N-cadherin-mediated interaction with multiple myeloma cells inhibits osteoblast differentiation

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Online 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 HECD-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 (5×10^5) 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 (Rozemuller *et al.*, 2008). 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-LightPLUS 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

Migration assays were performed in triplicate as described previously (de Gorter *et al.*, 2007), with transwells (8-μm 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 (5×10^5), 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 % H_2O_2 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/cm^2 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 (25×10^3/well of a 24-well plate for alkaline phosphatase expression; 10^6/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 8×10^4 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 10^6 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.

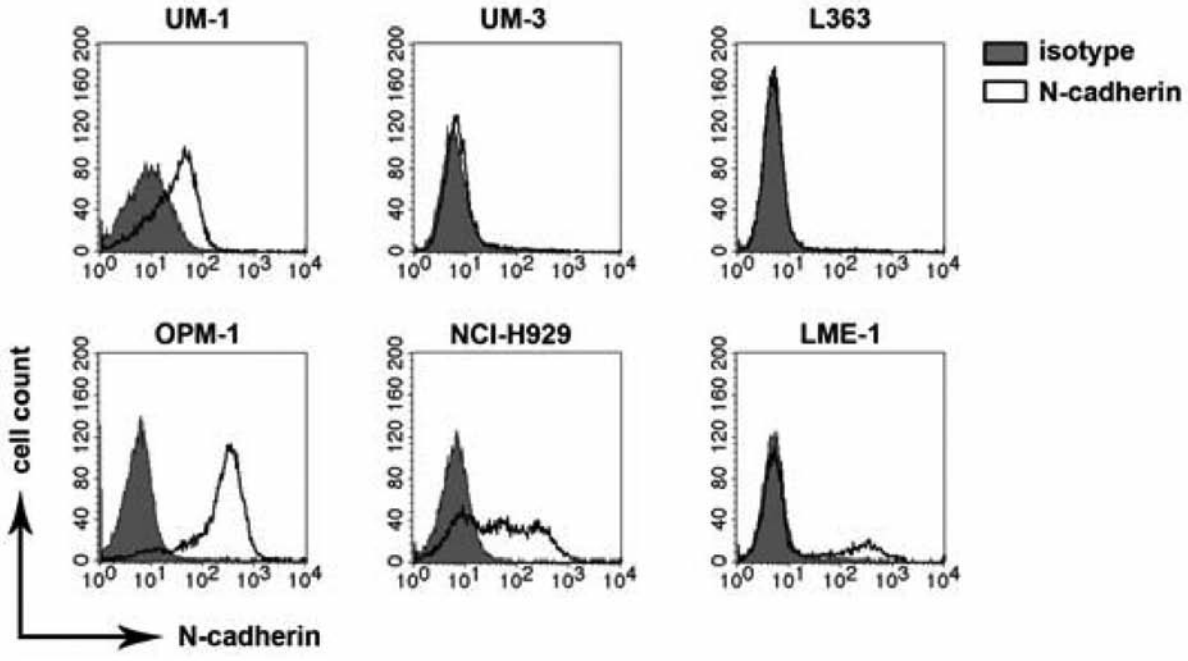
Mouse-specific primers were designed recognizing alkaline phosphatase (*Akp2*), osteocalcin (*Bglap*), collagen, type I, alpha1 (*Col1A1*), sp7 transcription factor 7/ osterix (*Osx*), runt-related transcription factor 2 (*Runx2*), vascular cell adhesion molecule 1 (*Vcam1*), tumor necrosis factor (ligand) superfamily member 11 aka Rankl (*Tnfsf1*), interleukin 6 (*Il6*), N-cadherin (*Cdh2*), and beta-2-microglobulin (*B2m*). Human primers were designed recognizing alkaline phosphatase (*ALPL*), collagen type I, alpha1 (*COL1A1*), osteocalcin (*BGLAP*), and beta-2-microglobulin (*B2M*). The sequences are shown in *Online Supplementary Tables S1 and S2*. 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.

Online Supplementary Table S1. Mouse-specific primer sets for quantitative PCR.

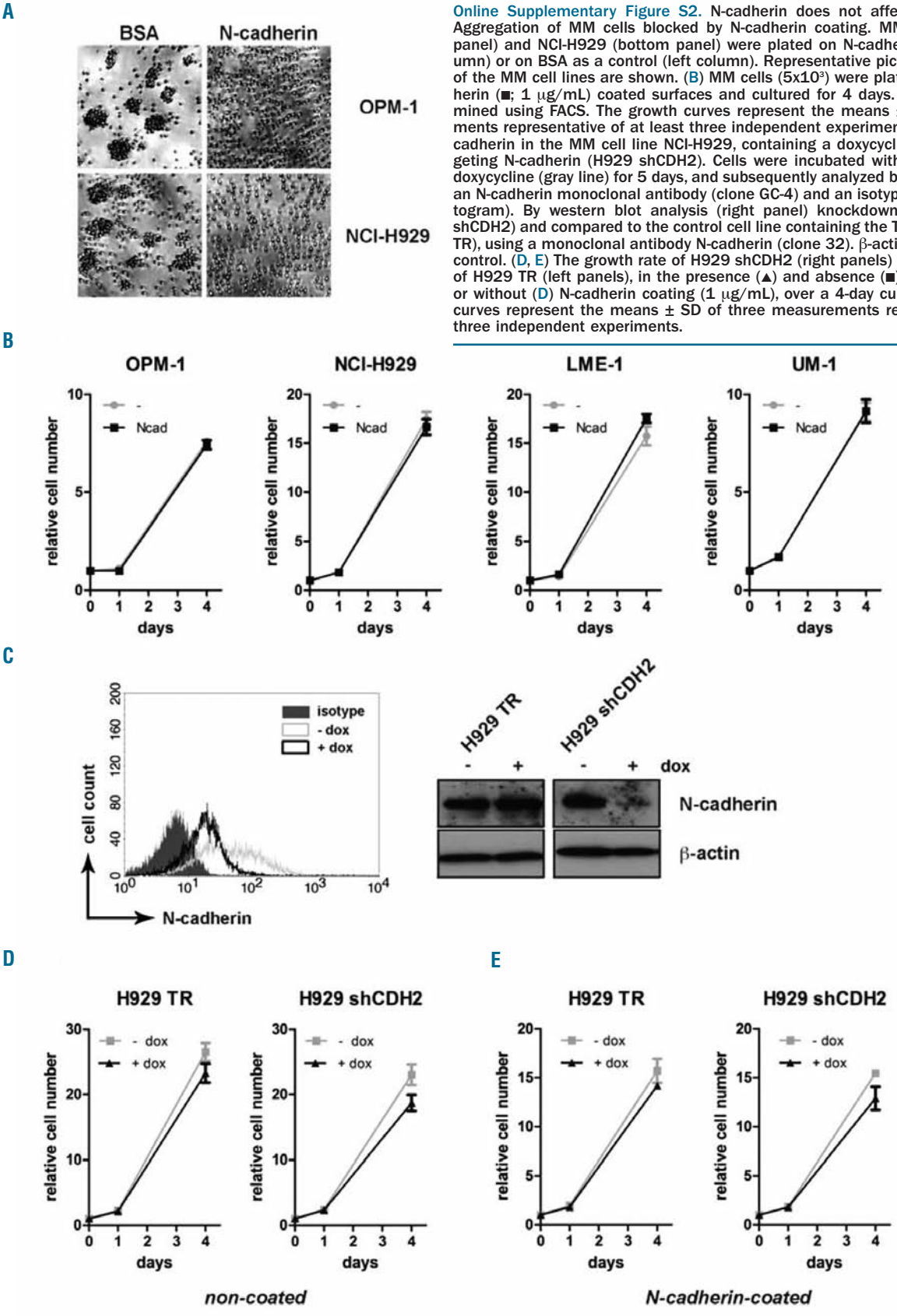
		Forward (5'-3')	Reverse (5'-3')
Alkaline phosphatase	*Akp2*	GGATAACGAGATGCCACC	CATCCAGTTCGTATTCCAC
Osteocalcin	*Bglap*	CAATAAGGTAGTGAACAGACTCC	CTGGTCTGATAGCTCGTCAC
Collagen, type I, alpha1	*Col1A1*	CAAAGGAGAACCCGGTGCTAC	GGTCCAGGCAGTCCGGAAG
Sp7 transcription factor 7/ osterix	*Sp7/Osx*	TCCCATTCTCCCTCCCTCT	GGGACTGGAGCCATAGTGAG
Runt-related transcription factor 2	*Runx2*	GATCTGAGATTTGTGGGC	CCACTGTCACTTTAATAGC
Interleukin 6	*Il6*	TGATGGATGCTACCAAACTGG	TTCATGTACTCCAGGTAGCTATGG
Tumor necrosis factor (ligand) superfamily, member 11	*Tnfsf11 primerset 1* *Tnfsf11 primerset 2* *Tnfsf11 primerset 3*	AAGACACACTACCTGACTCCTGC ACTCTGGAGAGTGAAGACACACTAC CCAGCCATTTGCACACCTC	CCACAATGTGTTGCAGTTCC CCATGAGCCTTCCATCATAG AGCAGGGAAGGGTTGGACA
Vascular cell adhesion molecule 1	*Vcam1*	TGGTGAAATGGAATCTGAACC	GACCCAGATGGTGGTTTCC
Cadherin 2	*Cdh2*	CCTCCATGTGCCGGATAG	CAATTTCACCAGAAGCCTCC
Beta-2-microglobulin	*B2m*	CTGGTGCTTGTCTCACTGACC	GGTGGAACTGTGTTACGTAGC

Online Supplementary Table S2. Human primer sets for quantitative PCR.

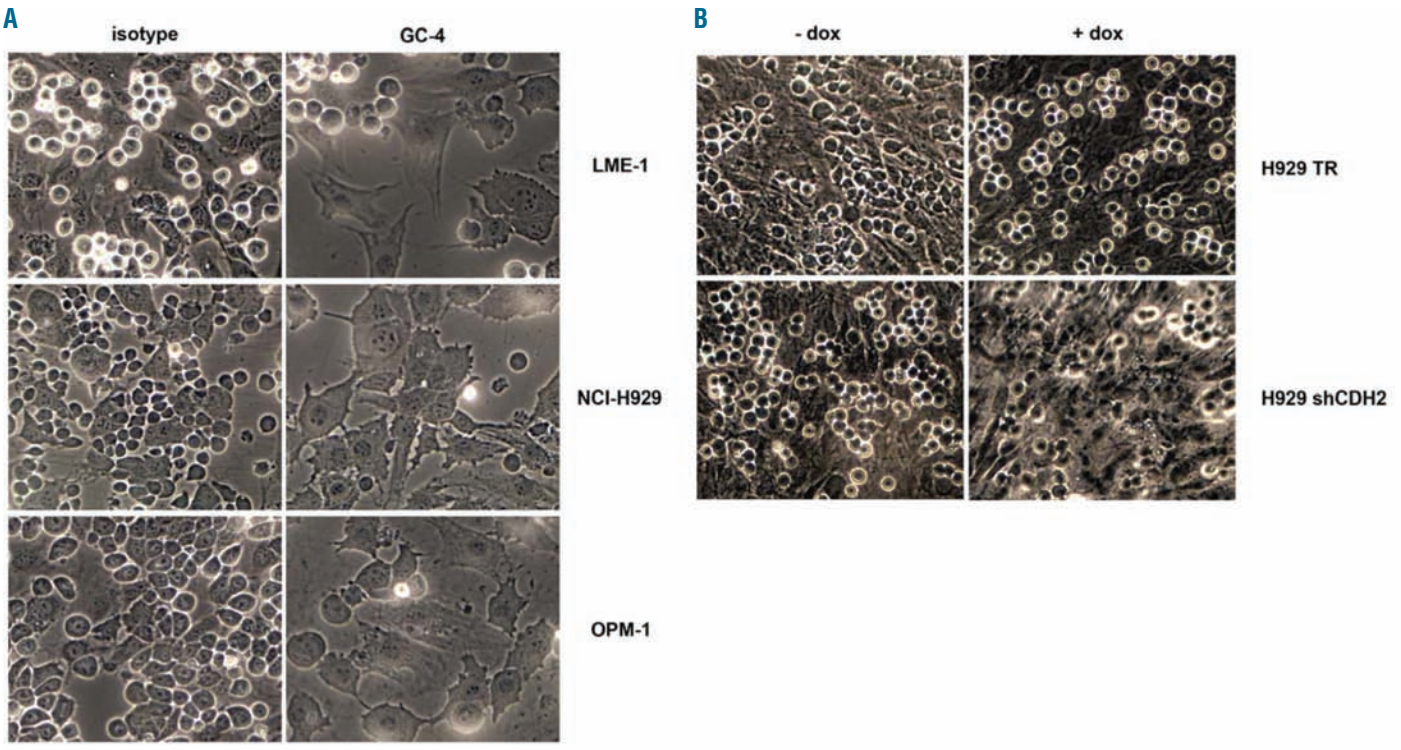
		Forward (5'-3')	Reverse (5'-3')
Alkaline phosphatase	*ALPL*	ACAAGCACTCCCACTTCATCTGGA	TCACGTTGTTCCTGTTCAGCTCGT
Osteocalcin	*BGLAP*	GGCAGCGAGGTAGTGAAGAG	GATGTGGTCAGCCAACTCGT
Collagen, type I, alpha1	*COL1A1*	AGGGCCAAGACGAAGACATC	AGATCACGTCATCGCACAACA
Beta-2-microglobulin	*B2M*	GTCTTTCAGCAAGGACTGGTC	CTTCAAACCTCCATGATGC



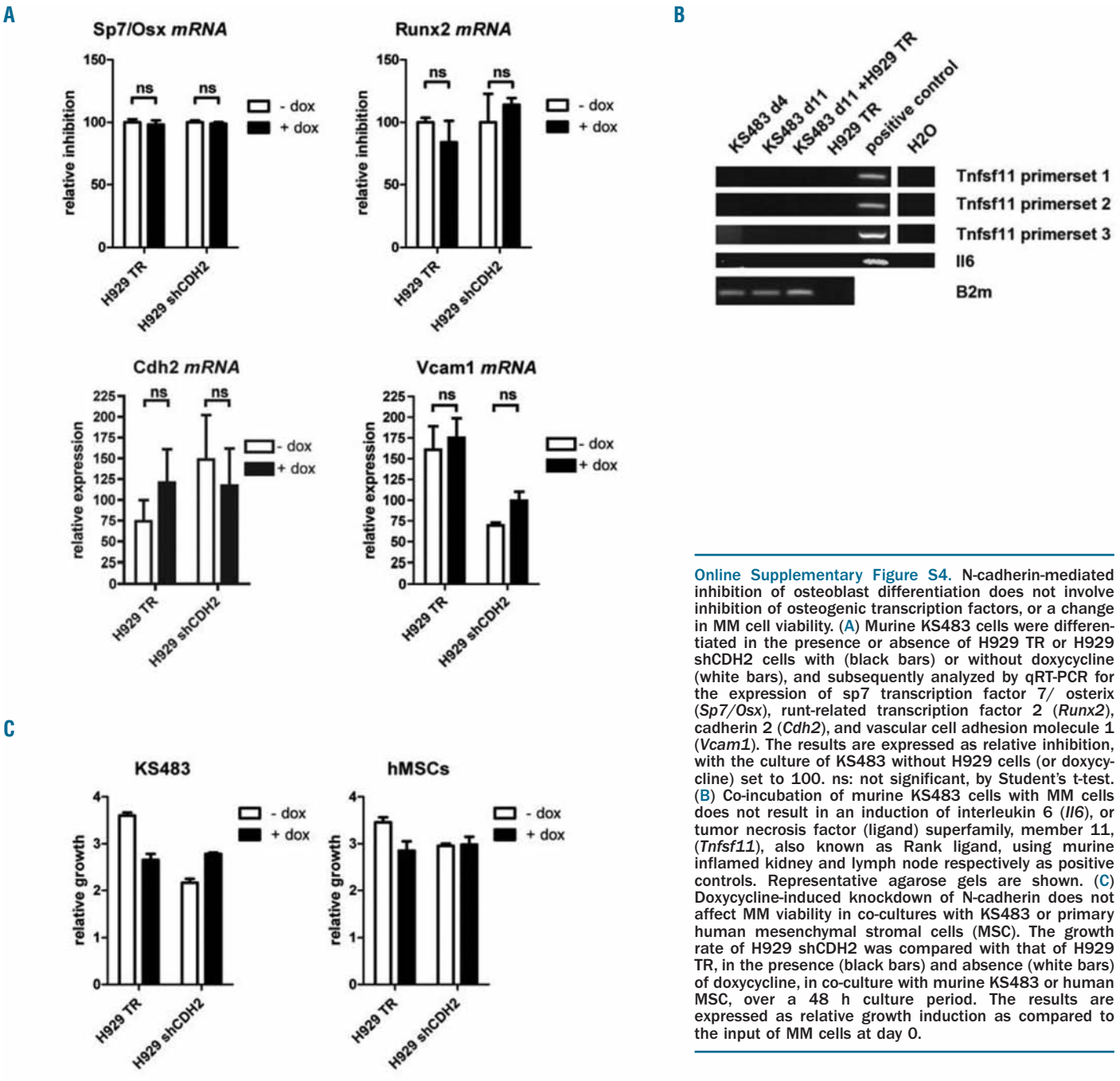
Online Supplementary Figure S1. Multiple myeloma cells express N-cadherin. Fluorescence-activated cell sorting (FACS) analysis for N-cadherin protein expression in MM cell lines. Cells were stained with anti-N-cadherin monoclonal antibody GC-4 (open histogram) or isotype control (filled histogram).



Online Supplementary Figure S2. 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 ($5x10^3$) 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.



Online Supplementary Figure S3. 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.



Online Supplementary Figure S4. 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, (*Tnfsf11*), 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.