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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Authors’ contributions
AF designed research, performed experiments and wrote the manuscript; MAF supervised experiments and analyzed data; MM, AC, SB, IC, AGS, DV, MMA, DL, AR, DR performed experiments; AZ, PD, EM contributed material; RR and VR commented on the manuscript; and AV supervised the experiments, provided financing and wrote the manuscript.

List of abbreviations
2-DE, two-dimensional gel electrophoresis; 7-ADD, 7-aminoactinomycin D; Abs, antibodies; ANXA2, annexin A2; ANXA4, annexin A4; BM, bone marrow; BMSCs, BM stromal cells; CAM, chorioallantoic membrane; CFSE, carboxyfluorescein succinimidyl ester; CM, conditioned medium; cMET, mesenchymal-epithelial transition factor; CPNS1, calpain small subunit 1; Ct, threshold cycle; CXCL16, chemokine ligand 16; DAPI, 4’,6-diamidino-2-phenylindole; DMEM, Dulbecco’s modified eagle’s medium; ECs, endothelial cells; FAK, focal adhesion kinase; FITC, fluorescein isothiocyanate; FGF-2, fibroblast growth factor-2; HGF, hepatocyte growth factor; IEF, isoelectric focusing; IL, interleukin; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MGECs, ECs of MGUS patients; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; MCP-1, monocyte chemotactic protein-1 (CCL2); MMECs, ECs of MM patients; MS, mass spectrometry; MVD, microvessel density; OD, optical density; p-cMET, phospho-cMET; PCs, plasma cells; PE, phycoerythrin; PHB, prohibitin; PI, proliferation index; PI3K, phosphatidylinositol 3-kinase; PMF, mass fingerprinting; PRDX6, peroxiredoxin-6; RT-PCR, reverse transcriptase-PCR; SC, scatter factor; SERPIN E1, serpin peptidase inhibitor,
clade E (nexin, plasminogen activator inhibitor type 1), member 1; SERPIN F1, serpin peptidase inhibitor, clade F (α2 antiplasmin, pigment epithelium derived factor), member 1; SFM, serum-free medium; TRITC, tetramethylrhodamine isothiocyanate.

STATEMENT OF TRANSLATIONAL RELEVANCE

Bone marrow (BM) angiogenesis is enhanced in multiple myeloma (MM) vs monoclonal gammopathy of undetermined significance (MGUS) and entails an attractive target for treatment. HGF/cMET pathway is implicated in the MM pathogenesis and progression. Here we show that BM derived endothelial cells (ECs) from patients with active MM (MMECs) but not those from patients with MGUS (MGECs) or with benign anemia (controls) 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, i.e., SU11274, tested singularly and in combination with bortezomib or lenalidomide, suggest that the HGF/cMET pathway of MMECs may be envisaged as a new therapeutic target for the antiangiogenic management of active MM patients.

ABSTRACT

Purpose: To investigate the angiogenic role of the HGF/cMET pathway and its inhibition in bone marrow (BM) endothelial cells (ECs) from patients with multiple myeloma (MM) vs those with monoclonal gammopathy of undetermined significance (MGUS) or benign anemia (controls).

Experimental Design: The HGF/cMET pathway was evaluated in ECs from MM patients (MMECs) 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 from controls. The effects of a selective cMET tyrosine kinase inhibitor (SU11274) on the MMECs angiogenic activities were studied in vitro and in vivo.
**Results:** MMECs express more HGF, cMET, and activated cMET (phospho (p)-cMET) at both RNA and protein level vs MGECs and control ECs. MMECs are able to maintain the HGF/cMET pathway activation in absence of external stimulation, while treatment with anti-HGF and anti-cMET neutralizing antibodies (Abs) is able to inhibit the cMET activation. The cMET pathway regulates several MMECs 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 MM angiogenesis, and represents an appealing new target to potentiate the antiangiogenic management of MM patients.

**INTRODUCTION**

Multiple myeloma (MM) is a clonal expansion of plasma cells (PCs) in the bone marrow (BM), where they proliferate and acquire resistance to apoptosis and drugs (1). MM angiogenesis results from interactions between PCs and BM microenvironment cells, and is a constant hallmark of disease progression since it correlates with tumor growth, relapse and drug resistance (2). The angiogenesis is enhanced in MM patients compared to those with monoclonal gammopathy of undetermined significance (MGUS) and normal controls (3); and it correlates with prognosis (4). MM 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).

Hepatocyte growth factor (HGF)/scatter factor (SF) is a potent angiogenic cytokine. Bussolino et al. first showed that HGF vividly induces endothelial cell (ECs) proliferation and migration in vitro by triggering their specific tirosine 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 vascular endothelial growth factor (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/MEK, 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 MM pathogenesis. Coexpression of HGF and cMET has been observed in MM PCs, implying the existence of an autocrine loop (9, 10). Since BM stromal cells (BMSCs) 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 BM blood of MM patients 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 MM 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 HGF/cMET pathway in MM-associated angiogenesis, and the effects of cMET inhibition by SU11274 on ECs of MM patients (MMECs) both in vitro and in vivo.

MATERIAL AND METHODS

Patients and ECs

Patients fulfilling the International Myeloma Working Group diagnostic criteria (15) for MM (n=32) and MGUS (n=24) were studied. MM patients (18 men/14 women), aged 41–82 (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). MGUS patients (15 men/9 women), aged 42–79 (median 60.5)
years, were IgG ($n=13$), IgA ($n=7$), or $\kappa$ or $\lambda$ ($n=4$). Control BM ECs were harvested from 10 subjects with anemia due to iron or vitamin B$_{12}$ deficiency (2). The study was approved by the Ethics Committee of the University of Bari Medical School, and all patients provided their informed consent according to the Declaration of Helsinki. MMECs, MGUS derived ECs (MGECs) and control ECs were harvested and cultured as described (16).

**ELISA, immunoprecipitation, western blot, and real time reverse transcriptase (RT)-PCR**

MMECs, MGECs and control ECs ($1 \times 10^6$ cells/mL) were cultured for 24 h 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$^{\text{\textregistered}}$ Human HGF, R&D Systems, Inc., Minneapolis, MN, USA). Protein lysates from all the ECs types ($7 \times 10^5$ cells/sample) were incubated with an anti-cMET antibody (Ab) (Cell Signaling Technology, Danvers, MA, USA), then antigen-Ab complexes immunoprecipitated by Protein G/agarose (Sepharose®, Sigma-Aldrich, St Louis, MO, USA). Protein aliquots (50 $\mu$g) were immunoblotted with anti-cMET and anti-phospho(p)-cMET (Tyr1349) Abs (Cell Signaling Technology). Immunoreactive bands were detected using enhanced chemiluminescence (LiteAblot, Euroclone, Milan, Italy) and the Gel-Logic1500 system (Eastman Kodak Co., Rochester, NY, USA), quantified by the Kodak Imaging software, and expressed as optical density (OD) units (17).

Real time RT-PCR was performed using primers (Invitrogen, Paisley, UK) shown in Supplementary Table 1, 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 $2^{\Delta\Delta CT}$ formula (19).

**Fluorescence-activated cell sorting (FACS)**

Three $\times 10^5$ MMECs, MGECs or normal ECs/tube were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-cMET and phycoerythrin (PE)-labeled anti-p-cMET (Y1234/1235) Abs (R&D Systems). At least 100,000 events/sample were acquired and analyzed using FACScantoII.
cytofluorimeter and the FACSDiva software (Becton Dickinson-BD, San Jose, CA, USA). Negative controls were stained with isotype-matched irrelevant antibodies (BD).

**Immunofluorescence**

Five×10³ MMECs, MGECs and control ECs/chamber were cultured on fibronectin-coated chamber slides (LabTek, Nalge Nunc International, Naperville, IL, USA), fixed (paraformaldehyde), permeabilized (Triton X-100), and incubated with an anti-cMET mouse monoclonal Ab and with an anti-p-cMET rabbit Ab (both from Abcam, Cambridge, UK), then with the anti-mouse IgG-FITC and with the anti-rabbit IgG-tetramethylrhodamine isothiocyanate (TRITC) Abs (both from Sigma-Aldrich). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, in Vectashield® Hard_Set™ mounting medium, Vector, Burlingame, CA, USA). Pictures were acquired by an Axioplan-2 microscope (Carl Zeiss, Jena, Germany), and analyzed by the Leica Application Suite Advanced Fluorescence software (Leica Microsystems, Wetzlar, Germany).

**Functional studies**

**Treatment of MMECs with HGF and cMET inhibitors and anti-MM drugs.** The anti-HGF and anti-cMET 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 MMECs. The cMET inhibitor SU11274 (Selleck Chemicals, Houston, TX, USA) was used at 0.5 and 1 µmol/L (14). Bortezomib (Velcade®, Millennium Pharmaceuticals Inc., Cambridge, MA, USA) and lenalidomide (Revlimid®, Celgene Corporation, Summit, NJ, USA) were dissolved in dimethylsulfoxide (Sigma-Aldrich) at final doses of 7.5 nmol/L *in vitro* /20 nmol/L *in vivo* (20), and 1.75 µmol/L (17), respectively.

**Cell viability.** Five×10³ cells/100 µL/well were plated in triplicate in 96-well plates in serum-free Dulbecco’s modified eagle’s medium (DMEM, Euroclone) alone (negative control), or supplemented with 20% fetal calf serum alone (positive control), or added with the neutralizing Abs (singularly and in combination), or with SU11274 for 24 h, then tested with the MTT assay (Cell Growth Determination kit, Sigma-Aldrich) on the last 4 h, and measured at 570 nm absorbance.
Angiogenesis on Matrigel. Two × 10⁴ MMECs/well were seeded in triplicate on Matrigel-coated (BD) 48-well plates in SFM alone (positive control), or added with the neutralizing Abs, or with SU11274 and/or bortezomib or lenalidomide. After 18 h the skeletonization of the mesh was followed by measurement of mesh areas and vessel length in three randomly-chosen ×200 fields on an EVOS digital inverted microscope (Euroclone) (18). Three different doses of SU11274 (0.1, 0.5, and 1 μmol/L), bortezomib (7.5, 10, and 20 nmol/L) and lenalidomide (0.5, 0.25, and 1.75 μmol/L) were singularly tested in the Matrigel assay. The antiangiogenic potency of the drug associations was assessed by combining the highest dose of SU11274 with the highest dose of bortezomib or lenalidomide.

“Wound” healing. Confluent MMECs on fibronectin (10 μg/mL)-coated (Sigma-Aldrich) 6 cm² dishes were scraped as a “wound” with a pipette tip, and left to move into the wound for 24 h in SFM alone (control), or added with the neutralizing Abs or with SU11274. Cells were fixed, and counted in at least three randomly chosen ×10 wound fields on the EVOS microscope (17).

Chemotaxis. By using the Boyden microchamber assay (16), 1 × 10⁵ MMECs/well were seeded in triplicate on the upper compartment of the chamber, exposed to the neutralizing Abs or SU11274, and left to migrate towards DMEM with 1.5% fetal calf serum (negative control) or added with VEGF (10 ng/mL, Sigma Chemical Co.) and FGF-2 (10 ng/mL, Peprotech Inc., Rocky Hill, NJ, USA) (positive control) in the lower compartment. After 8 h at 37°C, the migrated cells were fixed, stained (Snabb-Diff Kit, Labex AB, Helsingborg, Sweden), and counted on 3-4 ×400 fields/membrane using the EVOS microscope.

Adhesion to and spreading on fibronectin. Two × 10³ MMECs/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 min (to assess adhesion) or 90 min (to assess spreading), fixed (4% paraformaldehyde), and quantified by the crystal violet assay at 595 nm in a Microplate Reader (Molecular Devices Corp., Sunnyvale, CA, USA) (18).
Apoptosis. Five×10^5 MMECs untreated or treated with the neutralizing Abs or SU11274 were washed with ice-cold PBS without Ca^{2+} and Mg^{2+}, incubated with aminoactinomycin D (7-ADD) and FITC Annexin V (Becton Dickinson-BD Biosciences™, San Jose, CA), and analyzed on FACScantoII with the FACSDiva software (BD).

Proliferation assay with carboxyfluorescein succinimidyl ester (CFSE) staining. Five×10^5 MMECs untreated or treated with the neutralizing Abs or SU11274 were labeled with CFSE at 24 and 72 h by using CellTrace™ cell proliferation kit (Molecular Probes Inc., Eugene, OR, USA), acquired by the FACScantoII with the FlowJo software (Tree Star, Inc., Ashland, OR, USA), and shown as proliferation index (PI).

Angiogenesis assay

CM of MMECs untreated or treated with SU11274 was 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.

Two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) protein identification

The 2-DE was done in duplicate by isoelectric focusing (IEF), followed by SDS-PAGE. Gels were silver stained, and analyzed on PD-Quest software (Bio-Rad, Hercules, CA, USA) (21). Spots of interest were excised, destained, dehydrated in acetonitrile and digested with trypsin. Proteins were identified by peptide mass fingerprinting (PMF) and tandem MS/MS analysis with a matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF)/TOF Ultraflextreme (Bruker Daltonics, Billerica, MA, USA) (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 19-Mar-2014 of UniProtKB/TrEMBL, 54247468 sequence entries).

In vivo chorioallantoic membrane (CAM) 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 removed to detach the CAM. On day 8, the CAMs...
were implanted with 1 mm³ sterilized gelatin sponges (Gelfoam Upjohn Co., Kalamazoo, MI, USA) loaded with SFM alone (negative control) or with the CM of MMECs 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 ovo by a stereomicroscope (Olympus Italia Srl, Segrate, Milan, Italy).

RESULTS

Expression of HGF and cMET in the ECs types

MMECs (at diagnosis, at relapse, and in refractory disease), MGECs and control ECs were evaluated. MMECs expressed (as average) ~2-fold more HGF and cMET mRNA vs MGECs; and ~4-fold more vs control ECs (P<0.01; Fig. 1A). Accordingly, MMECs secreted ~2.5-fold more HGF than MGECs (P<0.03) and ~12-fold more than control ECs (P<0.01) in a 24 h serum-free medium (SFM) culture (Fig. 1B, left panel). Similarly, immunoprecipitated total cMET protein was ~3-fold more in MMECs vs MGECs and control ECs (P<0.01; Fig. 1B, right panel). The activation of cMET in MMECs was assessed by western blot and optical density (OD) of p-cMET: it was ~4-fold more vs MGECs and control ECs (P<0.01; Fig. 1B, right panel). FACS analysis (Fig. 1C) showed high variability in cMET and p-cMET expression among 1st diagnosed MM patients (20 ± 10% double positive MMECs), while 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 MMECs, but not in MGECs and control ECs.

Overall data suggest that MMECs, but not MGECs and control ECs, have an activated HGF/cMET pathway.

Existence of a HGF/cMET autocrine loop in MMECs 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 MMECs 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 ECs types starved in SFM for 24 h (Fig. 2A): while the p-cMET/cMET double positive cells decreased in MGECs and control ECs, they persisted in MMECs (18% vs 20% in a 1st diagnosed, 72% vs 80% in a refractory, and 70% vs 79% in a relapsed representative patients; Fig. 2A). Treatment of MMECs 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 1st diagnosed patients (data not shown).

The evidence that MMECs 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 MMECs of active patients.

**The HGF/cMET autocrine loop mediates MMECs migration and angiogenesis**

We next investigated whether the HGF/cMET pathway regulates the MMECs angiogenic activities. A 24 h treatment with anti-HGF and anti-cMET Abs in SFM did not modify neither MMECs viability (Fig. 3A), nor apoptosis, nor proliferation (Supplementary Fig. S1A; PI=1). Data were confirmed at 72 h (not shown). However, the spontaneous MMECs 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 MMECs; \( P<0.001 \); Fig. 3B).

Similarly, in the Boyden microchambers, the MMECs migratory activity towards VEGF+FGF-2 (both 10 ng/mL) as chemoattractants was inhibited by 40%, 37%, and 45% (\( P<0.01 \); Fig. 3C, left panel). The lack of differences between each single and combined treatments in absence of external stimulation further confirms the existence of an autocrine HGF/cMET loop in MMECs.

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

MMECs are able to spontaneously spread and form a closely knit capillary network when seeded on Matrigel surface due to their overangiogenic phenotype (Fig. 3D) (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, vs 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 MMECs.

Overall data suggest that HGF/cMET pathway regulates MMECs migratory activities, and that it is implicated in the whole MM angiogenesis.

Effects of cMET inhibition by SU11274 in vitro

MMECs were treated with SU11274 0.1-10 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 h. Similar data were obtained at 72 h (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) vs 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 panel). SU11274 impacted dose dependently with MMECs 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 panels). SU11274 was able to inhibit dose dependently the whole MMECs angiogenesis: 0.5 and 1 µmol/L reduced the mesh areas respectively by 58% and 80%; the vessel length by 61% and 83% vs 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% vs SU11274-treated cells (P<0.001; Fig. 4D).

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

Data suggest that the HGF/cMET pathway is implicated in regulating the overangiogenic MMECs profile.

SU11274 alone and in combination with anti-MM drugs changes the MMECs proteome
The MMECs proteome was studied following treatment with SU11274 alone or combined with bortezomib or lenalidomide vs 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), i.e., annexin A4 (ANXA4, #2), prohibitin (PHB, #3), peroxiredoxin-6 (PRDX6, #10), and annexin A2 (ANXA2, #12), which govern cell shape and migration, were downregulated; while one protein, i.e., 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).

**Antiangiogenic effect on MMECs in vivo**

When CAMs were implanted with a gelatine sponge soaked with the MMECs 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 physiological angiogenesis in serum-free medium; \( P < 0.001 \); Fig. 6, panels a and b). In contrast, MMECs treated with SU11274 were poorly angiogenic (16 ± 2; panel c), and even less with SU11274 plus bortezomib or lenalidomide in a statistically significant fashion (7 ± 2 and 8 ± 3, respectively; \( P < 0.001 \); Fig. 6, panels d and e).

**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 vascular endothelial growth factor (VEGF)/VEGF receptors (VEGFRs), whose VEGF-A/VEGFR2 autocrine loop has been found in ECs derived from BM of patients with active MM (MMECs) (30). Angiogenesis inhibitors targeting this pathway, both alone and combined with chemotherapeutics, are a well defined therapeutic option for cancer patients (31). So far, however, clinical trials have failed to show a therapeutic power of VEGF-A/VEGFR2 inhibitors in MM (32).
Tentatively, we suggest that the HGF/cMET pathway may be one promising new target for antiangiogenic management of MM patients. This pathway governs the tumor cell scattering, hence it regulates cell motility and metastatic spread (33). HGF is released by mesenchyma-derived cells, whereas cMET is expressed by several cell types, including vascular endothelial cells (34) and accessory cells (pericytes) (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 MM pathogenesis: its paracrine and autocrine activation have been found in both PCs and microenvironment cells (9, 10).

The HGF/cMET pathway plays a key role in angiogenesis, since 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 MMECs of patients with active disease (at first diagnosis, and on refractory and relapse phase), but not in ECs from MGUS patients (MGECs) and control (benign anemia) patients. The significant increase of cMET phosphorylation in MMECs vs MGECs/control ECs suggests that this pathway is constitutively activated in MM. Thus, parallel to the autocrine HGF/cMET loop found in PCs (9, 10) an autocrine loop occurs in MMECs 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 MMECs vs norm-angiogenic MGECs (30), the HGF/cMET autocrine loop may act as an additional angiogenesis amplifier for MMECs. Worth of note is that VEGF synergizes with HGF in inducing angiogenesis (37). Treatment with the neutralizing Abs to HGF and cMET interferes with MMECs spontaneous and chemo-induced migration, while MMECs adhesion and spreading are not impacted by external blockade of HGF. Perhaps an intracrine HGF secretion and stimulation of the cMET cytoplasmic moiety (24) may occur in MMECs. An intracrine VEGF-A/VEGFR1 signaling has been shown in human primary
PCs (38). Plausibly, autocrine and intracrine HGF/cMET loops may be operative in MMECs, hence the intracrine loop is sufficient to mediate cell adhesion and spreading. On the other hand, treatment of cells with the anti-cMET Ab (Fig. 3C) or with SU11274 (Fig. 4C) is able to inhibit both autocrine and intracrine loops, hence MMECs adhesion and spreading, which supports a role of HGF/cMET pathway also in these cell functions.

We found that the cMET pathway is overactivated in drug-resistant MM cell lines as well as in PCs from MM patients at relapse or on refractory phase (10). Rocci et al. (39) reported that the overexpression of the cMET oncogene in PCs indicates poor prognosis. Overall, an altered HGF/cMET pathway activation in MM PCs implies a less responsive disease. Accordingly, targeting of the HGF/cMET pathway may disrupt the interactions between tumor PCs and their microenvironment, which may significantly increase treatment efficacy in refractory disease (39).

Several compounds targeting cMET have been developed, and are now being tested in clinical trials. Results of a phase I trial using a selective oral cMET inhibitor (ARQ197) have been reported (40). Also, an anti-cMET antibody (METMab) significantly improves progression-free survival in patients with non-small-cell lung cancer with high cMET expression and receiving erlotinib, an epidermal growth factor receptor inhibitor (41). Here, we tested SU11274, a novel cMET tyrosine kinase inhibitor, and demonstrated its antiangiogenic activity on MMECs both singularly and in combination with other two antiangiogenic/anti-MM drugs, i.e., bortezomib (20) and lenalidomide (17), in a statistically significant fashion, as demonstrated in vitro (Matrigel assay) and in vivo (CAM assay). SU11274 interferes with the MMECs angiogenic activities, such as spontaneous and chemo-induced migration, adhesion, spreading, and whole angiogenesis, which are all partly mediated by the HGF promigratory effects (9). It inhibits three angiogenic cytokines, i.e., SERPIN E1 (25), CXCL16 (26) and MCP-1 (27) which are involved in adhesion, migration, chemotaxis and homing of MM cells, and upregulates the antiangiogenic cytokine SERPIN F1 (28), which is expressed by MMECs but not MGECs (42).
cMET inhibitor also downregulates angiogenic proteins, such as ANXA2 (43), ANXA4 (44), PRDX6 (45) and PHB (46), and upregulates CPNS1, a protein favouring cell damage and senescence (47).

In sum, SU11274 is able to exert a direct inhibitory effect on MMECs 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 MM patients to be further tested in clinical trials (48).

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LEGENDS

Figure 1

Expression of HGF and cMET in the ECs types.

A) HGF (left panel) and cMET (right panel) mRNA levels were analyzed by real time RT-PCR and normalized to GAPDH. B) HGF levels were assessed by ELISA (left panel); p-cMET and cMET by western blot and optical density (OD) of bands (right panel). Gene and protein expression fold change levels in control ECs were arbitrarily set as 1. Data are means ± SD of MMECs 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 MM, MGUS
patients, and control subjects. Merge (yellow signal) shows cytoplasmatic colocalization of cMET and p-cMET. Left panels: merged pictures of cMET, p-cMET and nuclei; middle panels: merged pictures of cMET and nuclei; right panels: merged pictures of p-cMET and nuclei, by an Axioplan-2 microscope. Original magnification ×63; Scale bar: 30 μm.

Figure 2
HGF/cMET autocrine loop in MMECs vs MGECs and control ECs.
A) FACS analysis on MMECs, MGECs and control ECs: percentages of double positive p-cMET/cMET cells at 24 h culture in complete or serum-free medium. B) Time and dose finding effects of anti-HGF and anti-cMET neutralizing Abs on MMECs. A representative relapsed patient is shown.

Figure 3
HGF/cMET autocrine loop mediates migration and angiogenesis of MMECs.
A) No effect of anti-HGF (80 ng/μL) and anti-cMET (40 ng/μL) neutralizing Ab on cell viability, but B) inhibitory effects on the cell spontaneous migration into the “wound”. C) Inhibition of chemotaxis (left panel) by the Abs. No effect with anti-HGF, but inhibition with anti-cMET on cell adhesion (middle panel) and spreading (right panel). 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 means ± SD of MMECs from relapsed (n=8) or refractory (n=9) patients. *P<0.03; and **P<0.01 by Wilcoxon signed-rank test.

Figure 4
Effects of the cMET inhibition by SU11274 and drug combinations on MMECs 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 MMECs “wound”; and C) on the cell chemotaxis (left panel), adhesion (middle panel) and spreading (right panel). 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 means ± SD of MMECs from relapsed (n=8) or refractory (n=9) patients. *P<0.03; and **P<0.01 by Wilcoxon signed-rank test.

Figure 5
SU11274 modifies MMECs angiogenesis-related cytokines and proteins.
A) The CM of SU11274-treated vs untreated MMECs was tested by a cytokine angiogenesis assay. Values from untreated cells were arbitrarily set as 0. Data are given as average of 3 independent experiments. Significant downregulation of three angiogenic cytokines (SERPIN E1, CXCL16, 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 vs untreated MMECs. 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 or upregulated proteins, respectively.

Figure 6
SU11274 antiangiogenic effect in vivo.
CAMs were incubated with gelatine sponges loaded with serum-free medium (physiological angiogenesis, panel a), with the CM of untreated MMECs (positive control, panel b) or treated with SU11274 alone (panel c) or combined with bortezomib (panel d) or lenalidomide (panel e). A
statistically significant antiangiogenic effect by the drug combinations is shown ($P<0.001$). A representative patient at relapse is shown. Original magnification $\times 50$ on a stereomicroscope.
Figure 1
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**In vivo CAM assay**

<table>
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<tr>
<th>Serum-free medium</th>
<th>plus SU11274</th>
<th>SU+BORT</th>
<th>SU+LEN</th>
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Vessel counts = 8 ± 3 = 27 ± 4 = 16 ± 2 = 7 ± 2 = 8 ± 3

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\text{P < 0.001}
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Figure 6
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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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