment. The interactions of MM cells with the BM microenvironment, either directly via adhesion molecules or indirectly via the ensuing stimulation of autocrine/paracrine production of cytokines, activate a broad range of proliferative and anti-apoptotic signaling pathways. Here, we identified a new interactant of MM cells with the BM microenvironment, i.e. N-cadherin.

We observed high expression of N-cadherin on the malignant plasma cells in a subset of approximately 50% of primary MM as well as MM cell lines, but not in normal BM plasma cells (Figures 1 and 3). The functionality of the expressed N-cadherin was initially demonstrated by means of in vitro homophilic adhesion assays (Figure 2). As expected, N-cadherin co-localizes and physically interacts with β-catenin (Figures 1 and 3). Furthermore, high levels of N-cadherin seem to correlate with high levels of β-catenin (Figure 1B). This increase in β-catenin protein levels might be explained by the “protective” effect of N-cadherin binding to β-catenin, preventing degradation of the latter.34,35

Our gene expression profiling analysis of a large group of MM patients revealed that CDH2, the gene encoding N-cadherin, is highly, but not exclusively, expressed in MM cells bearing a t(4;14)(p16;q32) translocation. Although this MM subtype is associated with poor prognosis,36,37 expression of CDH2 by itself does not predict prognosis. Notably, in a previous independent gene expression profiling study of 29 primary MM samples, CDH2 was among the genes up-regulated in five patients carrying the t(4;14) MMSET translocation.38 Moreover, CDH2 was among the
genes down-regulated upon silencing of the \textit{MMSET} gene in the KMS-11 cell-line.\textsuperscript{39} Also, in our panel of MM cell-lines, the two cell-lines that carry the t(4;14) translocation, \textit{i.e.}, OPM-1 and NCI-H929, displayed prominent N-cadherin expression (Figure 1). Taken together, these data indicate that the aberrant expression of N-cadherin in a subgroup of MM patients may, at least in part, be the indirect consequence of overexpression of the transcriptional repressor MMSET.

Increasing evidence indicates that the gain of N-cadherin expression in solid tumor cells is associated with enhanced invasive potential and metastasis.\textsuperscript{30,31,40} In line with these reports, we here show that N-cadherin potentiates basal MM cell motility as well as SDF-1-induced migration (Figure 4B-C), and contributes to the homing to and/or retention in the BM of MM cells (Figure 4A). In order to separate these two processes, we performed a transendothelial migration assay, showing that knockdown of N-cadherin and/or treatment with an N-cadherin blocking antibody, did not affect migration of MM cells across an endothelial barrier (Figure 4D), whereas a blocking antibody targeting α4-integrin did suppress migration of the cells. In this context it is important to note that endothelial cells mainly display extrajunctional localization of N-cadherin,\textsuperscript{41,42} which might facilitate adhesion rather than migration in these static migration assays. Nevertheless, our results favor the hypothesis that N-cadherin is involved in the retention of MM cells in the BM, which may be the consequence of homotypic and/or heterotypic cell-cell adhesion within the BM microenvironment.

Indeed, N-cadherin expression by MM cells participates in homophilic, heterotypic adhesion of MM cells with their environment, \textit{e.g.}, with osteoblasts (Figures 2 and 5). It has become apparent that the interplay between MM cells and bone is bi-directional. Important clinical sequelae of this bidirectional interaction is increased osteoclast activity and suppression of osteoblast function, resulting in osteolytic bone lesions.\textsuperscript{6,7} Besides inhibition by soluble factors, such as Wnt antagonists, activin A, interleukin-3 and interleukin-7, osteoblast function and maturation can also be suppressed through direct contact between MM cells and pre-osteoblastic cells mediated via α4β1-VCAM-1 and/or N-CAM-N-CAM interactions.\textsuperscript{43} The latter findings, in addition to the reported key role of cadherin-based interactions in osteoblast function and differentiation,\textsuperscript{9,32,33} prompted us to investigate whether N-cadherin-mediated adhesion of MM cells to osteoblasts (Figure 5B–C and Supplementary Figure S3) can contribute to inhibition of osteoblast differentiation. Indeed, silencing of N-cadherin in myeloma cells impaired these cells’ capacity to suppress osteoblast differentiation (Figure 5D-F). It should, however, be noted that although these data clearly show that N-cadherin-mediated adhesion of MM cells to osteoblasts contributes to inhibition of osteoblast differentiation, no cor-
relation could be found between \textit{CDH2} expression and the presence of osteolytic lesions (\textit{data not shown}), and N-cadherin expression was also (weakly) enhanced in some cases of MGUS (Figure 3A, right panel). This pre-malignant stage of disease is characterized by a small expansion of clonal plasma cells, diffusely located throughout the bone marrow, and by the absence of osteolytic lesions. Presumably, inhibition of osteoblast differentiation may require local high amounts of plasma cells. Notably, expression of another molecule associated with bone disease, DKK1, has also been observed in MGUS.\textsuperscript{11} Furthermore, since N-cadherin expression not only contributes to the inhibition of osteoblast differentiation, but also to migration and BM homing/retention (Figure 4), it may well be that in this pre-malignant stage of disease N-cadherin is mainly involved in determining the localization of the plasma cells. Together our results indicate that N-cadherin expression contributes to osteolysis, but is neither critical nor sufficient for this process.

In addition to the indirect role of N-cadherin discussed above, N-cadherin-mediated adhesion may also directly inhibit osteoblast differentiation. Since both loss of function and over-expression of N-cadherin have been shown to result in osteoporosis,\textsuperscript{32,33,44} two distinct mechanisms may account for this. The adhesion may induce relocation of N-cadherin from the intercellular osteoblast junctions to the site of MM contact, resulting in loss of osteoblast-osteoblast contact and osteoblast function. Alternatively, adhesion may enhance N-cadherin stability and expression, resulting in inhibition of osteoblast differentiation by interfering with Wnt signaling,\textsuperscript{33} a pathway playing a central role in the osteolytic bone disease observed in MM.\textsuperscript{11,12,43}

Apart from contributing to osteolytic bone disease, the N-cadherin-mediated interaction of MM with osteoblasts may also contribute to another sequela of MM, \textit{i.e.} the development of pancytopenia. Since osteoblasts have a central role in the organization of the endosteal stem cell niche,\textsuperscript{45} it is tempting to speculate that N-cadherin expression might enable MM cells to access this niche, leading to niche dysregulation and thereby contributing to the pancytopenia that is typically observed in MM patients.

In conclusion, our data indicate that N-cadherin-mediated interaction of MM cells with osteoblasts may be involved in bone marrow retention and results in reduced osteoblast differentiation, which may play a crucial role in the pathogenesis of myeloma bone disease. Targeting N-cadherin, \textit{e.g.}, with a recently developed small cyclic peptide ADH-1,\textsuperscript{46} may prove to be a successful novel means of therapeutic intervention in a subgroup of MM patients.
REFERENCES

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY DESIGN AND METHODS

ANTIBODIES
Monoclonal antibodies were: anti-N-cadherin, clone 32 (IgG1); anti-β-catenin, clone 14 (IgG1) (both from BD Biosciences, Erembodegem, Belgium); anti-β-actin, clone AC-15 (IgG1); anti-N-cadherin, clone GC-4 (IgG1) (both from Sigma-Aldrich, St Louis, MO, USA); anti-E-cadherin, clone HEC-D-1 (IgG1) (Takara Bio, Shiga, Japan); anti-CD138, clone B-B4 (IgG1) (IQ Products, Groningen, The Netherlands); and IgG1 control antibody (DAKO, Carpinteria, CA, USA).

Polyclonal antibodies used were: rabbit anti-human β-catenin, H-102 (Santa Cruz Biotechnology, Santa Cruz, CA; USA); horseradish peroxidase (HRP)-conjugated rabbit anti-mouse; R-phycoerythrin (RPE)-conjugated streptavidin (both from DAKO); biotinylated goat anti-mouse IgG1 (Southern Biotechnology, Birmingham, AL, USA); Alexa488-conjugated goat anti-mouse and Alexa568-conjugated goat anti-rabbit (both from Invitrogen Life technologies, Breda, The Netherlands).

CELL CULTURE
Multiple myeloma (MM) cell lines, UM-1, UM-3, L363, OPM-1, NCI-H929, XG-1 and LME-1 were cultured as described previously (Derksen et al., 2004). The murine cell lines C2C12 and C3H10T1/2 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies) and murine pre-osteoblastic KS483 cells were grown in minimum essential medium (MEM) alpha (Invitrogen Life Technologies), both supplemented with 10% fetal calf serum (FCS), penicillin (50 U/mL) and streptomycin (50 μg/mL) (both from Invitrogen Life Technologies).

CELL GROWTH ASSESSMENT
Cells were plated (5 × 10³) in a 96-well plate coated with recombinant N-cadherin/Fc chimera (1 μg/mL; R&D Systems, Abingdon, UK), or BSA as control. When assessing the growth of the doxycycline-inducible cell line, knockdown was obtained by incubating NCI-H929 shCDH2 cells for 5 days with 0.2 μg/mL doxycycline prior to the assay, and maintained by addition of 0.2 μg/mL doxycycline to the culture medium. Cell numbers and viability were determined by means of trypan blue staining (Sigma-Aldrich). The effect of co-culture with KS483 or human mesenchymal stem cells on MM growth was analyzed using luciferase-marked myeloma cells, which were generated as described previously.
Cell viability was determined with a luminometer as described by McMillin et al. (McMillin et al., 2010).

**IMMUNOPRECIPITATION AND IMMUNOBLOT ANALYSIS**
Immunoprecipitation and western blot analysis were performed as described previously (de Gorter et al., 2007). The immunoblots were stained with anti-N-cadherin (clone 32), anti-E-cadherin (HECD-1), or anti-β-catenin (clone 14). Equal loading was confirmed with anti-β-actin. Primary antibodies were detected by HRP-conjugated rabbit anti-mouse, followed by detection using Lumi-Light-PLUS western blotting substrate (Roche, Basel, Switzerland).

**CELL ADHESION ASSAYS**
Cell-substrate adhesion assays were done as described previously (Spaargaren et al., 2003), in triplicate on flat-bottom 96-well plates (Costar, Cambridge, MA, USA) coated overnight at 4°C with PBS containing 1 μg/mL recombinant N-cadherin/Fc chimera (R&D Systems), 4% BSA, or for 15 min at 37°C with 1 mg/mL poly-l-lysine (PLL), and blocked for 2 h at 37°C with 4% BSA in RPMI 1640. MM cells (10^5 cells/100 μL) were plated and incubated at 37°C for 20 min. To block N-cadherin-mediated adhesion, cells were incubated with the monoclonal antibody GC-4 (10 μg/mL) for 30 min at 4°C, prior to the adhesion assay. Results are presented as percentages of maximum adhesion, as measured by adhesion to the PLL-coated surface, and the bars represent the means ±SD of a triplicate experiment of at least three independent experiments. Malignant plasma cells were isolated from bone marrow aspirates from MM patients using magnetic activated cell sorting (MACS) as described elsewhere (Derksen et al., 2003).

For cell-cell adhesion assays, C3H10T1/2 osteoblastic cells were seeded at a density of 7500 cells/200 μL, in a 96-well flat-bottom tissue culture plates (Costar). Twenty-four hours after plating an adhesion assay was performed by adding MM cells (10^5 cells/100 μL) and incubating for 20 min either in culture medium as a control, or in Hanks’ balanced salt solution (HBSS) in the presence or absence of 2 mM calcium chloride, and in the presence of an N-cadherin blocking antibody (10 μg/mL), or an isotype antibody as a control. Images were captured using an EVOS original camera (AMG, Mill Creek, WA, USA) and processed with Adobe Photoshop. The results are expressed as relative adhesion with the adhesion of the non-pretreated MM cells to C3H10T1/2 cells in HBSS supplemented with calcium and the isotype control normalized to 100. The bars represent the means ± SD of four measurements, representative of at least three independent experiments.
Migration assays were performed in triplicate as described previously (de Gorter et al., 2007), with transwells (8μm pore size; Costar) coated with 1 μg/mL recombinant N-cadherin/Fc chimera (R&D Systems), sVCAM-1 (R&D Systems), or BSA (fraction V; Sigma-Aldrich) coating as a control.

Transendothelial migration was performed by growing a confluent layer of HUVEC cells on a fibronectin-coated transwell insert. Subsequently, H929 shCDH2 cells (5 \times 10^5), either induced with or without doxycycline, were added and allowed to migrate for 5 h towards 100 ng/mL SDF-1, in the presence or absence of blocking antibodies against N-cadherin (GC-4), or α4-integrin (HP2/1). The amount of viable migrating cells was determined by fluorescence-activated cell sorting (FACS) and expressed as a percentage of the input. The percentage of non-pretreated cells was normalized to 100%. The bars represent the means ± SD of three measurements, representative of at least three independent experiments.

Sample preparation and microarray hybridization and analysis
Isolation of plasma cells and RNA profiling were performed as described previously (Bergsagel et al., 2005). Gene expression was measured by U133 Plus2.0 Affymetrix oligonucleotide microarray probeset 203440_at of 559 newly diagnosed MM patients. Expression data were summarized with MAS5 using default parameters in Affymetrix GeneChip operating software, median normalized and plotted against genomic aberrations. This research study was performed with the approval of the institutional review board, and all subjects provided written informed consent in accordance with the Declaration of Helsinki. In addition, publicly available U133 Plus2.0 Affymetrix oligonucleotide microarray data, provided by the Donna D. and Donald M. Lambert Laboratory of Myeloma Genetics, were used to analyze the expression of N-cadherin on the plasma cells of 345 MM patients from the total therapy 2 (TT2) patient set. MAS5 summarized data have been deposited in the NIH Gene Expression Omnibus (GEO; National Center for Biotechnology Information [NCBI], http://www.ncbi.nlm.nih.gov/geo/ under accession number GSE2658.

Immunohistochemistry
Immunohistochemical staining was performed on formalin-fixed, plastic-embedded tissue sections. Endogenous peroxidase activity was blocked with 0.3% H2O2 in methanol. For antigen retrieval sections were boiled for 10 min in a Tris/EDTA buffer (respectively 10 mM/1 mM) pH9, after which they were blocked with 10%
normal goat serum. Followed by incubation for either 1 h at room temperature with mouse monoclonal antibody against CD138 or overnight at 4°C with mouse monoclonal antibodies against N-cadherin (clone 32) or β-catenin (clone 14). Binding of the antibody was visualized using the PowerVision plus detection system (Immunovision Technologies, Duiven, The Netherlands) and 3,3-diaminobenzidine (Sigma). The sections were counterstained with hematoxylin (Merck, Darmstadt, Germany), washed and protected with a cover slip.

**OSTEOBLAST DIFFERENTIATION**

KS483 cells were seeded at a density of 12000 cells/cm² and cultured until confluence. After confluence ascorbic acid (50 μg/mL) was added to the medium, and the medium was changed every 3 days. Co-cultures were initiated from the day of confluence, day 4, by addition of MM cells (25 × 10³/well of a 24-well plate for alkaline phosphatase expression; 10⁶/well of a 6-well plate for RNA samples) and maintained for 1 week, in the presence or absence of doxycycline (0.2 μg/mL; Sigma-Aldrich). Subsequently, cultures were stained for alkaline phosphatase expression as described by van der Horst et al. (van der Horst et al., 2002), or cells were lysed in Tri Reagent (Sigma-Aldrich).

Co-cultures with primary human mesenchymal stromal cells (MSC), obtained and expanded as described previously (Prins et al., 2009), were initiated by plating 8 × 10⁴ MSC in a 6-well plate in a platelet-lysate supplemented medium (Prins et al., 2009). After 24 h, osteogenic differentiation was started by changing the medium with NH OsteoDiff human medium (Miltenyi Biotec, Bergisch Gladbach, Germany), in the presence or absence of 10⁶ MM cells. Cultures were maintained for 1 week and medium was changed every 3 days. MSC were positively selected from the MM cells by MACS as described previously (Derksen et al., 2003), using anti-CD73 (clone AD2, BD Biosciences).

**REAL-TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION**

RNA isolation and cDNA synthesis were performed as described previously (Derksen et al., 2004). The quantitative reverse transcription polymerase chain reaction (qRT-PCR) runs were performed on a Roche LightCycler 1.5 using FastStart DNA Master SYBR Green I kit (Roche, Basel, Switzerland). Results were analyzed using LinReg PCR analysis software (version 7.5; Ramakers et al., 2003). Expression was normalized over beta-2-microglobulin expression.

Mouse-specific primers were designed recognizing alkaline phosphatase (Akp2), osteocalcin (Bglap), collagen, type I, alpha1 (Col1A1), sp7 transcription
factor 7/osterix (Osx), runt-related transcription factor 2 (Runx2), vascular cell adhesion molecule 1 (Vcam1), tumor necrosis factor (ligand) superfamily member 11 aka Rankl (Tnfsf11), interleukin 6 (Il6), N-cadherin (Cdh2), and beta-2-microglobulin (B2m). Human primers were designed recognizing alkaline phosphatase (ALPL), collagen type I, alphal (COL1A1), osteocalcin (BGLAP), and beta-2-microglobulin (B2M). The sequences are shown in Supplementary Tables S1 and S2.

Supplementary Table 1. Mouse-specific primer sets for quantitative PCR

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<td>Beta-2-microglobulin</td>
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All primers were manufactured by Sigma-Aldrich (Haverhill, UK). The results are expressed as relative inhibition, with the co-culture of KS483 (or primary MSCs) and H929 in the absence of doxycycline normalized to 100. The bars represent the means ± SD of three measurements, representative of at least three independent experiments.
Supplementary Table 2. Human primer sets for quantitative PCR

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<td>Beta-2-microglobulin</td>
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Supplementary Figure 1. Multiple myeloma cells express N-cadherin

Fluorescence-activated cell sorting (FACS) analysis for N-cadherin protein expression in MM cell lines. Cells were stained with anti-N-cadherin monoclonal antibody GC-4 (open histogram) or isotype control (filled histogram).

A

BSA  N-cadherin

OPM-1

NCI-H929
Supplementary Figure 2. N-cadherin does not affect MM cell growth

(A) Aggregation of MM cells blocked by N-cadherin coating. MM cell lines OPM-1 (top panel) and NCI-H929 (bottom panel) were plated on N-cadherin (1 μg/mL; right column) or on BSA as a control (left column). Representative pictures of the aggregation of the MM cell lines are shown. (B) MM cells (5 × 10^3) were plated on BSA (○) or N-cadherin (●: 1 μg/mL) coated surfaces and cultured for 4 days. Cell viability was determined using FACS. The growth curves represent the means ± SD of three measurements representative of at least three independent experiments. (C) Knockdown of N-cadherin in the MM cell line NCI-H929, containing a doxycycline-inducible shRNA targeting N-cadherin (H929 shCDH2). Cells were incubated with (black line) or without doxycycline (gray line) for 5 days, and subsequently analyzed by FACS (left panel) using an N-cadherin monoclonal antibody (clone GC-4) and an isotype control (filled gray histogram). By western blot analysis (right panel) knockdown was confirmed (H929 shCDH2) and compared to the control cell line containing the TET repressor only (H929 TR), using a monoclonal antibody N-cadherin (clone 32). β-actin was used as a loading control. (D, E) The growth rate of H929 shCDH2 (right panels) was compared with that of H929 TR (left panels), in the presence (●) and absence (○) of doxycycline, with (E) or without (D) N-cadherin coating (1 μg/mL), over a 4-day culture period. The growth curves represent the means ± SD of three measurements representative of at least three independent experiments.
Supplementary Figure 3. N-cadherin-mediated adhesion to osteoblasts

(A) MM cell lines were allowed to adhere to C3H10T1/2 cells in Hanks’ balanced salt solution (HBSS) in the presence of calcium in combination with an N-cadherin blocking antibody (GC-4) or isotype control antibodies. Representative pictures of the adhesion in HBSS supplemented with calcium and the isotype as a control (left column), and HBSS supplemented with calcium and the blocking antibody GC-4 (right column). (B) N-cadherin knockdown abolishes N-cadherin-mediated adhesion of MM cells to osteoblasts. H929 TR (top panel) and H929 shCDH2 cells (bottom panel) were incubated with (right column) or without (left column) doxycycline for 5 days, and subsequently allowed to adhere to C3H10T1/2 cells in HBSS in the presence of calcium. Representative pictures of the adhesion are shown.
Supplementary Figure 4. N-cadherin-mediated inhibition of osteoblast differentiation does not involve inhibition of osteogenic transcription factors, or a change in MM cell viability.

(A) Murine KS483 cells were differentiated in the presence or absence of H929 TR or H929 shCDH2 cells with (black bars) or without doxycycline (white bars), and subsequently analyzed by qRT-PCR for the expression of sp7 transcription factor 7/ osterix (Sp7/Osx), runt-related transcription factor 2 (Runx2), cadherin 2 (Cdh2), and vascular cell adhesion molecule 1 (Vcam1). The results are expressed as relative inhibition, with the culture of KS483 without H929 cells (or doxycycline) set to 100. ns: not significant, by Student’s t-test. (B) Co-incubation of murine KS483 cells with MM cells does not result in an induction of interleukin 6 (Il6), or tumor necrosis factor (ligand) superfamily, member 11, (Tnfsfll), also known as Rank ligand, using murine inflamed kidney and lymph node respectively as positive controls. Representative agarose gels are shown. (C) Doxycycline-induced knockdown of N-cadherin does not affect MM viability in co-cultures with KS483 or primary human mesenchymal stromal cells (MSC). The growth rate of H929 shCDH2 was compared with that of H929 TR, in the presence (black bars) and absence (white bars) of doxycycline, in co-culture with murine KS483 or human MSC, over a 48 h culture period. The results are expressed as relative growth induction as compared to the input of MM cells at day 0.