RESEARCH ARTICLE

Rescue Screens with Secreted Proteins Reveal Compensatory Potential of Receptor Tyrosine Kinases in Driving Cancer Growth

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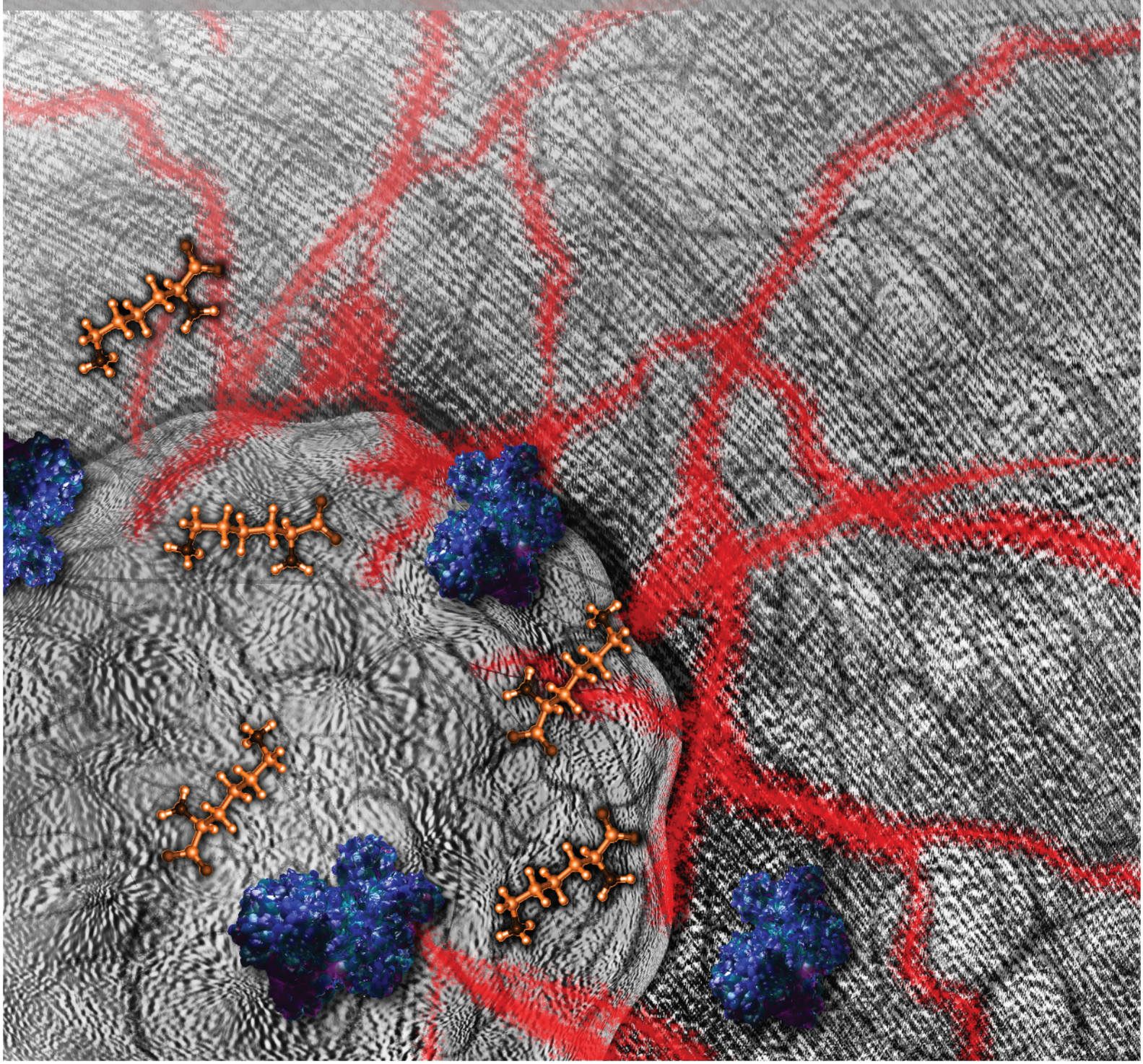

ABSTRACT

The overall power of kinase inhibitors is substantially overshadowed by the acquisition of drug resistance. To address this issue, we systematically assessed the potential of secreted proteins to induce resistance to kinase inhibitors. To this end, we developed a high-throughput platform for screening a cDNA library encoding 3,432 secreted proteins in cellular assays. Using cancer cells originally dependent on either *MET*, *FGFR2*, or *FGFR3*, we observed a bypass of dependence through ligand-mediated activation of alternative receptor tyrosine kinases (RTK). Our findings indicate a broad and versatile potential for RTKs from the HER and FGFR families as well as MET to compensate for loss of each other. We further provide evidence that combined inhibition of simultaneously active RTKs can lead to an added anticancer effect.

SIGNIFICANCE: Although initial tumor responses to kinase inhibitors can be significant, therapeutic benefit is often limited by the emergence of resistance (e.g., as a consequence of mutations in the drug target or through activation of alternative pathways to bypass dependence on the original target). Because the activation of alternative growth-promoting kinases by stimulation with their cognate ligands can constitute such a bypass mechanism, the identification of growth factors as possible mediators of resistance to kinase inhibitors is of clinical interest. *Cancer Discov; 2(10); 1–12. ©2012 AACR.*

INTRODUCTION

Genetic alterations, including point mutations, gene amplifications, and chromosomal translocations, can render kinases oncogenic (1, 2). The term "oncogene addiction" has been used to describe the phenomenon in which growth and survival of cancer cells becomes dependent on an aberrantly activated protein, for example, a kinase (3, 4). Oncogene addiction has been validated for several oncogenic "driver" kinases in preclinical models and in cancer patients using selective kinase inhibitors. Despite striking initial clinical activity, the emergence of resistance is becoming a common problem. One mechanism of resistance involves mutation of the target, thereby compromising binding and activity of the therapeutic agent. Efforts to understand the specific mechanisms of resistance to imatinib in chronic myelogenous leukemia have led to second-generation inhibitors (e.g., nilotinib) that treat and prevent resistance through increased potency. However, the discovery of parallel or downstream bypass mechanisms of resistance are motivating novel combination therapies as a means to prevent such bypass events.

A well-characterized example representing both types of mechanisms is acquired resistance to the EGF receptor (EGFR) inhibitors gefitinib and erlotinib in non–small cell lung cancers carrying activating *EGFR* mutations (5, 6). In the majority of patients, this occurs either by emergence of the secondary resistance mutation T790M in EGFR or by loss of EGFR dependence through activation of the receptor tyrosine kinase (RTK) MET (7–11). Although the EGFR-T790M mutation interferes with binding of EGFR inhibitors like gefitinib or erlotinib (12), MET activation can compensate for loss of EGFR activity by activating an overlapping set of downstream signaling molecules including HER3 that continue to provide growth-promoting signals (8). Interestingly, MET-driven resistance can occur because of *MET* gene amplification (8) as well as activation by its ligand hepatocyte growth factor (HGF; refs. 10, 13). On the other hand, it has been shown that *MET*-amplified cancer cell lines that are sensitive to MET inhibitors can be rescued by ligand-induced EGFR activation (14). EGFR and MET are, therefore, considered to be alternative driver kinases in the same cancer cells because dependence on one can be compensated by activation of the other. In such a setting, simultaneous inhibition of both kinases or intervention at a common downstream node is thus required for effective anticancer therapy.

Preclinical studies of resistance mechanisms have been conducted using several experimental approaches, including random mutagenesis (15–17), prolonged drug exposure of sensitive cancer models to evoke outgrowth of resistant subclones (18, 19), and expression of potential compensatory proteins in drug-exposed cancer cells using cDNA libraries (20). Prolonged drug-exposure experiments have successfully been used to recapitulate autocrine ligand–mediated resistance mechanisms that were previously found by hypothesis-driven testing (19, 21). However, the potential role of the tumor microenvironment in providing paracrine stimuli is not adequately modeled in such an experimental setup. The rationale for expressing cDNA

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Note: Supplementary data for this article are available at Cancer Discovery Online (http://www.cancerdiscovery.aacrjournals.org/).

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doi: 10.1158/2159-8290.CD-12-0237

libraries in drug-exposed cancer cells is that resistance to RTK inhibitors can occur because of ligand-mediated activation of compensatory kinases. Such rescue ligands can be produced by cancer cells themselves, leading to autocrine stimulation, or by stromal cells in the cancer microenvironment, leading to paracrine stimulation.

To systematically assess the capability of secreted proteins to drive resistance to kinase inhibitors, we established a high-throughput "secretome" screening platform. Secreted proteins are produced in cell media supernatants by transfection of a library encompassing 3,432 cDNAs. The resulting supernatants are then screened for their potential to rescue kinase-dependent cancer cells that are simultaneously treated with a relevant kinase inhibitor. Specifically, supernatants leading to the reversal of inhibitor-mediated proliferation in MET-dependent and FGFR-dependent cancer cell lines were discovered. Although the specific ligands of each inhibited RTK were not capable of reversing growth inhibition, a surprisingly high degree of flexibility was observed in bypassing oncogene addiction through activation of alternative RTKs. In particular, activation of HER family members could compensate for inhibition of either MET or FGFRs and activation of FGFR or MET could cross-rescue HER inhibition. These results suggest a specific and complementary role of these 3 growth factor pathways and suggest at least partial overlap of downstream signaling pathways and common cellular effects of each pathway.

RESULTS

Secretome Screening Suggests that RTKs from the HER and FGFR Families Can Replace MET in Driving Cancer Cell Growth

In order to discover potential modes of resistance to targeted therapies mediated by secreted proteins, a novel secretome screening platform of 3,432 cDNAs was used. These cDNAs, representing 2,803 genes predicted to encode secreted proteins, were individually transfected into HEK293T cells in 384-well plates to obtain cell culture supernatants where each well was expected to contain a defined secreted protein (Fig. 1A). The ability of these supernatants to abrogate growth inhibition was then tested by transferring the supernatants to wells containing cancer cells addicted to a specific oncogene and for which a selective inhibitor was available. Supernatants that induced resistance to inhibitor treatment were identified by quantification of cell growth after an incubation period of 4 days (Fig. 1A).

As a pilot screen, MKN-45 gastric cancer cells were treated with the novel MET inhibitor NVP-JAA120 (hereinafter referred to as JAA120) along with the secretome library. MKN-45 cells are highly *MET*-amplified, resulting in MET-dependent growth (22). JAA120 displays potent and highly selective activity against MET in biochemical and cellular assays (Supplementary Table S1). The screening concentration of 100 nmol/L JAA120 was found to fully inhibit MET in MKN-45 cells based on quantification of MET activation loop phosphorylation (data not shown). After incubation of MKN-45 cells with JAA120 and conditioned media from the secretome library for 4 days, relative cell growth was quantified using a CellTiter-Glo readout (Fig. 1B). The vast majority of secreted proteins did not significantly alter MKN-45 cell growth compared to a neutral control (vector only). However, a small subset of supernatants was able to partially rescue the effect of MET inhibition and promote growth of MKN-45 in the presence of JAA120 (Fig. 1B). In line with previously described concepts, members of the EGF family were among the ligands that mediated MET inhibitor rescue (Fig. 1B and Supplementary Table S2). In addition, we found that several members of the fibroblast growth factor (FGF) family could rescue MKN-45 cells. Among this family, FGF7-mediated rescue was most pronounced (Fig. 1B).

Similarly, secretome resistance screening was then carried out in 4 additional cancer cell lines in which MET activity was known to be a major driver of cell growth. Also in these additional lines, we observed that members of the EGF and/or FGF families could suppress the growth effects of JAA120 (Supplementary Table S2). These data suggest that downstream signals emerging from either HER or FGFR family members that promote cell growth and survival are to some extent qualitatively redundant with signals triggered by MET.

Secretome Screening Reveals Broad Compensatory Potential of HER, FGFR, and MET RTKs

Next, cancer cell lines that are dependent on a member of the FGFR family were examined. A first secretome rescue screen was carried out in the bladder cancer cell line RT-112. These cells harbor a focal *FGFR3* gene amplification, leading to overexpression of FGFR3 as well as FGFR3-dependent growth (23, 24). RT-112 cells were treated with the novel FGFR inhibitor NVP-BGJ398 (hereinafter referred to as BGJ398), a potent and selective inhibitor of FGFR1, FGFR2, FGFR3, and FGFR4 (23) that is currently in phase I clinical trials. The secretome library supernatants were added and cell growth was quantified as before. A small set of secreted factors was found to rescue RT-112 cells when FGFR3 was inhibited (Fig. 1C). Consistent with the notion that BGJ398 effectively blocks signaling from the FGF receptors, FGF ligands were not identified as rescue hits in the screen. As in the aforementioned MET screens, several of the identified rescuers were ligands binding to the HER family of RTKs. In addition, a strong rescuer in RT-112 cells was HGF, the only known ligand of MET. A second screen in the *FGFR2*-amplified gastric cancer cell line KATO III (25) produced overlapping hits, confirming the overall trend (Supplementary Table S3). In summary, secretome screens revealed that at least some cancer cells are highly flexible in bypassing RTK dependencies through activation of alternative RTKs.

Ligand-Mediated Rescue Can Be Prevented by Combining RTK Inhibitors

To validate the rescue effects observed in the high-throughput screens, the relevant recombinant proteins obtained from commercial sources were individually tested in the same cell proliferation assay. Specifically, a set of 18 recombinant FGFs as well as EGF and neuregulin 1-β (NRG1-β; a ligand for homo- and heterodimers containing HER2, HER3, and/or HER4) were tested for their ability to rescue MKN-45 in the presence of JAA120 (Fig. 2A). The previously observed proliferation rescue phenotypes could be confirmed with

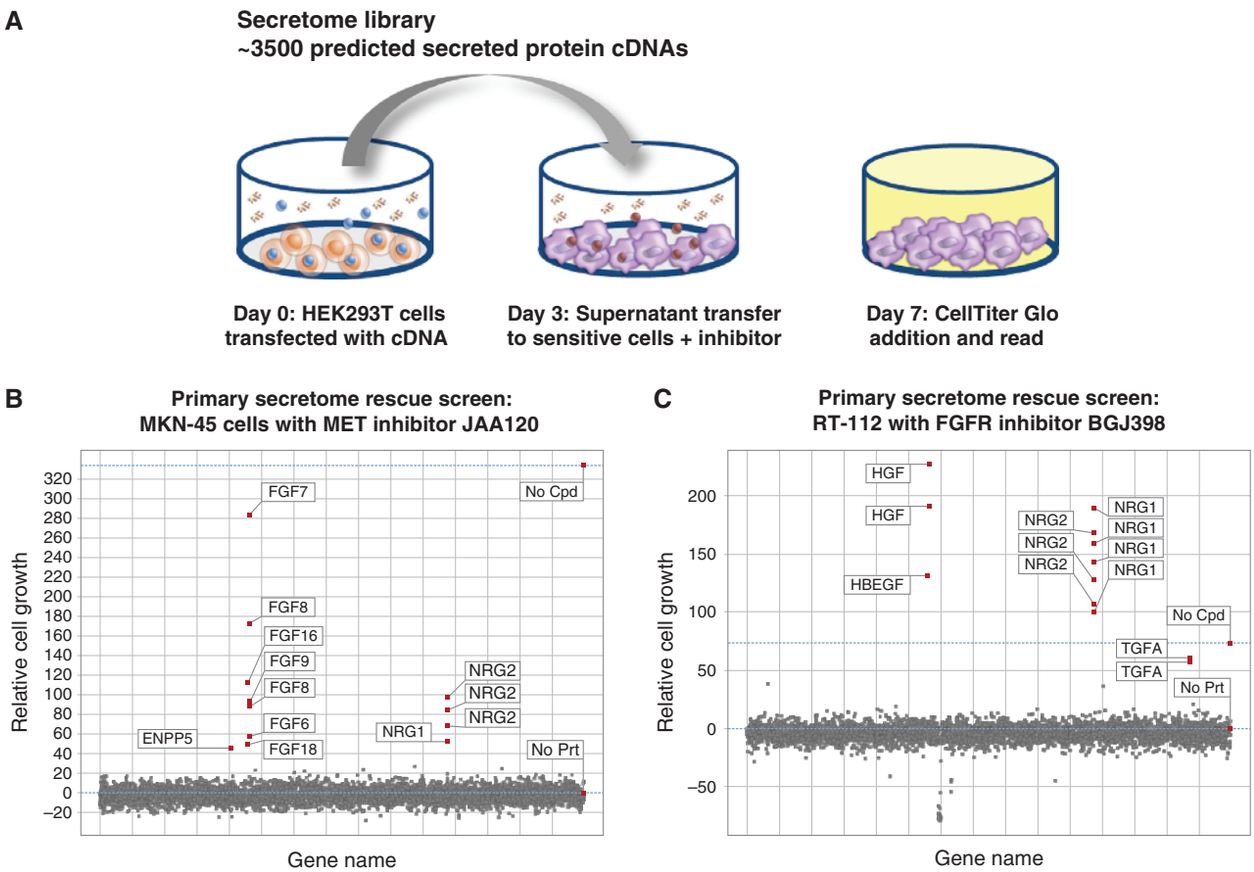

Figure 1. Secretome screening in oncogene-addicted cancer cell lines. **A,** secretomics platform schematics. **B,** screening data for *MET*-amplified MKN-45 cells treated with the MET inhibitor JAA120. Each dot represents the average of 3 replicate wells of cells treated with JAA120 and the supernatant derived from 1 cDNA; gene name (in alphabetical order) on the *x*-axis and relative cell growth on day 7 (CellTiter-Glo readout, arbitrary units) on the *y*-axis. Some genes are represented in our secretome library with multiple isoforms. "No Cpd," no compound (JAA120) and no cDNA, level marked by upper blue line; "no Prt," no cDNA-derived protein, but JAA120, level marked by lower blue line. **C,** screening data for *FGFR3*-amplified RT-112 cells treated with the FGFR inhibitor BGJ398. Representation as in **B**.

several FGF and EGF family members. EGF had not been part of the secretome library, but was tested here based on the published observation that EGF could rescue *MET*-amplified gastric cancer lines exposed to the MET inhibitor PHA-665752 (14). Importantly, none of the ligands had an effect on the growth of the cells when tested alone, thus excluding the possibility of a primary growth effect in the absence of the inhibitor (Supplementary Fig. S1). To ascertain whether ligand-mediated rescue was governed by activation of the cognate receptors, we next used specific inhibitors—BGJ398 for FGFR1/2/3/4 and lapatinib for HER1/2—to attempt to block or reverse ligand-mediated rescue (Fig. 2B). These experiments were done both with JAA120 (Fig. 2B, top) and the equally selective MET inhibitor INC280 (26), which is currently in clinical development (Fig. 2B, bottom). In MKN-45 cells, the antiproliferative activity of the 2 MET inhibitors was restored in the presence of FGF7 by cotreatment with the FGFR inhibitor BGJ398. Similarly, the antiproliferative activity of JAA120 and INC280 was restored in the presence of NRG1 by cotreatment with lapatinib (Fig. 2B).

To investigate whether common downstream signals were likely to underlie the observed rescue effects, the consequences of MET inhibition, ligand-mediated rescue, and inhibitor-mediated reversal on the level of protein phosphorylation were assayed by immunoblotting of the relevant protein extracts (Fig. 2C). In the absence of added ligands, the MET inhibitors JAA120 and INC280, but not BGJ398 or lapatinib, had a profound effect on the phosphorylation of MET and downstream MET signaling outputs including ERK1/2 and AKT. Surprisingly, the phosphorylation of the adaptor protein FRS2 was also profoundly downregulated by the MET inhibitors, but not by the FGFR inhibitors. Addition of FGF7 reactivated FRS2 phosphorylation, a downstream substrate of the FGFRs, and at the same time partially reactivated ERK1/2 phosphorylation with no or minimal effect on AKT phosphorylation. This rescue effect could be reversed by BGJ398 but not lapatinib, strongly suggesting that the addition of FGF ligands restores ERK signaling through the recruitment of FRS2. Notably, the rescue effects mediated through NRG1 were quite distinct. In this case, FRS2 and ERK phosphorylation were unchanged and instead AKT reactivation appeared to be the more pronounced signaling result. In turn, AKT activation mediated by NRG1 was again reversed by coadministration of lapatinib.

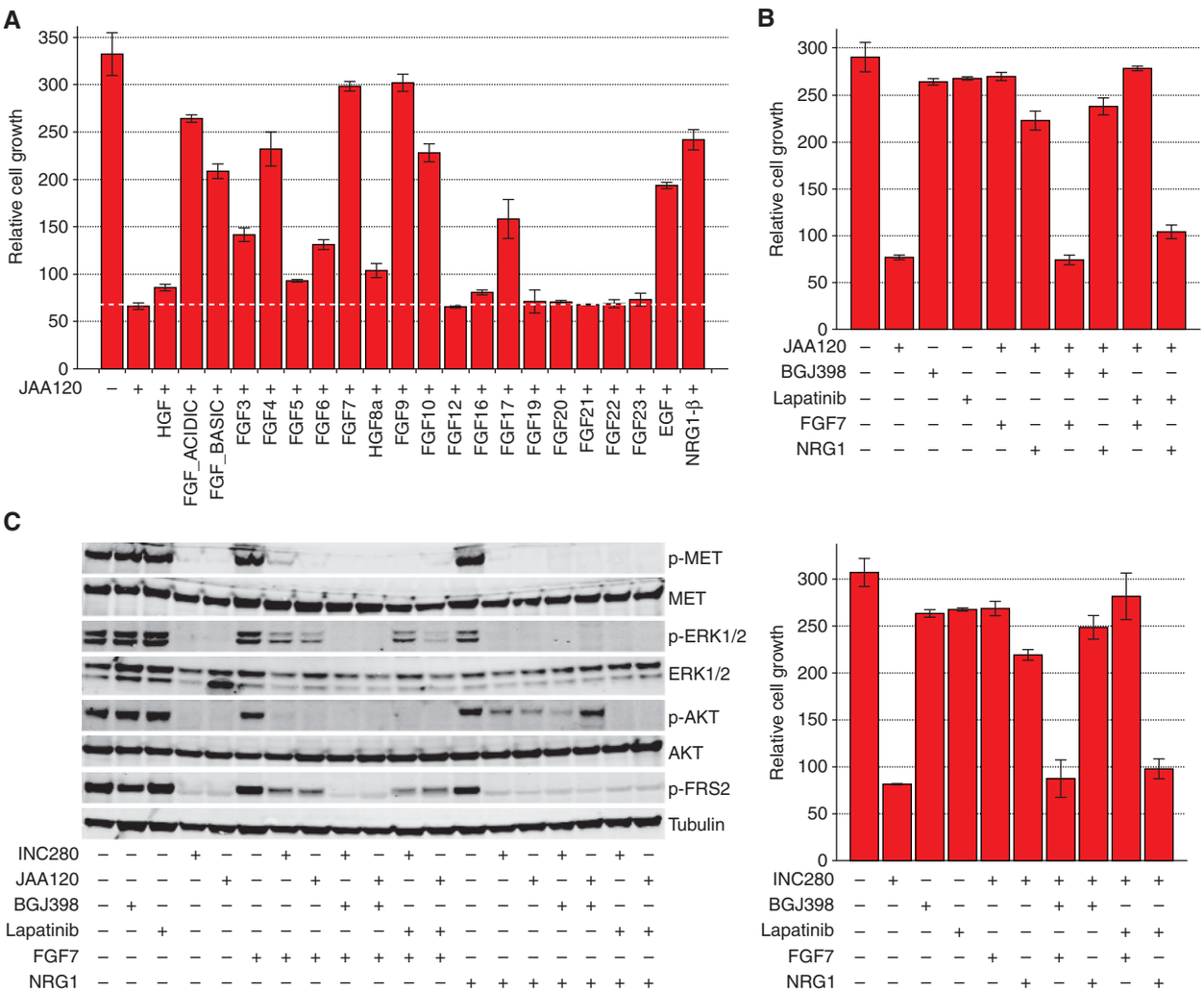

Figure 2. Validation of MKN-45 screening data using recombinant proteins and selective inhibitors. **A,** confirmation of hits with recombinant proteins and additional FGF family members. MKN-45 cells were treated with 0.1 μmol/L JAA120, and all recombinant proteins were added at 100 ng/mL. The dashed line indicates the level of cell growth in the presence of inhibitor but no rescue protein. **B,** reversal of rescue with selective inhibitors in MKN-45. JAA120 (top) and INC280 (bottom), tested at 0.1 μmol/L, BGJ398 tested at 0.5 μmol/L and lapatinib tested at 1.5 μmol/L final concentration. Recombinant human (rh) FGF7 and rhNRG1 tested at 250 ng/mL final concentration. **C,** Western blot analysis of protein phosphorylation in extracts from MKN-45 cells that were treated for 2 hours with the indicated compounds at concentrations as in **B**.

We conducted analogous validation experiments in the FGFR3-dependent cancer cell line RT-112. Recombinant HER ligands as well as HGF were found to reproduce the rescue effects observed in secretome screening (Fig. 3A and Supplementary Fig. S1). Furthermore, selective inhibition of the cognate RTKs could reverse rescue mediated by HGF or NRG1 (Fig. 3B). In the absence of ligands, JAA120 or INC280 specifically inhibited MET phosphorylation, whereas BGJ398 significantly reduced both FRS2 and ERK phosphorylation and modestly decreased phospho-AKT (Fig. 3C). Addition of HGF restored ERK phosphorylation in the presence of BGJ398, whereas simultaneous addition of JAA120 or INC280 prevented this rescue effect. Likewise, NRG1 caused a lapatinib-sensitive restoration of ERK phosphorylation and also stimulated phosphorylation of AKT. In RT-112 cells phosphorylation of ERK1/2 appeared to be better correlated with cell growth than AKT phosphorylation.

To investigate the specificity of the observed rescue phenotypes, we tested additional cell lines that are non–RTK-dependent as well as an EGFR-dependent cell line (Supplementary Fig. S2). As expected, the EGFR-dependent cell line, HCC-827, was rescued from its corresponding inhibitor (gefitinib) by the addition of HGF, whereas none of the other ligands were capable of promoting cell growth in the presence of the inhibitor. This compensatory relationship was further shown in an ALK-dependent cell line, NCI-H2228. In this case, when treated with a potent and selective ALK inhibitor (TAE684), both HGF and EGF rescued cells from TAE684-induced growth arrest. Importantly, the rescue effect mediated by HGF was abolished by the addition of crizotinib, a dual MET and ALK inhibitor. Finally, growth inhibition of other non–RTK-dependent cell lines—K562 (driven by BCR-ABL) and SET2 (driven by JAK2-V617F)—could not be reversed upon addition of recombinant ligands in the presence of their corresponding inhibitors (the

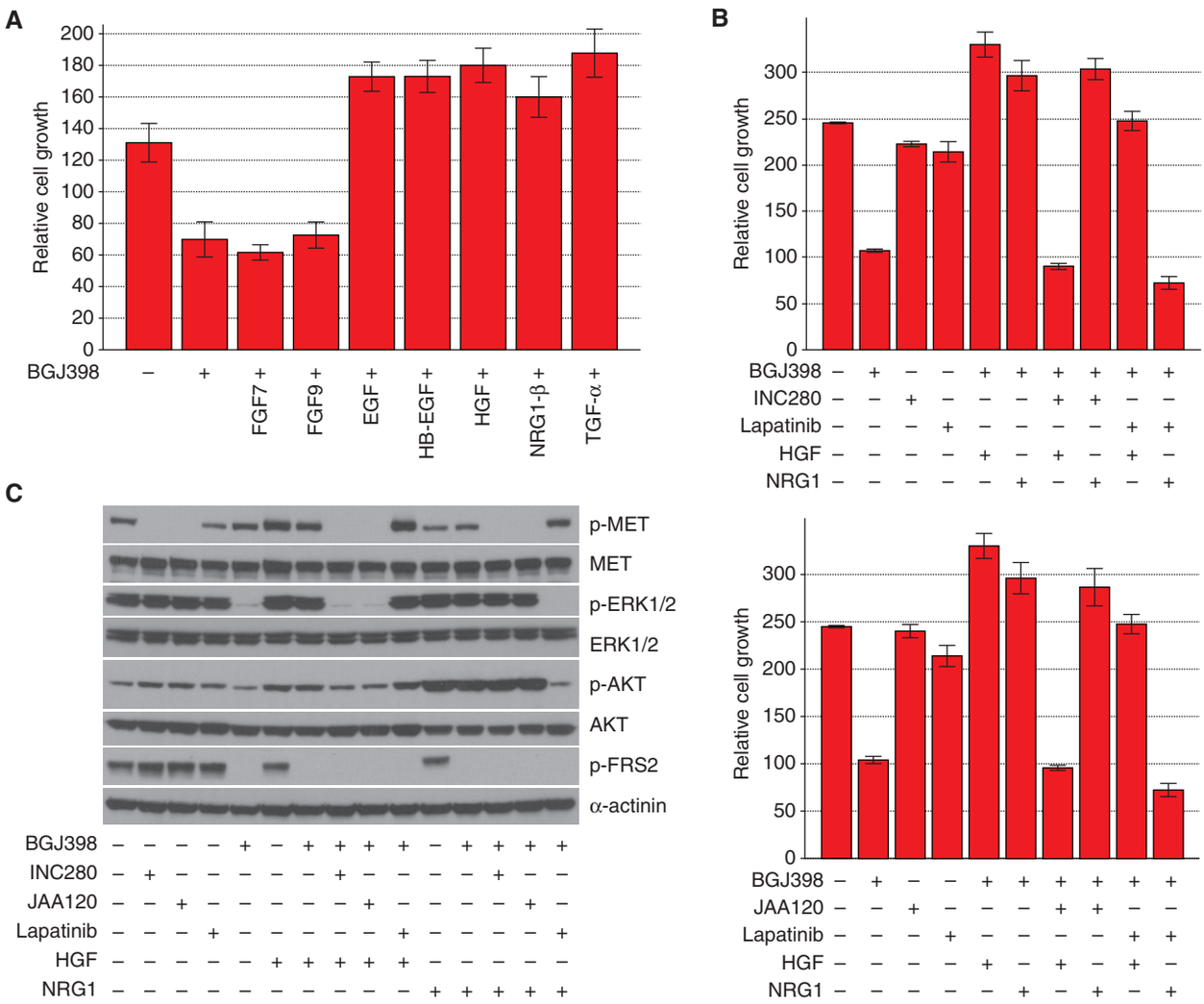

Figure 3. Validation of RT-112 screening data using recombinant proteins and selective inhibitors. **A,** confirmation of hits with recombinant proteins. RT-112 cells were treated with 0.1 μmol/L BGJ398, and all recombinant proteins were added at 100 ng/mL. **B,** reversal of rescue with selective inhibitors in RT-112. BGJ398 tested at 0.1 μmol/L, JAA120 and INC280 tested at 0.5 μmol/L, and lapatinib tested at 1.5 μmol/L final concentration. rhHGF and rhNRG1 tested at 250 ng/mL final concentration. **C,** Western blot analysis of protein phosphorylation in extracts from RT-112 cells that were treated for 2 hours with the indicated compounds at concentrations as in **B**.

ABL inhibitor nilotinib and the JAK2 inhibitor BVB808; ref. 27). This further highlights the complementary, yet specific, role of the HER, MET, and EGF kinases.

Altogether these results suggest that pair-wise combinations of HER, MET, and FGFR inhibitors may be useful for therapeutic efficacy if 2 of these RTKs are activated simultaneously by either genetic alterations or by cognate ligands. Although the ligands in our experiments have been derived from exogenous sources, the ligands in cancer patients may originate from the tumor itself (autocrine stimulation) or from other sources, such as tumor-associated stroma (paracrine stimulation).

Distinct Effects of Selective MEK and PI3K Inhibition in MKN45 and RT112 Cells

We further investigated to what extent the MEK/MAPK and phosphoinositide 3-kinase (PI3K)/AKT pathways contribute to the rescue effects of the growth factors using the MEK inhibitor GSK1120212 (28) and the PI3K inhibitor GDC-0941 (ref. 29; Supplementary Fig. S3). In both MKN-45 and RT-112 cell lines, MEK and AKT phosphorylation was optimally abrogated at 1 μmol/L for both GSK1120212 and GDC-0941, respectively (data not shown). In MKN-45 cells, PI3K inhibition on its own had no effect on growth and the previously described ligand-mediated rescue effects were not prevented by coadministration of the PI3K inhibitor (Supplementary Fig. S3A). In the case of NRG1, this was somewhat unexpected, given that this ligand appeared to specifically reactivate AKT phosphorylation but not ERK phosphorylation. On the contrary, MEK inhibition in MKN-45 cells was strongly growth inhibitory and also abrogated ligand-mediated rescue. In RT-112 cells, PI3K inhibition on its own had a partial effect on growth, but combination with BGJ398 inhibited growth much more strongly than either single agent (Supplementary Fig. S3B). This is in line with the marginal inhibition of AKT phosphorylation by BGJ398 alone observed in Fig. 3C. Both HGF and NRG1 could still

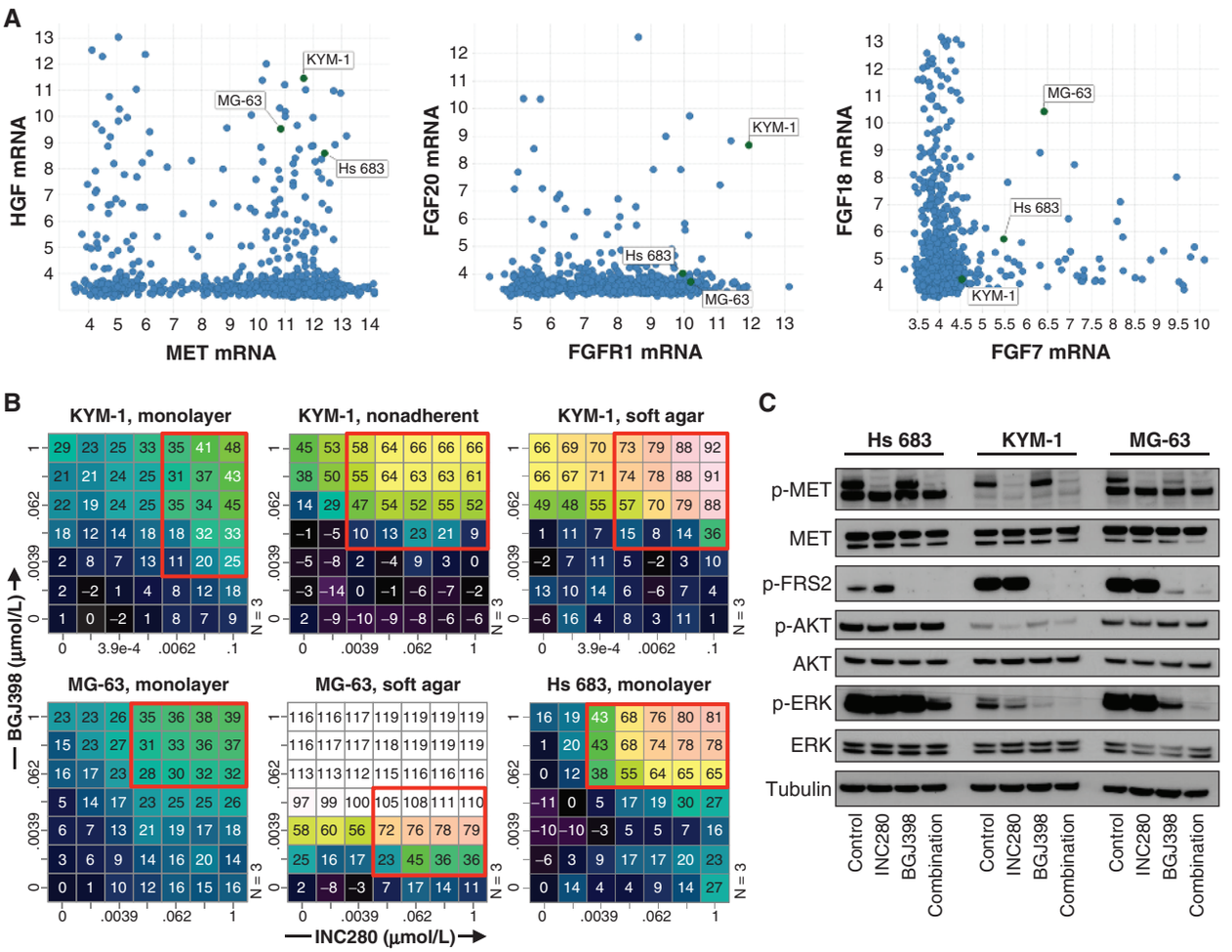

Figure 4. Simultaneous autocrine activation of FGFR and MET in cancer cell lines. **A,** gene expression patterns suggesting autocrine activation of MET and FGFR1. The levels of HGF and MET mRNA expression are displayed as a scatter plot, with the *x* and *y*-axes representing the Robust Multichip Analysis (RMA) value of probe 203510_at (MET) and 209960_at (HGF), respectively, measured by Affymetrix U133 microarray. Each dot represents an individual cell line. **B,** proliferation of indicated cancer cell lines treated with combinations of BGJ398 and INC280. Red boxes approximately mark the areas of synergy based on the analysis shown in Supplementary Fig. S4. **C,** analysis of protein phosphorylation in cells used in **B**. Numbers represent percent inhibition, where 0% = dimethyl sulfoxide (DMSO) control, and 100% = readout of seeded cells at the time of compound addition. Colors were added to visualize the extent of inhibition (e.g., green = moderate inhibition, red = strong inhibition). Cells were grown on adherent plates and treated for 24 hours with DMSO control, 1 μmol/L INC280, 1 μmol/L BGJ398, or a combination of both drugs. The indicated proteins were visualized by Western blotting.

partially rescue in the presence of a combination of BGJ398 and GDC-0941. An overall similar picture was obtained when testing the MEK inhibitor GSK1120212 under the same conditions. In summary, MKN-45 cells displayed a predominant dependence on MEK, whereas RT-112 showed contributions of both PI3K and MEK signaling to cell growth.

Coactivation of MET and FGFR Is Observed in Cancer Cell Lines

Simultaneous activity of 2 RTKs could be the cause of primary or acquired resistance to selective kinase inhibitors. To investigate whether coactivation of RTKs through autocrine loops or by other genetic alterations is found in established cancer cell lines and whether this modulates the response to selective RTK inhibitors, we analyzed available expression and copy number profiles from the Cancer Cell Line Encyclopedia (30). We focused our analysis on MET because of the relative simplicity—1 receptor, 1 ligand—as well as on the FGF/FGFR family because of the novel discovery of cross-talk with MET. Because high-level amplification of *MET* (as in MKN-45) and any of the *FGFRs* (as in RT-112 or KATO III) was found to be mutually exclusive, we turned our attention to potential autocrine loops. By applying a simple rank-order algorithm using expression values for MET, HGF, FGFRs, and FGFs, we identified cell lines with potential for dual autocrine RTK activation (Fig. 4A and Supplementary Table S4). Three promising and experimentally tractable candidates were then selected for *in vitro* combination studies. The KYM-1 rhabdomyosarcoma cell line was found to express high MET and HGF levels as well as high FGFR1 and FGF20 (Fig. 4A). We tested these cells in several experimental settings: first, we grew cells on adherent plates in monolayer. As a next step, we used nonadherent plates, either in the presence or absence of semisolid media. Under these conditions, especially in semisolid media, cells grew in a more clustered manner, potentially strengthening autocrine RTK stimulation and resembling more closely

the *in vivo* situation. We incubated cells with combinations of INC280 and BGJ398 in several concentrations using a "checkerboard" layout (Fig. 4B and Supplementary Fig. S4). Although MET inhibition on its own had no effect, FGFR inhibition led to partial growth suppression. Combined inhibition of both RTKs suppressed growth more profoundly than either single agent. This finding was more pronounced when cells grew in clusters and supports the hypothesis that coactivation of RTKs can alleviate the dependence on a single RTK. In the osteosarcoma cell line MG-63 (high MET/HGF, high FGFR1/FGF18; Fig. 4A), we observed a similar combination effect in monolayer proliferation assays. Unexpectedly, in semisolid media strong growth inhibition with BGJ398 alone was observed and combination with INC280 was only beneficial at low concentrations of BGJ398, where inhibition of FGFR1 may be incomplete. Although this result still argues for simultaneous activity of FGFR1 and MET in MG-63 cells, FGFR1 appears to be the dominant driver for growth. Lastly, we tested the glioma cell line Hs 683 (high MET/HGF, high FGFR1/FGF7) and observed clearly enhanced growth inhibition in monolayer assays when simultaneously inhibiting both RTKs (Fig. 4B). These cells did not grow on nonadherent plates, thus precluding further such experimental settings. Analysis of the effects of RTK inhibition on protein phosphorylation in these 3 cell lines was done by Western blotting (Fig. 4C). We found that each compound as a single agent effectively inhibited its own target or target substrate, but effects on the downstream signal transducers AKT and ERK were only partial at best. Combinatorial effects were apparent in each cell line on the level of ERK phosphorylation, whereas AKT phosphorylation was unaffected. When conducting the same analysis in KYM-1 cells grown in nonadherent plates, we observed a much more pronounced combination effect on ERK phosphorylation and a modest reduction in AKT phosphorylation with drug combination (Supplementary Fig. S5).

In Vivo Combination of Selective FGFR and MET Inhibitors Leads to Increased Antitumor Efficacy in a Xenograft Model of FGFR1 and MET Coactivation

Following the same strategy as above, we screened expression data from a set of primary xenograft models, that is, human tumor samples that have only been propagated as subcutaneous xenografts in immunocompromised mice. We identified a lung cancer model that displayed exceptionally high FGFR1 expression combined with high MET and HGF expression (Fig. 5A). Mice bearing xenografts derived from this model were randomized to 4 groups that were then treated with a vehicle control, INC280 as single agent, BGJ398 as single agent, or a combination of both drugs. Due to its relatively short half-life in mice, INC280 was orally administered at a dose of 10 mg/kg twice daily. BGJ398 was given orally at a dose of 40 mg/kg once daily, and the same regimen for both drugs was used in combination.

Compared to vehicle control, INC280 alone had only a very modest, statistically not significant antitumor effect (Fig. 5B and Supplementary Table S5). In contrast, treatment with BGJ398 as single agent led to tumor growth inhibition resulting in stable disease over a course of 18 days. The combination of both RTK inhibitors, however, led to substantial tumor regression (Fig. 5B). Statistical analysis using the method of Clarke (31) indicated synergy (Supplementary Table S5). Analysis of pharmacodynamic effects at the end of the study was conducted by ELISA for MET phosphorylation and by Western blotting for several other markers. As expected, application of INC280 alone or in combination led to profound suppression of MET phosphorylation at 2 hours after dosing, whereas recovery of MET phosphorylation was apparent at 12 hours (Fig. 5C). Likewise, dosing of BGJ398 led to full suppression of FRS2 phosphorylation at 2 hours, and recovery was apparent in some samples at 24 hours after dosing (Fig. 5D). Analysis of AKT phosphorylation revealed strong variability between samples and inhibition appeared to be driven primarily by BGJ398. Phosphorylation of ERK1/2 at 2 hours after dosing was unaffected by INC280 monotherapy, partially suppressed by BGJ398 monotherapy, and more potently inhibited by the combination of both agents. Hence, once again ERK1/2 phosphorylation appeared to correlate best with antitumor efficacy in this model. Pharmacokinetic analysis of plasma and tumor samples from tumor-bearing mice did not show any evidence for drug–drug interactions (Fig. 5E).

In summary, our secretome screening data together with the combination experiments conducted in selected cancer models indicate that simultaneously activated RTKs can independently contribute to growth in the same cancer cell. Coactivation of RTKs may thus contribute to acquired or primary resistance against single-agent kinase inhibitor therapy in a broader fashion than previously appreciated, providing a rationale for combination of selective kinase inhibitors where indicated by the according molecular alterations.

DISCUSSION

Here, we describe a technology platform that enables the screening of cellular phenotypes after treatment with freshly produced secreted proteins (i.e., secretomics screening). We generated secreted proteins by parallel transfection of 3,432 cDNAs into HEK293T cells. After a 3-day incubation, we assumed that the respective encoded protein had been produced and secreted, resulting in what we term "conditioned media." We have not quantified protein amounts in our conditioned media in a systematic manner, and we assume that not all proteins are produced and that the relative protein production efficiency is likely to vary. It is possible that only a subset of supernatants contains sufficient amounts of the respective secreted protein to evoke a biologic effect, and therefore, the true complexity of our secretome library is currently unknown. Regardless, the discovery that FGF and EGF ligands can, respectively, rescue MET and FGFR inhibition indicates that this platform is useful for identifying novel activities that were previously unknown. Similar secretome efforts that have been published used either conditioned supernatants to find novel cytokine activity (32) or used purified tagged proteins to identify novel mediators of stem cell pluripotency (33). In both cases, known and novel signaling molecules were reported as hits in their screens and there was no attempt to measure specific activity across their entire secretome collection. In addition, we have observed activity for many known signaling pathway ligands in our other screens, including WNT, TGF-β, FGF, EGF, interleukins/cytokines, insulin/IGF, and interferon, which leads us to believe that there is significant activity within the library (data not shown). As indicated, the secretome

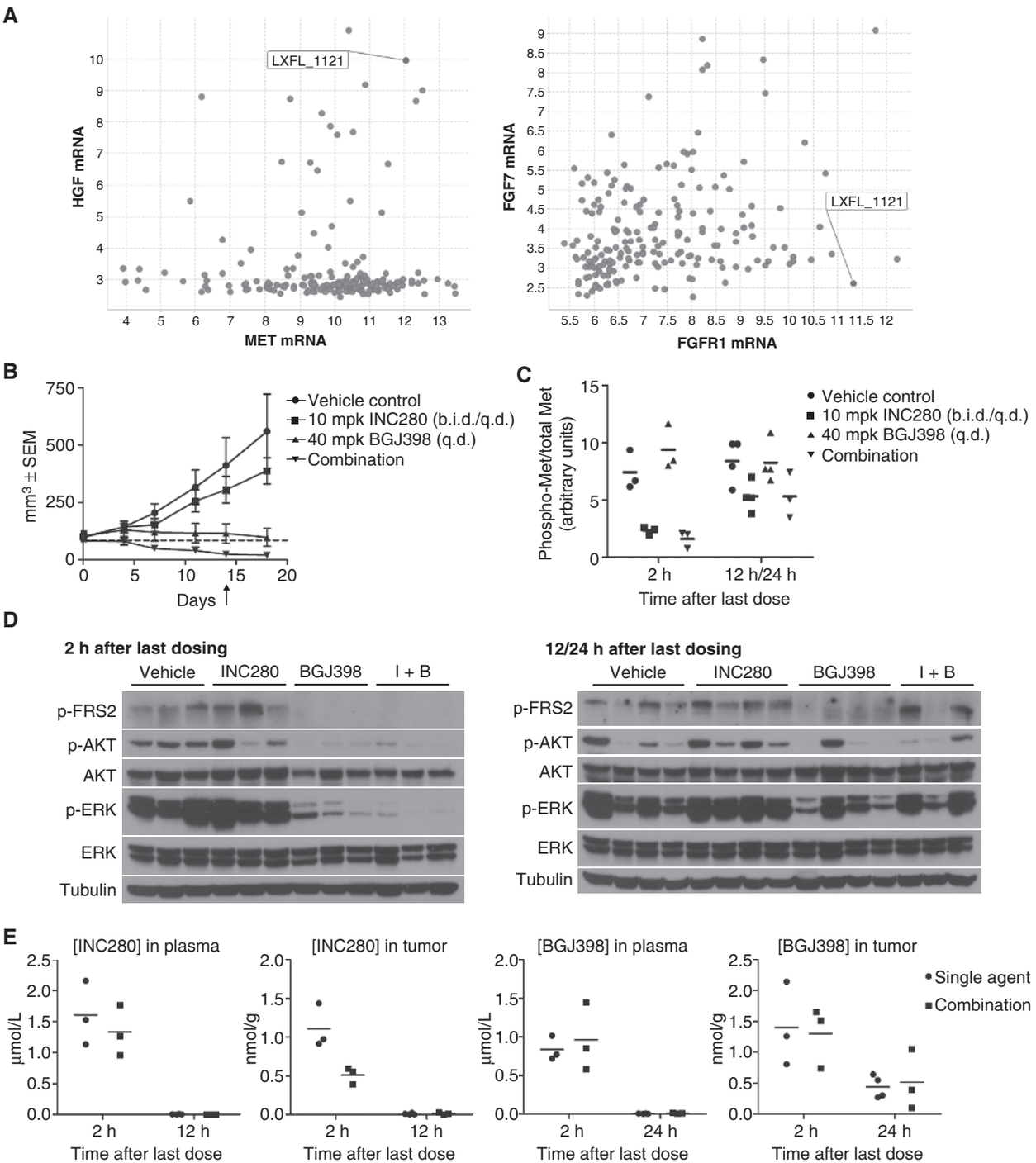

Figure 5. Antitumor activity of an FGFR/MET inhibitor combination in a primary lung cancer xenograft model. **A,** expression of selected RTKs and cognate ligands in a collection of primary xenografts. The levels of *HGF* and *MET* mRNA expression are displayed as a scatter plot along the *x* and *y*-axes, respectively. Each dot represents an individual xenograft. **B,** tumor growth curves in cohorts of tumor-bearing mice treated with the indicated regimens. The arrow marks a reduction in the frequency of INC280 dosing from twice (b.i.d.) to once (q.d.) daily. **C,** analysis of MET phosphorylation by ELISA in tumors 2 or 12 hours after the last INC280 dose. **D,** Western blot analysis of FRS2, AKT, and MAPK (ERK1/2) phosphorylation in tumors 2 hours after the last drug dose or at drug trough level (12 hours after administration of INC280 and 24 hours after administration of BGJ398). **E,** pharmacokinetics of INC280 and BGJ398 in tumor-bearing mice. Concentrations of INC280 and BGJ398 in plasma and tumor samples from tumor-bearing mice sacrificed are displayed at the indicated time points.

screening platform is generic and we have been able to screen many types of assay formats in addition to cell proliferation, such as reporter gene activity, endogenous mRNA, immunofluorescence (high-content screening), and fluorescence-activated cell-sorting–based cell typing.

In this report, we have used several secretome screens to identify novel modes of resistance to targeted cancer therapeutics. Our interest in resistance screening was triggered by several prior reports that implicated secreted ligands in driving resistance to RTK inhibitors. For example, hypoxia-mediated upregulation of proangiogenic factors including FGF family members was found to contribute to resistance against a VEGFR2 blocking antibody based on evidence from a pancreatic islet tumor model (34). It has also been shown that EGFR and HGF ligands produced by stromal cells may regulate the sensitivity of EML4–ALK-positive lung cancer cells to ALK inhibitors by triggering bypass survival signals (35). Likewise, a compensatory activation of MET was shown to bypass EGFR dependence in lung cancer (8). Although the initial discovery of this resistance mechanism involved MET activation through gene amplification, mechanistic studies showed that the MET ligand HGF can also drive resistance in this setting (10, 13). Interestingly, this RTK switch works in both directions: ligands that activate HER kinases can also mediate resistance to MET inhibitors (14). FGF-FGFR autocrine loops have also been observed in lung cancer samples, possibly driving resistance to EGFR inhibition (36). Indeed, FGF-mediated rescue of EGFR inhibitor-sensitive lung cancer lines was described in a recent report, where the authors also observed FGFR upregulation upon EGFR inhibition (37). In addition, interleukin-6 secretion triggered by TGF-β was suggested to drive resistance to EGFR inhibition (38). In principle, resistance-mediating ligands can be produced by cancer cells in an autocrine fashion—possibly under selective pressure by a targeted agent—or by other cells in the tumor microenvironment, leading to paracrine activation of the respective receptors. Notably, while this manuscript was under revision, 2 groups reported similar efforts on identifying ligand-mediated rescue to anticancer agents (39, 40). One group studied 6 RTK ligands across 41 cancer cell lines with known kinase dependency; the other identified rescue ligands by coculture of 23 different stromal cell types with 45 cancer cell lines in the presence of kinase inhibitors and other drugs. Although both studies focused primarily on HGF-mediated rescue of *BRAF*-mutant cancers, Wilson and colleagues (40) additionally reported rescue effects in MET- and FGFR-dependent cell lines that are in good alignment with our findings. We also extended our secretome screens to *BRAF*-mutant melanoma lines using the novel selective RAF inhibitor LGX818 (ref. 41; data not shown). In agreement with the 2 studies mentioned above (39, 40), we observed strong rescue with HGF. Further hits included NRG1 and FGFs.

We used our secretome screening platform to identify novel secreted factors that can mechanistically drive resistance to either MET or FGFR inhibitors. We found that in multiple cancer cell lines with primary dependence on either of these kinases, activation of the respective alternate kinase could rescue growth. Furthermore, activation of HER kinases led to rescue in either case. These results confirm previous data regarding reciprocal MET/EGFR switching, and also reveal an analogous MET/FGFR switch that to our knowledge has not yet been reported. More generally, our findings reveal that cancer cells can be highly flexible with respect to the particular RTK that drives their growth. Confirmation of this concept comes from the recent finding that in glioblastoma multiple RTKs can be activated by amplification in a mosaic fashion (42). Likely, signals emerging from MET, FGFR, HER, and possibly other RTKs are partially redundant because they converge on key downstream signal transduction nodes like the MEK/ERK or PI3K/AKT pathway. Crossphosphorylation of RTKs leading to additional activation of downstream signals has also been described. For example, HER family kinases have been shown to be downstream targets of amplified FGFR2 signaling in KATO III cells and to drive KATO III cell proliferation downstream of activated FGFR2 (25). In line with our secretome screening data, it is thus conceivable that upregulation of HER ligands in this setting would be an effective resistance mechanism to overcome FGFR inhibitory therapy. The differences in rescue potential that were observed with ligands belonging to the same family could reflect different receptor expression profiles on the respective cancer cells, together with ligand preferences for certain RTK family members.

Addressing the relevance or abundance of these novel compensatory kinase switches for acquired resistance in cancer patients is indeed of future interest. Clinical trials with highly selective and potent MET and FGFR inhibitors like INC280 and BGJ398 are ongoing, but resistance to such agents has not yet been investigated in patients. However, the clinical relevance of ligand-mediated resistance could be studied in cases involving anticancer agents that are in more advanced stages of clinical development. For example, Yano and colleagues (43) observed high-level HGF expression in a large fraction of *EGFR*-mutant tumors with intrinsic or acquired resistance to EGFR inhibitors. Likewise, HGF expression was found to inversely correlate with response to RAF inhibitors in melanoma patients (39, 40). In the present study, we made use of available gene expression profiles from several hundred cancer models to identify samples in which both MET and at least 1 of the FGFRs are likely to be active at the same time, suggesting primary resistance to MET or FGFR inhibitors as single agents, but sensitivity to a combination of both agents. Although we did not find cancer models that were fully resistant to both monotherapies, we observed additive or synergistic growth inhibition by combining MET and FGFR inhibitors in several cancer models *in vitro* and *in vivo*, including a xenograft model directly derived form a lung cancer patient. Such models appear to be rare in the absence of selective pressure by targeted therapeutics, but it is conceivable that their frequency will be substantially higher in a setting of acquired resistance.

Tumor-associated stromal fibroblasts have long been recognized as a potential source of paracrine factors stimulating cancer growth (44). Consequently, a role for fibroblast-derived factors in mediating resistance appears likely and has even been experimentally confirmed. For example, following the discovery that exogenous HGF could induce resistance to EGFR inhibitors in *EGFR*-mutant lung cancer (10), tumor-associated stromal fibroblasts were identified as a potential source of HGF in lung cancer patients (45). Another study also identified tumor-associated stroma as the cause of EGFR inhibitor resistance in an *in vivo* lung cancer model (46). Interestingly, *EGFR*-mutant lung cancer cell lines also attract fibroblasts via

an unidentified molecular mechanism that is enhanced under treatment with an EGFR inhibitor (45). As mentioned above, a recent systematic screen using a stromal cell–cancer cell coculture system revealed the potential of HGF-secreting stromal cells to rescue *BRAF*-mutant melanoma (39).

In summary, our findings suggest that clinical trials with RTK inhibitors would benefit from integration of biomarker assays to monitor the activity of putative compensatory RTKs. Accumulating clinical experience with highly selective RTK inhibitors may eventually reveal common RTK bypass mechanisms beyond EGFR/MET that can be tackled by an appropriate combination of selective agents.

METHODS

Creation of the Secretome Library

A bioinformatics pipeline was built to identify secreted and single-pass transmembrane proteins; it is similar to previously described methods (32, 33). In brief, all human RefSeq protein sequences (June 2004 version containing 27,959 proteins) were filtered through the databases SWISSPROT and INTERPRO for previous annotation as secreted or transmembrane (47, 48). Then, protein sequences were analyzed with algorithms that identify signal sequences and transmembrane helices: TMHMM, SIGNALP, and PHOBIUS (49–51). A total of 2,803 unique genes were selected and mapped to 3,432 clones; all were purchased from the Invitrogen Ultimate ORF collection and DNA isolated using standard techniques. pcDNA-DEST40 (Invitrogen) was the plasmid vector for all clones, and all clone inserts were confirmed by full sequencing.

Secretomics Screening

MET Inhibitor Rescue Screen Secretome library cDNAs were reverse transfected into HEK293T cells using FuGENE HD (4:1 ratio transfection reagent to DNA) and allowed to incubate 4 days to allow accumulation of secreted proteins in supernatant. The supernatant was then transferred to MKN-45 cells, followed by addition of NVP-JAA120 to a final concentration of 100 nmol/L. After 96 hours, growth was measured using CellTiter-Glo.

FGFR Inhibitor Rescue Screen The format was identical to the MET inhibitor rescue screen, substituting RT-112 cells as the target line and NVP-BGJ398 as the inhibitor.

Microarray gene expression data for the Cancer Cell Line Encyclopedia (30) are available at the Gene Expression Omnibus (accession number: GSE36139). The identity of all cell lines has been verified by SNP genotyping.

More details on the secretomics screens, confirmation experiments with recombinant proteins and selective kinase inhibitors, Western blotting, and *in vitro*/*in vivo* drug combination experiments can be found in the Supplementary Materials and Methods.

Disclosure of Potential Conflicts of Interest

All authors except for T. Metz are full-time employees of Novartis. No potential conflicts of interest were disclosed by T. Metz.

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Acknowledgments

The authors wish to acknowledge and thank Craig Mickanin, Frank Buxton, and the BioArchive team for procurement and handling of the secretome cDNA library, Amy Chen for initial development of the secretome screening processes, Omar Magid for his technical assistance within screening runs, Steve Cleaver and the Quantitative Biology Team for support, Alan Abrams for help with graphic design, and Deborah Castelletti for critical reading and helpful suggestions on the manuscript.

Received May 24, 2012; revised August 2, 2012; accepted August 2, 2012; published OnlineFirst August 8, 2012.

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