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Lenalidomide Restrains Motility and Overangiogenic Potential of Bone Marrow Endothelial Cells in Patients with Active Multiple Myeloma

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Abstract

Purpose: To determine the *in vivo* and *in vitro* antiangiogenic power of lenalidomide, a "lead compound" of IMiD immunomodulatory drugs in bone marrow (BM) endothelial cells (EC) of patients with multiple myeloma (MM) in active phase (MMEC).

Experimental Design: The antiangiogenic effect *in vivo* was studied using the chorioallantoic membrane (CAM) assay. Functional studies *in vitro* (angiogenesis, "wound" healing and chemotaxis, cell viability, adhesion, and apoptosis) were conducted in both primary MMECs and ECs of patients with monoclonal gammopathies (MGUS) of undetermined significance (MGEC) or healthy human umbilical vein endothelial cells (HUVEC). Real-time reverse transcriptase PCR, Western blotting, and differential proteomic analysis were used to correlate morphologic and biological EC features with the lenalidomide effects at the gene and protein levels.

Results: Lenalidomide exerted a relevant antiangiogenic effect *in vivo* at 1.75 $\mu\text{mol/L}$, a dose reached in interstitial fluids of patients treated with 25 mg/d. *In vitro*, lenalidomide inhibited angiogenesis and migration of MMECs, but not of MGECs or control HUVECs, and had no effect on MMEC viability, apoptosis, or fibronectin- and vitronectin-mediated adhesion. Lenalidomide-treated MMECs showed changes in VEGF/VEGFR2 signaling pathway and several proteins controlling EC motility, cytoskeleton remodeling, and energy metabolism pathways.

Conclusions: This study provides information on the molecular mechanisms associated with the antimigratory and antiangiogenic effects of lenalidomide in primary MMECs, thus giving new avenues for effective endothelium-targeted therapies in MM. *Clin Cancer Res*; 17(7); 1935–46. ©2011 AACR.

Introduction

Lenalidomide (CC-5013, Revlimid; Celgene Corporation) is an oral immunomodulatory drug (IMiD) with

shown efficacy and tolerability in patients with multiple myeloma (MM; refs. 1, 2). It is an analogue of thalidomide but has greater potency and a better toxicity profile (3). We have previously shown that thalidomide is antiangiogenic by downregulating key genes involved in autocrine and paracrine angiogenic loops of MM patients (4).

Earlier studies have shown that in the bone marrow (BM) microenvironment, TNF- α increases cell–cell adhesion by inducing NF- κ B-dependent upregulation of adhesion molecules on both MM and stromal cells. Although TNF- α only modestly triggers the MM plasma cell proliferation, the subsequent activation of NF- κ B stimulates interleukin (IL)-6—another key survival signal—in stromal cells, and both factors are targeted by IMiDs (5). Lenalidomide shows *in vivo* antitumor properties owing to stimulation of patients' T cells and IL-2/IFN- γ production and inhibition of TNF- α (6). In MM cells, it upregulates apoptotic genes, thus blocking growth (3, 7). It also inhibits MM cell adhesion to stromal cells via downregulation of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1. In peripheral blood mononuclear cells, it reduces the expression of angiogenic factors, such as VEGF and basic fibroblast growth factor (bFGF; ref. 3), TNF- α ,

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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Translational Relevance

Bone marrow (BM) angiogenesis is an attractive target for the treatment of multiple myeloma (MM). Here, we investigate the antiangiogenic power of lenalidomide, a molecule structurally similar to but less toxic than thalidomide. Lenalidomide impacts angiogenesis *in vivo* and *in vitro* and selectively blocks migration of BM endothelial cells (EC) of patients with active disease (MMEC). It downregulates key angiogenic genes and VEGF/VEGFR2-mediated downstream signaling pathways involved in MMEC migration and the NF- κ B pathway. Proteomics analysis shows that lenalidomide-treated MMECs specifically modulate the expression levels of angiogenesis-related molecules governing MMEC migration, cell shape and cytoskeletal remodeling, energy metabolism, and protein clearance. Altogether, these data imply the intrinsic complexity of the signaling pathways modulated by lenalidomide at the cellular and molecular levels. Data also suggest that lenalidomide, by targeting angiogenesis, may exert an indirect anti-MM effect. The identified genes and proteins offer candidate targets in MMECs for the development of targeted antiangiogenic therapies.

and IL-6 (8). However, its precise mechanisms of action and molecular targets in a well-defined subpopulation of BM stromal cells, such as MM endothelial cells (MMEC), are still elusive.

Methods

Reagents

Lenalidomide (Celgene Corporation) was dissolved in dimethyl sulfoxide (Sigma-Aldrich) as a stock solution of 10 mmol/L and stepwise diluted in culture medium before use: the 1.75 μ mol/L dose is the interstitial fluid concentration following the therapeutic oral dose of 25 mg/d for an adult patient weighing 70 kg. Recombinant human VEGF₁₆₅ and TNF- α were purchased from Sigma-Aldrich; heat-inactivated FBS, Dulbecco's modified Eagle's medium (DMEM), and RPMI 1640, antibiotic/antimycotic, glutamine, trypsin/EDTA, and PBS without Ca²⁺ and Mg²⁺ were from Euroclone.

Patients and EC cultures

Patients fulfilling the International Myeloma Working Group diagnostic criteria (9) for active MM ($n = 44$) and monoclonal gammopathy of undetermined significance (MGUS; $n = 31$) were studied. MM patients had a diagnosis of symptomatic disease and an increase in M-component level in the 3 months before analysis ($n = 28$) or were in relapse ($n = 12$) or in leukemic phase ($n = 4$). There were 24 men and 20 women, aged 44 to 75 (median = 61.5) years, staged as IIA ($n = 6$), IIB ($n = 8$), IIIA ($n = 23$), and IIIB ($n = 7$), and were not under

treatment with lenalidomide. MGUS patients were 18 men and 13 women, aged 43 to 78 (median = 65.7) years. The study was approved by the Ethics Committee of the University of Bari Medical School, and all patients provided their informed consent in accordance with the Declaration of Helsinki.

BM primary ECs from MM (MMEC) and MGUS (MGEC) patients were obtained by centrifugation on Ficoll gradient of heparinized BM aspirates, followed by incubation with magnetic microbeads coated with *Ulex europaeus* agglutinin-1 lectin (10). Control (healthy) HUVECs were purchased from American Type Culture Collection) and cultured in endothelial growth media-2 (Lonza) with 10% FBS.

Plasma cell conditioned media (CM) were prepared as previously described (10).

Chorioallantoic membrane assay

Fertilized white Leghorn chicken eggs were incubated at 37°C at constant humidity (11). On day 3, the shell was opened and 2 to 3 mL of albumen was removed to detach the chorioallantoic membrane (CAM). On day 8, the CAMs were implanted with 1-mm³ sterilized gelatin sponges (Gelfoam Upjohn) loaded with serum-free medium (SFM) alone (negative control) or CM of MM plasma cells alone (positive control) or with 1.75 μ mol/L lenalidomide. The angiogenic response was evaluated on day 12 as the number of vessels converging toward the sponge at 50 \times and photographed *in ovo* (Olympus stereomicroscope).

Functional studies

Cell viability and apoptosis assays. Cell viability was determined by MTT assay (12): cells ($5 \times 10^3/100 \mu$ L per well) were plated in triplicate in 96-well plates in serum-free DMEM (negative control), complete DMEM alone (positive control), or the lenalidomide doses for 72 hours and treated with MTT for the last 4 hours. The absorbance was measured at 570 nm, with 655 nm as a reference wavelength. Crystal violet assay was carried out as previously described (10). For apoptosis, 5.0×10^5 cells were washed with ice-cold PBS without Ca²⁺ and Mg²⁺, incubated with 7-AAD (7-aminoactinomycin D) and PE (phycoerythrin) Annexin V (BD Biosciences), and analyzed by flow cytometry (FACSCantoII; Becton Dickinson).

Chemotaxis. Chemotaxis was done in triplicate by the Boyden microchamber technique (13) toward SFM alone (negative control) or admixed with the CM of MM plasma cells alone (positive control) or with the lenalidomide doses. Cells were counted on 400 \times oil-immersion field/membrane.

"Wound" healing. ECs were grown to confluence on fibronectin-coated (10 μ g/mL) 6-cm² dishes. A "wound" was made by scraping the cell monolayer with a P200 pipette tip. Cells were exposed for 24 hours to SFM alone (negative control) or admixed with the CM of MM plasma cells alone (positive control) or with the lenalidomide doses, fixed, and quantified for motility by counting the cells migrating into the total wound area of each 10 \times field

with an EVOS digital inverted microscope (Euroclone). At least 3 different fields were randomly chosen across the wound length (12).

Adhesion assays. ECs were plated (2×10^3 cells per well) in triplicate in 96-well fibronectin-coated (10 $\mu\text{g}/\text{mL}$), vitronectin-coated (10 $\mu\text{g}/\text{mL}$), and uncoated plates in SFM alone (positive control) or with the lenalidomide doses, then fixed at 30 minutes with 2.5% glutaraldehyde, stained with crystal violet methanol, and counted as in the cell viability assay, reading absorbance at 595 nm (12).

Angiogenesis assay. ECs were plated (1×10^5 cells per well) in duplicate in Matrigel-coated (Becton Dickinson) 24-well plates in SFM alone (positive control) or with the lenalidomide doses. After 18 hours, the skeletonization of the mesh, acquired with the EVOS microscope, was followed by measurement of its topological parameters ("mesh areas," "length," and "branching points") with a computed image analyzer (14).

Western blotting

Total EC protein lysates were subjected to immunoblot with primary and secondary antibodies to the following: VEGFR-2, phospho (p)-extracellular signal regulated kinase (p-Erk)-1/2, Erk-1/2, VE-cadherin, Src, p-NF- κB p65, NF- κB p65, CXCL12/SDF-1, CCL2/MCP-1, myosin light chains (MLC), and p-MLCs (Cell Signaling Technology); p-VEGFR-2 (Y1054) and IEX-1 peptide (Abcam); p-VE-cadherin and p-Src (Invitrogen Corporation); BNIP3 (GeneTex, Inc.); VEGF, bFGF, and SEPW1 (Santa Cruz Biotechnology, Inc.); β -actin (Sigma-Aldrich); and mouse and rabbit horseradish peroxidase-conjugated IgG (Bio-Rad). Immunoreactive bands were visualized by enhanced chemiluminescence (LiteAblot extend substrate; Euroclone) and the Gel Logic 1500 Imaging System (Eastman Kodak Co.), quantified with the Kodak Molecular Imaging Software, and expressed as arbitrary optical density (OD).

MMEC proteomics

Two-dimensional gel electrophoresis, imaging, and quantification. Total lysates of MMECs and MGECs were prepared as previously described (12). Two-dimensional gel electrophoresis (2-DE) was done in duplicate by isoelectric focusing (IEF) on an IPG-phor system (GE Healthcare Bio-Sciences) with precast gel strips (pH 3–10), followed by SDS-PAGE (Hofer SE 600 Ruby; GE Healthcare Bio-Sciences), digitized by an ImageScanner (Amersham Biosciences), and analyzed by the ImageMaster 2D Platinum v.5.0 software (GE Healthcare Bio-Sciences) for spot detection and gels matching, as previously described (15). Spots exhibiting an intensity difference between untreated and lenalidomide-treated samples with a P value of 0.05 by the 2-tailored Student's t test for equal or unequal variance (depending on the calculated variance of spots) were considered to be differentially expressed.

In-gel trypsin digestion and analysis by mass spectrometry of selected protein spots. A preparative gel was obtained by loading whole MMEC lysates. Selected differential protein spots were excised out of the gel, destained with 15 mmol/L

$\text{K}_3\text{Fe}(\text{CN})_6$ and 50 mmol/L $\text{Na}_2\text{S}_2\text{O}_3$, washed with sequential incubation in 25 mmol/L NH_4HCO_3 in 50% ACN, and dried with 100% ACN. The gel proteins were subjected to cysteine reduction and alkylation with 10 mmol/L DTT (dithiothreitol) in 100 mmol/L ambic (ammonium bicarbonate) and 55 mmol/L iodacetamide in 100 mmol/L ambic, respectively, and subsequently reswollen with 10 ng/ μL trypsin in 50 mmol/L ambic (10 $\mu\text{g}/\text{mL}$) and digested overnight at 37°C. Two microlitres of 1% trifluoroacetic acid (TFA) was added to stop the enzymatic reaction. Peptides were purified and concentrated by solid-phase extraction (SPE) in ZipTip C18 pipette tips (Millipore) and spotted directly onto a matrix-assisted laser desorption/ionization (MALDI) ground still target (Bruker Daltonics Bremen) upon elution with CHCA matrix (5 mg/mL in 50% ACN and 0.1% TFA). MALDI-mass spectrometry (MS) and MALDI-MS-MS were done with the UltraFlex III MALDI-TOF (time-of-flight)/TOF mass spectrometer (Bruker Daltonics). MS data were acquired in the positive reflectron mode. All acquisitions were done in a mass range of 700 to 3,500 Thomson [relative intensity (m/z)] with voltages of 25 and 21.7 kV for the first and second ion extraction stages, 9 kV for the lens, and 26.3 and 13.8 kV for reflectors 1 and 2, respectively. Quadratic external calibration of TOF was done with monoisotopic mass of bradykinin (clip 1–7) $[\text{M} + \text{H}]^+$, angiotensin II $[\text{M} + \text{H}]^+$, angiotensin I $[\text{M} + \text{H}]^+$, substance P $[\text{M} + \text{H}]^+$, bombesin $[\text{M} + \text{H}]^+$, adrenocorticotropic hormone (ACTH; clip 1–17) $[\text{M} + \text{H}]^+$, ACTH (clip 18–39) $[\text{M} + \text{H}]^+$, and somatostatin $[\text{M} + \text{H}]^+$. MS spectra were analyzed by the Bruker Flex Analysis 3.0 software. MALDI-TOF/MS-MS analysis was carried out in LIFT mode. Precursor ions were selected manually. MS-MS spectra were acquired with 4,000 to 8,000 (maximum/maximum) laser shots by using the instrument calibration file. Peptide mass fingerprint obtained from MS analysis was used for protein identification in the Swiss-Prot database (SwissProt 2010_04.fasta; 516 081 sequences; 181 677 051 residues) by using the peptide search routine MASCOT 2.3 algorithm (<http://www.matrixscience.com>) restricted to *Homo sapiens* taxonomy with carbamidomethyl of cysteines as fixed modification and oxidation of methionines as variable modification. All peptide mass values were considered monoisotopic and mass tolerance was set at 50 ppm. MASCOT scores greater than 56 were considered significant ($P \leq 0.05$). A database search for MS-MS data was carried out using the same setting of MS analysis, with a fragment tolerance of 0.4 Da. The main cellular location, function, and biological relationships of identified proteins were searched and determined on PubMed with EndNote software v.5.0.

Real-time reverse transcriptase PCR

Total EC RNA was isolated using the RNeasy Mini kit (Qiagen) and reverse transcribed into total cDNA with the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was carried out using the "StepOne real-time RT-PCR system" (Applied Biosystems). The reaction mixture contained SYBR

Green PCR master mix (Applied Biosystems), cDNA, and the following specific (forward/reverse) primers (Invitrogen): VEGF (5'-AAGGAGGAGGGCAGAATCAT-3'/5'-CCAGGCCCTCGTCATTG-3'), bFGF (5'-CCCAGCGGCCGAGTTGAC-3'/5'-CACATTTAGAAGCCAGTAATCT-3'), CCL2 (5'-CAAGCAGAAGTGGGTTTCAGGAT-3'/5'-TCTTCGGAGTTTGGGTTTC-3'), CXCL12 (5'-TCAATTGCATCTCCCAGATAATGT-3'/5'-CACGCTGCGTATAGGAATTGG-3'), COL4A1 (5'-CGTAACTAACACACCCTGCTTCAT-3'/5'-CACTATTGAAAGCTTATCGCTGTCTT-3'), CRYAB (5'-AGGTGCATGAAAAACATGAAGA-3'/5'-GGAACCTCCCTGGAGATGAAACC-3'), Notch1 (5'-CATGGTACCAATCATGATCTTTGTT-3'/5'-CTGGAGGGACCAAGAAGTGTATAA-3'), BNIP3 (5'-ATATTCCTCCCAAGGAGTTCCT-3'/5'-ACGCTCGTTCCTCATGCT-3'), IER3 (5'-CCGCACTCCCAAAAAGA-3'/5'-GCTCTCGCGCACCAGGTA-3'), SEPW1 (5'-CGGCCGCCTGGACAT-3'/5'-AATCAACTTCCCGGCTACCA-3'), and control glyceraldehyde 3 phosphate dehydrogenase (GAPDH; 5'-GAAGGTGAAGTCCGGAGT-3'/5'-CATGGGTGGAATCATATTGGAA-3'). Reaction mixture and amplification conditions were maintained according to the manufacturer's instructions. Each RNA was tested in triplicate, and the threshold cycle values were normalized to GAPDH and averaged \pm SD. The relative gene expression (fold change) between untreated and lenalidomide-treated ECs was calculated with the $2^{-\Delta\Delta CT}$ method (16).

Immunofluorescence microscopy

MMECs plated on chamber slides (5×10^3 cells per chamber, LabTek; Nalge Nunc International) were treated with VEGF (10 ng/mL) alone or with 1.75 μ mol/L lenalidomide, fixed (4% paraformaldehyde), permeabilized (0.2% Triton X-100), blocked (3% BSA/PBS 1X), and then stained with a murine monoclonal antibody to p120 catenin (p120ctn, pp120 Src substrate; BD Transduction Laboratories) and fluorescein (FITC)-conjugated goat anti-mouse IgG (Sigma-Aldrich). Images were obtained using an Axioplan-2 microscope/63X oil-immersion objective (Carl Zeiss).

Results

Lenalidomide suppresses *in vivo* and *in vitro* MM-related angiogenesis

To investigate whether lenalidomide could directly inhibit the MM-related angiogenesis, we first examined the *in vivo* CAM assay. CAMs implanted with a gelatin sponge soaked with the CM of MM patients' plasma cells were surrounded by more newly formed capillaries converging radially toward the sponge in a "spoked-wheel" pattern (mean number of vessels 24 ± 7 ; Fig. 1A, middle) than with SFM (11 ± 4 ; Fig. 1A, left). When lenalidomide 1.75 μ mol/L was added, a significant inhibition in MM-induced angiogenesis was seen (5 ± 2 , $P < 0.01$; Fig. 1A, right).

Next, we examined whether lenalidomide could impact MMECs *in vitro* angiogenesis by the Matrigel assay. After 18 hours of incubation, MMECs spread throughout the Matrigel surface and aligned to form branching and anastomosing

thick tubes with multicentric junctions, which gave rise to a closely knit network of capillary-like structures (Fig. 1B, left). Lenalidomide dose dependently inhibited this network, which progressively became a poorly organized plexus with few and no anastomosed tubes with scarce junctions. The 0.25 and 0.50 μ mol/L concentrations significantly lowered the mesh areas (-34% and -48% , respectively), the vessel length (-45% and -58%), and the vessel branching points (-48% and -55% , $P < 0.05$; Fig. 1B and C). The MM therapeutic dose of 1.75 μ mol/L almost completely inhibited these angiogenic topological parameters (mesh area -67% , vessel length -76% , branching point -80% , $P < 0.001$; Fig. 1B and C). The 2.5 μ mol/L dose fully inhibited MMEC angiogenesis: cells were either isolated or aggregated in small clumps without junctions (mesh area -90% , vessel length and branching points -95% ; Fig. 1B and C). Similar, though fainter, effects were observed in the ECs of MGUS patients (MGECs; Supplementary Fig. S1A), whereas there were no significant effects in HUVECs (data not shown).

Lenalidomide affects MMEC functions involved in angiogenesis

MMEC migratory activity was significantly inhibited in a dose-dependent manner, as evidenced both in the "wound" healing assay (Fig. 2A) and in the chemotaxis assay (Fig. 2B). MMEC migration was stimulated by CM of MM plasma cells and reduced in the "wound" by 56% and 75% at 0.5 and 1.75 μ mol/L, respectively (number of migrated cells in the "wound" area: 42 ± 3 and 24 ± 2 ; $P < 0.03$ and $P < 0.01$), and in the Boyden microchamber, 0.5 and 1.75 μ mol/L inhibited by 27% ($P < 0.05$) and 55% ($P < 0.01$). No significant effect on MGECs chemotaxis (Supplementary Fig. S1B) and no effect on HUVECs were observed (data not shown).

Of note, independently of the dose (0.1–10 μ mol/L), lenalidomide did not induce cell death of MMECs (neither MGECs nor HUVECs), as assessed by both MTT and crystal violet assays, and did not significantly interfere with their capacity to attach to and spread on fibronectin and vitronectin surfaces and on uncoated dishes. Similar results were obtained with MGECs (Supplementary Fig. S1C) and HUVECs (data not shown). Apoptosis was also not induced (Fig. 2C).

Lenalidomide downregulates key genes involved in the MMEC angiogenic profile

To check possible targets of the lenalidomide antiangiogenic activity, we investigated whether it could modulate the expression profile of genes that are primarily involved in MM angiogenesis (VEGF and bFGF), MMEC migration (CCL2 and CXCL12), and several critical biological functions closely related to angiogenesis (BNIP3, IER3, SEPW1, COL4A1, CRYAB, and Notch1; ref. 17). A significant downregulation was observed only in active MMECs at the 1.75- μ mol/L therapeutic dose (Fig. 3). VEGF was 40% less expressed on average than untreated MMECs ($P < 0.03$); bFGF 50% ($P < 0.001$); CCL2 and CXCL12 approximately

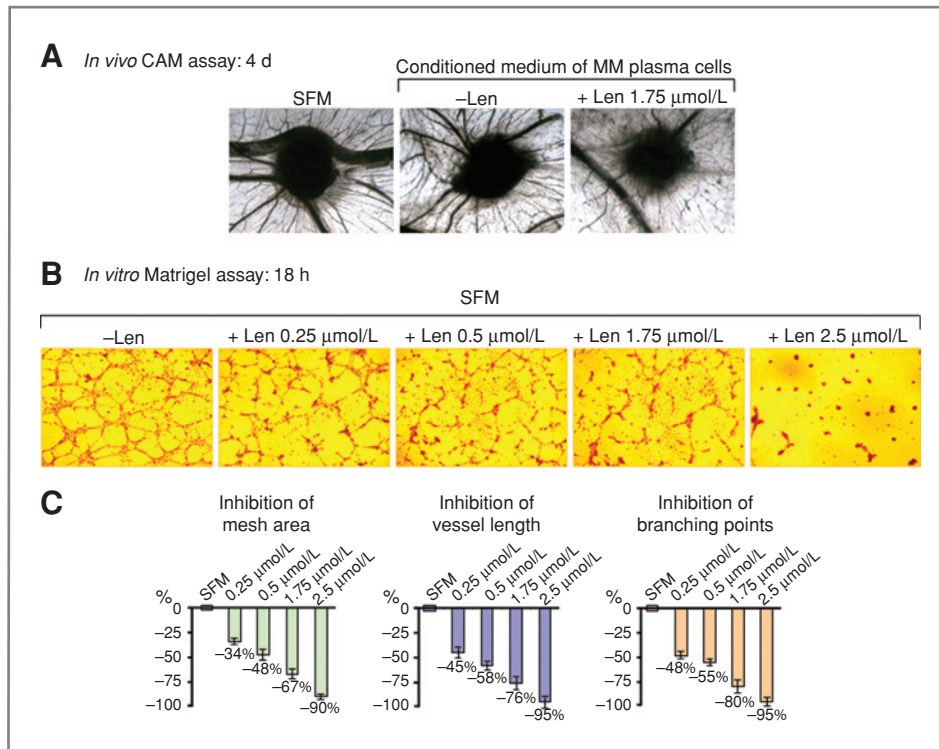


Figure 1. Lenalidomide inhibits angiogenesis in CAM and Matrigel. **A**, CAMs were incubated with gelatin sponges loaded with SFM (left) and with conditioned medium of MM plasma cells either alone (middle) or supplemented with 1.75 μmol/L lenalidomide (right). Note the inhibition of MM angiogenesis by the drug. Images were acquired with a stereomicroscope. Original magnification $\times 50$. **B**, lenalidomide inhibits MMEC angiogenesis in the Matrigel in a dose-dependent manner. MMECs arranged to form a closely knit capillary-like plexus (left), whereas the tube formation was gradually blocked with increasing lenalidomide doses with a full inhibition at 2.5 μmol/L (right). A representative patient is shown. Images were acquired with a digital inverted microscope. Original magnification $\times 80$. **C**, skeletonization of the mesh was followed by measurements of its topological parameters: mesh area, vessel length, and branching points. Data are presented as mean \pm SD of percent inhibition. Significance assessed by the Wilcoxon signed-rank test. Len, lenalidomide.

45% ($P < 0.001$); *BNIP3* 40% ($P < 0.03$); and *IER3* and *SEPWI* approximately 35% ($P < 0.03$), whereas *COL4A1*, *CRYAB*, and *Notch1* were not affected (Fig. 3). Immunoblotting analysis confirmed the relative protein inhibition (Fig. 3). In contrast, lenalidomide produced no or negligible gene/protein regulation in MGECs (Supplementary Fig. S1D) or HUVECs (data not shown).

Lenalidomide abolishes MMEC migration and angiogenesis by affecting VEGF/VEGFR2-mediated downstream signaling pathways

Lenalidomide was shown to exert an inhibitory effect on MMEC angiogenesis by targeting chemotaxis. We have previously found that MMEC functions, including chemotaxis, are mediated by a VEGF/VEGFR2 autocrine loop (12, 18). To assess whether lenalidomide could affect VEGF₁₆₅-triggered MMEC angiogenesis, serum-starved MMECs were exposed to VEGF₁₆₅ alone or with added lenalidomide. The VEGF₁₆₅-triggered VEGFR2 tyrosine phosphorylation lowered significantly and dose dependently with the drug (–25% and –48% with 0.5 and 1.75 μmol/L, $P < 0.03$ and $P < 0.01$, respectively; Fig. 4A).

Since the activation of the mitogen-activated protein kinases Erk-1/2 is closely involved in the migration and

tubular-like formation of ECs (19, 20), we investigated whether lenalidomide could inhibit Erk-1/2 in MMECs and found that it sizably affected, in a dose-dependent fashion, the VEGF₁₆₅-induced phosphorylation of Erk-1/2 (–34% and –80% at 0.5 and 1.75 μmol/L, $P < 0.003$ and $P < 0.001$, respectively, Fig. 4A). In addition, Erk-1/2 influences the cell motility machinery by phosphoactivating MLC kinase activity. This leads to phosphoactivation of MLCs which promotes cytoskeletal myosin-actin contraction needed for cell movement (21). Here, we found that lenalidomide also markedly reduces the VEGF₁₆₅-induced phosphorylation of MLCs in MMECs (–60% at 1.75 μmol/L, $P < 0.01$; Supplementary Fig. S2).

It is well documented that VEGFR2 is the principal receptor that mediates the mitogenic and chemotactic effects of VEGF on ECs (22) and that the VEGF/VEGFR2 loop stimulates the tyrosine phosphorylation of VE-cadherin, which is critical for the sprouting of capillary networks during angiogenesis and the metastatic spread of tumor cells (23, 24). Thus, we tested whether lenalidomide could impact VEGF₁₆₅-mediated VE-cadherin phosphorylation in MMECs. The VEGF₁₆₅ treatment significantly increased tyrosine phosphorylation of VE-cadherin (on Y⁶⁵⁸), which was effectively diminished by

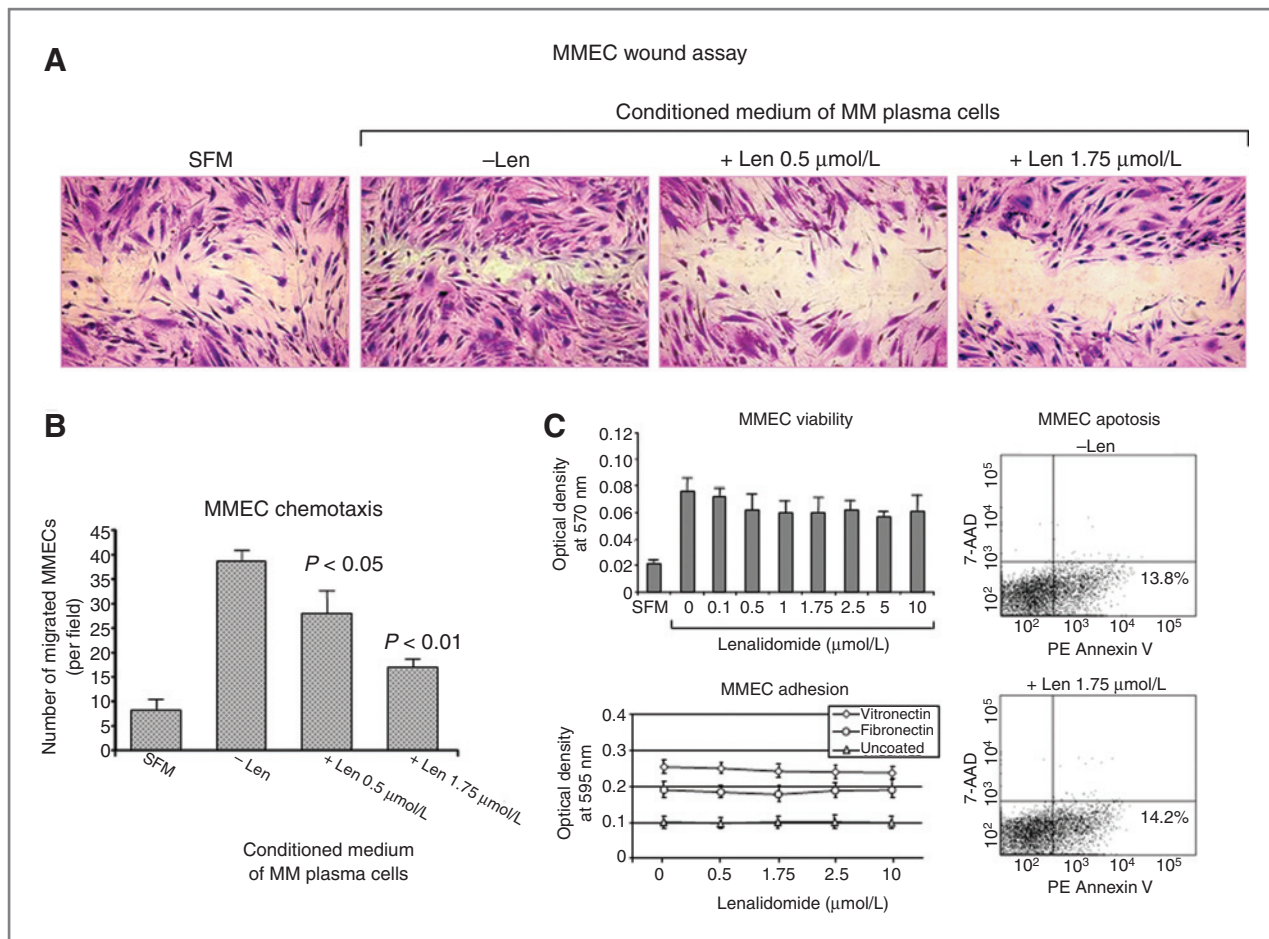


Figure 2. Lenalidomide affects MMEC functions associated with angiogenesis. It inhibits MMEC migration in a dose-dependent manner, using the "wound" healing (A) and the Boyden microchamber (B) assays. Images were acquired with a digital inverted microscope. Original magnification $\times 200$. MMEC chemotaxis columns/bars, means of migrated MMECs counted in five to eight $400\times$ fields per patient in 27 patients \pm SD. C, lenalidomide increasing doses do not affect MMEC viability and adhesion, nor induce cell apoptosis. MMEC viability columns/bars, means \pm SD in duplicate experiments per patient in 44 patients. MMEC adhesion symbols/bars, means \pm SD in duplicate experiments per patient in 32 patients. MMEC apoptosis is from 1 of 28 representative patients. Significance assessed by the Wilcoxon signed-rank test. Len, lenalidomide.

0.5 and 1.75 $\mu\text{mol/L}$ lenalidomide (-30% and -66% , respectively, $P < 0.01$; Fig. 4B). This phosphorylation is mediated by Src tyrosine kinase activity and the activation of the VE-cadherin–Src complex is increased in angiogenic tissues or VEGF-induced ECs, being a necessary step for the cell switch from the quiescent to the angiogenic phenotype (24, 25). We found that lenalidomide consistently reduced VEGF₁₆₅-induced Src phosphorylation (on Y⁴¹⁸) in a dose-dependent manner (-20% and -53% at 0.5 and 1.75 $\mu\text{mol/L}$, respectively, $P < 0.03$ and $P < 0.01$; Fig. 4B). Overall, data suggest that lenalidomide inhibition of VEGF₁₆₅-triggered angiogenesis in MMECs is mediated by disruption of both Src kinase and subsequent VE-cadherin tyrosine phosphorylation.

Since p120ctn is a VE-cadherin-binding protein in the adherent junctions and affects cell–cell contacts and motility (26), we wondered whether it was modified by lenalidomide. Immunofluorescence studies showed that in VEGF₁₆₅-stimulated confluent MMECs, the p120ctn-speci-

fic fluorescence is diffusely distributed through the cytoplasm, probably because p120ctn cytosolic localization mediates the VEGF-triggered MMEC migration. In contrast, 1.75 $\mu\text{mol/L}$ lenalidomide elicited a massive sequestration of p120ctn at the cell–cell junctions (Supplementary Fig. S3), which may contribute to the inhibition of MMEC motility.

Furthermore, since NF- κ B regulates critical genes associated with inflammation and cancer metastases (27), we investigated whether lenalidomide could also modulate the NF- κ B-mediated pathway. NF- κ B transcriptional activation occurs via heterodimerization of its subunits p50 and p65. Lenalidomide inhibited, in a dose-dependent manner, TNF- α -triggered phosphorylation of the p65 subunit (-65% and -86% at 0.5 and 1.75 $\mu\text{mol/L}$, respectively, $P < 0.003$ and $P < 0.001$; Fig. 4C), without affecting basal p65. However, lenalidomide exerts comparable effects, albeit not significant, in MGECS but not in HUVECS (Supplementary Fig. S4).

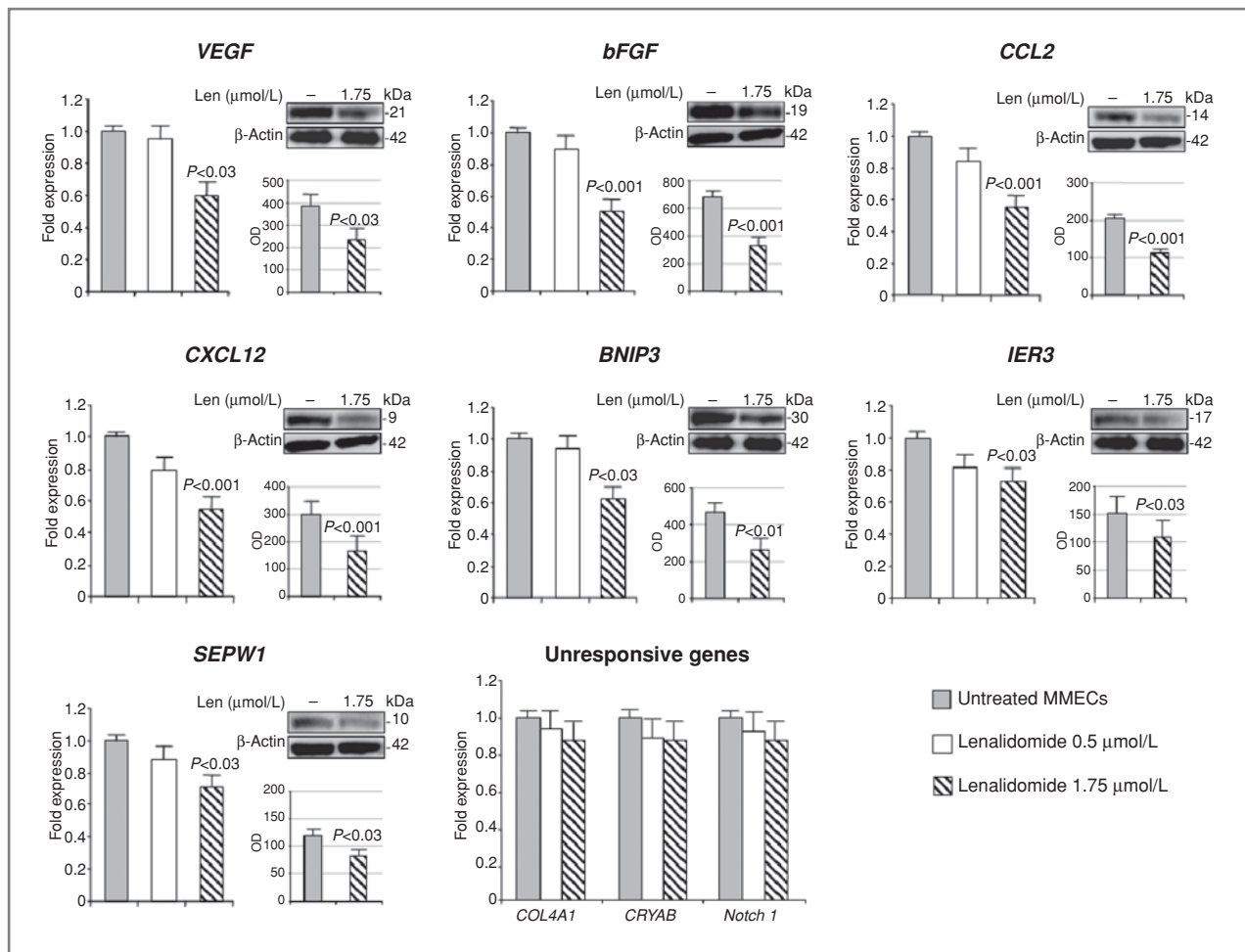


Figure 3. Lenalidomide downregulates key genes and proteins of MM angiogenesis. Expression levels of genes (real-time RT-PCR) and proteins (Western blotting) in MMECs untreated and treated with lenalidomide. Data are expressed as mean relative expression \pm SD of each gene/GAPDH mRNA levels of 3 independent experiments for each of 20 patients. Gene expression fold change in untreated MMECs was arbitrarily set as $1 \pm$ SD. Significance was assessed by the Wilcoxon signed-rank test. Len, lenalidomide.

Lenalidomide-induced changes in the MMEC proteome

To further dissect the inhibitory effects of lenalidomide at the molecular level, we pursued comparative proteomics analysis of MMECs of 2 patients (1 at diagnosis and 1 at relapse) cultured without (Fig. 5A) or with (Fig. 5B) lenalidomide 1.75 μ mol/L for 72 hours, and the same analysis was in parallel carried out for MMECs, as control BM-derived ECs (Fig. 5C and D). At least two 2-DE gels were run per sample, followed by computer-assisted spot matching and silver staining imaging to enable localization of variation spots. More than 500 protein spots were visualized per gel; 10 proteins that were differentially expressed by the treatment (2-fold change) in MMECs were identified by peptide sequencing on the basis of tandem mass spectrometry (MS-MS) and database searching (Fig. 6 and Supplementary Table S1). Among these proteins, 5 were downregulated, the cell membrane-embedded LBP/p40 (RSSA), cytoplasmatic vimentin and keratin2C1, mito-

chondrial DLDH, and glutamate dehydrogenase (DHE3), whereas 5 were upregulated, cytoplasmatic septin2 (SEPT2), γ -actin (ACTG), PNPB, the glycolytic enzyme ENOA, and PSB4. As shown in Figure 6, all the identified proteins were not significantly affected by lenalidomide treatment in MMECs. Interestingly, basal expression levels of vimentin, keratin2C1, mitochondrial DLDH, DHE3, and ENOA were increased in untreated MMECs versus their normal counterpart (Fig. 6A, for both MMECs and MMECs).

In accordance with the aforementioned antimigratory and antiangiogenic effects of lenalidomide, most of the differently expressed MMEC proteins in response to lenalidomide are angiogenesis-related agents controlling cell shape and invasiveness (LBP/p40; ref. 28), cytoskeletal dynamic remodeling (vimentin, keratin2C1, septin-2, and γ -actin; refs. 29–32), and coordinated events at the interface between cytoskeleton and membrane biology (polymerization of F-actin microfilaments and transport

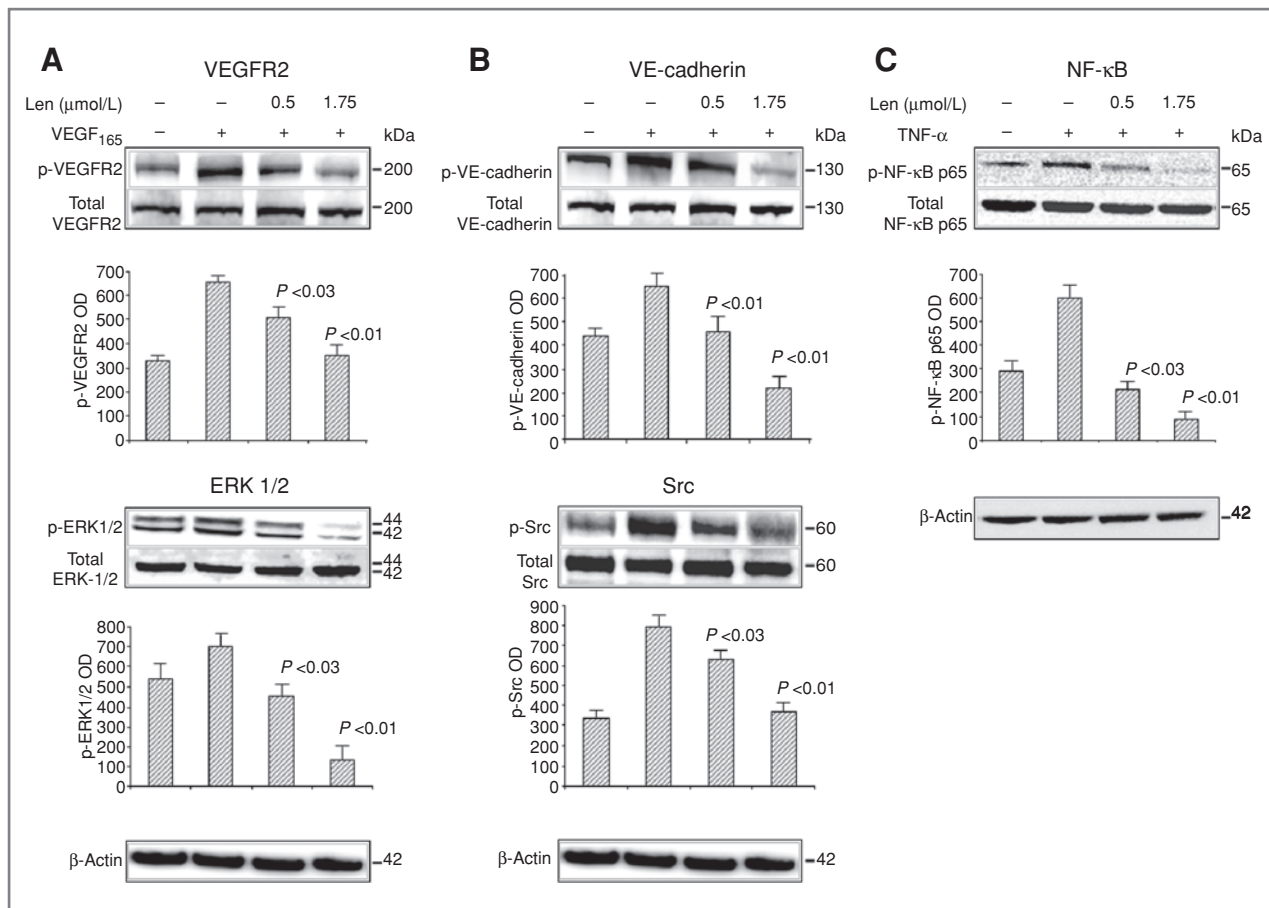


Figure 4. Lenalidomide impacts the VEGF₁₆₅/VEGFR2 downstream signaling in MMECs. Serum-starved MMECs from 16 patients were pretreated with the lenalidomide doses for 6 hours and then stimulated with 10 ng/mL VEGF₁₆₅ for 10 minutes or with TNF-α for 20 minutes. Whole cell lysates were prepared and probed with the indicated antibodies. The band intensity assessed with the Kodak Molecular Imaging Software as OD units is given as mean ± SD. Bands refer to a representative patient. Significance of changes was assessed by the Wilcoxon signed-rank test. Len, lenalidomide.

of organelle or ribosomal subunits during protein biogenesis). Alterations of enzymatic activities (DLDH, DHE3, PNPH, and ENOA; refs. 33, 34) are seemingly specific indicators of changes in energy metabolism pathways and oxidative stress responses, even in the absence of a significant apoptosis induction in lenalidomide-treated MMECs. Upregulated levels of PSB4 (35) could be part of a compensatory response to changes in cellular bioenergetics due to altered protein clearance and turnover.

Discussion

The clinical benefit of lenalidomide in treating myelodysplastic syndromes, MM, chronic lymphocytic leukemia, and non-Hodgkin's lymphoma has drawn heightened attention to its mechanism of action, because specific targets of IMiD compounds remain undefined. In MM plasma cells, lenalidomide inhibits cell growth and promotes apoptosis and blocks their adhesion to stromal cells in the BM milieu (3, 7). In stromal cells, lenalidomide reduces the expression of angiogenic factors and several

additional factors that support the plasma cell growth. In addition, lenalidomide stimulates T cells and natural killer cells (6). IMiDs, including lenalidomide, also have antiangiogenic properties that are independent of their immunomodulatory effects (36). We have already shown that thalidomide exerts a direct antiangiogenic activity on MMECs owing to downregulation of key genes mandatory for autocrine and paracrine loops of neovascularization, even though at approximately 10× higher doses than those of lenalidomide used here (4). However, although the IMiDs share many of biological properties of the thalidomide, the relative potency and range of these effects vary substantially from each other and from thalidomide. Thus, each molecule cannot be assumed to have the same overall biological effects or therapeutic properties as thalidomide or other IMiDs. In fact, as shown here, lenalidomide is antiangiogenic *in vivo* (CAM assay) and *in vitro*, hence overlapping thalidomide (37, 38). However, lenalidomide halts only MMEC chemotaxis but not proliferation whereas thalidomide halts both EC functions (38) and MMEC proliferation (10). Tentatively, we suggest that the differential

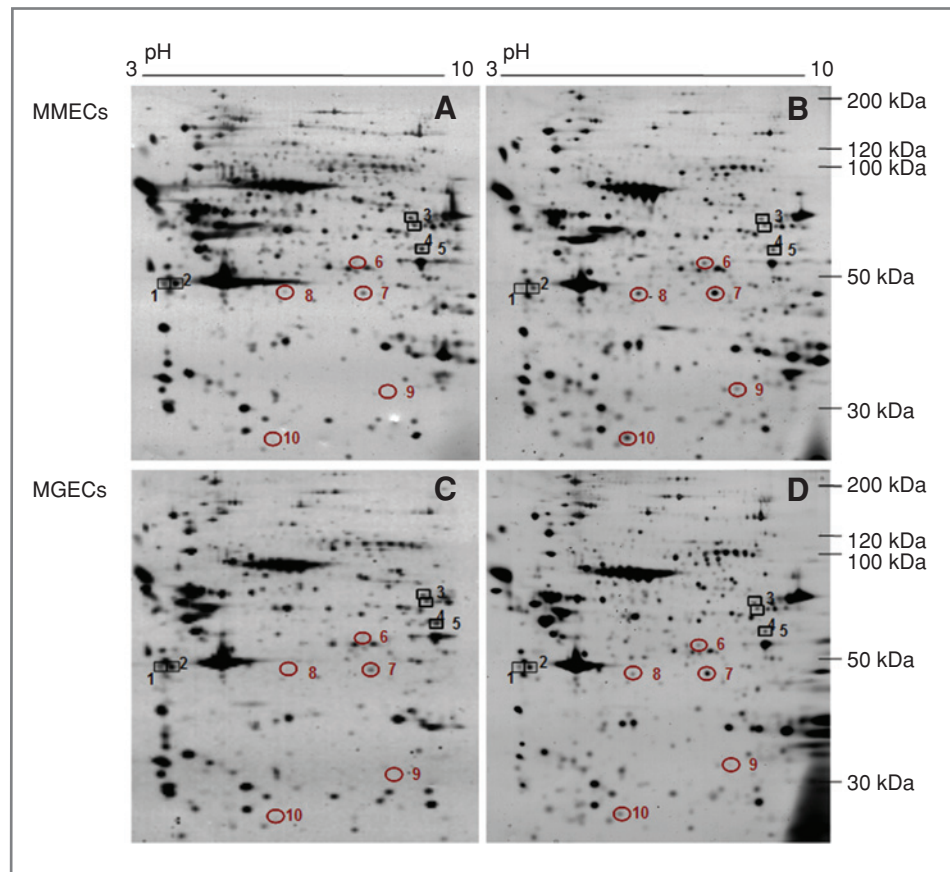


Figure 5. Lenalidomide-induced changes in the proteomic profiles of MMECs. 2-DE gels of whole protein lysates from MMECs and MGECs incubated without (A and C) or with (B and D) 1.75 $\mu\text{mol/L}$ lenalidomide for 72 hours, respectively. Over computer-assisted matching of about 500 protein spots visualized (silver staining images of 2 gels per sample), 10 proteins were found to be differentially expressed in lenalidomide-treated MMECs (2-fold change). Specifically, the squares and circles in gels indicate downregulated or upregulated proteins, respectively, upon lenalidomide treatment.

effects of the drug may derive from distinct mechanisms of action at the molecular level: thalidomide potently inhibits EC functions, possibly through preventing Erk-1/2 nuclear translocation and/or inhibition of Akt/PKB phosphorylation, thus halting mainly VEGF-mediated survival/proliferation (38) whereas lenalidomide inhibits markedly MLC phosphorylation in the cytoplasm (Supplementary Fig. S2), thus acting much more on cell migration. These observations further underscore striking pharmacodynamic differences between lenalidomide and thalidomide.

Accordingly, in this study, we first pointed out the strong potential of lenalidomide in inhibiting *in vivo* angiogenesis induced by the CM of MM plasma cells (CAM assay) at 1.75 $\mu\text{mol/L}$, which corresponds to the interstitial fluid level in patients treated with 25 mg/d per os. Then, we examined the activity of lenalidomide in targeting functions associated with *in vitro* angiogenesis and found that it impairs both MMEC angiogenesis and their migration activity, bringing on the maximum inhibition at the same dose effective for antiangiogenic activity *in vivo*. Of note, the angiogenic and chemotactic cell functions were significantly hampered only in MMECs derived from patients with active disease whereas the drug exerted only negligible or no effect on ECs from nonactive MM patients or MGECs, or on HUVECs. Previous studies (39) showed that lenalidomide inhibits VEGF-, bFGF-, and TNF- α -mediated

migration of HUVECs only at doses 10 to 100 times higher than those used here and inhibits angiogenesis in human umbilical artery explants, with approximately 50% inhibition at 1 $\mu\text{mol/L}$ (40). It is possible that the proangiogenic factors produced by the umbilical artery are either similar to those factors present in the MMEC culture or sufficient to reveal the antiangiogenic activity of lenalidomide.

Although lenalidomide produces *in vitro* growth arrest and apoptosis of MM plasma cells and a variety of solid tumor cell types (3), the drug does not affect viability and apoptosis of MMECs (neither MGECs nor HUVECs), thus indicating that the MMEC antiangiogenic effect does not correlate with those cell functions. Also, lenalidomide does not interfere with the MMEC fibronectin- and vitronectin-mediated adhesion. Data indicate that lenalidomide may inhibit MM angiogenesis because of an antimigratory rather than cytotoxic or apoptogenic mechanism on MMECs.

Accordingly, we investigated the molecular mechanisms involved in the inhibition of MM angiogenesis by lenalidomide. The drug induces inhibition of genes directly involved in the angiogenic cascade, such as VEGF and bFGF, and of the chemokines CCL2 and CXCL12 that are relevant for migration and homing of MMECs (41, 42). Lenalidomide displayed a selective effect on other angiogenesis-related genes shown to be upregulated in

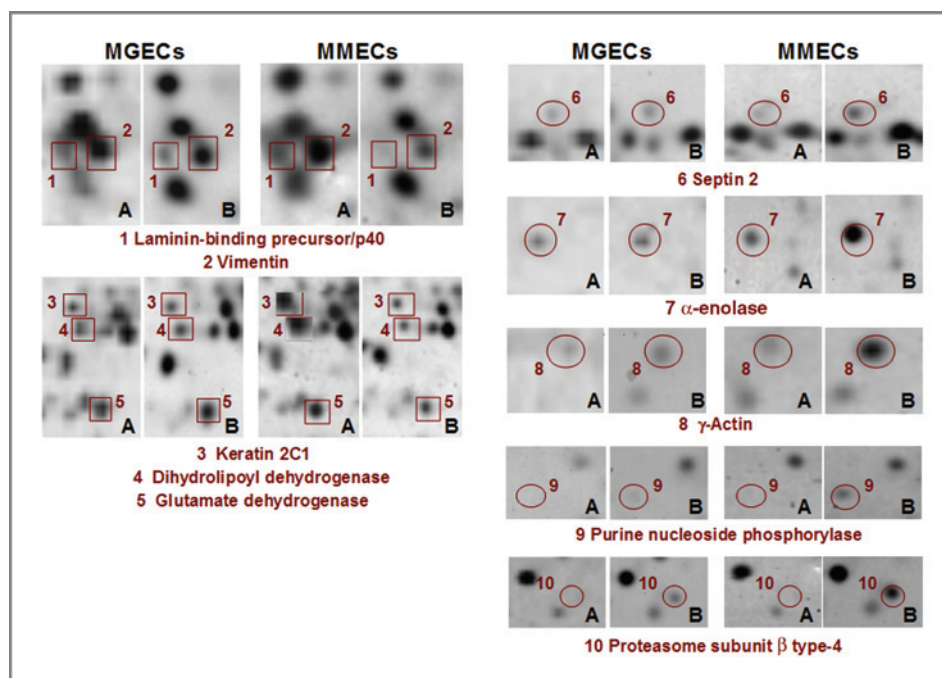


Figure 6. Representative areas of 2-DE gel maps. Enlarged panels of 2-DE spot patterns (Fig. 5) show differentially expressed proteins identified by MS (1–10) from untreated (A) and lenalidomide-treated (B) MMECs and MGECS.

active MMECs (17): inhibition of *BNIP3*, *IER3*, and *SEPW1* but no effect on *COL4A1*, *CRYAB*, and *Notch1*. *BNIP3* is overexpressed in many malignancies due to hypoxia (17, 43). Since hypoxia favors the malignant progression, the *BNIP3* overexpression indicates high metastatic ability and aggressive phenotype and implies a poor prognosis. Perhaps, the downregulation of *BNIP3* in active MMECs by lenalidomide can be explained as a way to overtake the hypoxia-mediated invasive potential, hence affecting their overangiogenic phenotype. *IER3* is a member of the "stress-associated early response gene" family involved both in a wide range of cell functions under stress conditions and in oncogenesis (44). Tentatively, *IER3* downregulation by lenalidomide in active MMECs may account for an effective way to block their overangiogenic phenotype, hence the MM plasma cell spreading and invasion. *SEPW1* has antioxidant function that protects cells from oxidative stress (45). Since *SEPW1*-mediated homeostasis in MMECs may be crucial for their life and survival, the lenalidomide-induced *SEPW1* downregulation may represent a way to overcome protection of the cells from oxidative stress, thus halting MM angiogenesis. Among the genes not regulated by lenalidomide, *CRYAB* is antiapoptotic (46) whereas *Notch1* drives proliferation of primary MM cells (47). Given their roles in proliferation and apoptosis, they are unlikely to be affected by lenalidomide, which, indeed, has no effects on these MMEC functions as shown here. Although *COL4A1* is a major component of the vascular basement membrane, involved in the migration (sprouting) of MM microvessels (10), the lenalidomide-mediated downregulation of other major genes governing the sprouting such as *VEGF*, *bFGF*, *CCL2*, *CXCL12*, *BNIP3*, *IER3*, and *SEPW1* can overcome the role of *COL4A1*.

We found that MMEC angiogenesis is sustained by the VEGF/VEGFR2 autocrine loop (18). VEGF strongly activates, via VEGFR2, the MAP kinase Erk-1/2, which is the last signaling mediator to the nucleus involved in cell proliferation and chemotaxis (19, 20). As a consequence, Erk-1/2-specific inhibitors reduce endothelial tubulogenesis *in vitro* (20). The block by lenalidomide in active MMECs of the VEGF₁₆₅-induced phosphorylation of both VEGFR2 and Erk-1/2 further confirms its antiangiogenic power in the MM BM milieu in which VEGF is highly represented (10).

VEGF increases tyrosine phosphorylation of the adherens junction components, such as VE-cadherin, β -catenin, plakoglobin, p120 catenin, and the cell–cell adhesion molecule PECAM-1 in cultured ECs (23), and the VEGF-mediated VE-cadherin phosphorylation causes the loosening of cell–cell contacts in established vessels to allow EC migration and vessel sprouting during angiogenesis (24). Src kinase is enriched at cell–cell contacts and implicated in regulating the phosphorylation of cadherins and catenins; the phosphorylation of VEGF-induced VE-cadherin tyrosine (on Y⁶⁸⁵) is mediated by Src kinase and is critical for the VEGF-triggered EC migration (48). Here, we show that lenalidomide inhibits VEGF-induced angiogenesis through blocking of Src kinase phosphoactivation and, consequently, tyrosine phosphorylation of VE-cadherin in MMECs, which underlies its antiangiogenic activity in MM. Overall, these data indicate that lenalidomide impacts on MMEC migration in active MM, thereby inhibiting angiogenesis. This inhibition is associated with, and partly mediated by, modifications of intracellular signals triggered by the VEGF-regulated VE-cadherin. Furthermore, we conducted immunolocalization studies on p120ctn,

which binds the cytoplasmic tail of cadherin and localizes into adherens junctions, thus playing a key role in cell–cell adhesion and motility (26). In epithelial cells, p120ctn shows a cytoplasmic diffuse distribution in sparse cultures that is indicative of the cell migratory state, whereas in confluent cells, the formation of cadherin-mediated cell–cell contacts is accompanied by sequestering p120ctn to the junction regions, thus abolishing its effects on cell migration (26). Our immunolocalization results suggest that in active MMECs, lenalidomide strongly overcomes VEGF-mediated localization of p120ctn in the cytoplasm by sequestering the protein at the cell–cell junctions. Perhaps, the VEGF-triggered cytoplasmic distribution of p120ctn could impair VE-cadherin–p120ctn binding, thus making ECs free to migrate, whereas lenalidomide-induced sequestration at cell–cell contacts may stabilize the p120ctn–VE-cadherin interaction, thus blocking the MMEC motility.

NF- κ B regulates several genes involved in angiogenesis including the chemokine genes governing EC migration (49). It stimulates angiogenesis by inducing IL-8 and VEGF expression (50). By demonstrating here that lenalidomide inhibits the activity of the p65 subunit of NF- κ B in active MMECs, we suggest its role in inhibiting cell migration and angiogenesis in MM.

We further extended these findings through a comparative proteomics analysis of the lenalidomide-treated MMECs versus untreated cells. The functional characterization of the identified proteins strengthens the inhibitory effects of lenalidomide on MMEC motility and angiogenic potential associated with changes in cytoarchitecture and mitochondrial energy metabolism. Some of the identified proteins (i.e., LBP/p40, vimentin, keratin2C1, septin2, and γ -actin) are closely correlated with tumor growth, invasion,

and metastasis (28–31). How MM signaling networks could affect turnover and the function of such proteins in MMECs remains a major challenge for future investigations. However, all these proteins could represent attractive biomarkers for monitoring resistance to antiangiogenic and cytoskeleton-disrupting drugs (32) in lenalidomide-treated MM patients.

In conclusion, our findings emphasize the intracellular mechanisms whereby lenalidomide exerts its antiangiogenic potential in active MM patients and indicate that this drug may be of improved therapeutic value and could enhance responses to chemotherapy and radiotherapy in MM.

Disclosure of Potential Conflict of Interest

The authors declare no competing financial interests. The sponsors of this study are public or nonprofit organizations that support science in general. They had no role in gathering, analyzing, or interpreting the data. The authors are fully responsible for the content and editorial decisions for the manuscript.

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References

- Dimopoulos M, Spencer A, Attal M, Prince HM, Harousseau JL, Dmoszynska A, et al. Lenalidomide plus dexamethasone for relapsed or refractory multiple myeloma. *N Engl J Med* 2007;357:2123–32.
- Lacy MQ, Gertz MA, Dispenzieri A, Hayman SR, Geyer S, Kabat B, et al. Long-term results of response to therapy, time to progression, and survival with lenalidomide plus dexamethasone in newly diagnosed myeloma. *Mayo Clin Proc* 2007;82:1179–84.
- Bartlett JB, Dredge K, Dalglish AG. The evolution of thalidomide and its IMiD derivatives as anticancer agents. *Nat Rev Cancer* 2004;4:314–22.
- Vacca A, Scavelli C, Montefusco V, Di Pietro G, Neri A, Mattioli M, et al. Thalidomide downregulates angiogenic genes in bone marrow endothelial cells of patients with active multiple myeloma. *J Clin Oncol* 2005;23:5334–46.
- Mitsiades N, Mitsiades CS, Poulaki V, Chauhan D, Richardson PG, Hideshima T, et al. Biologic sequelae of nuclear factor- κ B blockade in multiple myeloma: therapeutic applications. *Blood* 2002;99:4079–86.
- Marriott JB, Clarke IA, Dredge K, Muller G, Stirling D, Dalglish AG. Thalidomide and its analogues have distinct and opposing effects on TNF- α and TNFR2 during co-stimulation of both CD4⁺ and CD8⁺ T cells. *Clin Exp Immunol* 2002;130:75–84.
- Richardson PG, Schlossman RL, Weller E, Hideshima T, Mitsiades C, Davies F, et al. Immunomodulatory drug CC-5013 overcomes drug resistance and is well tolerated in patients with relapsed multiple myeloma. *Blood* 2002;100:3063–7.
- Corral LG, Haslett PA, Muller GW, Chen R, Wong LM, Ocampo CJ, et al. Differential cytokine modulation and T cell activation by two distinct classes of thalidomide analogues that are potent inhibitors of TNF- α . *J Immunol* 1999;163:380–6.
- International Myeloma Working Group. Criteria for the classification of monoclonal gammopathies, multiple myeloma and related disorders: a report of the International Myeloma Working Group. *Br J Haematol* 2003;121:749–57.
- Vacca A, Ria R, Semeraro F, Merchionne F, Coluccia M, Boccarelli A, et al. Endothelial cells in the bone marrow of patients with multiple myeloma. *Blood* 2003;102:3340–8.
- Ribatti D, Nico B, Vacca A, Presta M. The gelatin sponge-chorioallantoic membrane assay. *Nat Protoc* 2006;1:85–91.
- Coluccia AM, Cirulli T, Neri P, Mangieri D, Colanardi MC, Gnoni A, et al. Validation of PDGFR β and c-Src tyrosine kinases as tumor/vessel targets in patients with multiple myeloma: preclinical efficacy of the novel, orally available inhibitor dasatinib. *Blood* 2008;112:1346–56.
- Vacca A, Ribatti D, Presta M, Minischetti M, Iurlaro M, Ria R, et al. Bone marrow neovascularization, plasma cell angiogenic potential, and matrix metalloproteinase-2 secretion parallel progression of human multiple myeloma. *Blood* 1999;93:3064–73.
- Guidolin D, Vacca A, Nussdorfer GG, Ribatti D. A new image analysis method based on topological and fractal parameters to evaluate the angiostatic activity of docetaxel by using the Matrigel assay *in vitro*. *Microvasc Res* 2004;67:117–24.

15. Greco M, Mitri MD, Chiriaco F, Leo G, Brienza E, Maffia M, et al. Serum proteomic profile of cutaneous malignant melanoma and relation to cancer progression: association to tumor derived alpha-N-acetylgalactosaminidase activity. *Cancer Lett* 2009;283:222–9.
16. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-delta delta C(T))} method. *Methods* 2001;25:402–8.
17. Ria R, Todoerti K, Berardi S, Coluccia AM, De Luisi A, Mattioli M, et al. Gene expression profiling of bone marrow endothelial cells in patients with multiple myeloma. *Clin Cancer Res* 2009;15:5369–78.
18. Ria R, Vacca A, Russo F, Cirulli T, Massaia M, Tosi P, et al. A VEGF-dependent autocrine loop mediates proliferation and capillarogenesis in bone marrow endothelial cells of patients with multiple myeloma. *Thromb Haemost* 2004;92:1438–45.
19. Groskopf JC, Syu LJ, Saltiel AR, Linzer DI. Proliferin induces endothelial cell chemotaxis through a G protein-coupled, mitogen activated protein kinase-dependent pathway. *Endocrinology* 1997;138:2835–40.
20. Ilan N, Mahooti S, Madri JA. Distinct signal transduction pathways are utilized during the tube formation and survival phases of *in vitro* angiogenesis. *J Cell Sci* 1998;111:3621–31.
21. Viala E, Pouyssegur J. Regulation of tumor cell motility by ERK mitogen-activated protein kinases. *Ann N Y Acad Sci* 2004;1030:208–18.
22. Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J Biol Chem* 1994;269:26988–95.
23. Esser S, Lampugnani MG, Corada M, Dejana E, Risau W. Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. *J Cell Sci* 1998;111:1853–65.
24. Lambeng N, Wallez Y, Rampon C, Cand F, Christé G, Gulino-Debrac D, et al. Vascular endothelial-cadherin tyrosine phosphorylation in angiogenic and quiescent adult tissues. *Circ Res* 2005;96:384–91.
25. Eliceiri BP, Paul R, Schwartzberg PL, Hood JD, Leng J, Cheresh DA. Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. *Mol Cell* 1999;4:915–24.
26. Anastasiadis PZ, Reynolds AB. The p120 catenin family: complex roles in adhesion, signaling and cancer. *J Cell Sci* 2000;113:1319–34.
27. Sun SC, Xiao G. Deregulation of NF-kappaB and its upstream kinases in cancer. *Cancer Metastasis Rev* 2003;22:405–22.
28. Tanaka M, Narumi K, Isemura M, Abe M, Sato Y, Abe T, et al. Expression of the 37-kDa laminin binding protein in murine lung tumor cell correlates with tumor angiogenesis. *Cancer Lett* 2000;153:161–8.
29. Dutsch-Wicherek M. RCAS1, MT, and vimentin as potential markers of tumor microenvironment remodeling. *Am J Reprod Immunol* 2010;63:181–8.
30. Kirfel J, Magin TM, Reichelt J. Keratins: a structural scaffold with emerging functions. *Cell Mol Life Sci* 2003;60:56–71.
31. Sirajuddin M, Farkasovsky M, Zent E, Wittinghofer A. GTP-induced conformational changes in septins and implications for function. *Proc Natl Acad Sci U S A* 2009;106:16592–7.
32. Verrills NM, Po'uha ST, Liu ML, Liaw TY, Larsen MR, Ivery MT, et al. Alterations in gamma-actin and tubulin-targeted drug resistance in childhood leukemia. *J Natl Cancer Inst* 2006;98:1363–74.
33. Xu W, Koeck T, Lara AR, Neumann D, DiFilippo FP, Koo M, et al. Alterations of cellular bioenergetics in pulmonary artery endothelial cells. *Proc Natl Acad Sci U S A* 2007;104:1342–7.
34. Bzowska A, Kulikowska E, Shugar D. Purine nucleoside phosphor-ylases: properties, functions, and clinical aspects. *Pharmacol Ther* 2000;88:349–425.
35. Arit A, Bauer I, Schafmayer C, Tepel J, Muecköster SS, Brosch M, et al. Increased proteasome subunit protein expression and proteasome activity in colon cancer relate to an enhanced activation of nuclear factor E2-related factor 2 (Nrf2). *Oncogene* 2009;28:3983–96.
36. Dredge K, Marriott JB, Macdonald CD, Man HW, Chen R, Muller GW, et al. Novel thalidomide analogues display anti-angiogenic activity independently of immunomodulatory effects. *Br J Cancer* 2002;87:1166–72.
37. Therapontos C, Erskine L, Gardner ER, Figg WD, Vargesson N. Thalidomide induces limb defects by preventing angiogenic out-growth during early limb formation. *Proc Natl Acad Sci U S A* 2009;106:8573–8.
38. Rafiee P, Stein DJ, Nelson VM, Otterson MF, Shaker R, Binion DG, et al. Thalidomide inhibits inflammatory and angiogenic activation of human intestinal microvascular endothelial cells (HIMEC). *Am J Physiol Gastrointest Liver Physiol* 2010;298:G167–76.
39. Dredge K, Horsfall R, Robinson SP, Zhang LH, Lu L, Tang Y, et al. Orally administered lenalidomide (CC-5013) is anti-angiogenic *in vivo* and inhibits endothelial cell migration and Akt phosphorylation *in vitro*. *Microvasc Res* 2005;69:56–63.
40. Lu L, Payvandi F, Wu L, Zhang LH, Hariri RJ, Man HW, et al. The anti-cancer drug lenalidomide inhibits angiogenesis and metastasis via multiple inhibitory effects on endothelial cell function in normoxic and hypoxic conditions. *Microvasc Res* 2009;77:78–86.
41. Pellegrino A, Ria R, Di Pietro G, Cirulli T, Surico G, Pennisi A, et al. Bone marrow endothelial cells in multiple myeloma secrete CXC-chemokines that mediate interactions with plasma cells. *Br J Haematol* 2005;129:248–56.
42. Alsayed Y, Ngo H, Runnels J, Leleu X, Singha UK, Pittsillides CM, et al. Mechanisms of regulation of CXCR4/SDF-1 (CXCL12)-dependent migration and homing in multiple myeloma. *Blood* 2007;109:2708–17.
43. Burton TR, Gibson SB. The role of Bcl-2 family member BNIP3 in cell death and disease: NIPping at the heels of cell death. *Cell Death Differ* 2009;16:515–23.
44. Wu MX. Roles of the stress induced gene IEX-1 in regulation of cell death and oncogenesis. *Apoptosis* 2003;8:11–8.
45. Loflin J, Lopez N, Whanger PD, Kioussi C. Selenoprotein W during development and oxidative stress. *J Inorg Biochem* 2006;100:1679–84.
46. Kamradt MC, Lu M, Werner ME, Kwan T, Chen F, Strohecker A, et al. The small heat shock protein α B-crystallin is a novel inhibitor of TRAIL-induced apoptosis that suppresses the activation of caspase-3. *J Biol Chem* 2005;280:11059–66.
47. Jundt F, Pröbsting KS, Anagnostopoulos I, Muehlinghaus G, Chatterjee M, Mathas S, et al. Jagged1-induced Notch signaling drives proliferation of multiple myeloma cells. *Blood* 2004;103:3511–5.
48. Wallez Y, Cand F, Cruzalegui F, Wernstedt C, Souchelnytskyi S, Vilgrain I, et al. Src kinase phosphorylates vascular endothelial-cadherin in response to vascular endothelial growth factor: identification of tyrosine 685 as the unique target site. *Oncogene* 2007;26:1067–77.
49. Karin M, Cao Y, Greten FR, Li ZW. NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* 2002;2:301–10.
50. Huang S, Robinson JB, Deguzman A, Bucana CD, Fidler IJ. Blockade of nuclear factor-kB signaling inhibits angiogenesis and tumorigenicity of human ovarian cancer cells by suppressing expression of vascular endothelial growth factor and interleukin-8. *Cancer Res* 2000;60:5334–9.