tance in these tumors.[30] As the amounts of each clinical specimen were limited, we would like to perform further analyses in future studies should sufficient amounts of specimens become available.

Recent studies indicated that multiple resistance factors can be induced simultaneously in a single cancer. For example, Qi et al.[31] reported the simultaneous occurrence of *Met* mutation and activation of the EGFR pathway by ligand overexpression, similar to T790M mutation and HGF overexpression in EGFR mutant lung cancer, which caused resistance to Met-TKIs in gastric cancer. Katayama et al.[32] also reported that *ALK* gene amplification and gatekeeper mutation in *ALK* occurred simultaneously and conferred resistance to ALK inhibitors in EML4-ALK lung cancer. In this study, T790M secondary mutation and the high HGF expression level were simultaneously detected at high incidence (50%) in tumors with acquired resistance. Irreversible EGFR-TKIs were thought to have potential to control acquired resistance caused by T790M secondary mutation, but clinical responses were rarely observed in clinical trials.[33,34] We recently found that HGF induces resistance to not only reversible EGFR-TKIs but also irreversible EGFR-TKIs by activating the MET/PI3K/Akt pathway in *EGFR* mutant lung cancer cells with or without T790M secondary mutation.[26] Taken together, these observations suggest that HGF would be simultaneously expressed with T790M secondary mutation in tumors with acquired resistance and reduce the sensitivity to irreversible EGFR-TKIs in *EGFR* mutant lung cancer patients.

MET amplification has been detected in ~20% of tumors with acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer,[13,16,17] while the incidence reported in Japanese patients is rare.[14,18] Here, we detected *MET* amplification in two tumors (9%) with acquired resistance, suggesting that *MET* amplification can be detected in a significant proportion of tumors with acquired resistance even in Japanese patients. One case with high-level HGF expression and *MET* amplification (KZ-1) was treated with gefitinib and PFS was 254 days. The other case with low HGF and *MET* amplification (SG4) was treated with erlotinib and PFS was 60 days (Table 3). Although it is not possible to make definitive conclusions based on the data from only these two cases, the shorter PFS in the former case tentatively supports the observation that HGF accelerates expansion of preexisting clones with MET amplification.[16] Notably, simultaneous expression of these two factors was also detected in one tumor with intrinsic resistance (nonresponder). However, the mechanism by which HGF is induced in *EGFR* mutant lung cancer is still not well defined. Further examinations are warranted to elucidate the interaction between HGF expression and *MET* amplification in *EGFR* mutant lung cancer.

Among 68 resistant tumors, high-level HGF expression, T790M secondary mutation, and *MET* amplification were not detected in one tumor with acquired resistance and 31 tumors with intrinsic resistance, indicating the involvement of other mechanisms of resistance in these tumors. *EGFR* D761Y secondary mutation in exon 20 was detected in two tumors from the same patient.[24] *EGFR* D761Y mutation was originally identified in recurrent brain metastasis and was shown to induce intermediate-grade resistance to EGFR-TKIs.[35] In addition, rare secondary mutations (other than T790M and D761Y) or a preexisting resistance mutation in a minority of clones may also be involved in intrinsic resistance. Moreover, it was recently reported that a subpopulation of cancer cells that transiently exhibit a distinct phenotype characterized by engagement of IGF-1R activity, hypersensitivity to HDAC inhibition, and altered chromatin showed an intrinsic ability to tolerate exposure to EGFR-TKI.[36] Minor secondary mutations, a preexisting resistance mutation in a minority of clones, or chromatin-mediated drug resistance mechanisms may be involved in resistant tumors without high HGF expression, T790M secondary mutation, and *MET* amplification.

To overcome the HGF-induced resistance to EGFR-TKI in *EGFR* mutant lung cancer, double blockade of the EGFR pathway and HGF-MET pathway is therefore theoretically necessary.[14,16,27] To inhibit mutant EGFR with or without T790M secondary mutation, EGFR mutant-specific inhibitors were developed in addition to irreversible EGFR-TKIs.[37] To inhibit HGF-MET signaling, several inhibitors, including anti-HGF antibody, NK4 (natural antagonist of MET), and MET-TKIs, were developed.[16,25–27] Further studies are essential to determine optimal combined therapy with best efficacy and safety. In addition, a prospective study is required to determine whether immunohistochemical detection of HGF would be sufficiently reliable to identify patients with HGF-induced resistance to EGFR-TKIs. As levels of HGF in peripheral blood are correlated with clinical outcome to EGFR-TKIs in patients with non-small cell lung cancer,[38,39] such noninvasive methods may facilitate individual therapy for overcoming HGF-induced resistance to EGFR-TKIs in *EGFR* mutant lung cancer patients.

Recent studies indicated at least three important roles of HGF in EGFR-TKI resistance in *EGFR* mutant lung cancer. First, HGF induces resistance to reversible EGFR-TKIs, gefitinib, and erlotinib, by restoring MET/Gab1/PI3K/Akt pathways.[14,16] Second, HGF accelerates expansion of preexisting *MET*-amplified cancer cells and facilitates MET amplification-mediated resistance during EGFR-TKI treatment.[16] Third, after acquiring resistance to reversible EGFR-TKIs, HGF induces resistance of lung cancer cells with T790M secondary mutation to irreversible EGFR-TKIs.[24] Here, we detected high-level HGF expression frequently in tumors with intrinsic and acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer in Japanese patients. These findings indicate the value of HGF as a therapeutic target for EGFR-TKI-resistant *EGFR* mutant lung cancer. Therefore, combined therapy with EGFR-TKIs and HGF-MET inhibitors in patients with HGF-induced resistance may improve the clinical outcome of *EGFR* mutant lung cancer.

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Clinical Cancer Research

Met Kinase Inhibitor E7050 Reverses Three Different Mechanisms of Hepatocyte Growth Factor –Induced Tyrosine Kinase Inhibitor Resistance in *EGFR* Mutant Lung Cancer

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Cancer Therapy: Preclinical

Clinical Cancer Research

Met Kinase Inhibitor E7050 Reverses Three Different Mechanisms of Hepatocyte Growth Factor–Induced Tyrosine Kinase Inhibitor Resistance in *EGFR* Mutant Lung Cancer

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Abstract

Purpose: Hepatocyte growth factor (HGF) induces resistance to reversible and irreversible epidermal growth factor receptor–tyrosine kinase inhibitors (EGFR-TKI) in *EGFR* mutant lung cancer cells by activating Met and the downstream phosphoinositide 3-kinase (PI3K)/Akt pathway. Moreover, continuous exposure to HGF accelerates the emergence of EGFR-TKI–resistant clones. We assayed whether a new Met kinase inhibitor, E7050, which is currently being evaluated in clinical trials, could overcome these three mechanisms of resistance to EGFR-TKIs.

Experimental Design: The effects of E7050 on HGF-induced resistance to reversible (gefitinib), irreversible (BIBW2992), and mutant-selective (WZ4002) EGFR-TKIs were determined using the *EGFR* mutant human lung cancer cell lines PC-9 and HCC827 with an exon 19 deletion and H1975 with an T790M secondary mutation. PC-9 cells were mixed with HGF-producing fibroblasts, MRC-5 cells, and subcutaneously inoculated into severe combined immunodeficient mice, and the therapeutic effects of E7050 plus gefitinib were assayed.

Results: E7050 circumvented resistance to all of the reversible, irreversible, and mutant-selective EGFR-TKIs induced by exogenous and/or endogenous HGF in *EGFR* mutant lung cancer cell lines, by blocking the Met/Gab1/PI3K/Akt pathway *in vitro*. E7050 also prevented the emergence of gefitinib-resistant HCC827 cells induced by continuous exposure to HGF. In the *in vivo* model, E7050 plus gefitinib resulted in marked regression of tumor growth associated with inhibition of Akt phosphorylation in cancer cells.

Conclusions: A new Met kinase inhibitor, E7050, reverses the three HGF-induced mechanisms of gefitinib resistance, suggesting that E7050 may overcome HGF-induced resistance to gefitinib and next-generation EGFR-TKIs. *Clin Cancer Res; 18(6); 1663–71. ©2012 AACR.*

Introduction

The reversible epidermal growth factor receptor (EGFR)–tyrosine kinase inhibitors (TKI) gefitinib and erlotinib show dramatic therapeutic efficacy in patients with *EGFR*-activating mutations, such as in-frame deletions of exon 19 and the L858 point mutation in exon 21 (1, 2). Recent clinical trials have shown that these TKIs induced much higher response rates and longer progression-free survival than standard first-line cytotoxic chemotherapy in patients with *EGFR* mutant lung cancer (3, 4). Almost all patients, however, develop acquired resistance to EGFR-TKIs after varying periods of time (5). In addition, 20% to 30% of patients with *EGFR*-activating mutations show intrinsic resistance to EGFR-TKIs (5). Therefore, intrinsic and acquired resistances to EGFR-TKIs are major problems in the management of *EGFR* mutant lung cancer.

Three clinically relevant mechanisms have been reported to induce acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer—*EGFR* T790M secondary mutation (6, 7), *Met* gene amplification (8), and hepatocyte growth factor (HGF) overexpression (9). We found that HGF overexpression is involved not only in acquired but in intrinsic resistance to EGFR-TKIs (9). HGF has been shown to play at least 3 important roles in EGFR-TKI resistance in *EGFR* mutant lung cancer. First, HGF induces resistance to the reversible EGFR-TKIs gefitinib

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Translational Relevance

Hepatocyte growth factor (HGF) is involved in at least three important steps of epidermal growth factor receptor–tyrosine kinase inhibitor (EGFR-TKI) resistance in *EGFR* mutant lung cancer, inducing resistance to reversible EGFR-TKIs by restoring Met/Gab1/PI3K/Akt pathways, inducing resistance to next-generation EGFR-TKIs (irreversible TKI and mutant-selective EGFR-TKI), and accelerating the emergence of EGFR-TKI–resistant clones by continuous exposure to HGF. Therefore, HGF may be an ideal target for overcoming EGFR-TKI resistance in *EGFR* mutant lung cancer.

In preclinical experiments, we have tested whether a new Met kinase inhibitor, E7050, which is currently under evaluation in clinical trials, could overcome these three HGF-induced EGFR-TKI resistance mechanisms. Our findings suggest that E7050 may be useful for overcoming HGF-induced resistance to gefitinib and next-generation EGFR-TKIs in *EGFR* mutant lung cancer.

and erlotinib by restoring MetGab1/PI3K/Akt pathways (9, 10). Second, continuous exposure to HGF accelerates the expansion of preexisting *Met*-amplified cancer cells and facilitates *Met* amplification-mediated resistance during EGFR-TKI treatment (10). Third, after lung cancer cells acquire resistance to reversible EGFR-TKIs, HGF induces the resistance of cells with T790M secondary mutation to irreversible EGFR-TKIs (11). These findings indicate that HGF is an ideal target for overcoming EGFR-TKI resistance in *EGFR* mutant lung cancer.

There are several possible strategies for inhibiting HGF-Met signaling, including anti-HGF neutralizing antibody, HGF antagonist (NK4), Met tyrosine kinase inhibitors, and inhibitors of downstream molecules, such as phosphoinositide 3-kinase (PI3K), Akt, and mTOR (12). Previously, we showed that anti-HGF antibody (13), NK4 (13), and PI3K inhibitors (14) were effective in overcoming HGF-induced gefitinib resistance. Many Met-TKIs have therefore been developed and are expected to reverse HGF-induced resistance to EGFR-TKIs (10, 15).

E7050 is an orally active Met-TKI (16) that has been shown to inhibit the phosphorylation of Met, including amplified Met, and to suppress the growth of several types of cancer cells with *Met* amplification. On the basis of favorable preclinical data, a phase I clinical trial of E7050 is currently in progress. We have assessed whether E7050 can overcome the 3 HGF-induced resistance mechanisms to EGFR-TKIs.

Materials and Methods

Cell culture

The *EGFR* mutant human lung adenocarcinoma cell lines PC-9 and HCC827 were purchased from Immuno-Biological Laboratories Co. and the American Type Culture Collection, respectively. The human embryonic lung fibroblast cell line MRC-5 was purchased from Health Science Research Resources Bank. MRC-5 (P 30–35) cells were maintained in Dulbecco's modified Eagle's medium with 10% FBS. PC-9 and HCC827 cells were maintained in RPMI-1640 medium with 10% FBS.

Reagents

E7050 was synthesized by Eisai Co., Ltd (16). Gefitinib was obtained from AstraZeneca. The irreversible EGFR-TKI, BIBW2992, and the mutant-selective EGFR-TKI, WZ4002, were purchased from Selleck. Recombinant HGF and anti-human HGF antibody were prepared as described (17).

Cell growth assay

Cell growth was measured using the MTT dye reduction method (18). Tumor cells were plated at a density of 2×10^3 cells/100 μL/well into 96-well plates in RPMI-1640 medium with 10% FBS. After 24-hour incubation, various reagents were added to each well, and the cells incubated for a further 72 hours, followed by the addition of 50 μL of MTT solution (2 mg/mL; Sigma) to each well and further incubation for 2 hours. The media containing MTT solution were removed, and the dark blue crystals were dissolved by adding 100 μL of dimethyl sulfoxide. The absorbance of each well was measured with a microplate reader at test and reference wavelengths of 550 and 630 nm, respectively. The percentage of growth is shown relative to untreated controls. Each reagent and concentration was tested at least in triplicate during each experiment, and each experiment was conducted at least 3 times.

Antibodies and Western blotting

Cells were lysed in cell lysis buffer containing phosphatase and proteinase inhibitor cocktails (Sigma), and protein concentrations were determined using a BCA Protein Assay Kit (Pierce Biotechnology). For the detection of phosphorylated Met in subcutaneous tumors, 10 mg tumor lysates were immunoprecipitated with anti-Met (25H2) antibody. Total protein (40 μg per lane) was resolved by SDS-PAGE, and the proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad). After washing 4 times, the membranes were incubated with Blocking One (Nacalai Tesque Inc.) for 1 hour at room temperature, followed by overnight incubation at 4°C with primary antibodies to Met (25H2), phospho-Met (Y1234/Y1235; 3D7), phospho EGFR (Y1068), ErbB3 (1B2), phospho-ErbB3 (Tyr1289; 21D3), Gab1 (#3232), phospho-Gab1 (Y627; C32H2), Akt, and phospho-Akt (Ser473; 736E11; 1:1,000 each; Cell Signaling Technology); and anti-human EGFR (1 μg/mL) antibody (R&D Systems). After washing 3 times, the membranes were incubated for 1 hour at room temperature with species-specific horseradish peroxidase–conjugated secondary antibodies. Immunoreactive bands were visualized using SuperSignal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (Pierce Biotechnology). Each experiment was conducted at least 3 times independently.

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HGF production

Cells (2×10^5) were cultured in RPMI-1640 medium with 10% FBS for 24 hours, washed with PBS, and incubated for 48 hours in 2 mL of the same medium. The culture medium was harvested and centrifuged, and the supernatant was stored at −70°C until analysis. HGF concentrations were measured by IMMUNIS HGF EIA (Institute of Immunology, Tokyo, Japan), with a detection limit of 100 pg/mL, according to the manufacturer's instructions. All culture supernatants were tested in duplicate. Color intensity was measured at 450 nm using a spectrophotometric plate reader. Growth factor concentrations were determined by comparison with standard curves.

***HGF* gene transfection**

One day before transfection, aliquots of 1×10^5 HCC827 cells in 1 mL of antibiotic-free medium were plated on 6-well plates. Full-length *HGF* cDNA cloned into the BCMGSneo expression vector (19) was transfected using Lipofectamine 2000 in accordance with the manufacturer's instructions. After 24-hour incubation, the cells were washed with PBS and incubated for an additional 72 hours in antibiotic-containing medium, followed by selection in G418 sulfate (Calbiochem). After limiting dilution, HGF-producing cells, HCC827/HGF, were established. HGF production by HCC827/HGF cells was confirmed by ELISA.

RNA interference assay

Duplexed Stealth RNAi (Invitrogen) against *MET, ErbB3,* and *Gab1,* and Stealth RNAi Negative Control Low GC Duplex #3 (Invitrogen) were used for RNA interference assays. One day before transfection, aliquots of 2×10^4 tumor cells in 400 μL of antibiotic-free medium were plated on 24-well plates. After incubation for 24 hours, the cells were transfected with siRNA (50 pmol) or scrambled RNA using Lipofectamine 2000 (1 μL) in accordance with the manufacturer's instructions. After 24-hour incubation, the cells were washed with PBS and incubated with or without various reagents for an additional 72 hours in antibiotic-containing medium. Cell growth was measured using a Cell Counting Kit-8 (Dojin) in accordance with the manufacturer's instructions. Knockdown of *MET, ErbB3, Gab1,* and, *Shc1* was confirmed by Western blotting. Each reagent and concentration was tested at least in triplicate during each experiment, and each experiment was conducted at least 3 times.

Detection of *Met* amplification

Cell block sections (4-μm thick) were subjected to dual-color FISH using a MET/CEP7 probe cocktail (Kreatech Diagnostics) according to the manufacturer's instructions. Staining was evaluated as described (20).

Xenograft studies in SCID mice

Suspensions of PC-9 cells (5×10^6) mixed with MRC-5 cells (5×10^6) were injected subcutaneously into the backs of 5-week-old female severe combined immunodeficient (SCID) mice (Clea), as described (13). After 4 days (tumor diameter >5 mm), mice were randomly allocated into groups of 6 animals, each to receive E7050 (50 mg/kg/d) and/or gefitinib (25 mg/kg/d) by oral gavage. Tumor volume was calculated as $mm^3 = width^2 \times length/2$. All animal experiments were carried out in compliance with the Guidelines for the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (Approval number: AP-081088).

Immunohistochemistry

Frozen sections (5-μm thick) of xenograft tumors were fixed with cold acetone and washed with PBS. After blocking endogenous peroxidase activity with 3% aqueous H_2O_2 solution for 10 minutes, the sections were incubated with 5% normal horse serum, followed by overnight incubation at 4°C with anti–phospho-Akt antibody (Ser473; 736E11, 1:100 dilution). The sections were washed with PBS, incubated with biotin-conjugated anti-rabbit IgG (1:200 dilution) for 30 minutes at room temperature, and incubated for 30 minutes with avidin–biotin–peroxidase complex (ABC) using a Vectastain ABC Kit (Vector Laboratories). Staining was detected using the DAB (3,3′-diaminobenzidine tetrahydrochloride) Liquid System (DakoCytomation). Samples from which primary antibodies had been omitted served as negative controls.

Statistical analysis

Between-group differences were analyzed by one-way ANOVA, with P values less than 0.05 for overall comparisons tested by *post hoc* pairwise comparisons using the Newman–Keuls multiple comparison test. All statistical analyses were carried out using GraphPad Prism Ver. 4.01 (GraphPad Software, Inc.).

Results

E7050 reverses resistance to EGFR-TKIs induced by exogenous HGF

PC-9 and HCC827 cells were highly sensitive to gefitinib (Fig. 1A), whereas exogenously added HGF induced resistance to gefitinib in both cell lines (9, 13, 14). Although E7050 did not affect the growth of PC-9 or HCC827 cells at concentrations less than 3 μmol/L, the combination of E7050 with gefitinib reversed HGF-induced resistance of both cell lines in a concentration-dependent manner (Fig. 1B).

We previously reported that stromal fibroblasts are a source of exogenous HGF for EGFR-TKI naive non–small cell lung carcinoma (NSCLC) and that fibroblast-derived HGF induces resistance to gefitinib and erlotinib in PC-9 and HCC827 cells (13). Although E7050 had no effect on the growth or production of HGF or VEGF by MRC-5 cells (HGF-high producing fibroblasts) or PC-9 cells (data not shown), it reversed the gefitinib resistance of PC-9 cells induced by coculturing with MRC-5 cells (Fig. 1C), indicating that E7050 can reverse the EGFR-TKI resistance induced by exogenous HGF *in vitro*.

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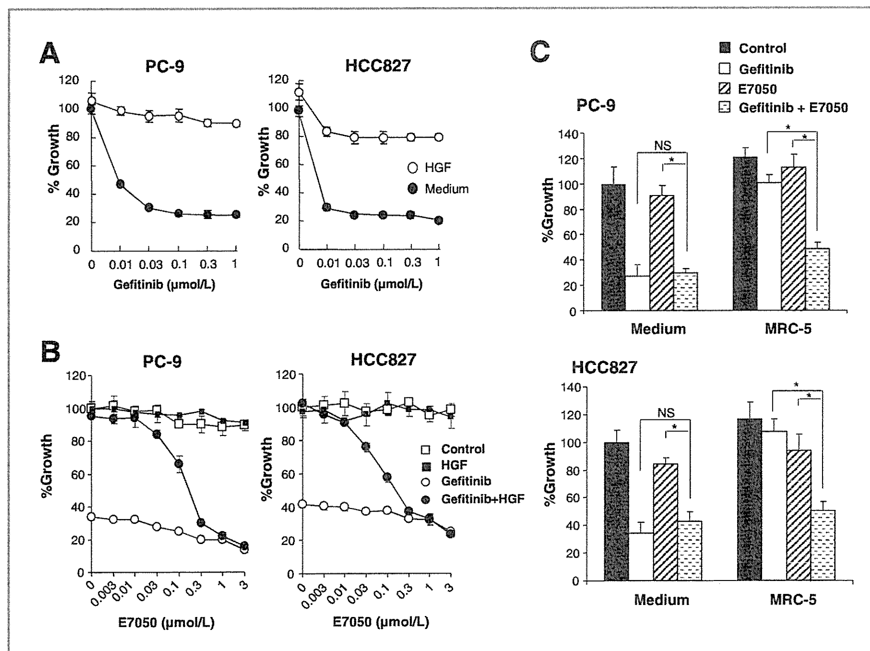

Figure 1. E7050 reverses resistance to EGFR-TKIs induced by exogenous HGF. A, PC-9 and HCC827 cells were incubated with various concentrations of gefitinib, with or without HGF (20 ng/mL). B, PC-9 and HCC827 cells were incubated with various concentrations of E7050, with or without HGF (20 ng/mL) and/or gefitinib (0.3 μmol/L). C, cells were cocultured in Transwell chambers separated by 8-μm pore filters. PC-9 and HCC827 cells (8×10^3 cells/700 μL) with gefitinib and or E7050 (0.3 μmol/L) were placed in the lower chambers and MRC-5 fibroblasts (10^4 cells/300 μL), producing high concentrations of HGF, were placed in the upper chambers. After 72 hours, the upper chambers were removed and cell growth was measured using the MTT assay. Bars indicate SD. *, $P < 0.01$.

E7050 reverses resistance to EGFR-TKIs induced by endogenous HGF

We have shown that HGF is present in tumor cells of NSCLC patients with acquired resistance to EGFR-TKIs, and that transient HGF gene transfection into PC-9 cells resulted in resistance to EGFR-TKIs (9). We therefore generated a stable HGF gene transfectant in HCC827 cells (HCC827/HGF) and assessed the effects of continuously produced endogenous HGF. HCC827/HGF, but not HCC827 or the vector control HCC827/Vec, cells secreted high levels of HGF and became resistant to gefitinib (Fig. 2A and B). Anti-HGF antibody reversed the gefitinib resistance of HCC827/HGF cells (Supplementary Fig. S1), indicating that endogenously produced HGF induced gefitinib resistance in this cell line. Although the combination of E7050 plus gefitinib successfully reversed the resistance of HCC827/HGF cells, E7050 alone did not inhibit the proliferation of HCC827/HGF cells (Fig. 2B).

Using Western blotting, we examined the effects of E7050 on signal transduction in HCC827/Vec and HCC827/HGF cells. We found that gefitinib inhibited the phosphorylation of EGFR and ErbB3 in HCC827/Vec cells, thereby inhibiting the phosphorylation of Akt and ERK1/2. However, gefitinib failed to inhibit phosphorylation of Akt in the presence of HGF. E7050 suppressed the constitutive phosphorylation of Met, but not of EGFR, ErbB3, and downstream Akt and ERK1/2. Whereas HGF stimulated the phosphorylation of Met, E7050 plus gefitinib inhibited this HGF-induced Met phosphorylation and strongly suppressed the phosphorylation of Gab1, Akt, and ERK1/2 (Fig. 2C).

The amount of Met protein was decreased in HCC827/HGF cells, compared with HCC827/Vec cells. This could be a result of Met downregulation by persistent HGF stimulation, as also observed in a previous report (21). In contrast, the degree of Met phosphorylation was higher in HCC827/HGF than in HCC827/Vec cells. Gefitinib inhibited the phosphorylation of EGFR and ErbB3, but not of Akt in HCC827/HGF cells. The combination of E7050 and gefitinib inhibited the phosphorylation of both Met and Akt (Fig. 2C). These results suggested that E7050 reversed HGF-induced gefitinib resistance by inhibiting the Met/Gab1/PI3K/Akt pathway.

To confirm that the E7050 reversal of gefitinib resistance in HCC827/HGF cells was due to the inhibition of Met/Gab1, we transfected cells with siRNA specific for *Met* or *Gab1*. Transfection of *ErbB3*, *Met*, or *Gab1* siRNA successfully knocked down the expression of the corresponding protein (Fig. 2D). Although scrambled or *ErbB3* siRNA did not reverse the gefitinib resistance of HCC827/HGF cells, siRNAs for *Met* and *Gab1* sensitized these cells to gefitinib (Fig. 2D), indicating that E7050 reverses gefitinib resistance in HCC827/HGF cells by inhibiting the Met/Gab1 pathway.

E7050 reverses HGF-induced resistance to next-generation EGFR-TKIs in H1975 cells

Next-generation EGFR-TKIs, irreversible TKIs (22–24), and mutant *EGFR*-selective TKIs (25) have been developed to treat gefitinib-resistant tumors caused by the *EGFR* T790M secondary mutation. H1975 cells with the *EGFR* mutations L858R and T790M mutations were resistant to reversible EGFR-TKIs, gefitinib, and erlotinib (data not

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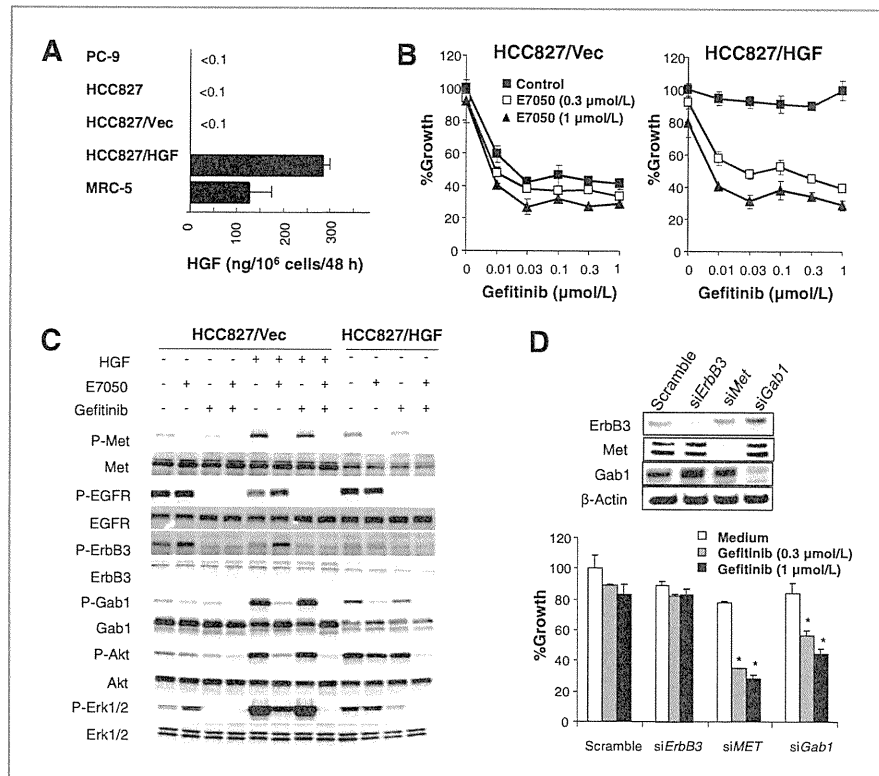

Figure 2. 7050 reverses resistance to EGFR-TKIs induced by endogenous HGF. A, cells (2×10^5/2 mL) were incubated for 48 hours and concentrations of HGF in the culture supernatants were determined by ELISA. B, HCC827/Vec and HCC827/HGF cells were incubated with various concentrations of gefitinib, with or without E7050. Cell growth was determined by MTT assays. C, HCC827/Vec and HCC827/HGF cells were incubated with HGF (20 ng/mL), E7050 (1 μmol/L), and/or gefitinib (1 μmol/L) for 1 hour. The cell lysates were harvested and phosphorylation of indicated proteins was determined by Western blotting. D, HCC827/HGF cells were treated with or without *ErbB3*, *Met*, or *Gab1* siRNA or scrambled siRNA for 24 hours, followed by further incubation in medium for 48 hours. The cell lysates were harvested and Western blotting was done to determine the expression of the indicated proteins. Cell growth after 72 hours was determined using MTT assays. Bars indicate SD. *, $P < 0.01$.

shown), but were sensitive to BIBW2992, an irreversible EGFR-TKI, and WZ4002, a mutant-selective EGFR-TKI (Fig. 3). HGF markedly induced resistance to BIBW2992 and WZ4002, whereas E7050 efficiently reversed the HGF-induced resistance to both BIBW2992 and WZ4002. These results indicated that E7050 can overcome HGF-induced resistance not only to gefitinib but to next-generation EGFR-TKIs, including irreversible and mutant-selective EGFR-TKIs.

E7050 prevents emergence of gefitinib-resistant HCC827 cells induced by continuous exposure to HGF

As HGF has been reported to accelerate the expansion of preexisting *Met*-amplified HCC827 cells and to facilitate *Met* amplification–mediated resistance during EGFR-TKI treatment (10), we examined the effects of E7050 on these phenomena. Although HCC827 cells did not produce viable colonies after 30 days of continuous exposure to gefitinib alone (Fig. 4A and B), these cells produced many colonies after exposure to both HGF and gefitinib. In contrast to previous findings (10), the percentage of cells with *Met* amplification was not increased when compared with parental HCC827 cells. The reason for this discrepancy remains unclear. Western blot analyses revealed that although the resultant cells expressed the same level of Met and Gab1 proteins compared with parental HCC827 cells, they expressed much higher levels of phosphorylated Met and Gab1 (Supplementary Fig. S2).

Importantly, E7050 prevented the emergence of viable clones even under conditions of continuous exposure to gefitinib and HGF (Fig. 4B). These results suggested the potential of E7050 to abrogate the effects resulting from continuous exposure to HGF.

E7050 circumvents HGF-induced resistance when combined with gefitinib *in vivo*

To investigate the therapeutic efficacy of E7050 *in vivo*, we used the gefitinib resistance model previously described (13). We mixed PC-9 cells with the HGF-high producing fibroblast cell line, MRC-5, and inoculated SCID mice subcutaneously with this mixture. Oral treatment with gefitinib and/or E7050 was started after the establishment of solid tumors on day 4. Consistent with previous observations, we found that treatment with gefitinib alone prevented the enlargement of tumors produced by the mixture of PC-9 and MRC-5 cells, but did not cause tumor regression. As gefitinib induces shrinkage of PC-9 tumors (13, 14), our results suggested that MRC-5 cells induced gefitinib resistance *in vivo*. Under these experimental conditions, treatment with E7050 alone did not inhibit tumor growth, whereas the combination of E7050 and gefitinib induced marked tumor regression (Fig. 5A and B).

To confirm that E7050 inhibits Met/PI3K/Akt signaling *in vivo*, we assessed expression of phosphorylated Met and Akt in the xenograft tumors. Immunoprecipitation

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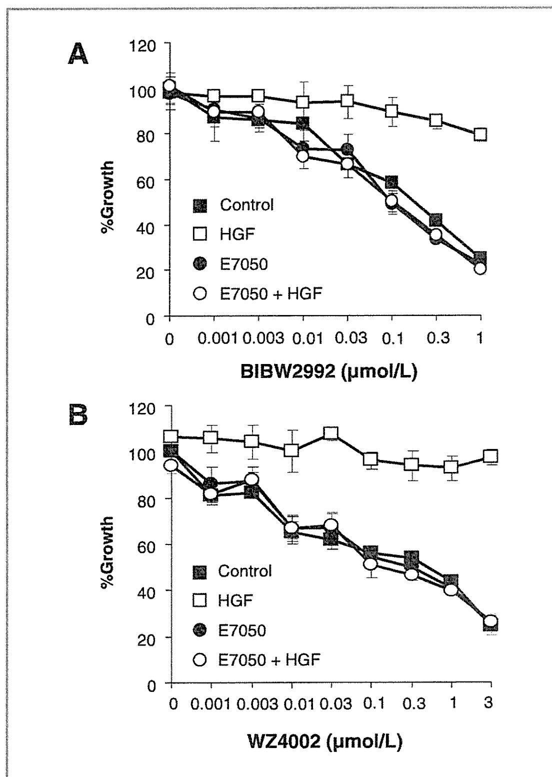

Figure 3. E7050 reverses HGF-induced resistance to next-generation EGFR-TKIs in H1975 cells. H1975 cells were incubated for 72 hours with various concentrations of BIBW2992 (A), an irreversible EGFR-TKI, and WZ4002 (B), a mutant-selective EGFR-TKI, in the presence or absence of HGF (20 ng/mL) and/or E7050 (1 μmol/L). Cell growth was determined by MTT assays. Bars indicate SD.

revealed that phosphorylated Met was detected in control tumors and gefitinib-treated tumors, but not in tumors treated with E7050 monotherapy or E7050 plus gefitinib (Fig. 5C), indicating efficacy of E7050 as a Met kinase inhibitor. Moreover, we observed higher levels of phosphorylated Akt in control cancer cells, with this phosphorylation slightly decreased by either E7050 or gefitinib alone and markedly inhibited by the combination of E7050 and gefitinib (Fig. 5D). In addition, there were no discernible differences in HGF concentrations between control and treated groups, when HGF protein concentrations were determined by EIA using lysates of tumors obtained after 5 days of treatment (Supplementary Fig. S3). These results suggested that E7050 overcame the gefitinib resistance associated with inhibition of the Met/Akt pathway.

Discussion

HGF is a multifunctional cytokine that can be produced not only by cancer cells but also by stromal cells, such as fibroblasts. The HGF receptor, Met, and EGFR interact with each other and mediate redundant signaling (26). Elevated serum concentrations of EGFR ligands and HGF were detected in patients with NSCLC, and HGF expression has been associated with poor prognosis in patients resected for NSCLC (27, 28). Although the role of HGF in *EGFR* mutant lung cancer remained unclear, we observed HGF-induced EGFR-TKI resistance in *EGFR* mutant lung cancers (9). Moreover, many studies have shown the important roles of HGF in sensitivity to molecular targeted drugs. Our observations with regard to EGFR-TKI in lung cancer were confirmed by subsequent studies (10, 29), and the concentrations of HGF in peripheral blood were found to be inversely correlated with clinical responses to EGFR-TKIs, in both *EGFR* mutant and wild-type lung cancer (30, 31). HGF was also found to cause resistance to sunitinib, a multikinase inhibitor, in renal cell carcinoma by compensating for inhibited angiogenesis (32). Taken together, these findings indicate the importance of HGF as a therapeutic target for drug resistance in cancer.

We have shown here that a new Met-TKI, E7050, reversed 3 HGF-induced resistance mechanisms in *EGFR* mutant lung cancer. First, E7050 reversed HGF-induced gefitinib resistance by inhibiting Met phosphorylation and thereby suppressing the downstream PI3K/Akt pathway. Second, E7050 inhibited the HGF-induced resistance to next-generation EGFR-TKIs, irreversible EGFR-TKIs, and mutant-selective EGFR-TKIs. Third, E7050 prevented the emergence of resistant clones induced by continuous exposure to HGF.

An interaction between HGF and *Met* amplification has been associated with EGFR-TKI resistance in lung cancer (10). In the presence of gefitinib, continuous exposure to HGF accelerated the expansion of preexisting *Met* amplified

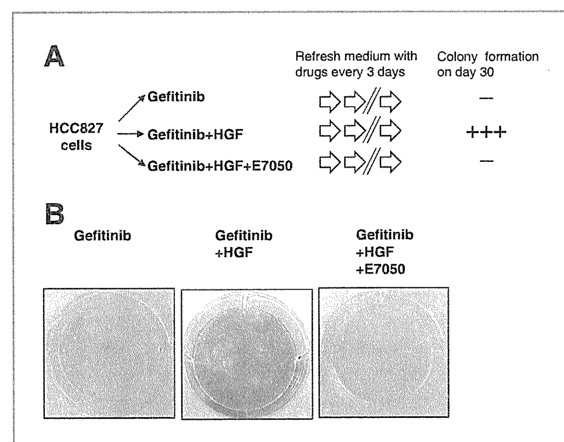

Figure 4. E7050 prevents the emergence of gefitinib-resistant HCC827 cells with amplified *Met* induced by continuous exposure to HGF. A, HCC827 cells were incubated with or without gefitinib (1 μmol/L), HGF (20 ng/mL), and/or E7050 (1 μmol/L), changing the medium every 3 days. After 30 days, viable colonies were stained with crystal violet. B, Representative cultures.

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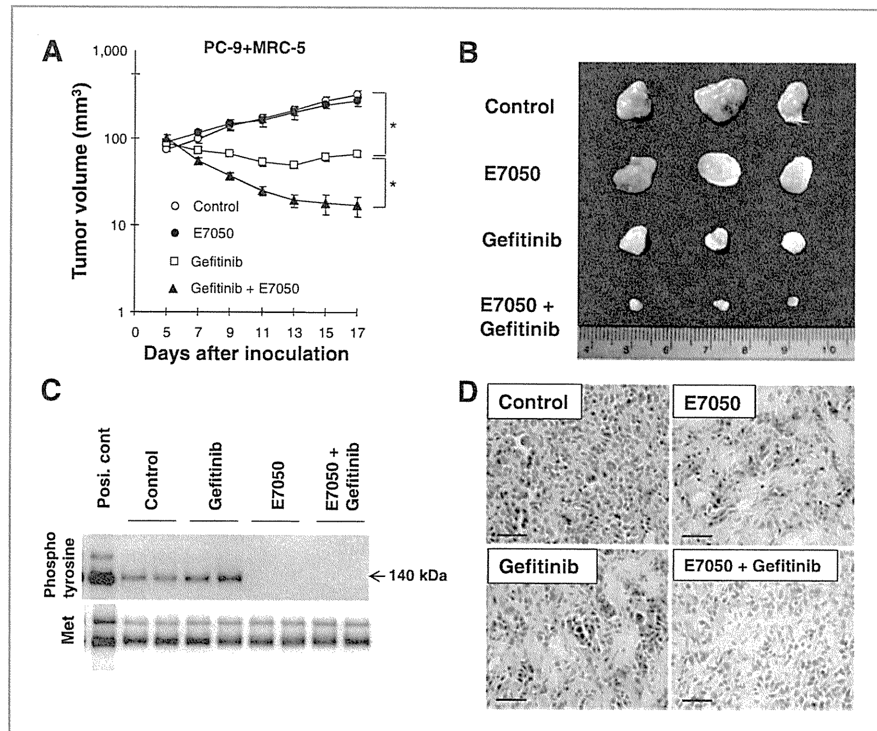

Figure 5. E7050 circumvents HGF-induced resistance when combined with gefitinib *in vivo*. A, PC-9 cells (5×10^6) with or without MRC-5 cells (5×10^6) were inoculated subcutaneously into SCID mice ($N = 6$) on day 0. The mice began treatment with oral gefitinib (25 mg/kg/d) and/or E7050 (50 mg/kg/d), on day 4. The tumor area was measured every 3 or 4 days and the tumor volume was calculated as described in Materials and Methods. Bars show SE of the means ± SD. *$P < 0.01$. B, macroscopic appearance of treated tumors harvested on day 17. C, expression of phosphorylated Met in harvested tumors. Met protein was immunoprecipitated by anti-Met antibody. Then, phosphorylated Met and Met protein were detected by anti-phosphotyrosine antibody and anti-Met antibody, respectively. D, expression of phosphorylated Akt in the harvested tumors. Frozen sections were immunohistochemically stained with anti-phospho Akt antibody. Original magnification, ×200.

HCC827 cells. Unexpectedly, when we cultured HCC827 cells with gefitinib and HGF for 30 days, we found that the percentage of cells with *Met* amplification was not increased. The reason we failed to detect expansion of clones with *Met* amplification, however, remains unclear. Transfection of the *HGF* gene into HCC827 cells produced HCC827/HGF cells, which constitutively produce HGF. These cells, however, were selected in the presence of geneticin but not gefitinib, with several clones showing amplification of *Met* (data not shown). Therefore, this phenomenon may be unique to a population of *EGFR* mutant lung cancer cells observed only under selection pressure with gefitinib plus an as yet unknown concentration of HGF.

Met was shown to be constitutively phosphorylated in human lung cancer cell lines, with the degree of phosphorylation not always correlated with susceptibility to EGFR-TKIs (33). Indeed, previous studies reported that the level of Met phosphorylation was higher in HCC827 cells than in other *EGFR* mutant cell lines (9, 10, 13, 29). Similar to these results, we also observed that the level of Met phosphorylation was higher in HCC827 cells than in PC-9 and Ma-1 cells (Supplementary Fig. S4). Although the bands for pMet in our study seem to be weaker than those in a previous study (34), ours and previous studies constantly showed that Met phosphorylation in HCC827 cells was higher than that in other *EGFR* mutant cells. Although the difference in the intensity of pMet bands between our study and the previous is unclear, it might be due to minor differences in experimental conditions, including the exposure time at Western blot and the cell culture conditions. With regard to HGF-triggered EGFR-TKI resistance, previous studies also support our findings that although HCC827 cells were highly sensitive to EGFR-TKIs, further Met activation or phosphorylation resulted in inducing resistance to EGFR-TKIs (10, 29, 35). We confirmed that knockdown of Met by siRNA canceled HGF-induced resistance in HCC827 cells (9). Moreover, it was reported that *Met* amplification resulted in increased level of Met phosphorylation and caused resistance to EGFR-TKIs in HCC827 cells (8). This accumulating evidence indicates that constitutive Met phosphorylation is insufficient and further activation by HGF or *Met* amplification may be necessary to induce EGFR-TKI resistance in HCC827 cells. Therefore, there may be a threshold level for Met phosphorylation to sufficiently cause EGFR-TKI resistance.

E7050 inhibits both Met and VEGFR2 kinases (16). *In vitro*, PC-9 and HCC827 cells express little VEGFR2 (data not shown). E7050 did not significantly inhibit the growth of these cell lines, and the anti-VEGF antibody bevacizumab did not augment the susceptibility of these cell lines to gefitinib (data not shown). These results suggest that the *in vitro* antitumor effects of E7050, when combined with gefitinib and HGF, may be largely due to Met inhibition. *In vivo*, we found that very high concentrations of HGF, obtained by *HGF* gene transfection into cancer cells, increased intratumor vessel density (submitted for publication elsewhere). However, HGF concentrations were

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lower in our xenograft model of mixed PC-9 and MRC-5 cells (fibroblasts) than in xenograft tumors produced by HGF gene–transfected lung cancer cells. We observed no difference in intratumor vessel density between tumors induced by PC-9 cells alone and tumors induced by PC-9 and MRC-5 cells (Supplementary Fig. S5). In addition, E7050 did not affect significantly the vessel density in tumors induced by PC-9 and MRC-5 cells. Collectively, these observations suggest that the antitumor effects of E7050 in this resistance model may not be predominantly due to angiogenesis inhibition.

The secondary T790M mutation in *EGFR* is the most prominent mechanism of acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer, with this mutation detected in about 50% of these patients (4). The T790M mutation increases the affinity of EGFR for ATP, decreasing the binding of EGFR to EGFR-TKIs and inducing resistance to the latter agents (36). *EGFR* mutant lung cancer cells with the T790M secondary mutation, however, remain susceptible to EGFR-mediating signaling and are thought to be manageable by inhibition of EGFR-mediated signaling (37). Preclinical studies have shown that next-generation EGFR-TKIs, irreversible TKIs, and mutant EGFR-selective TKIs have activity against gefitinib-resistant tumors with EGFR T790M secondary mutation (21–23). However, several irreversible EGFR-TKIs, including BIBW2992 (38) and HKI-272 (39), failed to meet primary endpoints in clinical trials of patients with EGFR-TKI–refractory lung cancer. High concentrations of HGF have been frequently detected in tumors with *EGFR*-T790M secondary mutations showing acquired resistance (10, 40, 41). In addition, we found previously (11) and confirmed here that HGF induces resistance to irreversible EGFR-TKIs in *EGFR* mutant lung cancer cells. Taken together, these observations suggest that HGF expressed in tumors with acquired resistance and *EGFR* T790M secondary mutations induce resistance to irreversible EGFR-TKI. As E7050 reversed the resistance to irreversible and mutant-selective EGFR-TKIs, it may augment the therapeutic efficacy of next-generation EGFR-TKIs in *EGFR* mutant lung cancer patients with acquired resistance to the *EGFR* T790M secondary mutation. These ideas further illustrate the necessity of methods to select patients who develop EGFR-TKI resistance due to HGF.

In conclusion, we have presented preclinical evidence showing that a new Met kinase inhibitor, E7050, may overcome HGF-induced resistance in *EGFR* mutant lung cancer. Further evaluation of E7050 in clinical trials is warranted to improve the outcomes of patients with *EGFR* mutant lung cancer.

Disclosure of Potential Conflicts of Interest

T. Uenaka and T. Nakagawa are employees of Eisai Co. S. Yano has received a commercial research grant from Eisai Co. and honoraria from speaker's bureau from Chugai Pharma.

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Paracrine Receptor Activation by Microenvironment Triggers Bypass Survival Signals and ALK Inhibitor Resistance in EML4-ALK Lung Cancer Cells

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Abstract

Purpose: Cancer cell microenvironments, including host cells, can critically affect cancer cell behaviors, including drug sensitivity. Although crizotinib, a dual tyrosine kinase inhibitor (TKI) of ALK and Met, shows dramatic effect against *EML4-ALK* lung cancer cells, these cells can acquire resistance to crizotinib by several mechanisms, including ALK amplification and gatekeeper mutation. We determined whether microenvironmental factors trigger ALK inhibitor resistance in *EML4-ALK* lung cancer cells.

Experimental Design: We tested the effects of ligands produced by endothelial cells and fibroblasts, and the cells themselves, on the susceptibility of *EML4-ALK* lung cancer cell lines to crizotinib and TAE684, a selective ALK inhibitor active against cells with *ALK* amplification and gatekeeper mutations, both *in vitro* and *in vivo*.

Results: *EML4-ALK* lung cancer cells were highly sensitive to ALK inhibitors. EGF receptor (EGFR) ligands, such as EGF, TGF-α, and HB-EGF, activated EGFR and triggered resistance to crizotinib and TAE684 by transducing bypass survival signaling through Erk1/2 and Akt. Hepatocyte growth factor (HGF) activated Met/Gab1 and triggered resistance to TAE684, but not crizotinib, which inhibits Met. Endothelial cells and fibroblasts, which produce the EGFR ligands and HGF, respectively, decreased the sensitivity of *EML4-ALK* lung cancer cells to crizotinib and TAE684, respectively. EGFR-TKIs resensitized these cells to crizotinib and Met-TKI to TAE684 even in the presence of EGFR ligands and HGF, respectively.

Conclusions: Paracrine receptor activation by ligands from the microenvironment may trigger resistance to ALK inhibitors in *EML4-ALK* lung cancer cells, suggesting that receptor ligands from microenvironment may be additional targets during treatment with ALK inhibitors. *Clin Cancer Res; 1–11. ©2012 AACR.*

Introduction

ALK fusion with EML4 in non–small cell lung cancer (NSCLC) was first detected in 2007 (1), with 3% to 7% of unselected NSCLCs having this fusion gene (1–4). EML4-ALK lung cancer is more frequently observed in patients with adenocarcinoma than with other histologies, in young adults than in older patients, and in never-smokers or light smokers (<15 pack-years) than in heavier smokers (2, 3). ALK kinase inhibitors show dramatic effects against lung cancers with *EMK4-ALK in vitro* and *in vivo* (3, 4). In a phase I–II trial with crizotinib, a dual tyrosine kinase inhibitor (TKI) of ALK and Met, the overall response rate was 47 of 82 (57%) patients with *EML4-ALK*–positive tumors (5). However, almost all patients who show a marked response to ALK-TKIs acquire resistance to these agents after varying periods of time (6, 7). Secondary mutations, including the gatekeeper L1196M mutation and others (F1174L, C1156Y, G1202R, S1206Y, 1151-T-ins, and G1269A), *ALK* amplification, *KIT* amplification, and autophosphorylation of EGF receptor (EGFR), were shown to be responsible for acquired resistance to crizotinib in ALK–translocated cancers (6–10).

Selective ALK inhibitors, including TAE684 and CH5424802, have been reported active against *EML4-ALK* lung cancer cells with *ALK* amplification and secondary mutations. These cells, however, may develop resistance to this class of inhibitor, due to several mechanisms, including novel *ALK* mutations (L1152R, L1198P, and D1203N), coactivation of EGFR and ErbB2, and EGFR phosphorylation (3, 11, 12).

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Translational Relevance

Although crizotinib, a dual inhibitor of ALK and Met, shows dramatic effects against *EML4-ALK* lung cancer cells, these cells can acquire resistance by several mechanisms, including ALK amplification and gatekeeper mutation. Selective ALK inhibitors may overcome crizotinib resistance due to these mechanisms, but these cells may become resistant to these inhibitors.

We show here that EGF receptor ligands produced by endothelial cells can cause *EML4-ALK* lung cancer cells to become resistant to crizotinib and selective ALK inhibitors, by triggering bypass survival signals. By contrast, hepatocyte growth factor produced by fibroblasts can induce resistance to selective ALK inhibitors, but not crizotinib. Because endothelial cells and fibroblasts are components of the microenvironment, our findings raise clinical questions about the class of ALK inhibitors more beneficial for *EML4-ALK* lung cancer patients. Moreover, our results provide a rationale for targeting receptor ligands in the microenvironment for more successful treatment with ALK inhibitors.

64 Most human cancers are composed of cancer cells that 65 coexist with a variety of extracellular matrix components 66 and cell types, including fibroblasts, endothelial cells, and 67 immune cells, which collectively form the tumor microen- 68 vironment (13). This microenvironment can influence the 69 growth, survival, invasiveness, metastatic ability, and drug 70 sensitivity of cancer cells within these tumors (14). Para- 71 crine signaling between cancer cells and host cells in the 72 microenvironment, mediated by cytokines, chemokines, 73 growth factors, and other signaling molecules, plays a 74 critical role in tumor growth (15). As receptors for these 75 factors, the EGFR family of receptors and Met are of par- 76 ticular interest in lung cancer (16). The EGFR family consists 77 of at least 4 receptor tyrosine kinases, including EGFR 78 (ErbB1), Her2/neu (ErbB2), HER3 (ErbB3), and HER4 79 (ErbB4). To date, 7 ligands for EGFR have been identified: 80 EGF, TGF-α; heparin-binding EGF-like growth factor (HB- 81 EGF); amphiregulin; betacellurin; epiregulin; and epigen 82 (17). By contrast, Met is the only specific receptor for 83 hepatocyte growth factor (HGF) and HGF binds only to 84 Met (18). Many lung cancer cells express EGFR and Met, 85 with these cells and others in their microenvironment 86 expressing their ligands (19, 20), suggesting that these 87 receptors and ligands modulate the sensitivity of cancer 88 cells to molecular targeted drugs in their microenviron- 89 ment. We previously showed that fibroblast-derived HGF 90 induces EGFR-TKI resistance in *EGFR*-mutant lung cancer 91 cells by activating Met and downstream pathways (21, 22). 92 However, the role of the microenvironment in the sensi- 93 tivity of EML4-ALK lung cancer cells to ALK-TKIs has not 94 been determined. We therefore examined whether factors in 95 the microenvironment of *EML4-ALK* lung cancer cells trig- 96 ger their resistance to crizotinib and TAE684, a selective ALK inhibitor, as well as clarifying their underlying mechanisms 98 of action. 99

Materials and Methods 100

Cell culture 101

The H2228 human lung adenocarcinoma cell line, with 102 the EML4-ALK fusion protein variant3 (E6;A20), the umbil- 103 ical vein endothelial cell line human umbilical vein endo- 104 thelial cells (HUVEC) and the human bronchial epithelial 105 cell line BEAS-2B, transformed with SV40 virus, were pur- 106 chased from the American Type Culture Collection. The 107 H3122 human lung adenocarcinoma cell line, with the 108 EML4-ALK fusion protein variant1 (E13;A20), was kindly 109 provided by Dr. Jeffrey A. Engelman of the Massachusetts 110 General Hospital Cancer Center, Boston, MA (3). The 111 MANA2 mouse lung adenocarcinoma cell line was estab- 112 lished in Jichi Medical University from a tumor nodule 113 developed in a transgenic mouse expressing *EML4-ALK* 114 variant 1 (E13;A20) (23). The MRC-5 and IMR-90 lung 115 embryonic fibroblast cell lines were obtained from RIKEN 116 Cell Bank. The human dermal microvessel endothelial cell 117 line HMVEC was purchased from Kurabo. The monocytic 118 leukemia cell line U937 was purchased from Health Science 119 Research Resources Bank. H2228 cells were cultured in 120 RPMI-1640 medium, MANA2 cells were cultured in 121 DMEM/F12+GlutaMAX-1, and MRC-5 (P 25–30) cells were 122 cultured in Dulbecco's modified Eagle's medium (DMEM) 123 medium, supplemented with 5% fetal bovine serum, 124 penicillin (100 U/mL), and streptomycin (50 μg/mL), in 125 a humidified CO_2 incubator at 37°C. HMVECs and 126 HUVECs were maintained in HuMedia-MvG with growth 127 supplements (Kurabo) and used for *in vitro* assays at 128 passages 2 to 5 and 2 to 4, respectively. BEAS-2B cells 129 were maintained in LHC9/RPMI-1640 medium, as 130 described (24), and used for *in vitro* assays at passages 131 42 to 46. Macrophage differentiation of U937 cells was 132 induced by incubation in RPMI-1640 medium containing 133 10 ng/mL phorbol 12-myristate 13-acetate (Sigma Chem- 134 ical Co.; ref. 25) for 5 days, with floating cells removed by 135 rinsing with PBS, as described (26). Differentiated U937 136 cells (PMA-U937 cells) attached to the dishes were used 137 for *in vitro* assays at passages 6 to 8. All cells were passaged 138 for less than 3 months before renewal from frozen, early- 139 passage stocks obtained from the indicated sources. Cells 140 were regularly screened for *Mycoplasma* using a MycoAlert 141 *Mycoplasma* Detection Kit (Lonza). 142

Reagents 143

TAE684, crizotinib, BIBW2992, and WZ4002 were pur- 144 chased from Seleck Chemicals. Erlotinib hydrochloride was 145 obtained from Chugai Pharmaceutical Co., Ltd. The anti- 146 human EGFR antibody cetuximab was obtained from 147 Merck Serono. E7050 was synthesized by Eizai Co., Ltd. 148 (27). Goat anti-human HGF antibody, control goat IgG, 149 recombinant EGF, TGF-α, HB-EGF, IGF-1, and PDGF-AA 150 were purchased from R&D Systems. Recombinant HGF was 151 prepared as described (28). 152

Cell growth assay

Cell proliferation was measured using the MTTdye reduction method (17). Tumor cells at 80% confluence were harvested, seeded at 2×10^3 cells per well in 96-well plates, and incubated in appropriate medium for 24 hours. Several concentrations of TAE684, crizotinib, erlotinib, BIBW2992, WZ4002, E7050, cetuximab, anti-HGF antibody, and/or EGF, TGF-α, HB-EGF, IGF-1, PDGF-AA, and HGF were added to each well, and incubation was continued for a further 72 hours. To each well was added 50 µL MTT (2 mg/mL; Sigma), followed by incubation for 2 hours at 37°C. The media were removed and the dark blue crystals in each well were dissolved in 100 µL of dimethyl sulfoxide (DMSO). Absorbance was measured with an MTP-120 Microplate reader (Corona Electric) at test and reference wavelengths of 550 and 630 nm, respectively. The percentage growth was calculated relative to untreated controls. Each assay was carried out at least in triplicate, with results based on 3 independent experiments.

Apoptosis assay

H2228 and H3122 cells (3×10^3 cells) were seeded in 96-well, white-walled plates and incubated overnight. The cells were treated with crizotinib (1 µmol/L) or vehicle (DMSO) for 48 hours. Cellular apoptosis was determined by measuring caspase-3/7 activity using a luminometric Caspase-Glo 3/7 assay (Promega) according to the manufacturer's protocol, with luminescence intensity measured using a Fluoroskan Ascent FL plate reader (Thermo Scientific). Cellular apoptosis was expressed relative to DMSO-treated control cells.

RNA interference

Duplexed Stealth RNAi (Invitrogen) against *EGFR*, *Met*, *ErbB3*, *Gab1*, *ALK*, and Stealth RNAi-negative control low GC Duplex #3 (Invitrogen) were used for RNA interference (RNAi) assays. Briefly, aliquots of 1×10^5 cells in 2 mL of antibiotic-free medium were plated into each well of a 6-well plate and incubated at 37°C for 24 hours. The cells were transfected with siRNA (250 pmol) or scrambled RNA using Lipofectamine 2000 (5 µL) in accordance with the manufacturer's instructions (Invitrogen). After 24 hours, the cells were washed twice with PBS and incubated with or without crizotinib (100 nmol/L), TAE684 (100 nmol/L), recombinant human EGF (100 ng/mL), TGF-α (100 ng/mL), HB-EGF (10 ng/mL), or HGF (50 ng/mL) for an additional 48 hours in antibiotic-containing medium. These tumor cells were then used for cell proliferation assays, with *EGFR*, *Met*, *ErbB3*, *Gab1*, and *ALK* knockdowns (#1, #2) confirmed by Western blotting.

The siRNA target sequences were as follows: *EGFR*, 5′-CGGAATAGGTATTGGTGAATTTAAA-3′ and 5′-UUUAAAUUCACCAAUACCUAUUCCG-3′, *Met*, 5′-UCCAGAAGAUCAGUUUCCUAAUUCA-3′ and 5′-UGAAUUAGGAAACUGAUCUUCUGGA-3′, *ErbB3*, 5′-GGCCAUGAAUGAAUUCUCUACUCUA-3′ and 5′-UAGAGUAGAGAAUUCAUUCAUGGCC-3′, *Gab1*, 5′-UAGAGUAGCAGAGGAUGAAUCUGCC-3′ and 5′-GGCAGAUUCAUCCUCUGCUACUCUA-3′, *ALK #1*, 5′-UCAUUAUCCGGUAUACAGGCCCAGG-3′ and 5′-CCUGGGCCUGUAUACCGGAUAAUGA-3′, and ALK #2, 5′-AAAGCUGCACUCCAGACCAUAUCGG-3′ and 5′-CCGAUAUGGUCUGGAGUGCAGCUUU-3′. Each assay was carried out at least in triplicate, with 3 independent experiments conducted.

Western blotting

SDS polyacrylamide gels (Bio-Rad) were loaded with 40 µg total protein per lane; following electrophoresis, the proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad), which were incubated with Blocking One (Nacalai Tesque) for 1 hour at room temperature, followed by overnight incubation at 4°C with anti-ALK (C26G7), anti–phospho-ALK (Tyr1604), anti–phospho-EGFR (Tyr1068), anti-STAT-3(79D7), anti–phospho-STAT-3 (Y705), anti-Akt, anti–phospho-Akt (Ser473), anti-ErbB4 (111B2), anti–phospho-ErbB4 (Tyr1284), anti-Met (25H2), anti–phospho-Met (Y1234/Y1235) (3D7), anti-Gab1 (#3232), anti-phospho-Gab1 (Tyr627) (C32H2), anti-ErbB3 (1B2), anti–phospho-ErbB3 (Tyr1289) (21D3), or anti-β-actin (13E5) antibodies (1:1,000 dilution each; Cell Signaling Technology), or with anti-human EGFR (1 µg/mL), anti-human/mouse/rat extracellular signal-regulated kinase (Erk)1/Erk2 (0.2 µg/mL), or anti-phospho-Erk1/Erk2 (T202/Y204) (0.1 µg/mL) antibodies (R&D Systems). After washing 3 times, the membranes were incubated for 1 hour at room temperature with secondary Ab (horseradish peroxidase–conjugated species-specific Ab). Immunoreactive bands were visualized with SuperSignal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (Pierce). Each experiment was carried out at least 3 times independently.

HGF, EGF, TGF-α, and HB-EGF production in cell culture supernatant

Cells (2×10^5) were cultured in 2 mL of RPMI-1640 or DMEM with 5% FBS for 24 hours. The cells were washed with PBS and incubated for 48 hours in RPMI-1640 or DMEM with 5% FBS. The culture medium was harvested and centrifuged, and the supernatant was stored at −70°C until analysis. HGF (Immunis HGF EIA; B-Bridge International), EGF, TGF-α, and HB-EGF (Quantikine ELISA kits; R&D Systems) were assayed by ELISA, in accordance with the manufacturer's procedures. All samples were run in triplicate. Color intensity was measured at 450 nm with a spectrophotometric plate reader. Growth factor concentrations were determined by comparison with standard curves. The detection limits for HGF, EGF, TGF-α, and HB-EGF were 0.1 ng/mL, 3.9 pg/mL, 15.6 pg/mL, and 31.2 pg/mL, respectively.

Coculture of lung cancer cells with fibroblasts or endothelial cells

Cells were cocultured in Transwell Collagen–Coated chambers separated by an 8-µm (BD Biosciences, Erembodegem) or 3-µm (Corning Costar) pore size filter. Tumor cells (8×10^3 cells/800 µL) with or without TAE684

269 (100 nmol/L) or crizotinib (100 nmol/L) in the lower chamber were cocultured with MRC-5 (1×10^4 cells/300 μL) or HMVEC (1×10^4 cells/300 μL) cells, with or without 2 hours of pretreatment with anti-human HGF antibody (2 μg/mL) or cetuximab (2 μg/mL) in the upper chamber for 72 hours. The upper chamber was then removed, 200 μL of MTT solution (2 mg/mL; Sigma) was added to each well and the cells were incubated for 2 hours at 37°C. The media were removed and the dark blue crystals in each well were dissolved in 400 μL of DMSO. Absorbance was measured with an MTP-120 Microplate reader (Corona Electric) at test and reference wavelengths of 550 and 630 nm, respectively. The percentage growth was measured relative to untreated controls. All samples were assayed at least in triplicate, with each experiment conducted 3 times independently.

Xenograft studies in SCID mice

Suspensions of H2228 cells (5×10^6), with or without MRC-5 cells (5×10^6), were injected subcutaneously into the backs of 5-week-old male severe combined immunodeficient (SCID) mice (Japan Clea). After 4 days (tumors diameter >4 mm), mice were randomly allocated into groups of 6 animals to receive TAE684 (1.25 mg/kg/d) or vehicle by oral gavage. Tumor size was measured with digital calipers, and tumor volume was calculated as $0.5 \times \text{length} \times (\text{width})^2$. All animal experiments complied with the Guidelines for the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (approval no. AP-081088).

HGF production in tumor tissues

Tumors obtained from SCID mice after 4 and 8 days were lysed in mammalian tissue lysis buffer containing a phosphatase and proteinase inhibitor cocktail (Sigma). HGF was quantitated by ELISA (Immunis HGF EIA; Institute of Immunology), with a detection limit of 0.1 ng/mL. All samples were assayed in triplicate.

Statistical analysis

Differences were analyzed by one-way ANOVA. All statistical analyses were carried out using GraphPad Prism Ver. 4.01 (GraphPad Software, Inc.). $P < 0.05$ was considered significant.

Results

HGF and/or EGFR ligands reduced the sensitivity of EML4-ALK lung cancer cells to ALK inhibitor *in vitro*

We first examined the sensitivity of human H2228, human H3122, and mouse MANA2 lung cancer cell lines, all containing EML4-ALK translocations, to the ALK inhibitors crizotinib and TAE684, and to various EGFR-TKIs. Human H2228 cells with *EML4-ALK* variant 3 (E6;A20) and H3122 cells with *EML4-ALK* variant 1 (E13;A20) were insensitive to the EGFR-TKIs erlotinib (a reversible EGFR-TKI) and WZ4002 (selective for mutant EGFR), but sensitive to the ALK-TKIs crizotinib and TAE684 (Fig. 1). MANA2 cells, established from lung tumors of an *EML4-ALK* variant 1 (E13;A20) transgenic mouse, were also sensitive to crizotinib and TAE684, although their viability was slightly inhibited by high concentrations (1 μmol/L) of EGFR-TKIs.

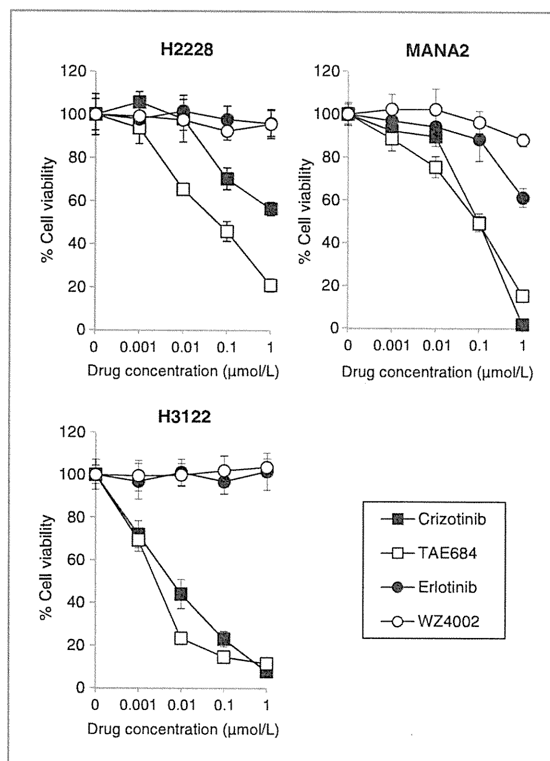

Figure 1. EML4-ALK lung cancer cells are highly sensitive to the ALK inhibitors, crizotinib, and TAE684. The sensitivity of EML4-ALK lung cancer cells, human H2228, human H3122, and mouse MANA2, to the ALK inhibitors, crizotinib, and TAE684 were determined by analyzing the effects of the EGFR-TKIs, erlotinib (reversible EGFR-TKI), and WZ4002 (mutant EGFR selective TKI). Tumor cell growth after 72 hours was measured by the MTT assay. Each sample was assayed in triplicate, with each experiment repeated at least 3 times independently. Q3

Because several growth factors have been associated with poor patient prognosis and/or drug resistance in lung cancer, we explored the effect of EGFR ligands (EGF, TGF-α, and HB-EGF), IGF-1, PDGF-AA, and HGF on the sensitivity of *EML4-ALK* lung cancer cells to ALK inhibitors. In the absence of ALK inhibitors, these growth factors slightly increased the viability of H2228, H3122, and MANA2 cells. In H2228 cells, all 3 EGFR ligands reduced sensitivity to crizotinib in a dose-dependent manner, but IGF-1, PDGF-AA, and HGF failed to do so (Fig. 2, Supplementary Fig. S1). Interestingly, HGF, as well as the EGFR ligands, reduced sensitivity to TAE684, but IGF-1 and PDGF-AA failed to do so. Similar results were observed in H3122 and MANA2 cells. To further confirm the effect of these growth factors on specific ALK inhibition, we knocked down ALK using 2 different specific siRNAs in H2228 cells. Whereas H2228 cells were highly sensitive to *ALK*-specific siRNAs, EGFR ligands and HGF restored cell viability inhibited by ALK knockdown (Supplementary Fig. S2). When we 344

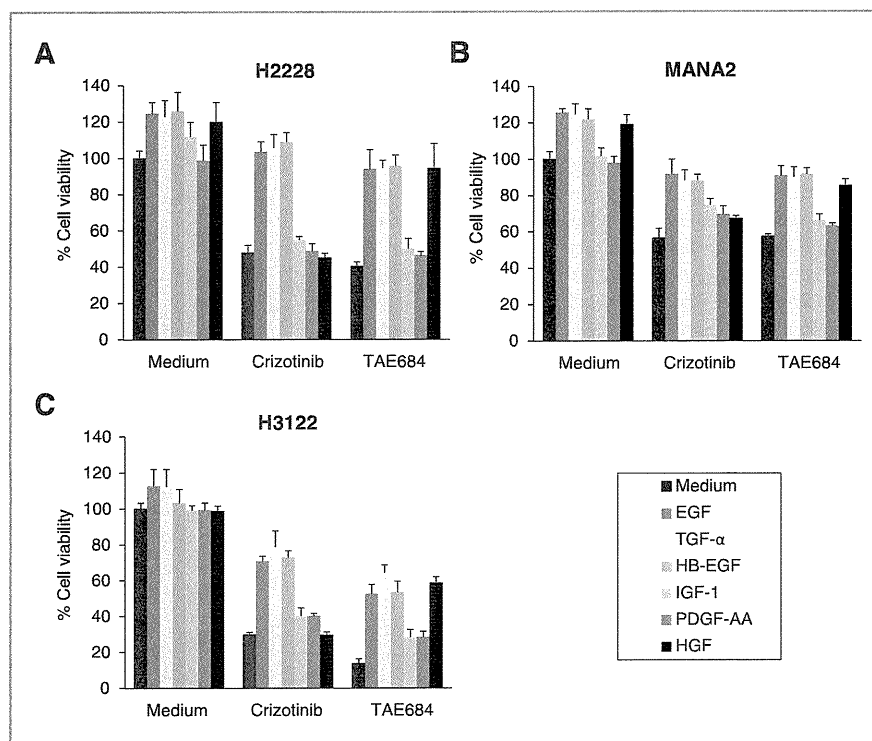

Figure 2. HGF and/or EGFR ligands (EGF, TGF-α, and HB-EGF) reduce the sensitivity of EML4-ALK lung cancer cells to ALK inhibitors *in vitro*. H2228, H3122, and MANA2 cells were incubated with or without crizotinib (100 nmol/L), TAE684 (100 nmol/L), and/or EGF, TGF-α, IGF-I, or PDGF-AA (100 ng/mL); HB-EGF (10 ng/mL), or HGF (50 ng/mL), with cell growth determined after 72 hours. The percentage growth is shown relative to untreated controls. Each sample was assayed in triplicate, with each experiment repeated at least 3 times independently.

assessed the ability of crizotinib to induce apoptosis in H2228 and H3122 cells, we found that crizotinib induced apoptosis in H3122, but not H2228, cells (Supplementary Fig. S3).

HGF and EGFR ligands trigger ALK inhibitor resistance via Met and EGFR, respectively

To assess the mechanism by which these growth factors reduced cell sensitivity to ALK inhibitors, we analyzed the phosphorylation status of ALK, receptors, and their downstream molecules in H2228, H3122, and MANA2 cells by Western blotting. Crizotinib inhibited ALK phosphorylation, thereby suppressing the phosphorylation of Akt, Erk1/2 and STAT-3, as described (ref. 11; Fig. 3A, Supplementary Fig. S4). The EGFR ligands, EGF, TGF-α, and HB-EGF stimulated EGFR phosphorylation. Crizotinib inhibited ALK and STAT-3 phosphorylation even in the presence of EGFR ligands, but failed to inhibit phosphorylation of EGFR and downstream Akt, and Erk1/2. Phosphorylation of ErbB4, a potential receptor for HB-EGF, was not affected by crizotinib or EGFR ligands. To further confirm the involvement of EGFR in crizotinib resistance induced by EGFR ligands, we knocked down EGFR by specific siRNAs in H2228 and H3122 cells (Fig. 3B). Although crizotinib markedly inhibited cell viability and all 3 EGFR ligands induced resistance in cells treated with scrambled siRNA, resistance to crizotinib was not induced by EGF, TGF-α, or HB-EGF in EGFR siRNA-treated cells, indicating that EGFR ligand-triggered crizotinib resistance is mediated by EGFR.

In parallel experiments, TAE684 inhibited ALK phosphorylation, thereby suppressing the phosphorylation of Akt, Erk1/2, and STAT-3 (Fig. 3C). HGF stimulated the phosphorylation of Met and its adaptor protein, Gab1, as described (29). TAE684 inhibited ALK and STAT-3 phosphorylation even in the presence of HGF, but failed to inhibit phosphorylation of Met and downstream Akt and Erk1/2. Phosphorylation of ErbB3, an adaptor of amplified, but not HGF-stimulated Met (30), was not affected by TAE684 or HGF. To further confirm the involvement of Met and Gab1 in HGF-induced TAE684 resistance, we knocked down Met, ErbB3, or Gab1 by specific siRNAs in H2228 and H3122 cells (Fig. 3D). TAE684 markedly inhibited the viability and HGF induced resistance in cells treated with scrambled siRNA. Importantly, treatment of cells with Met or Gab1, but not ErbB3, siRNA, induced TAE684 resistance, indicating the involvement of Met/Gab1 in HGF-induced resistance to TAE684.

Cross-talk of endothelial cells and fibroblasts reduces the sensitivity of EML4-ALK lung cancer cells to ALK inhibitors

To determine which types of host cells could produce EGFR ligands and HGF, we investigated production of these growth factors by various types of host stromal cells, comparing lung epithelial cells and cancer cells. The endothelial cell lines HMVEC produced discernible levels of EGFR ligands, including EGF, TGF-α, and HB-EGF, whereas fibroblasts produced a high level of HGF (Fig. 4A). EML4-ALK lung cancer cells (H2228, H3122, and

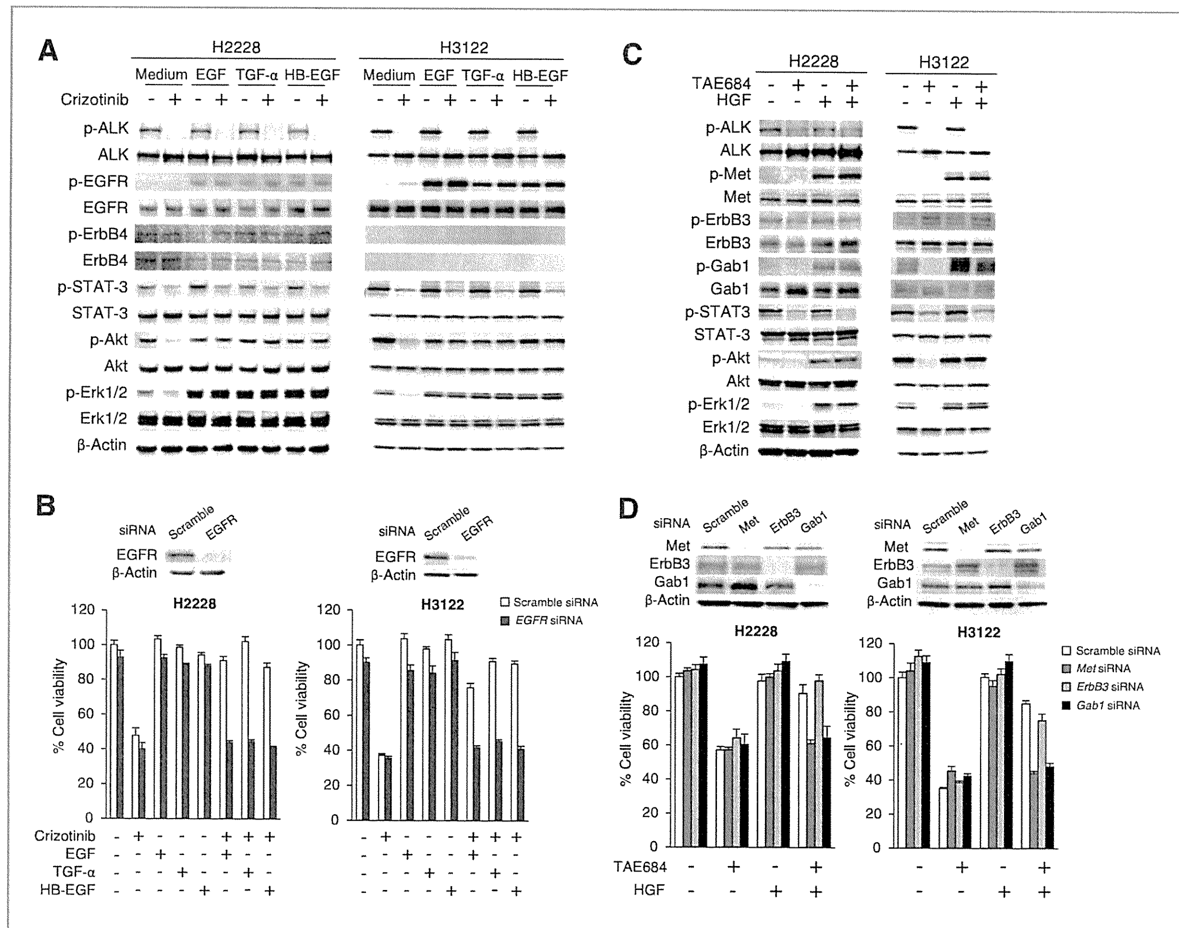

Figure 3. HGF and EGFR ligands trigger ALK inhibitor resistance via Met/Gab1 and EGFR, respectively. A, crizotinib inhibited the phosphorylation of ALK and STAT-3 but did not that of EGFR, Akt, and Erk1/2 in the presence of EGF, TGF-α, or HB-EGF. Tumor cells were treated with or without crizotinib (100 nmol/L) for 1 hour and/or EGF (100 ng/mL), TGF-α (100 ng/mL), or HB-EGF (10 ng/mL) for 15 minutes. The cells were lysed and the indicated proteins were detected by immunoblotting. The results shown are representative of 3 independent experiments. B, control or *EGFR*-specific siRNAs were introduced into H2228 and H3122 cells. After 24 hours, the cells were incubated with or without crizotinib (100 nmol/L), and/or EGF (100 ng/mL), TGF-α (100 ng/mL), or HB-EGF (10 ng/mL) for 72 hours and lung cancer cell growth was determined by MTT assays. *EGFR* knockdown was confirmed by immunoblotting. The percentage of growth is shown relative to untreated controls. Each sample was assayed in triplicate, with each experiment repeated at least 3 times independently. C, TAE684 inhibited the phosphorylation of ALK and STAT-3, but not of Met, Gab1, Akt, and Erk1/2 in the presence of HGF. Tumor cells were treated with or without TAE684 (100 nmol/L) for 1 hour and/or HGF (50 ng/mL) for 15 minutes. The cells were lysed and the indicated proteins were detected by immunoblotting. The results shown are representative of 3 independent experiments. D, control or *Met*, *ErbB3*, or *Gab1*-specific siRNAs were introduced into H2228 and H3122 cells. After 24 hours, the cells were incubated with or without TAE684 (100 nmol/L) and/or HGF (50 ng/mL) for 72 hours and lung cancer cell growth was determined by MTT assays. *Met*, *Gab1*, and *ErbB3* knockdowns were confirmed by immunoblotting. The percentage of growth is shown relative to untreated controls. Each sample was assayed in triplicate, with each experiment repeated at least 3 times independently.

MANA2) and lung epithelial cells (BEAS-2B) produced low or no detectable levels of EGFR ligands or HGF. Interestingly, coculture of H2228 or H3122 cells with fibroblasts (MRC-5) significantly reduced their sensitivity to TAE684, an effect abrogated by anti-HGF antibody (Fig. 4B). Coculture with endothelial cells (HMVEC) also reduced sensitivity to crizotinib, an effect inhibited by anti-EGFR antibody (Fig. 4C).

These results suggested that host stromal cells, such as endothelial cells and fibroblasts, may regulate sensitivity to ALK inhibitors by secreting EGFR ligands and HGF, respectively.

HGF derived from fibroblasts induces TAE684 resistance of EML4-ALK lung cancer cells *in vivo*

To investigate whether sensitivity to TAE684 could be affected by fibroblasts *in vivo*, we subcutaneously inoculated H2228 cells, with or without MRC-5 cells, into SCID mice. The tumors of mice injected with H2228 and MRC-5 cells grew slightly faster than those of mice injected with

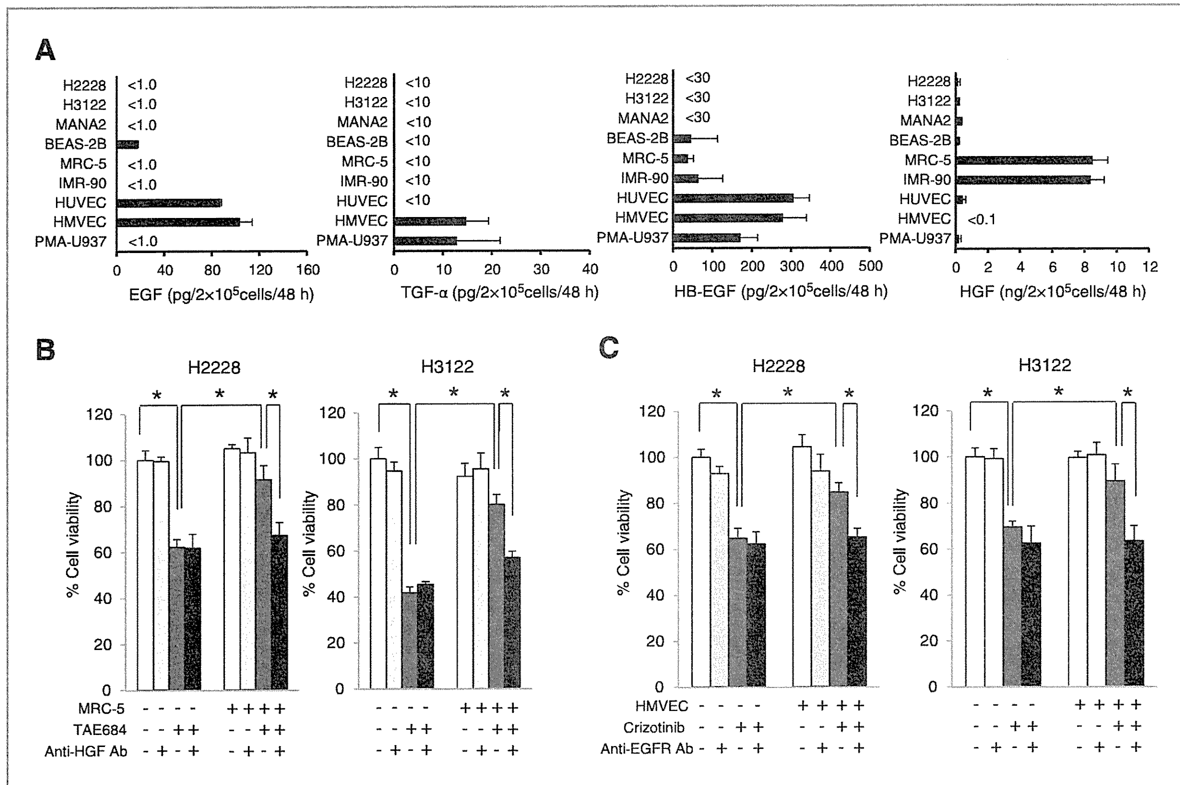

Figure 4. Cross-talk of endothelial cells and fibroblasts reduces sensitivity of EML4-ALK lung cancer cells to ALK inhibitors. A, receptor ligand production was assayed in lung cancer (H2228, H3122, and MANA2,), human bronchial epithelial cell (BEAS-2B), fibroblasts (MRC-5 and IMR-90), endothelial cells (HUVEC and HMVEC), and the macrophage differentiated cell line (PMA-U937). The cells were incubated in medium for 48 hours, culture supernatants were harvested, and EGF, TGF-α, HB-EGF, and HGF concentrations were determined by ELISA. All samples were assayed in triplicate. B, H2228 and H3122 cells were cocultured with or without fibroblasts, MRC-5 cells, and/or anti-HGF–neutralizing antibody (2 μg/mL), in the presence or absence of TAE684 (100 nmol/L) for 72 hours, with cell growth determined by MTT assays. *, $P < 0.05$ (one-way ANOVA). Each experiment included triplicate determinations, and each experiment was repeated at least 3 times independently. C, endothelial cell–derived EGFR ligands induced crizotinib resistance in lung cancer cells with EML4-ALK fusion protein, an induction abrogated by blockade of EGFR. H2228 and H3122 cells were cocultured with or without endothelial cells, HMVECs, and/or anti-EGFR–neutralizing antibody (2 μg/mL) in the presence or absence of crizotinib (100 nmol/L) for 72 hours, with cell growth determined as in B. *, $P < 0.05$ (one-way ANOVA). Each experiment included triplicate determinations, with each experiment repeated at least 3 times independently.

H2228 cells alone, but the difference was not statistically significant by day 8 (Fig. 5A). TAE684 treatment, beginning on day 4, caused marked regression of tumors in mice injected with H2228 cells alone, but not of tumors in mice injected with H2228 and MRC-5 cells, indicating that fibroblasts induced resistance to TAE684 *in vivo* (Fig. 5A). We confirmed that HGF was produced by MRC-5 cells *in vivo*. Although the tumors of mice injected with H2228 cells alone did not produce detectable levels of HGF, the tumors of mice injected with H2228 and MRC-5 cells produced high levels of HGF, started on day 4, but decreasing slightly on day 8 (Fig. 5B).

We further analyzed whether coinjection of MRC-5 cells restored the Akt pathway inhibited by TAE684 in the tumors. Western blotting showed that TAE684 treatment inhibited Akt phosphorylation, which was restored by coinjection of MRC-5 cells (Fig. 5C). These results suggested that fibroblasts produced HGF in the tumors and restored Akt phosphorylation as a survival signal, as well as inducing resistance to TAE684 in EML4-ALK lung cancer cells *in vivo*.

Ligand-triggered resistance to ALK inhibitors is abrogated by inhibitors of both HGF-Met and EGFR

To establish novel strategies to treat EGFR ligand- or HGF-triggered resistance to ALK inhibitors, we examined the effect of combinations of ALK inhibitors with EGFR inhibitors (anti-EGFR Abs and reversible EGFR-TKIs) and HGF-Met inhibitors (anti-HGF Abs and Met-TKIs). Combined treatment with erlotinib, a reversible EGFR-TKI and cetuximab, an anti-EGFR Ab, successfully resensitized H2228 and H3122 cells to crizotinib even in the presence of the EGFR ligands, EGF (Fig. 6A), TGF-α (Fig. 6B), and HB-EGF (Fig. 6C). Moreover, the combination of HGF with E7050 (Met-TKI) or anti-HGF Ab resensitized cells to TAE684 (Fig. 6D).

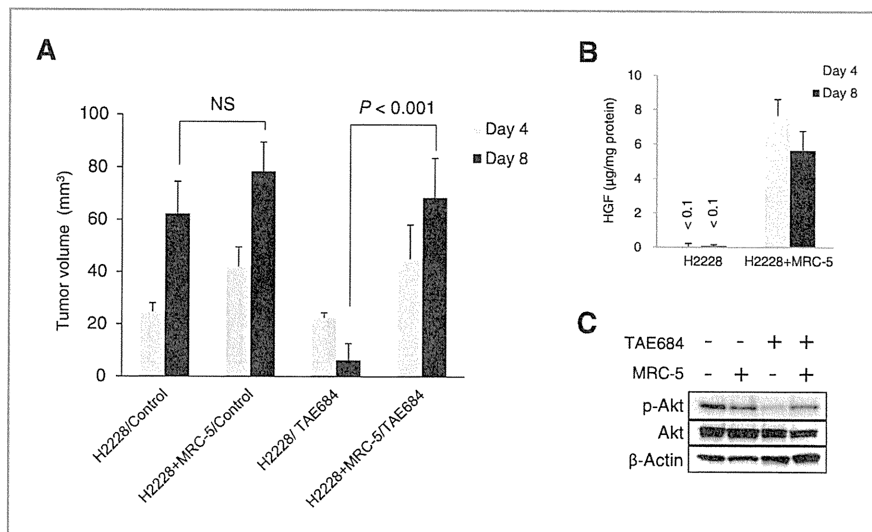

Figure 5. HGF derived from fibroblasts induces TAE684 resistance of EML4-ALK lung cancer cells *in vivo*. A, fibroblast-derived HGF induced TAE684 resistance in H2228 tumors in SCID mice. H2228 cells (5×10^6), with or without MRC-5 cells (5×10^6), were inoculated subcutaneously into SCID mice on day 0. Starting on day 4, mice received oral TAE684 (1.25 mg/kg/d) or vehicle alone, with tumor size measured on days 4 and 8. Tumor volumes were calculated as described in Materials and Methods. Data shown are the representative of 2 independent experiments. Error bars indicate SEs of 6 mice. $P < 0.05$ was considered significant by one-way ANOVA. NS, not significant. B, HGF production by tumor tissues. Tumors were harvested on days 4 and 8 and lysed, and HGFs in the lysates were assayed by ELISA. All samples were assayed in triplicate. C, fibroblast-derived HGF induced TAE684 resistance via the Akt signal pathway *in vivo*. Tumors were harvested 2 hours after treatment on day 7 and lysed, and the lysates were analyzed by immunoblotting with the indicated antibodies, as described in Materials and Methods. The results shown are representative of 2 independent experiments.

Discussion

We have shown here that endothelial cells and fibroblasts, both components of the tumor microenvironment, secreted EGFR ligands and HGF, respectively, causing resistance to the ALK inhibitors crizotinib and/or TAE684 by activating bypass survival signals.

Of the EGFR ligands, EGF and TGF-α bind predominantly to EGFR, whereas HB-EGF binds to EGFR and ErbB4 (17). H2228 cells expressed both EGFR and ErbB4. Our results suggested that the bypass survival signal induced by EGFR ligands is mediated mainly by EGFR, as EGFR ligands markedly activated the phosphorylation of EGFR, not ErbB4. Moreover, knockdown of EGFR abrogated resistance caused by all EGFR ligands tested. EGFR ligand–triggered resistance was canceled by erlotinib or cetuximab, an anti-EGFR Ab, drugs approved for the treatment of patients with NSCLC and colorectal cancer. In addition, AP26113, an inhibitor of both ALK and EGFR, has been reported active against *EML4-ALK* lung cancer cells with amplified *ALK* and secondary mutations (7). Therefore, clinical trials are warranted to evaluate the efficacy and feasibility of combinations of an ALK inhibitor and these EGFR inhibitors to overcome ALK inhibitor resistance.

HGF, the sole ligand of Met (29), is important in EGFR-TKI resistance in *EGFR*-mutant lung cancer. HGF derived from cancer cells or stromal fibroblasts activated Met phosphorylation and stimulated the downstream Akt and Erk1/2 pathways (21, 22, 30) using Gab1, an adaptor protein for Met (31), triggering resistance to both reversible and irreversible EGFR-TKIs. In our Japanese cohort study of patients with *EGFR*-mutant lung cancer, high HGF expression was detected in 61% of tumors with acquired resistance and in 29% of tumors with intrinsic resistance to EGFR-TKIs, suggesting the rationale of targeting HGF to overcome EGFR-TKI resistance (32). We also found that HGF triggered TAE684 resistance by activating Met and stimulating downstream Akt and Erk1/2 pathways using the adaptor protein Gab1. Because many anti-HGF Abs and Met-TKIs are being evaluated in clinical trials, HGF-triggered resistance to selective ALK inhibitors may be controlled by their combinations in the near future.

EGFR and Met have been shown to interact with each other and to mediate redundant signaling in lung cancer cells (33). In *EGFR*-mutant lung cancer cells, *Met* amplification causes EGFR-TKI resistance by triggering bypass survival signals using ErbB3, an adaptor protein (34). Met activation by HGF also triggers resistance to EGFR-TKIs that use Gab1 as an adaptor. In *EML4-ALK* lung cancer cells, both novel ALK second mutations and autocrine EGFR activation causes resistance to ALK inhibitors (11). We found that paracrine HGF and EGFR ligands could trigger ALK inhibitor resistance. Taken together, these findings suggest that signaling by EGFR and Met is crucial for the survival of lung cancer cells with *EGFR* mutations and *EML4-ALK* translocations under inhibition of these driver oncogenes.

We found that resistance to TAE684 was induced by both EGFR ligands and HGF, whereas crizotinib resistance was induced by EGFR ligands alone, a finding that may be due to the dual activities of crizotinib on ALK and