Published OnlineFirst September 11, 2014; DOI: 10.1158/1078-0432.CCR-14-0847

Biology of Human Tumors

Clinical Cancer Research

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. *Clin Cancer Res; 20(22); 5796–807. ©2014 AACR.*

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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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doi: 10.1158/1078-0432.CCR-14-0847

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).

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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/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 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 B_{12} 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 (1×10^6 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 (7×10^5 cells/sample) were incubated with an anti-cMET antibody (Ab; Cell Signaling Technology), then antigen–Ab complexes immunoprecipitated by Protein G/agarose (Sepharose, Sigma-Aldrich). Protein aliquots (50 μg) were immunoblotted with anti-cMET and anti-phospho(p)-cMET (Tyr1349) Abs (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 (C_t) method with *GAPDH* as the reference gene and with the $2^{-\Delta\Delta Ct}$ formula (19).

Fluorescence-activated cell sorting

A total of 3×10^5 multiple myeloma ECs, MGECs, or normal ECs/tube were incubated with FITC-conjugated anti-cMET and phycoerythrin (PE)-labeled anti-p-cMET

Published OnlineFirst September 11, 2014; DOI: 10.1158/1078-0432.CCR-14-0847

(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). Negative controls were stained with isotype-matched irrelevant antibodies (BD).

Immunofluorescence

A total of 5×10^3 multiple myeloma ECs, MGECs, and control ECs/chamber were cultured on fibronectin-coated chamber slides (LabTek, Nalge Nunc International), 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), 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). Pictures were acquired by an Axioplan-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 cMET inhibitors and anti-multiple myeloma 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 multiple myeloma ECs. The cMET 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 vivo* (20), and 1.75 μmol/L (17), respectively.

Cell viability. A total of 5×10^3 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 Growth Determination Kit, Sigma-Aldrich) on the last 4 hours, and measured at 570 nm absorbance referenced to 655 nm (17).

Angiogenesis on Matrigel. A total of 2×10^4 multiple myeloma ECs/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 hours, 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; ref. 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 multiple myeloma ECs on fibronectin (10 μg/mL)-coated (Sigma-Aldrich) 6 cm^2 dishes were scraped as a "wound" with a pipette tip, and left to move into the wound for 24 houra 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), 1×10^5 multiple myeloma ECs/well were seeded in triplicate on the upper compartment of the chamber, exposed to the neutralizing Abs or SU11274, and left to migrate toward DMEM with 1.5% FCS (negative control) or added with VEGF (10 ng/mL, Sigma Chemical Co.) and FGF-2 (10 ng/mL, Peprotech Inc.; positive control) in the lower compartment. After 8 hours at 37°C, the migrated cells were fixed, stained (Snabb-Diff Kit, Labex AB), and counted on three to four ×400 fields/membrane using the EVOS microscope.

Adhesion to and spreading on fibronectin. A total of 2×10^3 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 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).

Apoptosis. A total of 5×10^5 multiple myeloma ECs 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), and analyzed on FACScantoII with the FACSDiva software (BD).

Proliferation assay with carboxyfluorescein succinimidyl ester staining. A total of 5×10^5 multiple myeloma ECs untreated or treated with the neutralizing Abs or SU11274 were labeled with carboxyfluorescein succinimidyl ester (CFSE) at 24 and 72 hours by using CellTrace cell proliferation kit (Molecular Probes Inc.), acquired by the FACScantoII with the FlowJo software (Tree Star, Inc.), and shown as proliferation index (PI).

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 PD-Quest software (Bio-Rad; ref. 21). Spots of interest were excised, destained, dehydrated in acetonitrile, 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)/

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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 19-Mar-2014 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 mm^3 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 ovo* 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 ($20 \pm 10\%$ double positive multiple myeloma ECs), whereas refractory and relapsed patients gave substantially higher expression ($60 \pm 15\%$ and $80 \pm 20\%$ vs. $9 \pm 7\%$ MGECs and $2 \pm 1\%$ 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 ECs 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 chemoattractants 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,

Published OnlineFirst September 11, 2014; DOI: 10.1158/1078-0432.CCR-14-0847

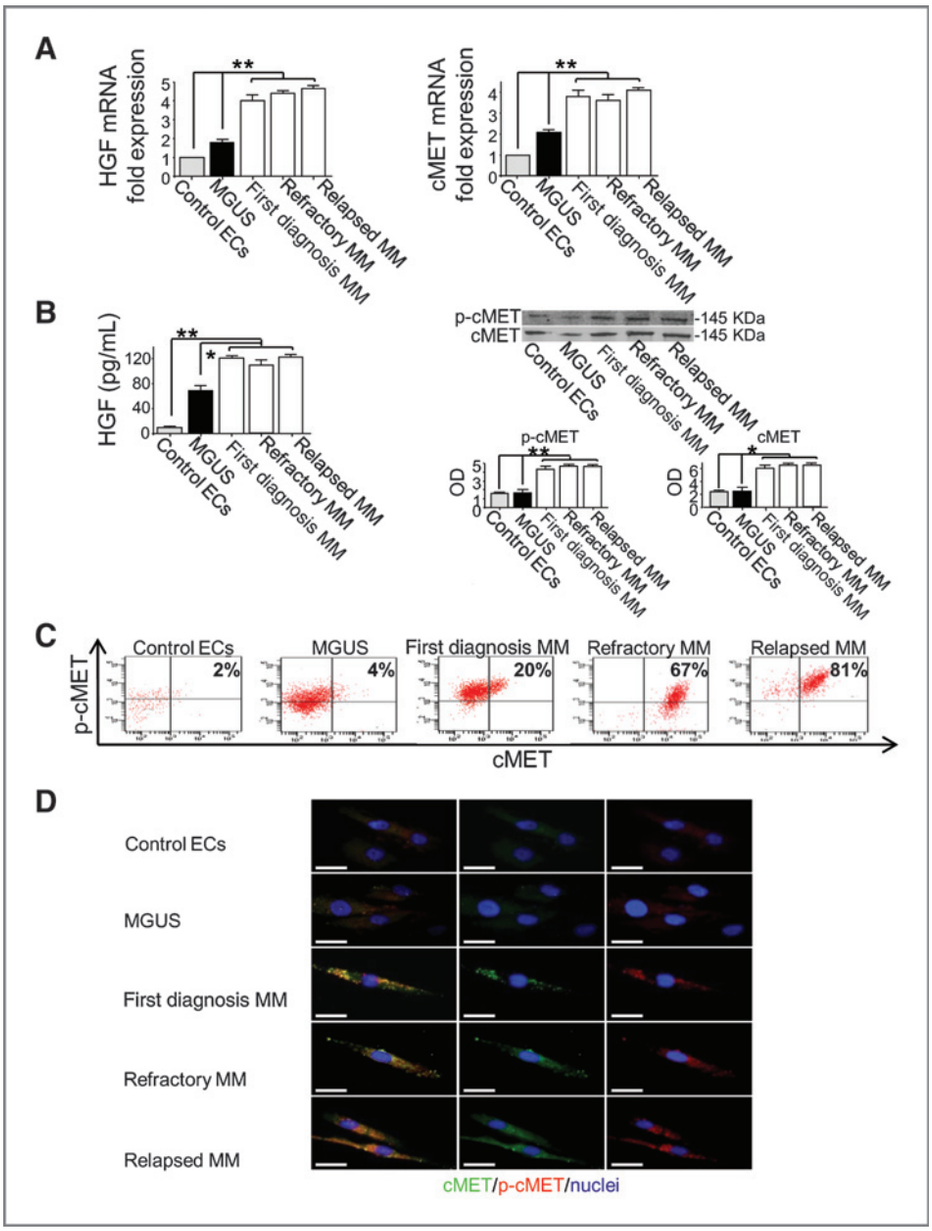

Figure 1. Expression of HGF and cMET in each EC type. A, *HGF* (left) and *cMET* (right) mRNA levels were analyzed by real-time RT-PCR and normalized to *GAPDH*. B, HGF levels were assessed by ELISA (left); p-cMET and cMET by Western blot analysis and optical density (OD) of bands (right). Gene and protein 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.

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 it 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

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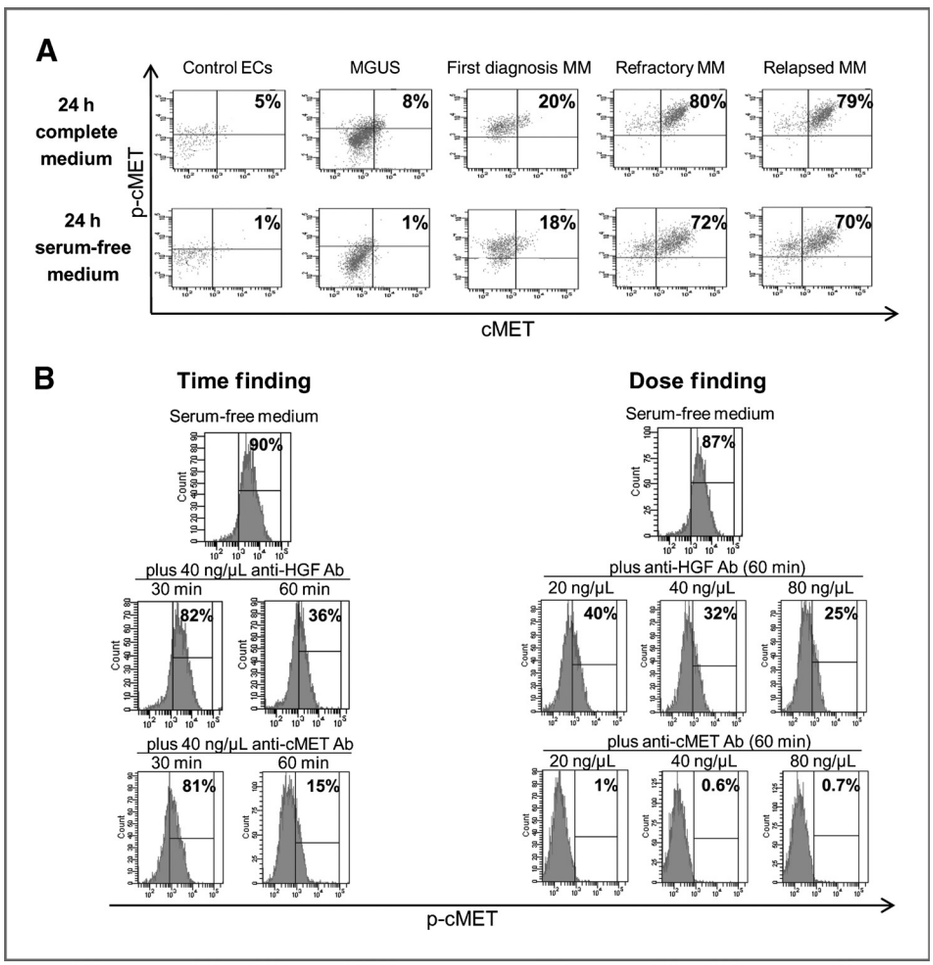

Figure 2. HGF/cMET autocrine loop in multiple myeloma (MM) ECs versus MGECs and control ECs. A, FACS analysis on multiple myeloma ECs, MGECs, and control ECs; percentages of double positive p-cMET/cMET cells at 24-hour culture in complete or SFM. B, time- and dose-finding effects of anti-HGF and anti-cMET neutralizing Abs on multiple myeloma ECs. A representative relapsed patient is shown.

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 untreatred 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

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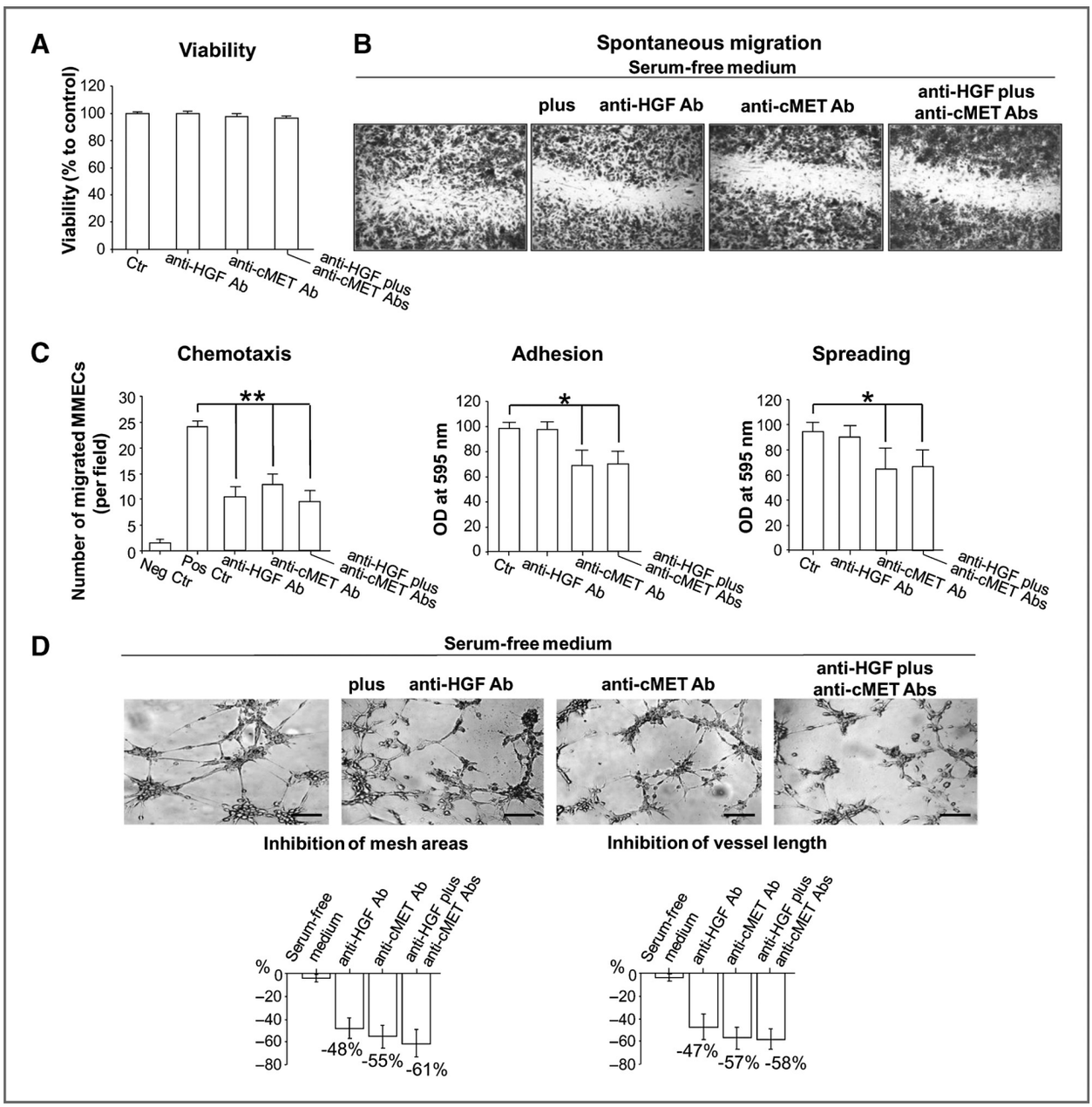

Figure 3. HGF/cMET autocrine loop mediates migration and angiogenesis of multiple myeloma (MM) ECs. A, no effect of anti-HGF (80 ng/μL) and anti-cMET (40 ng/μL) neutralizing Ab on cell viability, but (B) inhibitory effects on the cell spontaneous migration into the "wound." C, inhibition of chemotaxis (left) 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.

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).

Published OnlineFirst September 11, 2014; DOI: 10.1158/1078-0432.CCR-14-0847

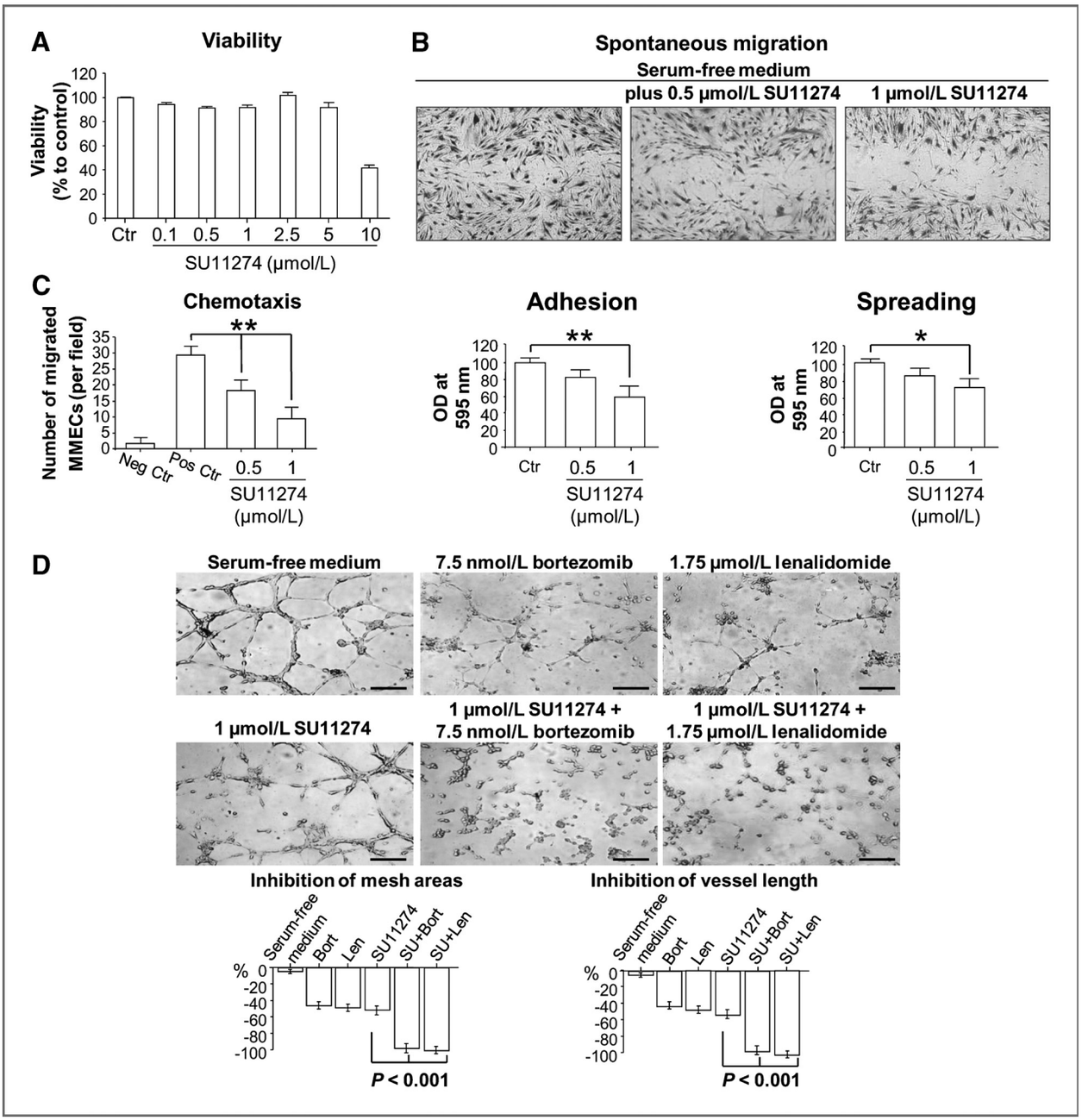

Figure 4. Effects of the cMET inhibition by SU11274 and drug combinations on multiple myeloma (MM) ECs *in vitro*. A, no effects of SU11274 increasing doses on cell viability except for the highest dose due to toxicity. B, dose-dependent inhibition by SU11274 of the cell spontaneous migration into the 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.

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).

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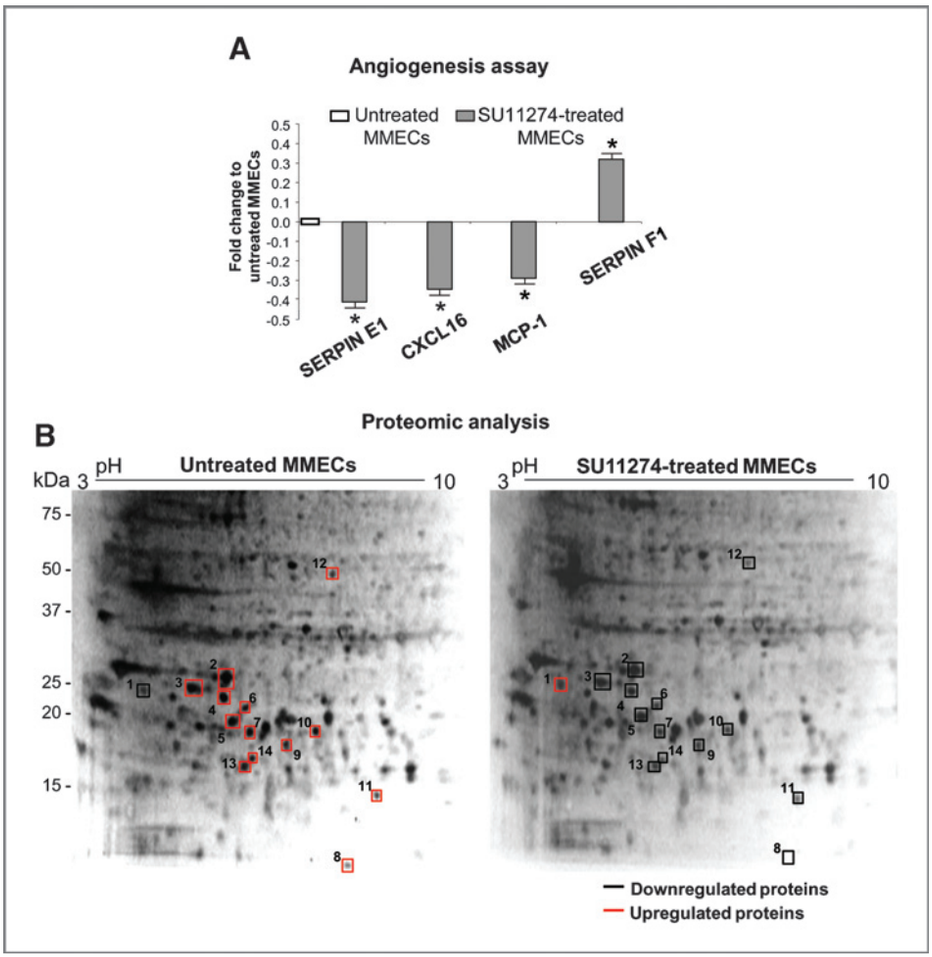

Figure 5. SU11274 modifies multiple myeloma (MM) ECs' angiogenesis-related cytokines and proteins. A, the conditioned media (CM) of SU11274-treated versus-untreated multiple myeloma ECs was tested by a cytokine angiogenesis assay. Values from untreated cells were arbitrarily set as 0. Data are given 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.

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

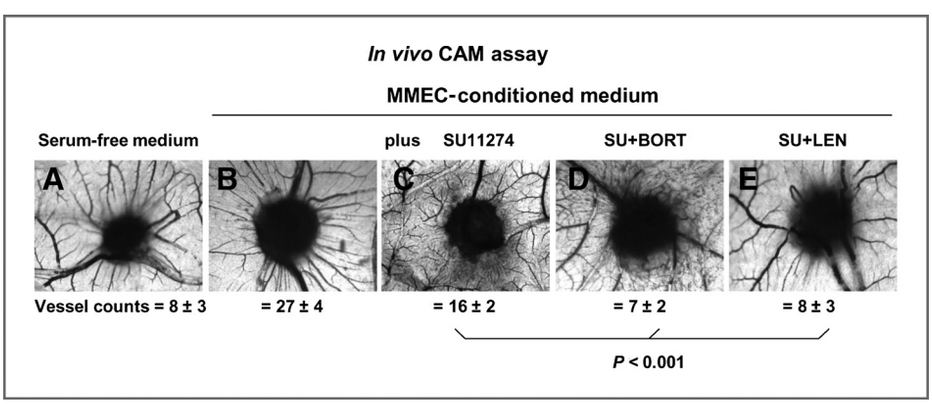

Figure 6. SU11274 antiangiogenic effect *in vivo*. Chorioallantoic membranes (CAM) were incubated with gelatine sponges loaded with SFM (physiologic angiogenesis, A), with the conditioned media (CM) of untreated multiple myeloma (MM) ECs (positive control), B, treated with SU11274 alone (C), or combined 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 ×50 on a stereomicroscope.

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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 normangiogenic 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 spontaneous and chemo-induced migration, whereas multiple myeloma ECs 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 multiple myeloma ECs. 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 multiple myeloma ECs; 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 multiple myeloma ECs adhesion and spreading, which supports a role of the HGF/cMET pathway also in these cell functions.

We found that the cMET pathway is overactivated in drug-resistant multiple myeloma cell lines as well as in PCs from patients with multiple myeloma at relapse or on refractory phase (10). Rocci and colleagues (39) reported that the overexpression of the cMET oncogene in PCs indicates poor prognosis. Overall, an altered HGF/cMET pathway activation in multiple myeloma 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 antiboby (MET-Mab) significantly improves PFS in patients with non–small cell lung cancer with high cMET expression and receiving erlotinib, an EGFR inhibitor (41). Here, we tested SU11274, a novel cMET tyrosine kinase inhibitor, and demonstrated its antiangiogenic activity on multiple myeloma ECs both singularly and in combination with other two antiangiogenic/anti-multiple myeloma drugs, that is, 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 multiple myeloma ECs 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, that is, SERPIN E1 (25), CXCL16 (26), and MCP-1 (27), which are involved in adhesion, migration, chemotaxis and homing of multiple myeloma cells, and upregulates the antiangiogenic cytokine SERPIN F1 (28), which is expressed by multiple myeloma ECs but not by MGECs (42).

cMET inhibitor also downregulates angiogenic proteins, such as ANXA2 (43), ANXA4 (44), PRDX6 (45), and PHB (46), and upregulates CPNS1, a protein favoring cell damage and senescence (47).

In sum, SU11274 is able to exert a direct inhibitory effect on 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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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Ferrucci, A.G. Solimando

Published OnlineFirst September 11, 2014; DOI: 10.1158/1078-0432.CCR-14-0847

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Grant Support

This work was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC, Milan), Investigator grant 2013 (no. 14095) and the 5 per thousand Molecular Clinical Oncology Special Program (grant no. 9965; to A. Vacca), the European Commission's 7th Framework Programme 2007–2013 (EU FP7) under grant agreement no. 278706 (OVER-MyR; to A. Vacca), and EU FP7 under grant agreement no. 278570 (to D. Ribatti), and grants from MIUR PRIN 2009WCNS5C_004 (to R. Ria) and 2010NECHBX (to A. Vacca).

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Received April 7, 2014; revised July 18, 2014; accepted August 4, 2014; published OnlineFirst September 11, 2014.

References

1. Palumbo A, Anderson K. Multiple myeloma. N Engl J Med 2011;364:1046–60.
2. Vacca A, Ribatti D, Roncali L, Ranieri G, Serio G, Silvestris F, et al. Bone marrow angiogenesis and progression in multiple myeloma. Br J Haematol 1994;87:503–8.
3. Rajkumar SV, Mesa RA, Fonseca R, Schroeder G, Plevak MF, Dispenzieri A, et al. Bone marrow angiogenesis in 400 patients with monoclonal gammopathy of undetermined significance, multiple myeloma, and primary amyloidosis. Clin Cancer Res 2002;8:2210–6.
4. Munshi NC, Wilson C. Increased bone marrow microvessel density in newly diagnosed multiple myeloma carries a poor prognosis. Semin Oncol 2001;28:565–9.
5. Podar K, Tai YT, Hideshima T, Vallet S, Richardson PG, Anderson KC. Emerging therapies for multiple myeloma. Expert Opin Emerg Drugs 2009;14:99–127.
6. Bussolino F, Di Renzo MF, Ziche M, Bocchietto E, Olivero M, Naldini L, et al. Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. J Cell Biol 1992;119:629–41.
7. Zhang YW, Su Y, Volpert OV, Vande Woude GF. Hepatocyte growth factor/scatter factor mediates angiogenesis through positive VEGF and negative thrombospondin 1 regulation. Proc Natl Acad Sci U S A 2003;100:12718–23.
8. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. Nat Rev Mol Cell Biol 2003;4:915–25.
9. Børset M, Lien E, Espevik T, Helseth E, Waage A, Sundan A. Concomitant expression of hepatocyte growth factor/scatter factor and the receptor c-MET in human myeloma cell lines. J Biol Chem 1996;271:24655–61.
10. Moschetta M, Basile A, Ferrucci A, Frassanito MA, Rao L, Ria R, et al. Novel targeting of phospho-cMET overcomes drug resistance and induces anti-tumor activity in multiple myeloma. Clin Cancer Res 2013;19:4371–82.
11. Takai K, Hara J, Matsumoto K, Hosoi G, Osugi Y, Tawa A, et al. Hepatocyte growth factor is constitutively produced by human bone marrow stromal cells and indirectly promotes hematopoiesis. Blood 1997;89:1560–5.
12. Seidel C, Børset M, Turesson I, Abildgaard N, Sundan A, Waage A. Elevated serum concentrations of hepatocyte growth factor in patients with multiple myeloma. The Nordic Myeloma Study Group. Blood 1998;91:806–12.
13. Ma PC, Jagadeeswaran R, Jagadeesh S, Tretiakova MS, Nallasura V, Fox EA, et al. Functional expression and mutations of c-Met and its therapeutic inhibition with SU11274 and small interfering RNA in non-small cell lung cancer. Cancer Res 2005;65:1479–88.
14. Koon EC, Ma PC, Salgia R, Welch WR, Christensen JG, Berkowitz RS, et al. Effect of a c-Met-specific, ATP-competitive small-molecule inhibitor SU11274 on human ovarian carcinoma cell growth, motility, and invasion. Int J Gynecol Cancer 2008;18:976–84.
15. 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.
16. 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.
17. De Luisi A, Ferrucci A, Coluccia AML, Ria R, Moschetta M, De Luca E, et al. Lenalidomide restrains motility and overangiogenic potential of bone marrow endothelial cells in patients with active multiple myeloma. Clin Cancer Res 2011;17:1935–46.
18. Berardi S, Caivano A, Ria R, Nico B, Savino R, Terracciano R, et al. Four proteins governing overangiogenic endothelial cell phenotype in patients with multiple myeloma are plausible therapeutic targets. Oncogene 2012;31:2258–69.
19. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Methods 2001;25:402–8.
20. Roccaro AM, Hideshima T, Raje N, Kumar S, Ishitsuka K, Yasui H, et al. Bortezomib mediates antiangiogenesis in multiple myeloma via direct and indirect effects on endothelial cells. Cancer Res 2006;66:184–91.
21. Ria R, Catacchio I, Berardi S, De Luisi A, Caivano A, Piccoli C, et al. HIF-1α of bone marrow endothelial cells implies relapse and drug resistance in patients with multiple myeloma and may act as a therapeutic target. Clin Cancer Res 2014;20:847–58.
22. Vergara D, Chiriacò F, Acierno R, Maffia M. Proteomic map of peripheral blood mononuclear cells. Proteomics 2008;8:2045–51.
23. Ribatti D, Nico B, Vacca A, Presta M. The gelatin sponge-chorioallantoic membrane assay. Nat Protoc 2006;1:85–91.
24. Re RN, Cook JL. An intracrine view of angiogenesis. BioEssays 2006;28:943–53.
25. Purdue MP, Lan Q, Menashe I, Zheng T, Zhang Y, Yeager M, et al. Variation in innate immunity genes and risk of multiple myeloma. Hematol Oncol 2011;29:42–6.
26. Nakayama T, Hieshima K, Izawa D, Tatsumi Y, Kanamaru A, Yoshie O. Cutting edge: profile of chemokine receptor expression on human plasma cells accounts for their efficient recruitment to target tissues. J Immunol 2003;170:1136–40.
27. Aggarwal R, Ghobrial IM, Roodman GD. Chemokines in multiple myeloma. Exp Hematol 2006;34:1289–95.
28. Chen L, Zhang SS, Barnstable CJ, Tombran-Tink J. PEDF induces apoptosis in human endothelial cells by activating p38 MAP kinase dependent cleavage of multiple caspases. Biochem Biophys Res Commun 2006;348:1288–95.
29. Ribatti D, Basile A, Ruggieri S, Vacca A. Bone marrow vascular niche and the control of angiogenesis in multiple myeloma. Front Biosci 2014;19:304–11.
30. 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.
31. Cesca M, Bizzaro F, Zucchetti M, Giavazzi R. Tumor delivery of chemotherapy combined with inhibitors of angiogenesis and vascular targeting agents. Front Oncol 2013;3:259.
32. Lopuch S, Kawalec P, Wiśniewska N. Effectiveness of targeted therapy as monotherapy or combined therapy in patients with relapsed or refractory multiple myeloma: a systematic review and meta-analysis. Hematology 2014 Feb 28. [Epub ahead of print].
33. Corso S, Comoglio PM, Giordano S. Cancer therapy: can the challenge be MET? Trends Mol Med 2005;11:284–92.
34. Ding S, Merkulova-Rainon T, Han ZC, Tobelem G. HGF receptor up-regulation contributes to the angiogenic phenotype of human endothelial cells and promotes angiogenesis *in vitro*. Blood 2003;101:4816–22.

Published OnlineFirst September 11, 2014; DOI: 10.1158/1078-0432.CCR-14-0847

35. Liu Y, Wilkinson FL, Kirton JP, Jeziorska M, Iizasa H, Sai Y, et al. Hepatocyte growth factor and c-Met expression in pericytes: implications for atherosclerotic plaque development. J Pathol 2007;212:12–9.
36. Mahtouk K, Tjin EP, Spaargaren M, Pals ST. The HGF/MET pathway as target for the treatment of multiple myeloma and B-cell lymphomas. Biochim Biophys Acta 2010;1806:208–19.
37. Xin X, Yang S, Ingle G, Zlot C, Rangell L, Kowalski J, et al. Hepatocyte growth factor enhances vascular endothelial growth factor-induced angiogenesis *in vitro* and *in vivo*. Am J Pathol 2001;158:1111–20.
38. Vincent L, Jin DK, Karajannis MA, Shido K, Hooper AT, Rashbaum WK, et al. Fetal stromal-dependent paracrine and intracrine vascular endothelial growth factor-A/vascular endothelial growth factor receptor-1 signaling promotes proliferation and motility of human primary myeloma cells. Cancer Res 2005;65:3185–92.
39. Rocci A, Gambella M, Aschero S, Baldi I, Trusolino L, Cavallo F, et al. MET dysregulation is a hallmark of aggressive disease in multiple myeloma patients. Br J Haematol 2014;164:841–50.
40. Yap TA, Olmos D, Brunetto AT, Tunariu N, Barriuso J, Riisnaes R, et al. Phase I trial of a selective cMET inhibitor ARQ 197 incorporating proof of mechanism pharmacodynamic studies. J Clin Oncol 2011;29:1271–9.
41. Spigel DR, Ervin TJ, Ramlau R, Daniel DB, Goldschmidt JH, Blumenschein GR, et al. Final efficacy results from OAM4558g, a randomized phase II study evaluating MetMAb or placebo in combination with erlotinib in advanced NSCLC. J Clin Oncol 29: 2011 (suppl; abstr 7505).
42. Ria R, Todoerti K, Berardi S, Coluccia AML, 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.
43. Bao H, Jiang M, Zhu M, Sheng F, Ruan J, Ruan C. Overexpression of Annexin II affects the proliferation, apoptosis, invasion and production of proangiogenic factors in multiple myeloma. Int J Hematol 2009;90:177–85.
44. Mogami T, Yokota N, Asai-Sato M, Yamada R, Koizume S, Sakuma Y, et al. Annexin A4 is involved in proliferation, chemo-resistance and migration and invasion in ovarian clear cell adenocarcinoma cells. PLoS ONE 2013;8:e80359.
45. Kalinina EV, Berezov TT, Shtil' AA, Chernov NN, Glazunova VA, Novichkova MD, et al. Expression of peroxiredoxin 1, 2, 3, and 6 genes in cancer cells during drug resistance formation. Bull Exp Biol Med 2012;153:878–81.
46. Guo S, Zou J, Wang G. Advances in the proteomic discovery of novel therapeutic targets in cancer. Drug Des Devel Ther 2013;7:1259–71.
47. Demarchi F, Schneider C. The calpain system as a modulator of stress/damage response. Cell Cycle 2007;6:136–8.
48. Gherardi E, Birchmeier W, Birchmeier C, Woude GV. Targeting MET in cancer: rationale and progress. Nat Rev Cancer 2012;12:89–103.

Published OnlineFirst September 11, 2014; DOI: 10.1158/1078-0432.CCR-14-0847

Clinical Cancer Research

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Clin Cancer Res 2014;20:5796-5807. Published OnlineFirst September 11, 2014.