Microenvironment-Derived HGF Overcomes Genetically Determined Sensitivity to Anti-MET Drugs

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Abstract

Cell-based drug screenings indicate that tumors displaying c-MET gene amplification are "addicted" to MET signaling and therefore are very sensitive to MET-targeted agents. However, these screenings were conducted in the absence of the MET ligand, hepatocyte growth factor (HGF), which is abundant in the tumor microenvironment. Sensitivity of six MET-addicted human tumor cells to three MET kinase inhibitors (JNJ-38877605, PHA-665752, crizotinib) and one antagonistic anti-MET antibody (DN30 Fab) was analyzed in the absence or presence of HGF, in a stroma–tumor coculture system, and by combining anti-MET drugs with an HGF neutralizing antibody (ficlatuzumab) in human HGF knock-in mice bearing c-MET–amplified tumors. In all models examined, HGF promoted resistance to MET-targeted agents, affecting both their potency and efficacy. HGF-induced resistance was due to restoration of physiologic GAB1–mediated PI3K activation that compensated for loss of aberrant HER3-dependent PI3K signaling. Ficlatuzumab restored sensitivity to MET-targeted agents in coculture systems and overcame resistance to JNJ-38877605, crizotinib, and DN30 Fab in human HGF knock-in mice. These data suggest that c-MET–amplified tumor cells—which normally exhibit ligand-independent, constitutive MET activation—become dependent on HGF for survival upon pharmacologic MET inhibition. Because HGF is frequently overexpressed in human cancer, this mechanism may represent a major cause of resistance to anti-MET therapies. The ability of ficlatuzumab to overcome HGF-mediated resistance generates proof of principle that vertical inhibition of both a tyrosine kinase receptor and its ligand can be therapeutically beneficial and opens new perspectives for the treatment of MET-dependent tumors. Cancer Res; 74(22): 6598–609. ©2014 AACR.
HER3 transphosphorylation, thus permitting PI3K activation in the presence of EGFR inhibitors (13). Interestingly, MET-mediated resistance to EGFR inhibitors in lung cancer can also occur in the absence of c-MET amplification via hepatocyte growth factor (HGF)-induced activation of the PI3K–AKT pathway (17, 18). In this case, however, PI3K signaling is sustained by recruitment of the GAB1 adaptor protein—the physiological MET signal transducer—and not by transphosphorylation of HER3 (19). Consistent with a role of HGF in mediating resistance to EGFR inhibitors, acquired resistance to gefitinib and erlotinib in patients with lung cancer is associated with high HGF levels in the tumor microenvironment (17, 20) and in plasma (21).

Recently, 2 studies systematically investigating the effect of growth factors on the response to targeted anticancer agents using a wide panel of oncogene-addicted human cancer cells identified HGF as the most relevant microenvironment-borne source of resistance (22, 23). Both these works recognized a recurrent theme in HGF-mediated resistance: PI3K re-activation, compensating for the survival signal transduced by the targeted kinase. Interestingly, PI3K also represents a key mediator of survival in MET-addicted tumor cells (13, 24). However, surprisingly, the role of HGF in innate or acquired resistance to MET-targeted agents has not been investigated.

In this study, we analyzed the influence of tumor microenvironment–derived HGF on the response to MET-targeted therapy using a panel of human tumor c-MET–amplified cell lines and 4 different MET inhibitors, including JNJ-38877605, PHA-66572, crizotinib, and DN30 Fab. To this end, we measured sensitivity of MET-addicted cells to anti-MET drugs in the presence of HGF and in coculture systems using HGF-secreting human fibroblasts. Because mouse HGF does not activate human MET, we also used genetically engineered human HGF knock-in (hHGFKI) SCID mice that express human HGF in place of mouse HGF. Using ficituzumab, a neutralizing anti-HGF antibody (25), we examined the in vivo relevance of microenvironment-derived HGF in determining the response of c-MET–amplified human tumors to anti-MET drugs.

Materials and Methods

Cell culture

MKN-45, Hs746T, SNU-5, H1993, A549, A2780, U87-MG, and MRC-5 cells were obtained from the European Collection of Cell Cultures and cultured as suggested by the supplier. EBC-1 cells were obtained from the Japanese Collection of Research Bioresources. GTL-16 cells have been described before (26). EBC-1 and GTL-16 cells were maintained in RPMI (Sigma Life Science) supplemented with 10% FBS and 2 mmol/L glutamine (Sigma Life Science). GTL-16 and EBC-1 cells were engineered to express luciferase by lentiviral vector technology using the pRRL-luciferase vector as described (27). Genetic identity of each cell line was confirmed by short tandem repeat profiling using Cell ID System (Promega) in November 2013.

Cell-based drug-sensitivity assays

For HGF assays, cells were seeded in 96-well plates (1,000 cells per well). The day after, cells were treated with increasing concentrations of the indicated drug in the presence of the appropriate concentration of recombinant human HGF (R&D Systems). After 3 days, cell number was determined using Cell Titer Glo (Promega) with a Victor X4 multilabel plate reader (Perkin Elmer). For ficituzumab assays, GTL-16 and EBC-1 cells were seeded in 96-well plates (1,000 cells per well) in the presence of ficituzumab as indicated. The day after, cells were treated with increasing concentrations of JNJ-38877605 or DN30 Fab plus the appropriate concentration of HGF (R&D Systems). Cell viability was determined 3 days later as above. For coculture experiments, MRC-5 fibroblasts (1,000 cells per well) and GTL-16-luc or EBC-1-luc (1,000 cells per well) were seeded concomitantly in 96-well plates in the presence of ficituzumab. The day after, cell mixtures were incubated with increasing concentrations of JNJ-38877605 as indicated. Cell viability was determined 3 days later by measuring luciferase activity using a Luciferase Reporter Assay System kit (Promega). Samples were analyzed with a GloMax 96 Microplate Luminometer (Promega). Cell proliferation data were analyzed and fit using Prism software (GraphPad). HGF concentration in conditioned medium was determined by ELISA using a human HGF Quantikine ELISA kit (R&D Systems).

Signal transduction analysis

GTL-16, MKN-45, and H1993 cells were incubated with the indicated drug concentrations for 24 hours (JNJ-38877605) or 48 hours (DN30 Fab) in the absence or presence of 100 ng/mL HGF (R&D Systems) and then processed for immunoblotting as described (28). Total cell lysates were analyzed by Western blotting using the following antibodies: anti-phospho-MET, anti-phospho-HER3, anti-phospho-HER2, anti-phospho-AKT, anti-AKT, anti-phospho-ERK, anti-ERK, anti-phospho-S6, and anti-S6 (Cell Signaling Technology). For assessment of PI3K-associated phospho-proteins, cell lysates were immunoprecipitated as described (28) using the DQ13 anti-phospho-HER3 antibodies (Cell Signaling Technology); anti-MET (Life Technologies); anti-HER3 (Santa Cruz Biotech). MKN-45 and H1993 cell lysates were also analyzed by Western blotting using anti-GAB1 antibodies and anti-phospho-GAB1 antibodies (Cell Signaling). For assessment of MET-associated phospho-proteins, cell lysates were immunoprecipitated as described (28) using the DQ13 anti-MET antibody (29) followed by Western blotting with anti-phospho-HER3 antibodies (Cell Signaling), anti-phospho-GAB1 antibodies (Cell Signaling), and anti-PI3K antibodies (Millipore). For the assessment of PI3K-associated phospho-proteins, cell lysates were immunoprecipitated as above using anti-PI3K antibodies (Millipore) followed by Western blotting with anti-phospho-HER3 antibodies (Cell Signaling), anti-phospho-GAB1 antibodies (Cell Signaling), and anti-PI3K antibodies (Millipore). MET shedding was analyzed as described (6).

Mouse models of cancer

Wild-type CB17 SCID mice and hHGFKI SCID mice were obtained from Charles River and AVEO Pharmaceuticals, respectively (see online Supplementary Methods). In all in vivo experiments, tumor growth was monitored over time using a caliper and the formula \( V = \frac{4}{3}\pi (x/2)^2 (y/2)^2 (z/2)^2 \), where \( x, y, \) and \( z \) are the 3 dimensions of the tumor. Cell injection, animal randomization, and treatment were performed as indicated in the Results. The M162 human colon carcinoma sample has been described before (30). Statistical
analysis was performed as described in online Supplementary Methods.

Results

HGF protects MET-addicted cancer cells against MET TKI–induced growth inhibition

To determine whether tumor microenvironment–borne signals could influence genetically determined sensitivity to anti-MET drugs, we incubated a panel of human tumor cell lines displaying c-MET gene amplification (GTL-16, MKN-45, SNU-5, Hs746T gastric carcinoma; H1993, EBC-1 lung carcinoma) with increasing concentrations (0–1,000 nmol/L) of a MET-selective TKI (JNJ-38877605; ref. 9) in the presence of 0, 10, 30, or 100 ng/mL recombinant human HGF. As control, we also incubated 2 human tumor cell lines bearing normal c-MET gene copy number (A549 lung carcinoma; U87-MG glioblastoma) and 1 human tumor cell line not expressing MET (A2780 ovary carcinoma) in the same conditions. Cell viability was determined after 3 days by measuring total ATP content (Fig. 1).

In the absence of HGF, JNJ-38877605 inhibited MET-addicted cell growth with IC50 ranging from 11 nmol/L (SNU-5) to 50 nmol/L (H1993) and EMAX varying between 53% (Hs746T) and 91% (MKN-45) but did not affect the growth of control cells. In the presence of HGF, however, the sensitivity of c-MET–amplified cells to JNJ-38877605 decreased substantially, whereas the response profile of control cells remained unaltered. HGF protected all MET-addicted cells against JNJ-38877605–induced growth inhibition in a dose-dependent fashion. At the maximum concentration tested, HGF caused an IC50 increase ranging from 5 times (H1993) to 23 (Hs746T) but did not substantially change the EMAX (Supplementary Table S1). An

Figure 1. HGF protects MET-addicted cancer cells against MET TKI–induced growth inhibition. The indicated cell lines displaying c-MET gene amplification (GTL-16, MKN-45, SNU-5, Hs746T, H1993, EBC-1) were incubated with increasing concentrations of JNJ-38877605 in the presence of HGF as indicated. As control, two cell lines bearing normal c-MET gene copy number (A549, U87-MG) and one cell line not expressing MET (A2780) were also incubated in the same conditions. Cell number was determined after 3 days by measuring total ATP content and expressed as percentage relative to the untreated control.
identical analysis was performed on selected cell lines (GTL-16, MKN-45, EBC-1) using two other small-molecule MET TKIs (PHA-665752, ref. 2; crizotinib, ref. 10). In analogy to the results obtained with JNJ-38877605, HGF decreased sensitivity of c-MET–amplified cells to these drugs in a dose-dependent fashion (Supplementary Fig. S1A and S1B). At 100 ng/mL HGF, the IC_{50} of PHA-665752 increased by 5 times (GTL-16), 9 times (MKN-45), and 13 times (EBC-1), whereas the IC_{50} of crizotinib increased by 5 times (GTL-16 and MKN-45) and 24 times (EBC-1; Supplementary Table S1). In contrast, the E_{\text{MAX}} values did not show any substantial change. These results indicate that HGF significantly reduces the potency of MET TKIs on MET-addicted cancer cells.

**HGF sustains HER3-independent PI3K signaling in c-MET–amplified cells exposed to MET TKIs**

In cells with normal c-MET gene copy number, MET signaling typically occurs via the multi-adaptor protein GAB1 (31). In contrast, MET signal transduction in c-MET–amplified tumor cells is characterized by transphosphorylation of HER3, which in turn activates various downstream pathways independently of GAB1 (13, 24). To cast light onto the molecular mechanisms underlying HGF-induced growth rescue, we incubated GTL-16 cells with increasing concentrations (0–200 nmol/L) of JNJ-38877605 in the presence of 100 ng/mL HGF. Cell lysates were analyzed by Western blotting using antibodies directed against different signaling molecules (Fig. 2A). In the absence of HGF, 50 nmol/L JNJ-38877605 inhibited constitutive MET autophosphorylation to an extent sufficient for completely abrogating its downstream signal transduction, including phosphorylation of HER3, AKT, ERK, and S6 kinase. Interestingly, HGF partially rescued activation of MET, AKT, ERK, and S6 but did not rescue HER3 phosphorylation (Fig. 2A). To determine the signaling pathways activated by HGF, we immunoprecipitated cell lysates with antibodies directed against MET or PI3K and examined coprecipitating phosphotyrosine proteins by Western blotting. This analysis revealed that HGF stimulation in the presence of JNJ-38877605 promotes de novo MET association with phospho-GAB1 (Fig. 2B), which becomes associated with PI3K at the expenses of HER3 (Fig. 2C). Similar results were also obtained by analyzing MET signal transduction in MKN-45 and H1993 cells (Supplementary Fig. S2). Taken together, these results suggest that HGF antagonizes JNJ-38877605–induced growth inhibition by promoting PI3K–GAB1 association rather than by rescuing PI3K–HER3 interaction.

**HGF protects MET-addicted cancer cells against anti-MET antibody-induced growth inhibition**

Next, we measured the ability of HGF to interfere with growth inhibition mediated by an anti-MET antibody Fab fragment that promotes MET "shedding" and downregulation (DN30 Fab; ref. 6). To this end, we incubated the same panel of MET-addicted human tumor cells with increasing concentrations (0–1,600 nmol/L) of DN30 Fab in the presence of 0, 10, 30, or 100 ng/mL HGF. Cell viability was determined after 3 days as described above (Fig. 3). In the absence of HGF, DN30 Fab inhibited the growth of cells bearing multiple c-MET gene copies with IC_{50} ranging from 81 nmol/L (SNU-5) to 380 nmol/L (HS576T) and E_{\text{MAX}} varying between 30% (H1993) and 87% (GTL-16) but did not affect proliferation of control cells. Remarkably, the sensitivity of MET-addicted cells to HGF was significantly reduced in the presence of HGF (Fig. 3).

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**Figure 2.** HGF sustains HER3-independent PI3K signaling in c-MET–amplified cells exposed to MET TKIs. A, GTL-16 cells were incubated with increasing concentrations of JNJ-38877605 in the absence or presence of HGF. Cell lysates were analyzed by Western blotting using antibodies directed against different signaling molecules (indicated with the "p" prefix). B, GTL-16 cells were incubated as above with the indicated concentrations of JNJ-38877605 in the absence or presence of HGF. Cell lysates were immunoprecipitated using anti-MET antibodies and analyzed by Western blotting with antibodies directed against phospho-HER3, phospho-GAB1, and MET. C, GTL-16 cells were incubated as in B in the absence or presence of HGF. Cell lysates were immunoprecipitated using anti-PI3K antibodies and analyzed by Western blotting with antibodies directed against phospho-HER3, phospho-GAB1, and PI3K.
DN30 Fab decreased significantly in the presence of HGF. In GTL-16 and MKN-45 cells, an HGF concentration of 10 ng/mL increased the IC$_{50}$ by 3 and 2 times, respectively. In the remaining four MET-addicted cell types, the lowest HGF dose was sufficient to prevent DN30 Fab from causing more than 50% growth inhibition, thus precluding IC$_{50}$ calculation (Supplementary Table S1). In contrast to MET TKIs that displayed reduced potency but unaltered efficacy, DN30 Fab showed both increased IC$_{50}$ and decreased $E_{\text{MAX}}$ (Supplementary Table S1). In the presence of 100 ng/mL HGF, DN30 Fab inhibited GTL-16 and MKN-45 cell growth by 30% and 35%, respectively, whereas it did not show any measurable inhibitory activity in SNU-5, Hs746T, H1993, and EBC-1 cells (Fig. 3 and Supplementary Table S1). These data suggest that HGF can rescue MET-addicted cells from growth inhibition induced by different types of MET-targeted agents, including small-molecule kinase inhibitors and monoclonal antibodies.

**HGF restores physiologic MET signaling in anti-MET antibody-treated MET-addicted cells**

Analysis of MET levels in DN30 Fab–treated cells revealed that HGF does not interfere with DN30 Fab–induced MET shedding (Fig. 4A). The rescuing activity of HGF could instead be explained by the very high MET levels in c-MET–amplified cells. In fact, DN30 Fab completely abrogated MET expression in cells displaying normal MET levels, but it only reduced MET expression in MET-addicted cells.
resulting in physiologic receptor levels (Fig. 4B). We hypothesized that these levels may still be competent for transducing HGF-mediated signals, thus allowing HGF to sustain PI3K activation in DN30 Fab–treated MET-addicted cells. To test this hypothesis, we incubated GTL-16 cells with increasing concentrations (0–1,600 nmol/L) of DN30 Fab in the absence or presence of 100 ng/mL HGF and analyzed cell lysates by Western blotting using antibodies directed against different signaling molecules (Fig. 4C). In the absence of HGF, DN30 Fab caused dose-dependent reduction of total MET, resulting in lower phosphorylation of MET, HER3, and AKT. HGF did not affect the ability of DN30 Fab to
downregulate MET and to block HER3 phosphorylation but partially rescued phosphorylation of MET and AKT. To investigate the molecular signals linking MET to AKT in the absence of phosphorylated HER3, we immunoprecipitated cell lysates with anti-MET antibodies or with anti-P13K antibodies and analyzed coprecipitating phosphotyrosine proteins by Western blotting. This analysis revealed that, in analogy to the results obtained with JNJ-38877605, HGF did not rescue MET-mediated transphosphorylation of HER3 but rather promoted de novo association and transphosphorylation of GAB1 (Fig. 4D), which in turn recruited PI3K (Fig. 4E). Similar signal transduction data were also obtained using MKN-45 and H1993 cells (Supplementary Fig. S3). These results support our hypothesis that residual MET expression on MET-addicted cells allows HGF to sustain PI3K signaling in the presence of DN30 Fab, thus permitting cell survival and growth.

Neutralization of HGF restores sensitivity to MET-targeted agents in a stroma–epithelium coculture system

To investigate whether HGF-induced resistance to MET-targeted agents can be reverted by neutralizing HGF, we incubated GTL-16 gastric and EBC-1 lung carcinoma cells with increasing concentrations of JNJ-38877605 (0–1,000 nmol/L) or DN30 Fab (0–1,600 nmol/L) in the presence of 50 ng/mL HGF plus 0, 15, 30, or 60 µg/mL flicatuzumab, a monoclonal antibody directed against HGF (25). Total ATP content analysis revealed that flicatuzumab restored sensitivity to JNJ-38877605 in a dose-dependent fashion (Fig. 5A). Ficlatuzumab-mediated rescue was more potent in GTL-16 cells, which are less sensitive to HGF-mediated resistance than EBC-1 cells. Ficlatuzumab also restored sensitivity to DN30 Fab in GTL-16 cells (Fig. 5B, left) but not in EBC-1 cells (Supplementary Fig. S4A). In the latter cell model, the rescuing activity of flicatuzumab could be unmasked by lowering HGF concentration to 5 ng/mL.

Figure 5. Ficlatuzumab rescues sensitivity to MET-targeted agents by neutralizing HGF. A, GTL-16 and EBC-1 cells were incubated with increasing concentrations of JNJ-38877605 in the presence of the indicated concentrations of HGF and flicatuzumab. As control, cells were incubated with the same concentrations of JNJ-38877605 in the absence of HGF. Cell number was determined after 3 days by total ATP content analysis and expressed as percentage relative to the untreated control. B, GTL-16 and EBC-1 cells were incubated with increasing concentrations of DN30 Fab in the presence of HGF and flicatuzumab as indicated. As control, cells were incubated with the same concentrations of DN30 Fab in the absence of HGF. Cell number was determined, expressed, and analyzed as in A. C, luciferase-expressing GTL-16 gastric and EBC-1 lung carcinoma cells were cocultured with immortalized human fibroblasts secreting HGF. Mixed cultures of stroma–tumor cells were incubated with increasing concentrations of JNJ-38877605 in the presence of flicatuzumab as indicated. Luciferase-expressing tumor cells alone were also treated with the same JNJ-38877605 concentrations as control. Tumor cell number was determined 3 days after by measuring luciferase activity. Data were expressed and analyzed as in A.
incubated with increasing concentrations (0-1,000 nmol/L) of HGF. Ficlatuzumab restored sensitivity of EBC-1 and GTL-16 gastric and EBC-1 lung carcinoma cells to MET-targeted agents in a dose-dependent fashion. These results suggest that paracrine secretion of HGF represents a relevant source of resistance in this system, although it is likely that other stroma-secreted cytokines also contribute to promoting tumor cell survival and growth.

Ficlatuzumab sensitizes MET-addicted tumors to MET-targeted agents in hHGF KI SCID mice

It is well established that mouse HGF binds to human MET with lower affinity and fails to activate it (33, 34). To overcome this limitation and to explore the therapeutic potential of ficlatuzumab in the appropriate context, we used hHGF KI SCID mice, which express human HGF in place of mouse HGF. We also used a high expression model of stroma in human tumor models. In the first model, EBC-1 lung carcinoma cells were injected subcutaneously into hHGF KI mice and into wild-type SCID mice as control. When experimental tumors reached a volume of approximately 50 mm³, mice were randomly assigned to the following treatment arms: control; 30 mg/kg ficlatuzumab; 5 mg/kg PEGylated DN30 Fab; 5 mg/kg JNJ-38877605; 5 mg/kg crizotinib 20 mg/kg, and tumor cell viability was determined 3 days after drug administration continued for a total of 34 days. B, EBC-1 cells were injected subcutaneously into hHGF KI SCID mice. When experimental tumors reached a volume of approximately 50 mm³, mice were randomly assigned to the following treatment arms: control; 30 mg/kg ficlatuzumab; 5 mg/kg PEGylated DN30 Fab; 5 mg/kg JNJ-38877605; 5 mg/kg crizotinib 20 mg/kg, and tumor cell viability was determined 3 days after drug administration continued for a total of 33 days. C, Hs746T cells were injected subcutaneously into hHGF KI SCID mice, and the animals were immediately divided into four treatment groups: control; 30 mg/kg ficlatuzumab; 5 mg/kg PEGylated DN30 Fab; 5 mg/kg JNJ-38877605; 5 mg/kg crizotinib 20 mg/kg, and tumor cell viability was determined 3 days after drug administration continued for a total of 35 days. D, M162 cells were injected subcutaneously into hHGF KI SCID mice, and the animals were immediately divided into four treatment groups: control; 30 mg/kg ficlatuzumab; 5 mg/kg PEGylated DN30 Fab; 5 mg/kg JNJ-38877605; 5 mg/kg crizotinib 20 mg/kg, and tumor cell viability was determined 3 days after drug administration continued for a total of 35 days.

Figure 6. Ficlatuzumab sensitizes MET-addicted tumors to MET-targeted agents in hHGF KI SCID mice. A, EBC-1 cells were injected subcutaneously into wild-type SCID mice. When experimental tumors reached a volume of approximately 50 mm³, mice were randomly assigned to the following treatment arms: control (CTRL); ficlatuzumab 30 mg/kg (F30); JNJ-38877605 5 mg/kg (J5); JNJ-38877605 5 mg/kg + ficlatuzumab 30 mg/kg (F30 + J5). Drug administration continued for a total of 34 days. B, EBC-1 cells were injected subcutaneously into hHGF KI SCID mice. Tumor-bearing animals were randomized, treated, and analyzed as described in A. Statistical significance of (without ficlatuzumab vs. with ficlatuzumab) was calculated by the Student t test. C, Hs746T cells were injected subcutaneously into hHGF KI SCID mice, and the animals were immediately divided into four treatment groups: control (CTRL); ficlatuzumab 30 mg/kg (F30); PEGylated DN30 Fab 5 mg/kg (D5); ficlatuzumab 30 mg/kg + PEGylated DN30 Fab 5 mg/kg (F30 + D5). Drug administration continued for 35 days. Statistical significance as in A, C, the M162 tumor was amplified subcutaneously in human hHGF KI SCID mice. Animals bearing tumors of approximately 400 mm³ were randomly divided into six treatment arms: control (CTRL); ficlatuzumab 30 mg/kg (F30); crizotinib 20 mg/kg (C20); crizotinib 40 mg/kg (C40); crizotinib 20 mg/kg + ficlatuzumab 30 mg/kg (C20 + F30); crizotinib 40 mg/kg + ficlatuzumab 30 mg/kg (C40 + F30). Treatment continued for a total of 53 days.
mg/kg JNJ-38877605 and 5 mg/kg JNJ-38877605 + 30 mg/kg ficlatuzumab. Ficlatuzumab was administered twice weekly by intraperitoneal injection; JNJ-38877605 was administered daily by oral gavage. Drug administration continued for approximately 1 month. Tumor volume measurement over time revealed that JNJ-38877605 potently inhibited tumor growth in wild-type SCID mice (84% inhibition; Fig. 6A), whereas it only partially reduced it in HGF-KI SCID mice (49% inhibition; Fig. 6B). Ficlatuzumab alone did not have any effect in either wild-type SCID mice or HGF-KI mice. However, combination of JNJ-38877605 and ficlatuzumab significantly blocked tumor growth (83% inhibition) in HGF-KI mice, resulting in stable disease (Fig. 6B). ELISA of tumor cell extracts with phospho-AKT antibodies confirmed our *in vitro* observation that HGF-mediated resistance to JNJ-38877605 correlates with sustained PI3K activity (Supplementary Fig. S6A). In the second model, Hs746T gastric carcinoma cells were injected into HGF-KI SCID mice as above, and mice were immediately divided into four treatment groups: control; 30 mg/kg ficlatuzumab; 5 mg/kg PEGylated DN30 Fab; and 30 mg/kg ficlatuzumab + 5 mg/kg PEGylated DN30 Fab. Both drugs were administered twice weekly by intraperitoneal injection for a period of 35 days. In this system, ficlatuzumab and DN30 Fab reduced tumor growth by 50% and 63%, respectively. However, their combination was much more efficient, achieving 80% tumor inhibition and demonstrating a significant cooperative effect (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcinoma “xenopatient” library (35) that bears (Fig. 6B). In the last model, a human tumor sample (M162) from colorectal carcin...
antibody concentrations, these MET levels may still guarantee a normal HGF signal transduction, leading to effective compensation for loss of aberrant, HER3-mediated PI3K activation.

The peculiar biology of the MET ligand makes it highly likely that microenvironment-derived HGF represents an important source of innate and acquired resistance to MET-targeted therapies. In fact, HGF is secreted by cells of mesenchymal origin—including fibroblasts, macrophages, and endothelial cells—as an inactive precursor (pro-HGF) that accumulates in the extracellular matrix of tissues owing to its high avidity for glycosaminoglycans. Moreover, during tissue repair and cancer invasion, several cytokines in the reactive interstitial compartment induce transcriptional upregulation of pro-HGF in both fibroblasts and resident macrophages (38, 39). Conversion of pro-HGF into an active, heterodimeric α-β ligand is catalyzed by specific proteases abundant in the tumor microenvironment, including urokinase-type plasminogen activator, HGF activator, a variety of proteases of the blood clotting cascade and multiple metalloproteases (40, 41). Inflammation further enhances pro-HGF conversion by upregulating matriptase and proteases of the plasminogen activation system (42).

The importance of environmental HGF in the resistance to targeted agents and in cancer biology in general has been historically underestimated in preclinical studies due to the fact that mouse HGF does not activate human MET (33, 34). Therefore, all in vivo studies investigating the role of MET in tumor progression and therapy conducted using human xenografts in mice favored the identification of cell-autonomous, HGF-independent processes but inhibited the study of ligand-dependent phenomena. To overcome this limitation, we used an engineered mouse model in which the mouse HGF gene was replaced by a human HGF cDNA by homologous recombination. Unlike transgenic mice over-expressing human HGF on top of the endogenous ligand (34), HGF KI mice express HGF driven by the endogenous mouse promoter and therefore maintain physiologic expression levels (Supplementary Fig. S5).

The ability of HGF-neutralizing antibodies such as flicatumzumab to sensitize c-MET–amplified tumors to MET-targeted agents generates proof of concept that vertical inhibition of a tyrosine kinase receptor and its ligand can be therapeutically beneficial. From a clinical perspective, this possibility can be exploited by two different approaches. It is well established that small-molecule ATP-competitive inhibitors give rise to off-target toxicity at high doses. In many cases, this toxicity poses a relevant limitation to their clinical use and may lead to clinical development interruption. Combination of an anti-MET drug with flicatumzumab or another anti-HGF antibody (43, 44) would allow achieving the same therapeutic goal using a lower dose of MET inhibitor, thereby reducing off-target side effects. Alternatively, combination with anti-HGF antibodies could be envisioned to enhance the pharmacologic effect of a MET-selective drug, thus accomplishing a more favorable therapeutic response at the highest tolerated dose. With respect to the latter possibility, it should be kept in mind that only a minor fraction of patients eligible for a targeted therapy based on genetic testing respond clinically to pharmacologic blockade of the corresponding target (45). Considering the high levels of HGF frequently found in the tumor microenvironment, innate resistance to MET inhibitors in a significant percentage of these nonresponders could probably be overcome by HGF neutralization using agents like flicatumzumab, thereby contributing to increasing the overall response rate.

Disclosure of Potential Conflicts of Interest
M. Han has ownership interest (including patents) in Aveo. J. Gyuris received other commercial research support from and has ownership interest (including patents) in Aveo Oncology, Inc. T. Perera is the Scientific Director and a fellow at, has ownership interest (including patents) in Janssen, and has provided expert testimony for Janssen. P. Michieli is a consultant/advisory board member for arGEN-X. No potential conflicts of interest were disclosed by the other authors.

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