A HGF/cMET Autocrine Loop Is Operative in Multiple Myeloma Bone Marrow Endothelial Cells and May Represent a Novel Therapeutic Target

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Abstract

Purpose: The aim of this study was to investigate the angiogenic role of the hepatocyte growth factor (HGF)/cMET pathway and its inhibition in bone marrow endothelial cells (EC) from patients with multiple myeloma versus from patients with monoclonal gammopathy of undetermined significance (MGUS) or benign anemia (control group).

Experimental Design: The HGF/cMET pathway was evaluated in ECs from patients with multiple myeloma (multiple myeloma ECs) at diagnosis, at relapse after bortezomib- or lenalidomide-based therapies, or on refractory phase to these drugs; in ECs from patients with MGUS (MGECS); and in those patients from the control group. The effects of a selective cMET tyrosine kinase inhibitor (SU11274) on multiple myeloma ECs' angiogenic activities were studied in vitro and in vivo.

Results: Multiple myeloma ECs express more HGF, cMET, and activated cMET (phospho (p)-cMET) at both RNA and protein levels versus MGECS and control ECs. Multiple myeloma ECs are able to maintain the HGF/cMET pathway activation in absence of external stimulation, whereas treatment with anti-HGF and anti-cMET neutralizing antibodies (Ab) is able to inhibit cMET activation. The cMET pathway regulates several multiple myeloma EC activities, including chemotaxis, motility, adhesion, spreading, and whole angiogenesis. Its inhibition by SU11274 impairs these activities in a statistically significant fashion when combined with bortezomib or lenalidomide, both in vitro and in vivo.

Conclusions: An autocrine HGF/cMET loop sustains multiple myeloma angiogenesis and represents an appealing new target to potentiate the antiangiogenic management of patients with multiple myeloma.

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Introduction

Multiple myeloma is a clonal expansion of plasma cells (PC) in the bone marrow, where they proliferate and acquire resistance to apoptosis and drugs (1). Multiple myeloma angiogenesis results from interactions between PCs and bone marrow microenvironment cells, and is a constant hallmark of disease progression because it correlates with tumor growth, relapse, and drug resistance (2). The angiogenesis is enhanced in patients with multiple myeloma compared with those with monoclonal gammopathy of undetermined significance (MGUS) and normal controls (3), and it correlates with prognosis (4). Multiple myeloma remains an incurable malignancy, despite important advances in conventional as well as high-dose chemotherapies supported by autologous stem cell transplantation. To overcome drug resistance and to improve clinical response, novel therapeutic approaches halting both PCs and angiogenesis are under experimental and clinical studies (5).
Translational Relevance

Bone marrow angiogenesis is enhanced in multiple myeloma as compared with monoclonal gammopathy of undetermined significance (MGUS) and therefore constitutes an attractive target for treatment. The hepatocyte growth factor (HGF)/cMET pathway is implicated in multiple myeloma pathogenesis and progression. Here, we show that bone marrow-derived endothelial cells (EC) from patients with active multiple myeloma but not from patients with MGUS or with benign anemia present the HGF/cMET pathway constitutively activated and operative in an autocrine fashion as an angiogenesis amplifier. In vitro and in vivo studies of a novel selective cMET inhibitor—that is, SU11274, tested singularly and in combination with bortezomib or lenalidomide—suggest that the HGF/cMET pathway of multiple myeloma ECs may be envisaged as a new therapeutic target for the antiangiogenic management of active patients with multiple myeloma.

Hepatocyte growth factor (HGF)/scatter factor (SF) is a potent angiogenic cytokine. Bussolino and colleagues first showed that HGF vividly induces endothelial cell (EC) proliferation and migration in vitro by triggering their specific tyrosine kinase receptor mesenchymal-epithelial transition factor (cMET), and angiogenesis in vivo using the rodent cornea assay (6). It also enhances the expression of other angiogenic factors, including VEGF and its receptors, and suppresses the expression of thrombospondin 1, an endogenous angiogenesis inhibitor (7). Aberrant HGF/cMET pathway activation has been described in both solid (8) and blood tumors (9) in which it triggers several signaling pathways, including Src/FAK, p120/STAT3, PI3K/Akt, and Ras/MAPK, which results in cell proliferation, migration, invasion, and resistance to apoptosis. The HGF/cMET pathway is thus important for tumor growth, angiogenesis, and metastatic spread.

The HGF/cMET pathway is involved in the multiple myeloma pathogenesis. Coexpression of HGF and cMET has been observed in multiple myeloma PCs, implying the existence of an autocrine loop (9, 10). Because bone marrow stromal cells (SC) produce HGF (11), a paracrine stimulation of PCs within their microenvironment may occur, and provide mitogenic, migratory, and morphogenic effects (9). HGF levels are significantly increased in peripheral and bone marrow blood of patients with multiple myeloma at diagnosis compared with healthy controls, and represent a negative prognostic factor (12). We have shown that the HGF/cMET pathway is constitutively activated in PCs from relapsed and resistant patients, and that it mediates the multidrug resistance (10). We have also shown a therapeutic activity of SU11274, a specific adenosine triphosphate-competitive small-molecule cMET inhibitor in a multiple myeloma xenograft model (10). Furthermore, SU11274 is able to inhibit cMET phosphorylation and cMET-dependent motility, invasion, and proliferation in preclinical models of lung (13) and ovarian (14) carcinomas.

Data to be presented show the role of the HGF/cMET pathway in multiple myeloma-associated angiogenesis, and the effects of cMET inhibition by SU11274 on ECs of patients with multiple myeloma both in vitro and in vivo.

Materials and Methods

Patients and endothelial cells

Patients fulfilling the International Myeloma Working Group diagnostic criteria (15) for multiple myeloma (n = 32) and MGUS (n = 24) were studied. Patients with multiple myeloma (18 men/14 women), ages 41 to 82 (median 61.5) years, were at first diagnosis (n = 9), on refractory phase to bortezomib- or lenalidomide-based chemotherapies (n = 12), or at relapse after these therapies (n = 11). The M-component was IgG (n = 18), IgA (n = 8), and κ or λ (n = 6). Patients with MGUS (15 men/9 women), ages 42 to 79 (median 60.5) years, were IgG (n = 13), IgA (n = 7), or κ or λ (n = 4). Control bone marrow ECs were harvested from 10 subjects with anemia due to iron or vitamin B12 deficiency (2). The study was approved by the Ethics Committee of the University of Bari Medical School (Bari, Italy), and all patients provided their informed consent according to the Declaration of Helsinki. Multiple myeloma ECs, MGUS-derived ECs (MGECS), and control ECs were harvested and cultured as described (16).

ELISA, immunoprecipitation, Western blot analysis, and real-time RT-PCR

Multiple myeloma ECs, MGECS, and control ECs (1 × 10⁶ cells/ml) were cultured for 24 hours in serum-free medium (SFM) 1% glutamine, and their conditioned media (CM) were prepared (16). HGF was quantified in the CM by an ELISA (Quantikine Human HGF, R&D Systems, Inc.). Protein lysates from all the ECs types (7 × 10⁶ cells/sample) were incubated with an anti-cMET antibody (Ab; Cell Signaling Technology), then antigen–Ab complexes (100 µg) were immunoblotted with anti-cMET and phycoerythrin (PE)-labeled anti-p-cMET (Cell Signaling Technology). Immunoreactive bands were detected using enhanced chemiluminescence (LiteAblot, Euroclone) and the Gel-Logic1500 system (Eastman Kodak Co.), quantified by the Kodak Imaging software, and expressed as optical density (OD) units (17).

Real-time RT-PCR was performed using primers (Invitrogen) shown in Supplementary Table S1, and the Applied Biosystems methodology (18). Relative quantification of the mRNA was performed using the comparative threshold cycle (Ct) method with GAPDH as the reference gene and with the 2⁻ΔΔCt formula (19).

Fluorescence-activated cell sorting

A total of 3 × 10⁵ multiple myeloma ECs, MGECS, or normal ECs/tube were incubated with FITC-conjugated anti-cMET and phycoerythrin (PE)-labeled anti-p-cMET

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treatment of multiple myeloma ECs with HGF and c-mET inhibitors and anti-multiple myeloma drugs. The anti-HGF and anti-c-mET neutralizing Abs (R&D Systems) were titrated and used at final doses of 80 ng/µL and 40 ng/µL, respectively, which were capable to significantly decrease the p-cMET expression in multiple myeloma ECs. The c-MET inhibitor SU11274 (Selleck Chemicals) was used at 0.5 and 1 µmol/L (14). Bortezomib (Velcade, Millennium Pharmaceuticals Inc.) and lenalidomide (Revlimid, Celgene Corporation) were dissolved in dimethylsulfoxide (Sigma-Aldrich). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, in Vectashield Hard Set mounting medium, Vector). Pictures were acquired by an Axiosplan-2 microscope (Carl Zeiss), and analyzed by the Leica Application Suite Advanced Fluorescence software (Leica Microsystems).

Functional studies

Treatment of multiple myeloma ECs with HGF and c-MET inhibitors and anti-multiple myeloma drugs. The anti-HGF and anti-c-MET neutralizing Abs (R&D Systems) were titrated and used at final doses of 80 ng/µL and 40 ng/µL, respectively, which were capable to significantly decrease the p-cMET expression in multiple myeloma ECs. The c-MET inhibitor SU11274 (Selleck Chemicals) was used at 0.5 and 1 µmol/L (14). Bortezomib (Velcade, Millennium Pharmaceuticals Inc.) and lenalidomide (Revlimid, Celgene Corporation) were dissolved in dimethylsulfoxide (Sigma-Aldrich) at final doses of 7.5 nmol/L in vitro/20 nmol/L in vitro (20), and 1.75 µmol/L (17), respectively.

Cell viability. A total of 5 × 10³ cells/100 µL/well were plated in triplicate in 96-well plates in serum-free DMEM (Euroclone) alone (negative control), or supplemented with 20% FCS alone (positive control), or added with the neutralizing Abs (singularly and in combination), or with SU11274 for 24 hours, then tested with the MTT assay (Cell proliferation kit (Molecular Probes Inc.), acquired by the FACScantoII with the FlowJo software (Tree Star, Inc.), and analyzed on FACScantoll with the FACSDiva software (BD).

Apoptosis. A total of 5 × 10³ multiple myeloma ECs untreated or treated with the neutralizing Abs or SU11274 for 30 (to assess adhesion) or 90 minutes (to assess spreading), fixed (4% paraformaldehyde), and quantified by the crystal violet assay at 595 nm in a Microplate Reader (Molecular Devices Corp.; ref. 18).

Adhesion to and spreading on fibronectin. A total of 2 × 10⁴ multiple myeloma ECs/well were plated in triplicate on fibronectin-coated 96-well plates in DMEM alone (control) or added with the neutralizing Abs or SU11274 for 30 to 90 minutes to assess spreading, fixed (4% paraformaldehyde), and quantified by the crystal violet assay at 595 nm in a Microplate Reader (Molecular Devices Corp.; ref. 18).

Angiogenesis assay

CM of multiple myeloma ECs untreated or treated with SU11274 were tested for the expression of 55 human angiogenesis-related proteins by using the Human Angiogenesis Western blot Array (R&D Systems). Spots were detected and quantified as for Western blot analysis.

Two-dimensional gel electrophoresis and mass spectrometry protein identification

The two-dimensional gel electrophoresis (2-DE) was done in duplicate by isoelectric focusing, followed by SDS-PAGE. Gels were silver stained and analyzed on PDQuest software (Bio-Rad; ref. 21). Spots of interest were excised, destained, dehydrated in acetoniite, and digested with trypsin. Proteins were identified by peptide mass fingerprinting and tandem MS/MS analysis with a matrix-assisted laser desorption/ionization-time of flight (TOF)/
TOF Ultraflextreme (Bruker Daltonics; ref. 22). Data were exported by the BioTools software (version 3.2, Bruker Daltonics), subjected to database search by using the Matrix Science system (www.matrixscience.com), and referred for mass to the SwissProt human protein database (release 2014_03 of UniProtKB/TrEMBL, 54247468 sequence entries).

**In vivo chorioallantoic membrane assay**

Fertilized white Leghorn chicken eggs were incubated at 37°C at constant humidity (23). On day 3, the shell was opened and 2- to 3 mL of albumen were removed to detach the chorioallantoic membrane (CAM). On day 8, the CAMs were implanted with 1 mm³ sterilized gelatin sponges (Gelfoam Upjohn Co.) loaded with SFM alone (negative control) or with the CM of multiple myeloma ECs untreated (positive control) or treated with SU11274 alone or added with bortezomib and/or lenalidomide. On day 12, the angiogenic response was evaluated as the number of vessels converging toward the sponge at ×50 and photographed in vivo by a stereomicroscope (Olympus Italia Srl).

**Results**

**Expression of HGF and cMET in EC types**

Multiple myeloma ECs (at diagnosis, at relapse, and in refractory disease), MGECS, and control ECs were evaluated. Multiple myeloma ECs expressed (as average) approximately 2-fold more HGF and cMET mRNA versus MGECS, and approximately 4-fold more versus control ECs ($P < 0.01$; Fig. 1A). Accordingly, multiple myeloma ECs secreted approximately 2.5-fold more HGF than MGECS ($P < 0.03$) and approximately 12-fold more than control ECs ($P < 0.01$) in a 24-hour SFM culture (Fig. 1B, left). Similarly, immunoprecipitated total cMET protein was approximately 3-fold more in multiple myeloma ECs versus MGECS and control ECs ($P < 0.01$; Fig. 1B, right). The activation of cMET in multiple myeloma ECs was assessed by Western blot analysis and OD of p-cMET: it was approximately 4-fold more versus MGECS and control ECs ($P < 0.01$; Fig. 1B, right). FACS analysis (Fig. 1C) showed high variability in cMET and p-cMET expression among first diagnosed patients with multiple myeloma (20% ± 10% double positive multiple myeloma ECs), whereas refractory and relapsed patients gave substantially higher expression (60% ± 15% and 80% ± 20% vs. 9% ± 7% MGECS and 2 ± 1% control ECs; $P < 0.001$; Fig. 1C). Immunofluorescence studies (Fig. 1D) confirmed these data, and showed a cytoplasmatic colocalization of both molecules in multiple myeloma ECs, but not in MGECS and control ECs.

Overall data suggest that multiple myeloma ECs, but not MGECS and control ECs, have an activated HGF/cMET pathway.

**Existence of a HGF/cMET autocrine loop in multiple myeloma ECs but not in MGECS and control ECs**

The simultaneous expression of both HGF and cMET, and the evidence of a constitutive p-cMET expression in multiple myeloma ECs suggest that a HGF/cMET autocrine loop is operative in these cells. To validate this hypothesis, p-cMET was evaluated by flow cytometry in all EC types starved in SFM for 24 hours (Fig. 2A); although the p-cMET/cMET double positive cells decreased in MGECS and control ECs, they persisted in multiple myeloma ECs (18% vs. 20% in a first diagnosed, 72% vs. 80% in a refractory, and 70% vs. 79% in a relapsed representative patients; Fig. 2A). Treatment of multiple myeloma ECs from refractory and relapsed patients with anti-HGF (80 ng/µL) or anti-cMET (40 ng/µL) neutralizing Abs reduced sizeably the p-cMET expression (Fig. 2B). Similar data were obtained in first diagnosed patients (data not shown).

The evidence that multiple myeloma ECs unchange the p-cMET expression on starvation (i.e., in absence of external stimulation), together with its inhibition by anti-HGF and anti-cMET Abs suggest that an autocrine HGF/cMET pathway is operative in multiple myeloma ECs of active patients.

**The HGF/cMET autocrine loop mediates multiple myeloma EC migration and angiogenesis**

We next investigated whether the HGF/cMET pathway regulates multiple myeloma ECs’ angiogenic activities. A 24-hour treatment with anti-HGF and anti-cMET Abs in SFM did not modify neither multiple myeloma ECs viability (Fig. 3A) nor apoptosis nor proliferation (Supplementary Fig. S1A; PI = 1). Data were confirmed at 72 hours (not shown). However, the spontaneous multiple myeloma ECs migration into the “wound” lowered by 78% and 80% (as average) with anti-HGF or anti-cMET Abs, and by 82% with both (migrated cells: 20% ± 3, 18% ± 2, and 16% ± 2 vs. 88% ± 15 in untreated multiple myeloma ECs; $P < 0.001$; Fig. 3B). Similarly, in the Boyden microchambers, the multiple myeloma ECs migratory activity toward VEGF-FGF-2 (both 10 ng/mL) as chemottractants was inhibited by 40%, 37%, and 45% ($P < 0.01$; Fig. 3C, left). The lack of differences between each single and combined treatment in the absence of external stimulation further confirms the existence of an autocrine HGF/cMET loop in multiple myeloma ECs.

Multiple myeloma ECs adhesion and spreading were inhibited by 30% with the anti-cMET Ab ($P < 0.03$; Fig. 3C, middle and right) but not with anti-HGF Ab, perhaps because an intracrine secretion of HGF stimulating the intracellular moiety of cMET (24) takes place in multiple myeloma ECs, and contributes to these cell functions.

Multiple myeloma ECs are able to spontaneously spread and form a closely knit capillary network when seeded on Matrigel surface due to their overangiogenic phenotype (Fig. 3D; ref. 16). The anti-HGF and anti-cMET Abs significantly inhibited the angiogenic network, since they reduced the mesh areas by 48% and 55%, respectively, and the vessel length by 47% and 57%, respectively, versus untreated cells. When Abs were combined, both areas and length were inhibited approximately at the same extent,
which further corroborates the existence of an autocrine HGF/cMET loop in multiple myeloma ECs.

Overall data suggest that the HGF/cMET pathway regulates multiple myeloma ECs migratory activities, and that is implicated in the whole multiple myeloma angiogenesis.

**Effects of cMET inhibition by SU11274 in vitro**

Multiple myeloma ECs were treated with SU11274 0.1 to 10 μmol/L: no effect on cell viability, except for the highest (toxic) dose (Fig. 4A), nor on cell apoptosis, nor proliferation (Supplementary Fig. S1B; PI = 1) was observed at 24 hours. Similar data were obtained at 72 hours (not shown). Of note, 0.5 and 1 μmol/L were able to decrease the p-cMET expression, respectively, by 23% and 40% (Supplementary Fig. S2A).

Much in the same way as the Abs, SU11274 reduced dose dependently both spontaneous and chemotactic migration: at 0.5 and 1 μmol/L, the former ("wound" assay; Fig. 4B) lowered, respectively, by 60% and 87% (as average) versus untreated cells (migrated cells: 40 ± 7 and 14 ± 4 vs. 90 ± 11; P < 0.001); the latter by 36% and 70% (P < 0.01; Fig. 4C, left). SU11274 impacted dose dependently with multiple myeloma ECs attachment to and spread on fibronectin: at 1 μmol/L, these cell activities were reduced, respectively, by 42% and 30% (P < 0.01 and P < 0.03; Fig. 4C, middle and right). SU11274 was able to inhibit dose dependently the whole multiple myeloma angiogenesis.

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**Figure 1.** Expression of HGF and cMET in each EC type. A, HGF (left) and cMET (right) mRNA levels were analyzed by real-time RT-PCR and normalized to GAPDH. B, HGF levels were assessed by ELISA (left); p-cMET and cMET by Western blot analysis and optical density (OD) of bands (right). Gene and protein expression fold-change levels in control ECs were arbitrarily set as 1. Data are mean ± SD of multiple myeloma (MM) ECs from patients at first diagnosis (n = 9), on refractory phase to bortezomib- or lenalidomide-based therapies (n = 12), or at relapse after these therapies (n = 11); of MGECs (n = 24) and of control ECs (n = 10). * P < 0.03 and ** P < 0.01 by Wilcoxon signed-rank test. C, FACS analysis shows percentages of p-cMET/cMET double positive ECs in the patients’ groups. D, immunofluorescence for cMET (green signal), p-cMET (red signal), and nuclei (blue signal) in ECs from representative multiple myeloma, MGUS patients, and control subjects. Merge (yellow signal) shows cytoplasmatic colocalization of cMET and p-cMET. Left, merged pictures of cMET, p-cMET, and nuclei; middle, merged pictures of cMET and nuclei; right, merged pictures of p-cMET and nuclei, by an Axioplan-2 microscope. Original magnification, ×63; scale bar, 30 μm.
ECs angiogenesis: 0.5 and 1 μmol/L reduced the mesh areas, respectively, by 58% and 80%; the vessel length by 61% and 83% versus untreated cells (Supplementary Fig. S2B).

SU11274 enhances the antiangiogenic power of bortezomib and lenalidomide. In fact, SU11274 (1 μmol/L), bortezomib (7.5 nmol/L; 20), and lenalidomide (1.75 μmol/L; 17) gave an overlapping antiangiogenic effect since inhibited the mesh areas by 48%, 42%, and 45%, respectively; the vessel length by 53%, 41%, and 43% (Fig. 4D). When SU11274 was combined with bortezomib or lenalidomide, it gave statistically significant antiangiogenesis: inhibition of mesh areas by 92% and 95%, respectively; of vessel length by 96% and 98% versus SU11274-treated cells (P < 0.001; Fig. 4D).

cMET inhibition leads to a particular modulation of angiogenesis-related cytokines in multiple myeloma ECs

To further study the activity of the HGF/cMET pathway on multiple myeloma angiogenesis, multiple myeloma ECs CM were tested for 55 angiogenesis-related cytokines upon the SU11274 treatment (1 μmol/L for 24 hours). The drug was able to significantly modulate four cytokines (P < 0.03 or better; Fig. 5A): three were angiogenic, that is, SERPIN E1 [serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1; ref. 25], CXCL16 (chemokine ligand 16; ref. 26), and MCP-1 (monocyte chemotactic protein-1, or CCL2; ref. 27), and were reduced, respectively, by 42%, 38%, and 32% (as average); one was antiangiogenic, that is, SERPIN F1 [serpin peptidase inhibitor, clade F (α2 antiplasmin, pigment epithelium-derived factor), member 1; ref. 28], and was increased by 30%. The drug modulation of these cytokines was also confirmed by real-time RT-PCR analysis of SU11274-treated versus untreated multiple myeloma ECs (Supplementary Fig. S4A).

Data suggest that the HGF/cMET pathway is implicated in regulating the over-angiogenic multiple myeloma ECs profile.

SU11274 alone and in combination with anti-multiple myeloma drugs changes the multiple myeloma ECs proteome

The multiple myeloma ECs proteome was studied following treatment with SU11274 alone or combined with bortezomib or lenalidomide versus untreated cells (control). At least three 2-DE gels were run per sample, and...
analyzed by computer-assisted spot matching (to identify spot variations), peptide sequencing, and MS–MS followed by database searching. Fourteen proteins varied significantly (2-fold changes vs. control) upon treatment with SU11274 alone (Fig. 5B) and in combination (Supplementary Fig. S3): four angiogenic proteins (Supplementary Table S2), that is, annexin A4 (ANXA4, #2), prohibitin (PHB, #3), peroxiredoxin-6 (PRDX6, #10), and annexin A2 (ANXA2, #12), which govern cell shape and migration, were downregulated, whereas one protein, that is, calpain small subunit 1 (CPNS1, #1), which regulates cell senescence, was upregulated. A real-time RT-PCR for these last five proteins showed a symmetric gene modulation following the cMET inhibition (Supplementary Fig. S4B).

Figure 3. HGF/cMET autocrine loop mediates migration and angiogenesis of multiple myeloma (MM) ECs. A, no effect of anti-HGF (80 ng/mL) and anti-cMET (40 ng/mL) neutralizing Ab on cell viability, but (B) inhibitory effects on the cell spontaneous migration into the “wound.” C, inhibition of chemotaxis (left) by the Abs. No effect with anti-HGF, but inhibition with anti-cMET on cell adhesion (middle) and spreading (right). D, Matrigel angiogenesis assay: inhibition of the whole angiogenesis by the Abs. Original magnification ×200; scale bar: 50 μm. Measurement of mesh areas and vessel length by the EVOS image software: histograms are mean ± SD of multiple myeloma ECs from relapsed (n = 8) or refractory (n = 9) patients. *, P < 0.03 and **, P < 0.01 by Wilcoxon signed-rank test.
Antiangiogenic effect on multiple myeloma ECs in vivo

When CAMs were implanted with a gelatine sponge soaked with the multiple myeloma ECs CM, many newly formed capillaries converging radially toward the sponge in a "spoked-wheel" pattern were seen (vessel count = 27 ± 4 vs. 8 ± 3 of physiologic angiogenesis in SFM; P < 0.001; Fig. 6A and B). In contrast, multiple myeloma ECs treated with SU11274 were poorly angiogenic (16 ± 2; Fig. 6C), and even less with SU11274 plus bortezomib or lenalidomide in a statistically significant fashion (7 ± 2 and 8 ± 3, respectively; P < 0.001; Fig. 6D and E).

Figure 4. Effects of the cMET inhibition by SU11274 and drug combinations on multiple myeloma (MM) ECs in vitro. A, no effects of SU11274 increasing doses on cell viability except for the highest dose due to toxicity. B, dose-dependent inhibition by SU11274 of the cell spontaneous migration into the multiple myeloma ECs “wound” and (C) of the cell chemotaxis (left), adhesion (middle), and spreading (right). D, Matrigel angiogenesis assay showing inhibitory effects of SU11274 and even more by its combination with bortezomib or lenalidomide. Original magnification ×200; scale bar: 50 μm. Measurement of mesh areas and vessel length by the EVOS image software showing statistically significant decrease by the drug combinations: histograms are mean ± SD of multiple myeloma ECs from relapsed (n = 8) or refractory (n = 9) patients. *, P < 0.03 and **, P < 0.01 by Wilcoxon signed-rank test.
Discussion

Tumor angiogenesis is driven by several growth factors that activate receptor tyrosine kinases and different signaling pathways in ECs (29). A key pathway is VEGF/VEGFRs, whose VEGF-A/VEGFR2 autocrine loop has been found in ECs derived from bone marrow of patients with active multiple myeloma (multiple myeloma ECs; ref. 30). Angiogenesis inhibitors targeting this pathway, both alone and combined with chemotherapeutics, are a well-defined therapeutic option for patients with cancer (31). So far, however, clinical trials have failed to show a therapeutic power of VEGF-A/VEGFR2 inhibitors in multiple myeloma (32). Tentatively, we suggest that the HGF/cMET pathway may be one promising new target for antiangiogenic management of patients with multiple myeloma. This pathway governs the tumor cell scattering, hence it regulates cell

Figure 5. SU11274 modifies multiple myeloma (MM) ECs’ angiogenesis-related cytokines and proteins. A, the conditioned media (CM) of SU11274-treated versus untreated multiple myeloma ECs was tested by a cytokine angiogenesis assay. Values from untreated cells were arbitrarily set as 0. Data are given as average of three independent experiments. Significant downregulation of three angiogenic cytokines (SERPIN E1, CXCL16, and MCP-1) and upregulation of an antiangiogenic cytokine (SERPIN F1) are shown. * , $P < 0.03$ or better; Wilcoxon signed-rank test. B, proteome analysis of SU11274-treated versus untreated multiple myeloma ECs. Silver-stained 2-DE gels of whole-protein lysates from a representative refractory patient out of 2 refractory and 3 relapsed patients. Black and red squares indicate downregulated and upregulated proteins, respectively.

Figure 6. SU11274 antiangiogenic effect in vivo. Chorioallantoic membranes (CAM) were incubated with gelatine sponges loaded with SFM (physiologic angiogenesis, A), with the conditioned media (CM) of untreated multiple myeloma (MM) ECs (positive control), B, treated with SU11274 alone (C), or combined with bortezomib (D) or lenalidomide (E). A statistically significant antiangiogenic effect by the drug combinations is shown ($P < 0.001$). A representative patient at relapse is shown. Original magnification $\times50$ on a stereomicroscope.
motility and metastatic spread (33). HGF is released by mesenchymal-derived cells, whereas cMET is expressed by several cell types, including vascular endothelial cells (34) and accessory cells (pericytes; ref. 35). Activation of cMET in tumors most often occurs through ligand-dependent autocrine or paracrine mechanisms. Either HGF is released from the surrounding stromal cells, resulting in a constitutive paracrine cMET activation (11); or coexpression of HGF and cMET leads to autocrine activation, as found in carcinomas, sarcomas, gliomas, and B-cell tumors (8, 9, 36). The HGF/cMET pathway is also involved in the multiple myeloma pathogenesis: its paracrine and autocrine activation has been found in both PCs and microenvironment cells (9, 10).

The HGF/cMET pathway plays a key role in angiogenesis because it stimulates ECs both directly and indirectly by enhancing the expression of the VEGF-A/VEGFR2 pathway and downregulating thrombospondin 1, an angiogenesis inhibitor (7). Here, we show that the HGF/cMET pathway is activated in multiple myeloma ECs of patients with active disease (at first diagnosis, and on refractory and relapse phase), but not in ECs from patients with MGUS and control patients with benign anemia. The significant increase of cMET phosphorylation in multiple myeloma ECs versus MGECS/control ECs suggests that this pathway is constitutively activated in multiple myeloma. Thus, parallel to the autocrine HGF/cMET loop found in PCs (9, 10), an autocrine loop occurs in multiple myeloma ECs too, as confirmed by experiments with anti-HGF and anti-cMET neutralizing Abs. Therefore, much in the same way as the VEGF-A/VEGFR2 autocrine loop entails the overangiogenic phenotype of active multiple myeloma ECs versus normoangiogenic MGECS (30), the HGF/cMET autocrine loop may act as an additional angiogenesis amplifier for multiple myeloma ECs. Worth of note is that VEGF synergizes with HGF in inducing angiogenesis (37). Treatment with the neutralizing Abs to HGF and cMET interferes with multiple myeloma ECs of patients on the active phase, in whom, the endothelial HGF/cMET autocrine loop is operative. This drug may be thus envisaged as a possible antiangiogenic option for patients with multiple myeloma to be further tested in clinical trials (48).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Disclaimer
The sponsors of this study are public or non-profit 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 this article.

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5805
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