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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.

Introduction

Several independent studies have identified *c-MET* gene amplification as the most predictive genetic event associated with high sensitivity to MET-targeted agents (1). In a screen for human tumor cell lines sensitive to PHA-66572, a MET-selective, ATP-competitive tyrosine kinase inhibitor (TKI; ref. 2), 5 of 5 gastric carcinoma cell lines bearing multiple *c-MET* gene copies (MKN-45, GTL-16, SNU-5, Hs746T, KATO II) displayed high

sensitivity to MET inhibition (3). A similar study identified the same gastric carcinoma cell lines plus 2 additional lung cell lines (EBC-1, H1993), also displaying *c-MET* gene amplification, as highly susceptible to PHA-66572 inhibition (4). RNAi confirmed that lung carcinoma cells bearing multiple copies of *c-MET* strictly depend on MET signaling for survival and growth (5). The same *c-MET*-amplified tumor cells (GTL-16, Hs746T, SNU-5, EBC-1, H1993) were found to be highly sensitive to DN30 Fab, the Fab fragment of a monoclonal anti-MET antibody inducing MET “shedding” (6). Recently, we (7) and others (8) analyzed the activity of different MET-targeted agents, including PHA-66572, JNJ-38877605 (an ATP-competitive, MET-selective TKI; ref. 9), crizotinib (a MET-ALK dual inhibitor; ref. 10), and DN30 Fab, on different human tumor cell lines, either bearing normal *c-MET* gene copy number or displaying *c-MET* amplification. Both these studies confirmed that only *c-MET*-amplified tumor cells are sensitive to MET inhibition in proliferation assays, independently of whether this was achieved by tyrosine kinase blockade or receptor downregulation. Consistent with these experimental findings, crizotinib displayed therapeutic activity in patients with lung carcinoma (11) and esophagogastric adenocarcinoma (12) bearing *c-MET*-amplified tumors.

Amplification of *c-MET* also accounts for resistance to anti-EGF receptor (EGFR) therapies in a significant fraction of patients treated with gefitinib or erlotinib (13–15). In EGFR-addicted tumors, survival and growth rely on EGFR-mediated transactivation of the HER3-PI3K pathway (16). Focal amplification of *c-MET* has been shown to result in MET-dependent 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 (hHGF KI) SCID mice that express human HGF in place of mouse HGF. Using ficlatuzumab, 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 ficlatuzumab assays, GTL-16 and EBC-1 cells were seeded in 96-well plates (1,000 cells per well) in the presence of ficlatuzumab 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 ficlatuzumab. 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-AKT, anti-AKT, anti-phospho-ERK, anti-ERK, anti-phospho-S6, and anti-S6 (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 hHGF KI 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 $V = \frac{4}{3}\pi \times (1/2x) \times (1/2y) \times (1/2z)$, 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 IC_{50} ranging from 11 nmol/L (SNU-5) to 50 nmol/L (H1993) and E_{MAX} 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 IC_{50} increase ranging from 5 times (H1993) to 23 (Hs746T) but did not substantially change the E_{MAX} (Supplementary Table S1). An 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_{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.

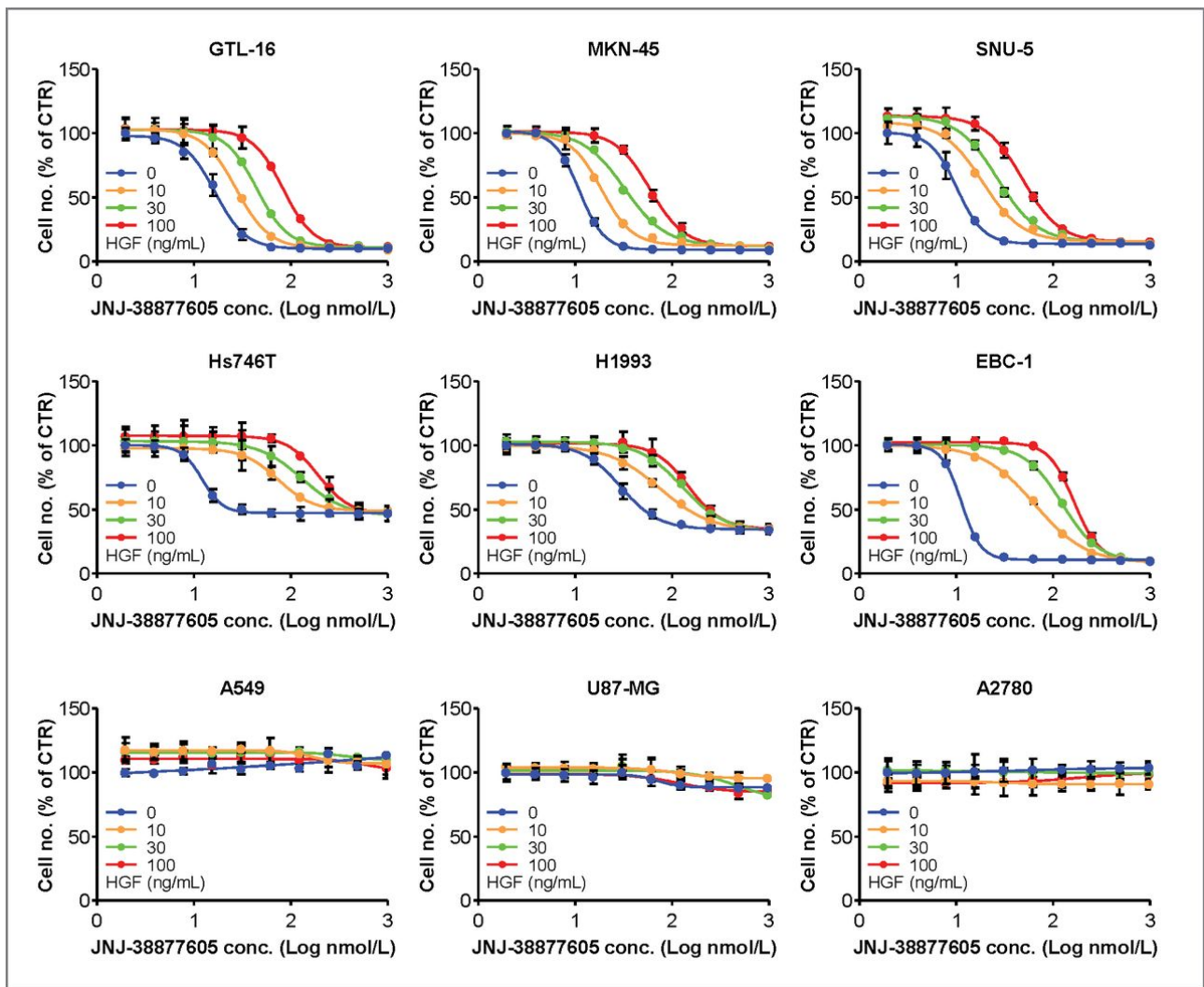


Figure 1.

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.

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 absence or 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.

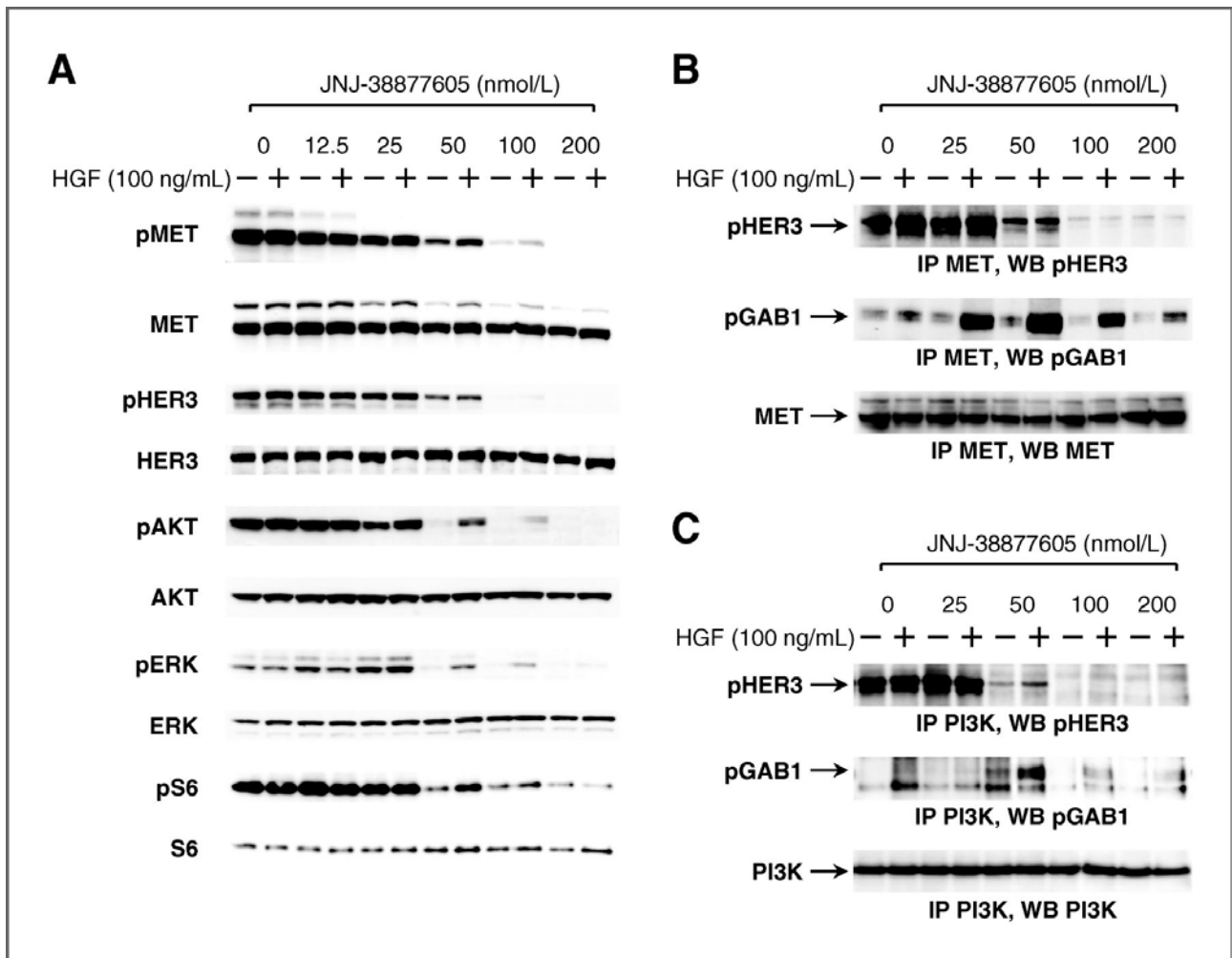


Figure 2.

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 the indicated signaling proteins and their phosphorylated forms (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.

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 (Hs746T) and E_{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 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_{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.

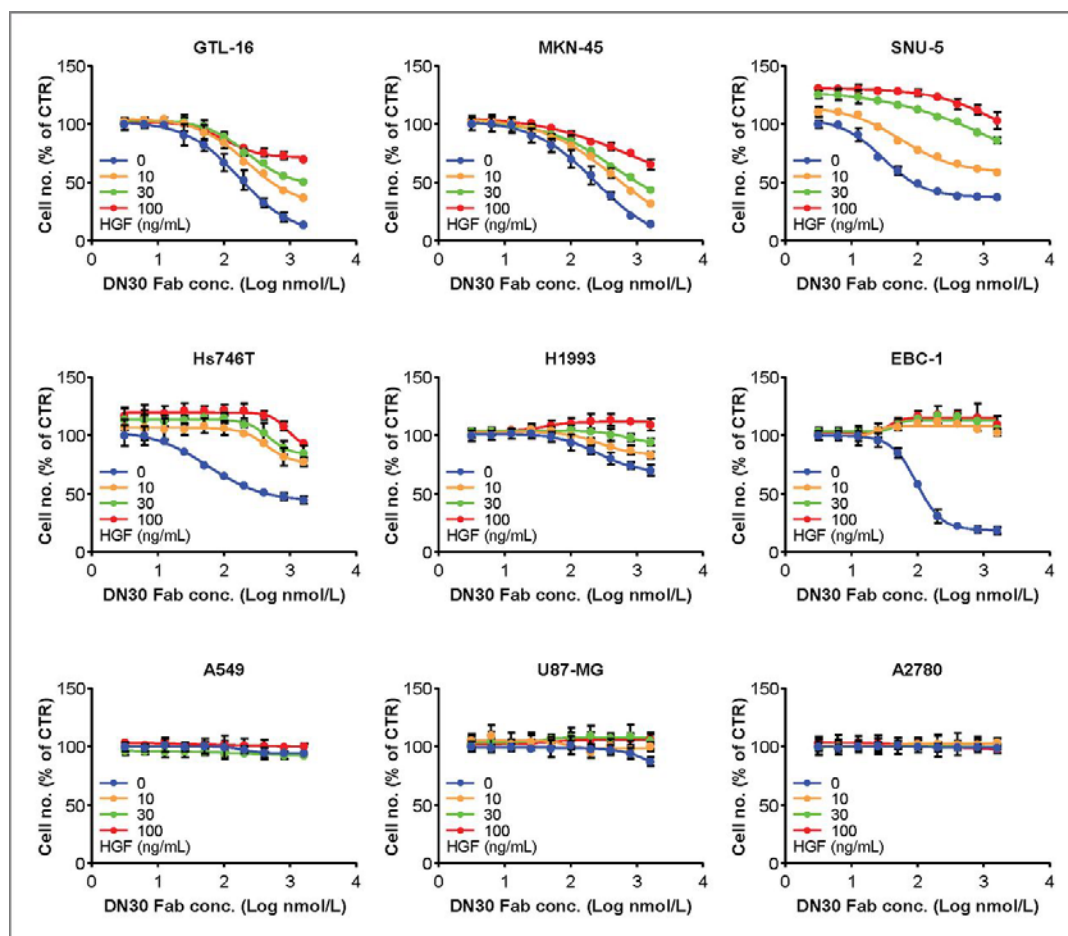


Figure 3.

HGF protects MET-addicted cancer cells against anti-MET antibody-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 the anti-MET antibody Fab fragment DN30 (DN30 Fab) 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.

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-PI3K 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.

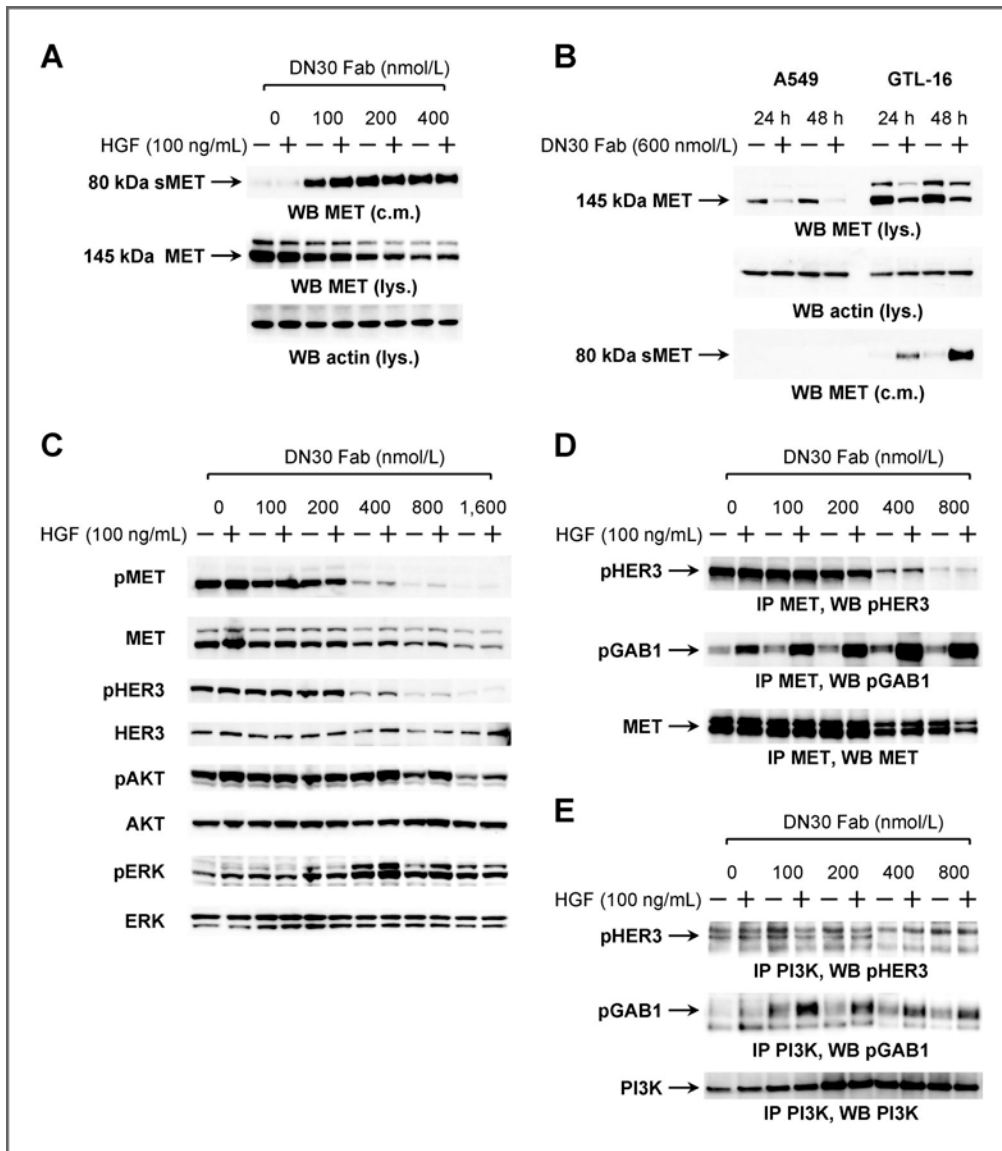


Figure 4.

HGF restores physiologic MET signaling in anti-MET antibody-treated MET-addicted cells. A, HGF does not affect DN30 Fab-induced MET shedding and inactivation. GTL-16 cells were incubated with increasing concentrations of DN30 Fab in the absence or presence of HGF. After 48 hours, conditioned medium (c.m.) and cell lysates (lys.) were analyzed by Western blotting using antibodies directed against the extracellular domain of MET. Soluble MET (sMET; 80 kDa) is the result of MET shedding. In the lysates, MET is present in two forms (unprocessed MET, 170 kDa; mature MET, 145 kDa). B, DN30 Fab abrogates MET expression in cells expressing moderate MET levels but not in cells overexpressing MET. A549 human lung carcinoma cells and GTL-16 cells were incubated with the indicated concentrations of DN30 Fab in the absence or presence of HGF. After 48 hours, cell lysates were analyzed by Western blotting using anti-MET antibodies and anti-actin antibodies. Conditioned medium was analyzed by Western blotting as described in A. C, GTL-16 cells were incubated with increasing concentrations of DN30 Fab in the absence or presence of HGF. Cell lysates were analyzed by Western blotting using antibodies directed against the indicated signaling proteins and their phosphorylated forms (indicated with the "p" prefix). D, GTL-16 cells were incubated as above with the indicated concentrations of DN30 Fab 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. E, GTL-16 cells were incubated as in D 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.

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 ficlatuzumab, a monoclonal antibody directed against HGF (25). Total ATP content analysis revealed that ficlatuzumab 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 ficlatuzumab could be unmasked by lowering HGF concentration to 5 ng/mL (Fig. 5B, right). This is explained by the fact that in EBC-1 cells, a concentration of 50 ng/mL HGF is well above the saturation threshold (Supplementary Fig. S4B). Because HGF is typically provided paracrinally by the tumor stroma (32), we further explored the therapeutic potential of ficlatuzumab in an *in vitro* model of stroma–tumor cell coculture. Luciferase-expressing GTL-16 gastric and EBC-1 lung carcinoma cells were cocultured with immortalized human fibroblasts secreting HGF, which reached a concentration of 100 ng/mL after 3 days of coculture. Mixed cultures of stroma–tumor cells were incubated with increasing concentrations (0–1,000 nmol/L) of JNJ-38877605 in the presence of 0, 15, 30, or 60 µg/mL ficlatuzumab, and tumor cell viability was determined 3 days after by measuring luciferase activity. Luciferase-expressing tumor cells alone were also subjected to the same treatment, and drug sensitivity in the presence of stromal cells was compared with that of tumor cells alone (Fig. 5C). Remarkably, and consistent with the HGF concentrations measured in the coculture system, stromal cells determined a decrease in drug sensitivity comparable with that observed with the higher doses of recombinant HGF. Ficlatuzumab restored sensitivity of GTL-16 and EBC-1 cells to JNJ-38877605 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.

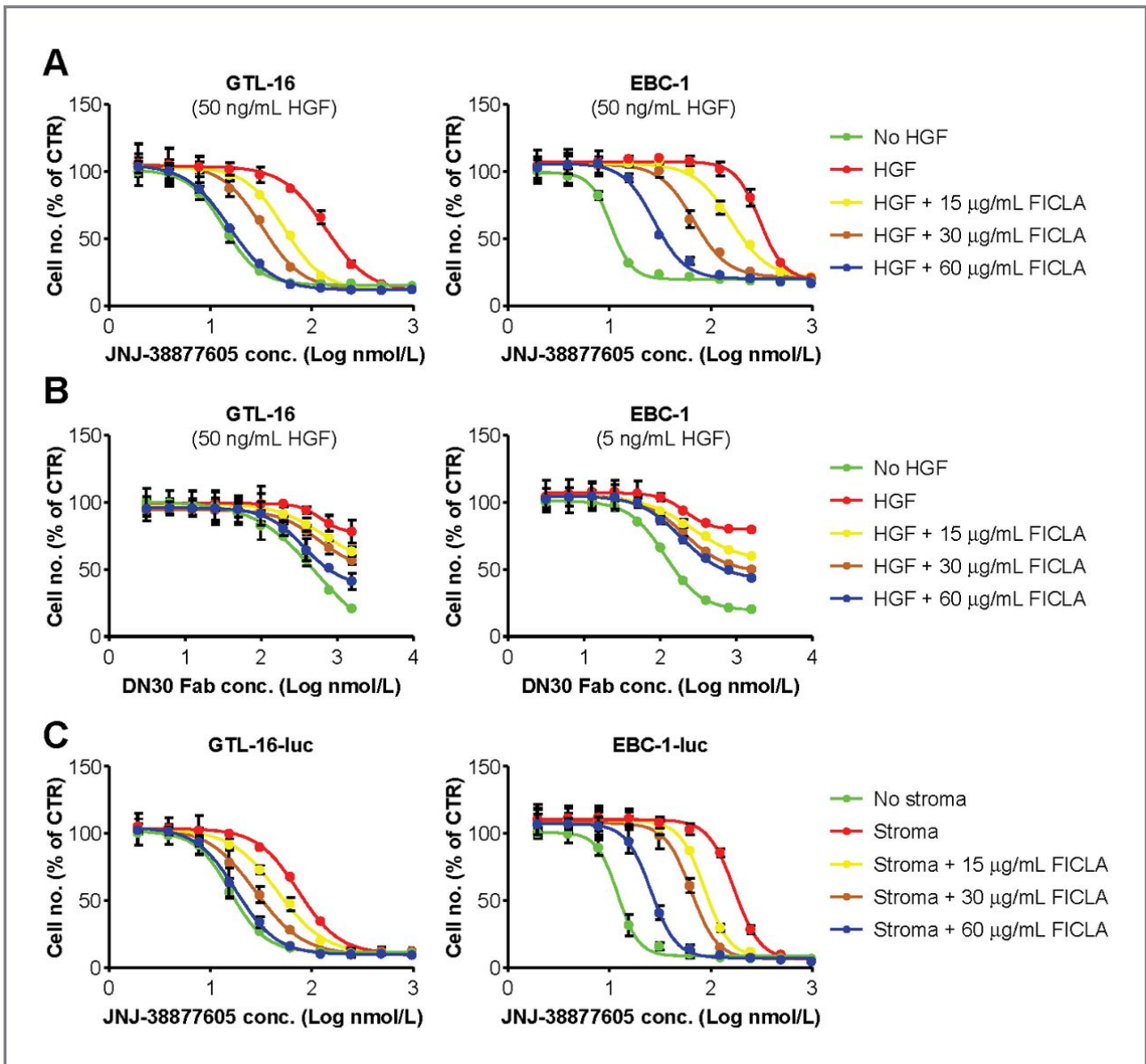


Figure 5.

Ficlatusumab 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 ficlatusumab. 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 ficlatusumab 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 ficlatusumab 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.

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 (Supplementary Fig. S5). The ability of ficlatuzumab to cooperate with different MET-targeted agents was analyzed using 3 distinct *c-MET*-amplified 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 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 hHGF KI SCID mice (49% inhibition; Fig. 6B). Ficlatuzumab alone did not have any effect in either wild-type SCID mice or hHGF KI mice. However, combination of JNJ-38877605 and ficlatuzumab significantly blocked tumor growth (83% inhibition) in hHGF 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 hHGF 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 a colorectal carcinoma “xenopatient” library (35) that bears approximately 30 *c-MET* gene copies (30) was propagated subcutaneously in human hHGF KI SCID mice. Animals bearing tumors of approximately 400 mm³ were randomly divided into 6 treatment arms: control; 30 mg/kg ficlatuzumab; 20 mg/kg crizotinib; 40 mg/kg crizotinib; 20 mg/kg crizotinib + 30 mg/kg ficlatuzumab; and 40 mg/kg crizotinib + 30 mg/kg ficlatuzumab. Crizotinib was administered daily by oral gavage; ficlatuzumab was administered twice weekly by intraperitoneal injection. Treatment continued for a total of 53 days. Analysis of tumor burden over time revealed that ficlatuzumab alone and 20 mg/kg crizotinib did not elicit any effect on tumor growth. Increasing crizotinib concentration to 40 mg/kg achieved 55% tumor inhibition, although drug toxicity reached its maximum tolerability at this dose in this mouse strain. Interestingly, the combination with 30 mg/kg ficlatuzumab sensitized tumors to 20 mg/kg crizotinib (32% inhibition) and cooperated with 40 mg/kg crizotinib, leading to tumor stabilization (78% inhibition). Immunohistochemical analysis of tumor sections revealed that crizotinib, could not effectively reduce phospho-S6 kinase levels in the absence of ficlatuzumab (Supplementary Fig. S6B), thus further strengthening the idea that activation of the PI3K pathway plays a crucial role in HGF-induced resistance to anti-MET drugs.

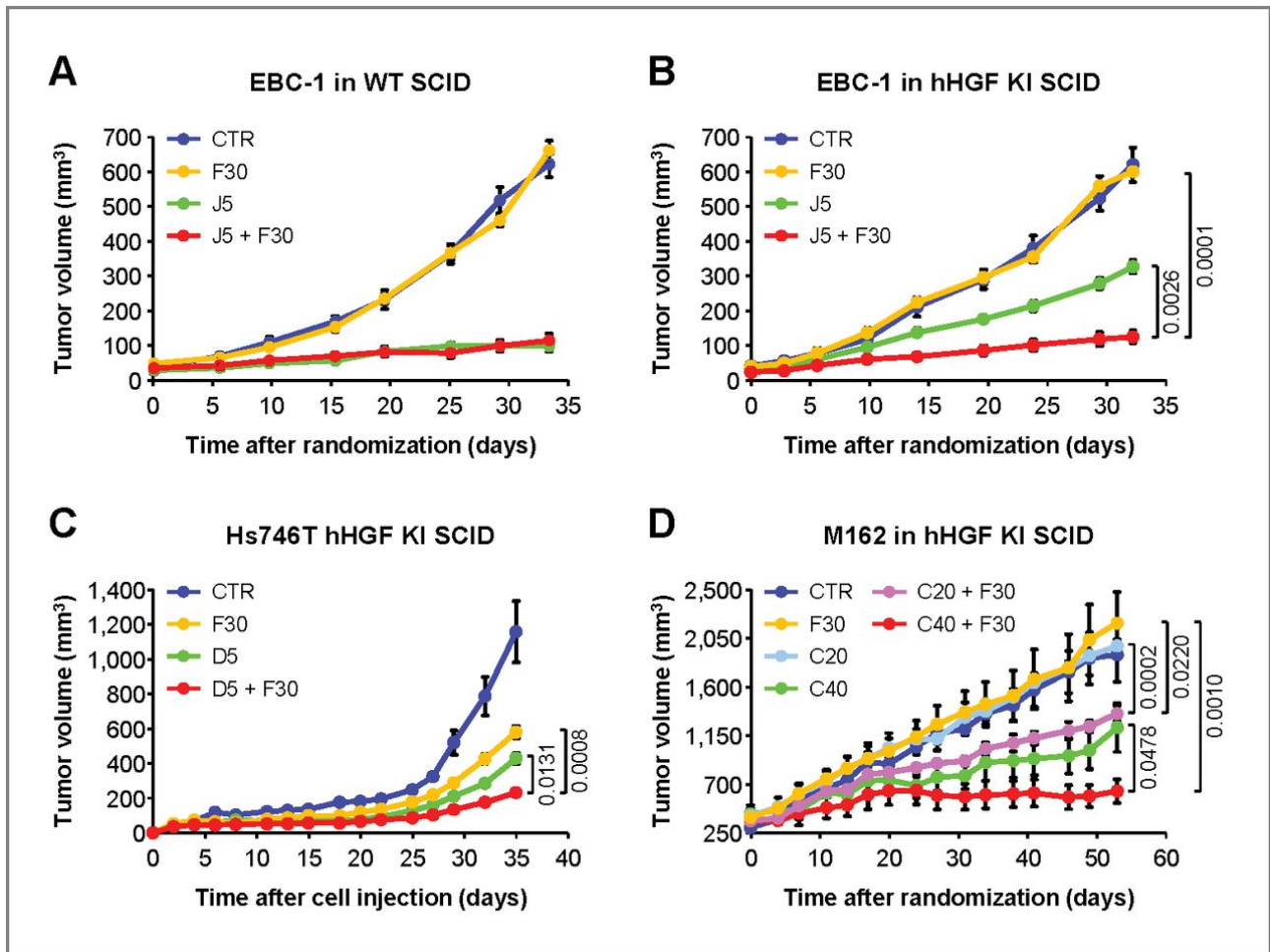


Figure 6.

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 (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. D, 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.

Discussion

In the present study, we report that HGF causes resistance of *c-MET*-amplified cancer cells to treatment with small-molecule MET TKIs and anti-MET antagonistic antibodies, bypassing genetically determined sensitivity to MET-targeted agents. Given that cells displaying high-grade, focal amplification of *c-MET* express high levels of the MET protein, resulting in ligand-independent, constitutive MET kinase activation (1), this finding is

somehow counterintuitive. Yet the data presented here explain this apparent paradox at a molecular level, providing insights into the biology of *c-MET* oncogene addiction.

Tumor cells bearing normal *c-MET* gene copy number are strictly dependent on HGF for MET activation that leads to execution of a variety of HGF-dependent biologic programs, but they do not rely on MET signaling for intrinsic survival and growth (1). Accordingly, cells not displaying *c-MET* gene amplification are insensitive to MET inhibition in proliferation assays (3, 4, 7, 8), regardless of the presence or absence of HGF (see Figs. 1 and 3 and Supplementary Table S1). In contrast, *c-MET*-amplified, MET-overexpressing tumor cells are “addicted” to MET signaling, which occurs independently of ligand stimulation in normal conditions. In these cells, the survival signal originating from MET is transduced through an aberrant pathway that involves interaction with a partner receptor tyrosine kinase, typically HER3 (13). Indeed, inhibition of MET in these cells resulted in HER3 inactivation and downstream PI3K signal silencing. HGF did not rescue HER3 activation but restored PI3K signaling through recruitment of the physiologic GAB1 adaptor (Figs. 2 and 4). This signaling adaptor shift may be explained by the different mechanisms of MET activation involved. MET protein overexpression increases the probability of receptor–receptor collision, therefore equally favoring both homodimerization (MET with MET) and heterodimerization (MET with other receptors, including HER3). In contrast, HGF stimulation conceivably results primarily in stabilization of MET homodimers. This could explain why HGF-induced MET activation is qualitatively different from overexpression-sustained MET activation, and the two signals behave de facto as two distinct redundant pathways in terms of cell survival.

The above-described mechanism makes MET-addicted cells become “hooked on” paracrine HGF for survival when they are challenged with MET-targeted agents. But how does HGF activate MET in the presence of a MET inhibitor? The molecular mechanisms underlying this phenomenon are different for small-molecule kinase inhibitors and antagonistic antibodies. In the case of MET TKIs, HGF-induced MET activation—due to ligand-mediated stabilization of MET homocomplexes—is conceivably less sensitive to MET kinase inhibition than MET-induced HER3 transactivation. Consistent with this hypothesis, HGF caused dose-dependent increase of MET TKI IC₅₀ but did not affect their *E*_{MAX} (i.e., it reduced their potency and not their efficacy). However, HGF induced an IC₅₀ increase ranging from 5 to 24 times (Supplementary Table S1), a change that is in line with the IC₅₀ shifts induced by multiple rescuing cytokines in a recent major study on targeted drug resistance (23). In the case of antagonistic antibodies that reduce MET expression by inducing “shedding” or internalization (6, 36, 37), the mechanism responsible for HGF-induced rescue may rely on the fact that expression of MET in *c-MET*-amplified cells is way above the physiologic level, and antagonistic antibodies decrease MET expression down to normal levels rather than abrogating it (Fig. 4). Hence, even in the presence of saturating 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 overexpressing human HGF on top of the endogenous ligand (34), hHGF 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 ficlatuzumab 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 ficlatuzumab 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 ficlatuzumab, 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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