Lung Cancer Cell Lines Harboring *MET* Gene Amplification Are Dependent on Met for Growth and Survival

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Abstract

Recent clinical successes of small-molecule epidermal growth factor receptor (EGFR) inhibitors in treating advanced non–small cell lung cancer (NSCLC) have raised hopes that the identification of other deregulated growth factor pathways in NSCLC will lead to new therapeutic options for NSCLC. Met, the receptor for hepatocyte growth factor, has been implicated in growth, invasion, and metastasis of many tumors including NSCLC. To assess the functional role for Met in NSCLC, we evaluated a panel of nine lung cancer cell lines for *Met* gene amplification, Met expression, Met pathway activation, and the sensitivity of the cell lines to short hairpin RNA (shRNA)–mediated Met knockdown. Two cell lines, EBC-1 and H1993, showed significant Met gene amplification and overexpressed Met receptors which were constitutively phosphorylated. The other seven lines did not exhibit *Met* amplification and expressed much lower levels of Met, which was phosphorylated only on addition of hepatocyte growth factor. We also found a strong up-regulation of tyrosine phosphorylation in β-catenin and p120/δ-catenin in the Met-amplified EBC-1 and H1993 cell lines. ShRNA-mediated Met knockdown induced significant growth inhibition, G_1-S arrest, and apoptosis in EBC-1 and H1993 cells, whereas it had little or no effect on the cell lines that do not have *Met* amplification. These results strongly suggest that *Met* amplification identifies a subset of NSCLC likely to respond to new molecular therapies targeting Met. [Cancer Res 2007;67(5):2081–8]

Introduction

Lung cancer is one of the most prevalent malignancies and the leading cause of cancer-related mortality in North America and throughout the world. Non–small cell lung cancer (NSCLC) accounts for ~80% of lung cancer (1). Standard therapies for advanced NSCLC include radiotherapy and chemotherapy, which confer palliative and moderate survival benefits with significant toxicities (1). To address the substantial unmet medical needs in NSCLC, extensive efforts have been made to identify molecular defects underlying the genesis and progression of NSCLC and to discover novel therapeutic agents targeting these defects. Recent clinical success of epidermal growth factor receptor (EGFR) inhibitors in refractory, advanced NSCLC (2) have raised hopes that targeting other deregulated growth factor signaling, such as the hepatocyte growth factor (HGF)/Met pathway, will lead to new therapeutic options for NSCLC.

Met is a heterodimeric transmembrane receptor tyrosine kinase composed of an extracellular α-chain disulfide-bonded to a membrane-spanning β-chain (3, 4). The tyrosine kinase domain resides in the cytosolic domain of the β-chain (3, 4). Binding of HGF/SF to Met induces receptor dimerization and trans-phosphorylation, triggering conformational changes that activate Met tyrosine kinase activity (5). The resultant tyrosine phosphorylation of a multi-substrate docking site, located near the COOH terminus of the Met β-chain, mediates the activation of a number of signaling pathways, including phosphoinositide-3-kinase/phosphoinositide-dependent protein kinase 1/AKT, Ras-Rac/Rho, Ras-mitogen-activated protein kinase, and phospholipase C-γ pathways (5). In addition to proliferative and antiapoptotic activities that are common to many growth factors, HGF/Met elicits unique motogenenic and morphogenic effects by stimulating cell-cell detachment, migration, invasiveness, and tubule formation and branching (5).

Aberrant Met signaling has been implicated in the development, maintenance, and progression of cancers in both animals and humans (5). Transgenic mice overexpressing HGF develop tumors in a number of tissues, and tissue-specific transgenic overexpression of Met or HGF causes or promotes malignant transformation of hepatocytes, airway epithelium, and mammary epithelium (5). In humans, Met plays a central role in hereditary papillary renal carcinoma, which is causally related to gain-of-function germ line mutations in the Met tyrosine kinase domain (6). Gain-of-function Met mutations have also been found in several nonrenal tumors, including lung cancers (5, 7). Notably, a somatic intronic mutation in the *Met* gene has been identified recently in lung cancer, resulting in a deletion in the juxtamembrane domain and stimulation of Met-transforming activity *in vitro* (8). In addition to mutations, several reports have shown genomic amplification of *Met* to occur in 5% to 10% of gastric cancers (9–11). Recent work has also identified *Met* amplification in 4% of esophageal (12) and 4% of lung cancers (13). While this work was in progress, gastric cancer cell lines with *Met* amplification were reported to be dependent on the amplified Met kinase for growth *in vitro*, indicating a critical role for Met in *in vitro* cell growth and survival (14). *Met* amplification also has clinical significance in that both *Met* gene amplification as well as Met and/or HGF overexpression have been correlated with poor clinical outcome in a variety of human cancers (5).

To assess the functional roles of Met in NSCLC, we evaluated a panel of lung cancer cell lines for *Met* gene amplification, Met expression, Met pathway activation, and the sensitivity of the cell lines to short hairpin RNA (shRNA)–mediated Met knockdown. ShRNA-mediated Met knockdown induced significant growth inhibition, G_1-S arrest, and apoptosis in cell lines harboring *Met*

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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gene amplification, whereas it had little or no effects on the cell lines that do not have *Met* amplification. These results suggest that *Met* amplification may identify a subset of NSCLC sensitive to emerging Met inhibitors.

Materials and Methods

Cell lines. Cell lines A549, H226, H596, Calu-1, SW900, SK-MES-1, H1048, and H1993 were obtained from American Type Culture Collection (Manassas, VA). EBC-1 was from the Health Science Research Resources Bank (Japan Health Sciences Foundation, Tokyo, Japan). Cells were maintained in Iscoves DMEM plus 10% FCS and 40 μg/mL gentamicin (Sigma, St. Louis, MO). H1048 growth medium was supplemented with 8 μg/mL insulin (Invitrogen, Grand Island, NY). GTL-16 cells were a gift from S. Giordano and P.M. Comoglio (University of Torino Medical School, Candiolo, Italy).

Quantitative PCR for analysis of *Met* genomic amplification. Quantitative PCR was done on genomic DNA purified using the DNA easy kit (Qiagen, Valencia, CA). Primers and probe used for MET are (5′ to 3′) F-TGTTGCCAAGCTGTATTCTGTTTAC; R-TCTCTGAATTAGAGCGATGTT-GACA, VIC-TGGATAATTGTGTCTTTCTCTAG-MGBNFQ (14). Primers and probe for single-copy reference gene RNase P, which encodes the RNA moiety for the RNase P enzyme, are a product of Applied Biosystems (Foster City, CA), as well as TaqMan Universal PCR reagent mix. Reactions were done in quadruplicate with 20 ng of genomic DNA, primers at 900 nmol/L, and probes at 250 nmol/L under standard thermocycling conditions (2 min at 50°C; 10 min at 95°C; 40 cycles of 15 s 92°C; and 1 min 60°C) Data were normalized to RNase P and then to the calibrator sample (normal lung genomic DNA; BioChain Institute, Hayward, CA).

ShRNA production and infection. shRNA sequences for Met were M1: GAAGATCACGAAGATCCCATTCAAGAGATGGGATCTTCGTGATCTTC; M2: GATCTGGGCAGTGAATTAGTTCAAGAGACTAATTCACTGCCCA-GATC; M3: GAGCTGTGATAATATACACTTCAAGAGAGTGTATATTCTCA-CAGCTC. For FGFR2, GCCAACCTCTCGAACAGTATTCAAGAGATACTGTT-CGAGAGGTTGGC, and for luciferase: CACCGGTGTTGTAACAATATCGAC-GAATCGATATTGTTACAACACCAAAA. These oligonucleotides were annealed to make double-stranded oligonucleotides with a 5′ *Bbs*I overhang and a 3′ *Spe*I overhang. The annealed oligonucleotides were cloned into a proprietary *Bbs*I/*Spe*I cut ENTR plasmid (expression from mouse U6 promoter) Gateway technology (Invitrogen) was used to convert into Plenti6/Block-iT-DEST (Invitrogen).

Virus was produced using the Invitrogen lentiviral expression system as directed by the supplier with the following exceptions. Plasmids were transfected into 2.5×10^7 293FT cells on 15-cm polylysine-coated plates, and the supernatant was collected at 48 and 72 h posttransfection. Titer was determined using colony formation under blasticidin selection as described by Invitrogen (Invitrogen lentiviral expression system). Lentiviral infection was at a multiplicity of infection (MOI) of 10 for EBC-1 and MOI of 20 for the remaining cell lines. For growth analysis, cells were seeded at 4000 cells per well in 96-well plates, whereas for Western analysis, cells were seeded at 40,000 cells in 12-well plates. Viral supernatants were added for 14 to 16 h in the presence of 6 μg/mL polybrene, at which point viral supernatants were removed, and cells were washed once with PBS and replaced with growth medium containing 15% fetal bovine serum.

Proliferation analysis. Cell growth was analyzed using the Vialight assay kit (Cambrex, Rockland, ME). For some assays, independent analysis by cell counting on a hematocytometer confirmed the results of Vialight.

Western blotting, immunoprecipitation, antibodies, and growth factors. After removing growth medium, tissue culture dishes were placed on ice and lysis buffer containing 30 mmol/L Tris-HCl (pH, 7.5), 50 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L NaF, 30 mmol/L NaPPi, 1% Triton, 0.5% IGEPAL, 10% glycerol, 1 mmol/L vanadate, 1 mmol/L bpPhen (Calbiochem, San Diego, CA), and protease inhibitor cocktail (Roche, Indianapolis, IN) was added with shaking for 10 min at 4°C. Cells were scraped from dishes, transferred to Eppendorf tubes followed by freezing at −70°C, thawing, and clarification of cellular debris by centrifugation at 20,000 × *g* for 5 min at 4°C. SDS-PAGE was on 40 μg of cell lysates, and Western blotting followed standard procedures. For β-catenin immunoprecipitation, the cell lysate was incubated with 2 μg of antibody for 1 h, then 15 μL of agarose-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) was added, and the incubation was continued overnight. The next day, the beads were pelleted and washed thrice in lysis buffer plus phosphatase and protease inhibitors. Proteins were eluted by boiling in Laemmli buffer followed by SDS-PAGE and Western blotting. The following antibodies were used: Met 25H2 (Cell Signaling Technology, Danvers, MA) or AF276 (R&D Systems, Minneapolis, MN); Y1349 Met, Y1234,1235 Met, Y845 EGFR, phosphorylated AKT (pAKT) 473, AKT, pERK, extracellular signal-regulated kinase (ERK), poly(ADP-ribose) polymerase (PARP), caspase-3 (8G10), and β-actin antibodies were from Cell Signaling Technology. Y1173 EGFR antibody was from Biosource Invitrogen (Carlsbad, CA). 4G10 phosphotyrosine was from Upstate (Charlottesville, VA). Antibody to Y228 δ-catenin was from BD Transduction Laboratories (San Jose, CA). Antibody to delta catenin was C4864 from Sigma-Aldrich (St. Louis, MO). β-Catenin antibody sc7199 and isoform-specific antibodies for K-ras (sc-30), H-ras (sc-520), and N-ras (sc-31) were from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant HGF was from R&D Systems.

Met protein levels were quantitated relative to actin using densitometry (Molecular Dynamics, Sunnyvale, CA) and Image Quant software.

Ras activation assays. Ras activation was assayed using a Ras activation kit (Upstate), which uses glutathione *S*-transferase-Raf to precipitate active Ras from cell lysates. Three hundred to 600 μg of cell lysates were used, and binding and washing were according to kit procedures.

Flow cytometry. A Becton Dickinson (Franklin Lakes, NJ) FACS Calibur was used for flow cytometry. A total of 500,000 cells were infected with virus and analyzed at the indicated time points. Nonadherent cells were collected and combined with trypsinized adherent cells. Cells were centrifuged at 250 × *g*, washed in PBS, and fixed overnight in 1 mL ice-cold 70% ethanol. Cells were then washed in PBS and stained with PI/RNase solution (BD PharMingen, San Diego, CA) for 1 h overnight. ModFit software was used to determine the relative distribution in G_1, S, G_2-M, and manual gating to determine the sub-G_1 content.

Receptor tyrosine kinase array. Array 001 (R&D Systems) was used according to supplier protocols to detect tyrosine-phosphorylated receptor tyrosine kinases. This array contains 42 receptor tyrosine kinase capture antibodies spotted in duplicate. Briefly, 50 μg of cell lysates were incubated with the membrane overnight in the provided buffer. Target proteins are captured by their respective antibodies. After washing, incubation with a phosphotyrosine antibody conjugated to horseradish peroxidase allows the detection of captured receptor tyrosine kinases that are tyrosine phosphorylated. A more detailed description of this array is provided in the Supplementary Data.

Results

We determined the protein expression levels and activation status of Met in nine NSCLC cell lines (Fig. 1*A*). The H1993 and EBC-1 cell lines expressed high levels of Met and contained high basal phosphorylation in both activation loop tyrosines (Y1234/1235) and a docking site tyrosine (Y1349). The high basal phosphorylation at Y1349 was minimally stimulated by HGF (2.5-fold and 1.7-fold in EBC-1 and H1993, respectively), whereas the high levels of Y1234/1235 phosphorylation were not further stimulated by HGF in either cell line. By contrast, the other cell lines in our panel expressed much lower levels of Met, which was phosphorylated at Y1234/1235 and Y1349 only on ligand addition. Met expression relative to actin is indicated in Fig. 1*A* below the Met panel. H1993 has been reported to contain genomic amplification of *Met* (13), whereas a comparative genomic hybridization database (link provided in Supplementary Data) revealed high copy number at the *Met* locus in the EBC-1 cell line. We verified *Met* amplification in EBC-1 and H1993, similar to GTL-16 gastric cancer cells previously shown to have *Met*

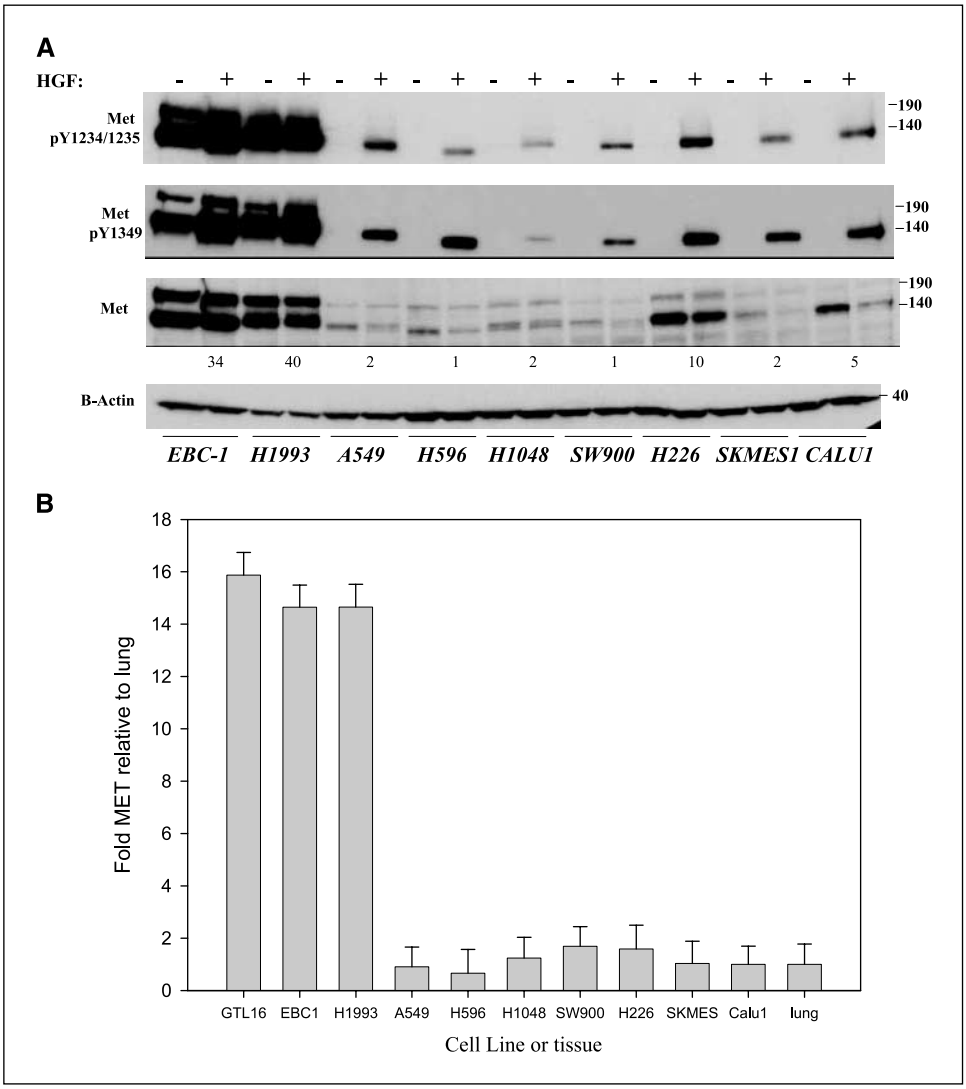

Figure 1. Met is overexpressed and contains high levels of tyrosine phosphorylation in EBC-1 and H1993 cells. *A,* cell lysates from 5×10^5 cells in six-well plates (prepared according to Materials and Methods) from untreated or HGF-treated (100 ng/mL, 5 min) cells were analyzed by SDS-PAGE and Western blotted with antibodies specific for Y1234/1235 Met, Y1349 Met, total Met, and β-actin as an internal control. The numbers on the right are molecular weights in kilodaltons. The numbers below the Met Western row indicate Met expression relative to actin, setting the lowest expressing lines arbitrarily as 1. *B, Met* is amplified in H1993 and EBC-1 cells. DNA was isolated from the indicated cell lines and subjected to quantitative PCR analysis for Met gene copy number relative to RNase P copy number, as described in Materials and Methods. GTL-16 is a gastric cancer cell line previously shown to contain *Met* gene amplification (15).

amplification (15), and found no amplification in the remaining cell lines (Fig. 1*B*).

We determined whether signaling pathways active in the ligand-independent *Met*-amplified cells are similar to the pathways activated by HGF in the non–Met-amplified cells. Although both EBC-1 and H1993 cells contain basal phosphorylation in pERK and pAKT that is not stimulated by HGF, the levels of activated ERK were much higher in nonamplified lines stimulated by HGF (Fig. 2*A*). Also in striking contrast to the nonamplified lines, we found highly elevated tyrosine phosphorylation in β-catenin and δ-catenin only in the *Met*-amplified lines (Fig. 2*A*). In addition, we observed high levels of activated ras in EBC-1 and H1993 cells, although this was similar to other cells in the panel (Fig. 2*B*). Interestingly, the amount of activated ras in EBC-1 and H1993 was similar to levels found in A549, which is homozygous for mutant K-ras, as well as Calu-1, which contains a heterozygous mutant of K-ras (16). EBC-1 has wild-type ras isoforms,[1] and H1993 K-ras was also reported be wild type (17). Using isoform-specific antibodies, we found that the activated ras isoform in EBC-1 and H1993 is exclusively K-ras (data not shown). Thus, both EBC-1 and H1993 cells have activated signaling pathways that, in some cases, are qualitatively and quantitatively dissimilar from pathways activated by HGF stimulation.

We next determined whether Met was important for NSCLC cell line growth. We used lentiviral vectors to deliver Met shRNA to EBC-1 and H1993 cells and found efficient knockdown of Met protein 72 h after infection (Fig. 3*A*). We also observed protein knockdown as early as 24 h after virus removal (see Fig. 4*B*). The Met shRNA M3 was highly efficient in decreasing Met protein (Fig. 3*A*), with M1 and M2 having less effect. A time course of growth inhibition with the potent Met shRNA M3 in H1993 and EBC-1 cells revealed that treated EBC-1 cells sustained a loss of ~50% of the starting cell number within 48 to 72 h of virus removal (Fig. 3*C, EBC-1*). Although Met knockdown in H1993 cells did not result in a decrease in starting cell numbers, there was still no significant increase in cell number from the time of virus removal (Fig. 3*B, H1993*). To test for specificity of growth inhibition by Met shRNA, we treated the remaining cell lines in our panel with the M3 shRNA, and we tested each of the three Met shRNAs for growth inhibition in EBC-1, H1993, and A549 cells. Met shRNA treatment resulted in growth inhibition in EBC-1 and H1993 cells

[1] S. Forbes, B. Choudhury, personal communication.

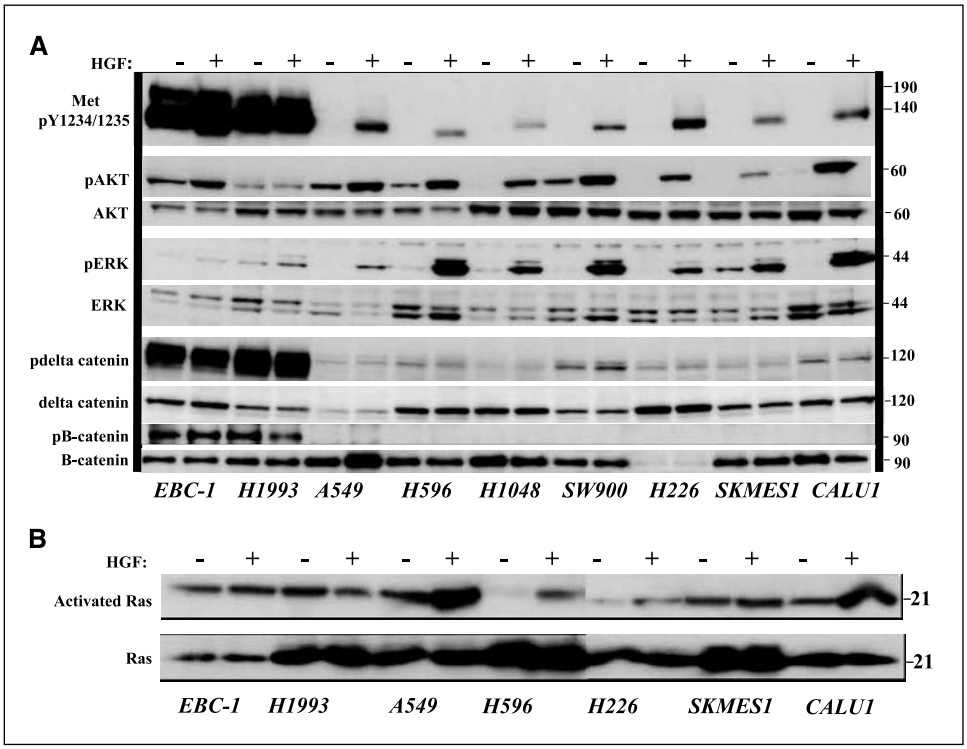

Figure 2. Met downstream signaling is activated in EBC and H1993 cells. *A,* lysates used in Fig. 1 were analyzed by Western blotting for the indicated phosphorylated or total proteins (Akt, Erk, p120 δ-catenin). β-Catenin was immunoprecipitated from the indicated lysates (100 μg) and Western blotted with 4G10 for phosphotyrosine content. *B,* Ras activation was done on separate samples (5×10^6 cells) using the lysis buffer provided by the kit as described in Materials and Methods. H1048 and SW900 lysates were not available for analysis of Ras activation. This panel is an adjoining of two noncontinuous regions of the same blot. Numbers on the right are molecular weights in kilodaltons.

that corresponded to the amount of protein knockdown in Fig. 3*A*. Proliferation in A549 and the remaining cell lines was not inhibited by Met shRNA treatment (Fig. 3*C*), despite efficient knockdown of Met (Fig. 3*D*). Although H1048 and Calu-1 showed minor growth inhibition, we note that the cell numbers in these lines increased 4-fold when treated with M3 shRNA (data not shown), which is in contrast to the potent inhibition seen in H1993 and EBC-1.

In addition to the effects on cell growth, Met shRNA resulted in a dramatic morphologic change in H1993 and EBC-1 cells (Supplementary Fig. S1); at 12 h after virus removal, the majority of cells had converted from rounded and poorly adherent to a flattened and adherent appearance. These morphology changes were not observed in untreated or control (luciferase or FGFR2 hairpin) lentivirus-treated cells.

Finally, flow cytometry was used to characterize the cell cycle profile of EBC-1 and H1993 cells treated with Met shRNA M3 (Fig. 4*A*). Within 24 h of virus removal, both EBC-1 and H1993 cells sustained a 3- to 4-fold reduction of cells in the S phase, such that the percentage of S-phase cells was decreased from 19% (in EBC-1) and 17% (in H1993) to 5% in each. This decrease in S phase persisted through the time course, suggesting that the cell cycle was blocked at G_1-S. At 48 and 72 h, there was an increase in cells in sub-G_1 that was most prominent for EBC-1 (6-fold induction at 72 h), indicative of cell death. This increase in the percentage of sub-G_1 cells is consistent with the decrease in cell number observed in Fig. 3*B*. In addition, both cell lines had a strong decrease in the G_2-M population at 48 and 72 h, consistent with a loss of cycling cells, but also suggesting that Met inhibition does not result in a block at G_2-M. The increased sub-G_1 population prominent in EBC-1 cells suggested an increase in apoptosis in these cells. Western blotting in EBC-1 cells indeed revealed the presence of cleaved PARP, an indicator of apoptosis, at 24 and 48 h (Fig. 4*B*). H1993 also revealed cleaved PARP, but to a lesser extent than EBC-1 cells, starting at 72 h after virus removal (Fig. 4*B*). The cleaved PARP was also accompanied by cleaved caspase-3 (data not shown).

Having shown that EBC-1 and H1993 cells require Met for growth, we next determined whether key signaling pathways were also inhibited after Met shRNA treatment. Twenty-four hours after M3 virus removal, the knockdown of Met protein was accompanied by a loss of tyrosine phosphorylation in δ-catenin and β-catenin, pAKT, and pERK, each accompanied by only minimal changes in protein levels (Fig. 5). There was also an almost complete loss of activated ras upon knockdown of Met (Fig. 5). These results reveal that for H1993 and EBC-1 cells, the loss of a single kinase, Met, causes a cessation of multiple signaling pathways and results in profound growth and morphology changes.

Given the role of receptor tyrosine kinase signaling in cell growth, we were interested to determine if other receptor tyrosine kinases were also active in the Met-amplified cells. We used a receptor tyrosine kinase array (Fig. 6*A*) and found that EGFR1 and Met were the predominant activated receptor tyrosine kinases in EBC-1 and H1993 cells. Surprisingly, the basal levels of phosphorylation in the EGFR activation site (Y845) and the SHC/PTP1 docking site (Y1173) were lost upon Met knockdown, with minimal change in the EGFR protein levels (Fig. 6*B*). Thus, the activation of EGFR1 in H1993 and EBC-1 cell lines was not a result of EGF present in serum, which is also supported by our finding that A549 growing in 10% serum require exogenous EGF to attain significant phosphorylation of EGFR (data not shown). Furthermore, this result suggests EGFR is a downstream signaling component of amplified Met kinase.

Discussion

In this work, we describe a critical function for the Met kinase in cancer cell proliferation and viability. For the subset of lung cancer cell lines with *Met* gene amplification, Met is highly activated, and

cell proliferation and survival are dependent on this activated Met kinase. By contrast, cells with non-amplified *Met* have very low levels of basal Met activation and are not dependent on Met for growth. The non–*Met*-amplified H596 cell line included in our analysis contains an oncogenic juxtamembrane deletion in Met (consistent with reduced SDS-PAGE mobility in Fig. 1; ref. 8). Although this mutation can enhance Met transformation in response to HGF stimulation (8) and may be a factor for *in vivo* tumor growth, it does not constitutively activate Met kinase activity and is not required for *in vitro* proliferation under standard growth conditions.

Our work thus defines the importance of amplified *Met* for NSCLC growth. While this work was in progress, another group

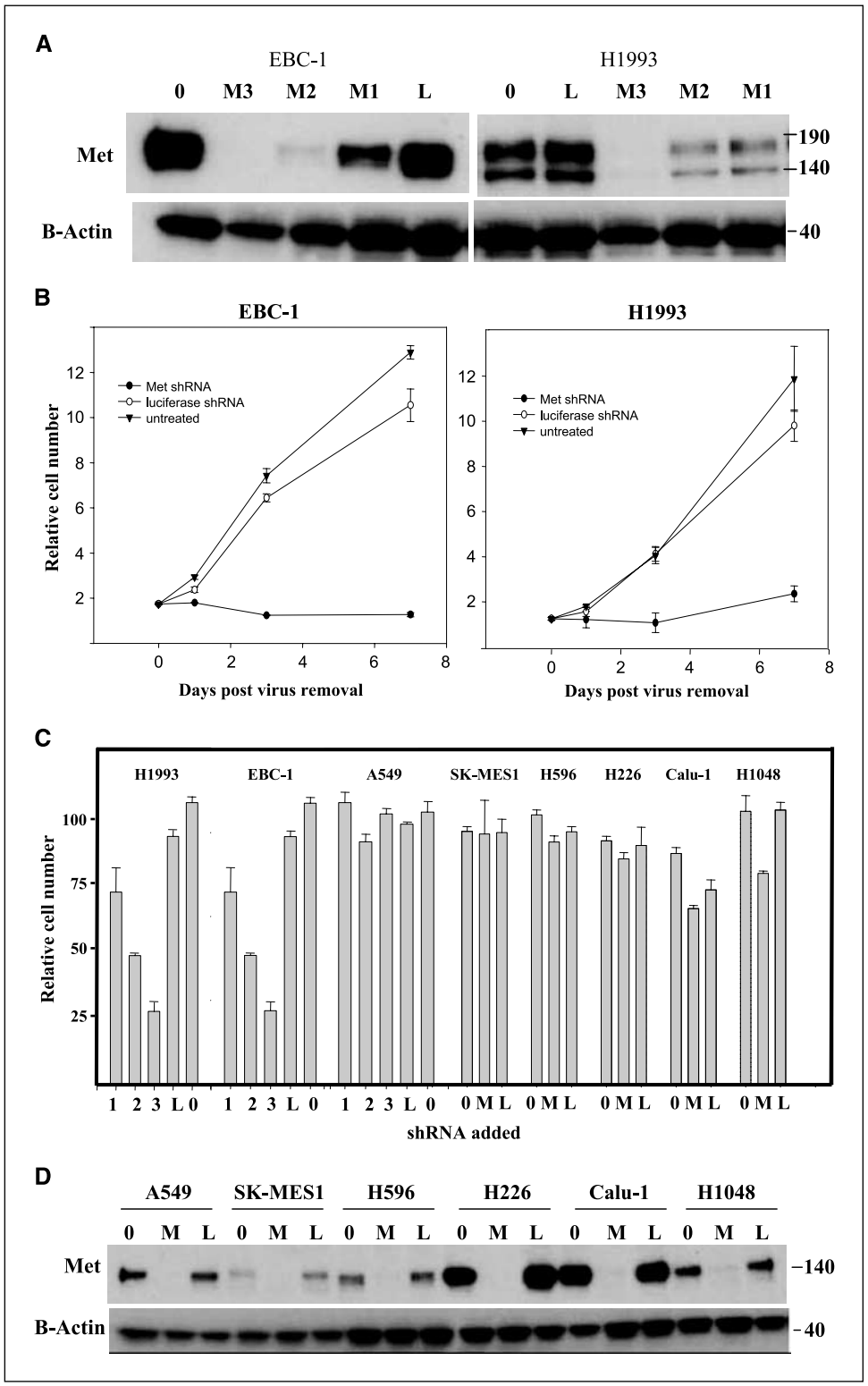

Figure 3. Inhibition of Met protein expression inhibits growth of EBC-1 and H1993 cells. *A,* ShRNA-mediated knockdown of Met in EBC-1 and H1993 cells. ShRNAs were added to cells, and 72 h later, lysates were prepared, equalized, and separated on SDS-PAGE followed by transfer to nylon membranes. Met was detected by Western blotting with 25H2 antibody. *0*, untreated cells; *M1*, *M2,* and *M3,* cells treated with three different Met hairpins. *L,* a control hairpin against luciferase. shRNAs against FGFR2 also were used as a control and did not affect Met expression or cell growth. *B,* time course of growth inhibition in EBC-1 and H1993 cells. Cells were plated in triplicate wells at 4,000 cells per well in a 96-well plate, treated with either luciferase shRNA or Met shRNA M3 (*Met shRNA*), as described in Materials and Methods, or left untreated (*untreated*). Cells were analyzed with Vialight reagent at 1, 3, and 7 d after virus removal. *C,* selective growth inhibition in Met-amplified cell lines. Cells in medium containing 10% serum were treated with the indicated shRNAs. H1993, EBC-1, and A549 were treated with each of three Met shRNAs [M1, M2, M3 in (*A*), indicated with 1, 2, and 3, respectively], luciferase shRNA (*L*) or left untreated (*0*), whereas the remaining cell lines were treated with the Met shRNA M3 (*M*) along with luciferase or left untreated. Cells were analyzed using Vialight reagents on day 4. *D,* ShRNA knockdown of Met in lung cancer cell lines. Cell lines were treated with the Met shRNA M3, Luciferase shRNA or left untreated for 72 h as described in Materials and Methods. Cell lysates were prepared, analyzed by SDS-PAGE, and probed for Met protein using Met antibody AF276. Numbers on the right are molecular weights in kilodaltons.

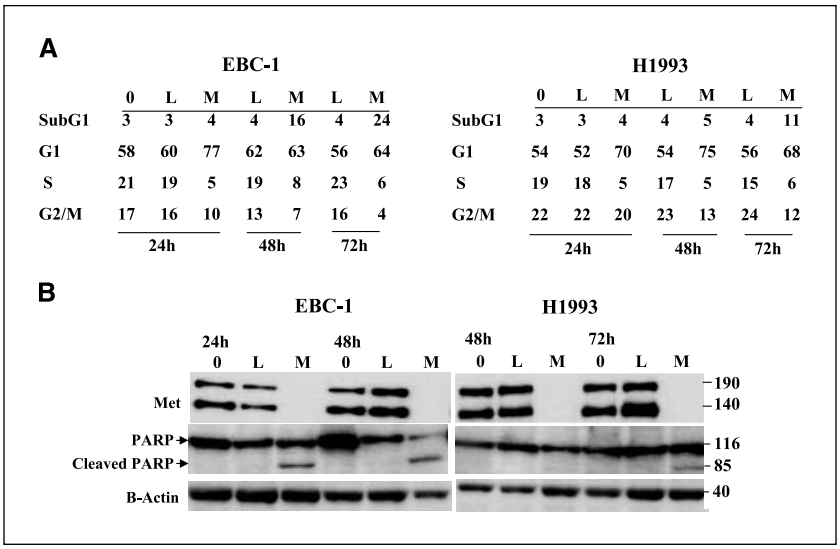

Figure 4. Met knockdown leads to growth arrest and accumulation of cells in sub-G_1. *A,* cells were treated with Met shRNA M3, luciferase shRNA, or left untreated and processed for flow cytometry at 24, 48, and 72 h after shRNA lentivirus removal, and DNA content analysis by flow cytometry was done as described in Materials and Methods. Tabular representation of EBC-1 and H1993 profiles as determined by ModFit software and manual gating. *B,* Met inhibition leads to induction of cleaved PARP. Duplicate cells treated as in (*A*) were lysed and processed for blotting with anti-PARP antibodies as described in Materials and Methods. The top row was probed for Met, whereas the bottom row was probed with anti-PARP. The position of full-length and cleaved PARP is indicated. Numbers on the right are molecular weights in kilodaltons.

found that *Met* amplification is critical for gastric cancer cell growth (14). The expression levels of Met in H1993 and EBC-1 are similar to Met expression levels in the *Met*-amplified gastric cancer cell lines SNU5, MKN45, HS746t, and GTL-16 (data not shown). Neither these gastric cell lines nor EBC-1 and H1993 cell lines secrete HGF as assayed by a lack of scatter activity from cell supernatants (data not shown). Mutation does not account for activation because *Met* is wild type in GTL-16 cells (15), and Smolen et al. (14) reported that *Met* is wild type in SNU5, MKN45, HS746T, and KATOII *Met*-amplified gastric cancer cell lines. Thus, the activation of amplified *Met* could result from highly expressed receptor clustering independent of ligand.

Met has previously been reported to contribute to the growth of non–*Met*-amplified cell lines, including the colon cancer cell lines KM20 (18), prostate cancer cell line PC3 (19), and the rhabdomyosarcoma cell line SJRH30 (20). Both KM20 and PC3 cells contain low levels of basal Met phosphorylation, and a proprietary Met small-molecule inhibitor does not inhibit the growth of PC3, KM20, or SJRH30 cell lines, whereas both EBC-1 and H1993 (and *Met*-amplified gastric cancer cell lines) are potently growth inhibited (data not shown). It is possible that Met protein-protein interactions are important for the growth of KM20, PC3, and SJRH30 cell lines, such that the loss of Met protein (as opposed to the loss of kinase activity) is required to inhibit growth *in vitro*.

Interestingly, we found that the amplified *Met* kinase can activate signaling pathways differently from HGF stimulation. Although ERK signaling is activated in the *Met*-amplified lines, the level of activation is much lower than that found in HGF-stimulated non–*Met*-amplified lines. By contrast, both p120/δ-catenin and β-catenin contained a striking increase in tyrosine phosphorylation only in the *Met*-amplified cell lines. HGF has been suggested to activate β-catenin signaling in primary cells (21), and a link between constitutively activated Met and tyrosine-phosphorylated β-catenin was suggested by Danilkovitch-Miagkova et al. (22). In this work exogenous expression of a mutationally activated Met (M1268T mutant) activates tyrosine phosphorylation in β-catenin, resulting in nuclear translocation and activation of target genes such as *myc* (22). In addition to a role in proliferation, β-catenin can function in cell adhesion through interactions with cadherin family members and the actin cytoskeleton (23, 24). Increased tyrosine phosphorylation in β-catenin has also been reported to correlate with a disruption of cell adhesion (25). In contrast to β-catenin, a link between Met and p120/δ-catenin has not been previously described. Although the function of p120/δ-catenin has been less well characterized, this catenin family member has recently been shown to cooperate with β-catenin to

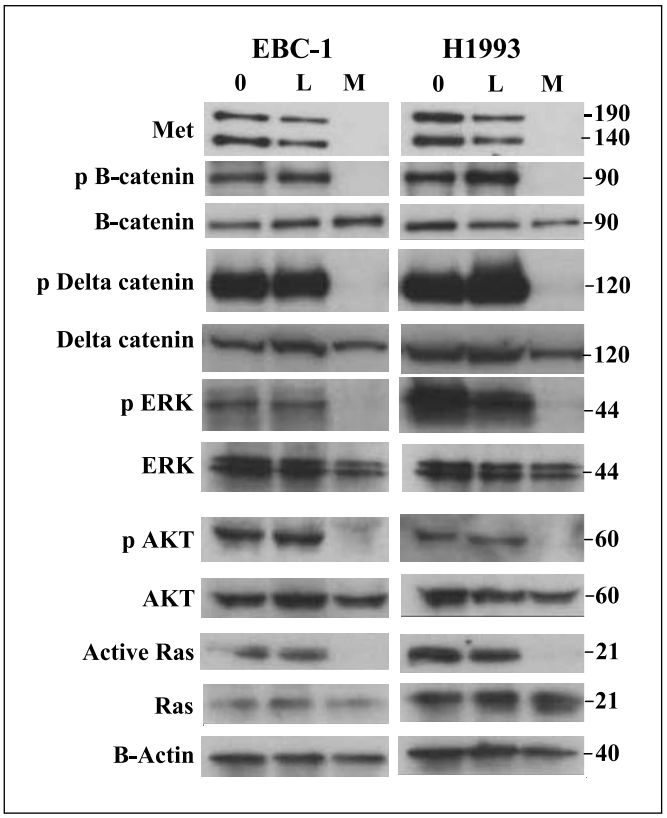

Figure 5. Loss of downstream signaling after Met inhibition. Lysates were prepared 24 h after Met shRNA M3, luciferase shRNA virus removal, or left untreated and processed for Western blotting with the indicated antibodies. β-Catenin was immunoprecipitated from indicated lysates and Western blotted for phosphotyrosine with 4G10. Numbers on the right are molecular weights in kilodaltons.

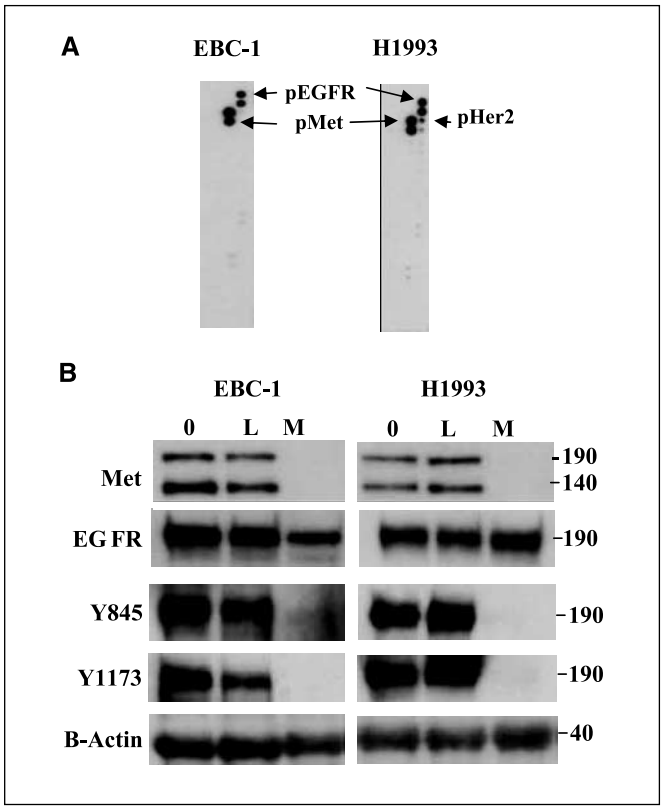

Figure 6. Met is required for basal tyrosine phosphorylation in EGFR. *A,* 20 μg cell lysate from H1993 and EBC-1 cells was incubated overnight with the ARY001 membrane, followed by washing and detection as described in Materials and Methods. *B,* cells were treated with Met shRNA M3, luciferase shRNA or left untreated, and 24 h postinfection, lysates were prepared. About 40 μg of lysates was analyzed by SDS-PAGE and Western blotting with the indicated EGFR antibodies. Numbers on the right are molecular weights in kilodaltons.

activate β-catenin target gene transcription (26). These studies warrant further investigation of amplified Met kinase in the function of cell-cell adhesion components.

Because we did not observe tyrosine phosphorylation in β-catenin or p120/δ-catenin after HGF stimulation, it is possible that the combined activation of EGFR and Met may be required for this effect. Surprisingly, we found that the basal level of phosphorylation in the activation site and the Y1173 docking site in EGFR was dependent on Met in EBC-1 and H1993 cells. This suggests that EGFR is phosphorylated by Met in *Met*-amplified cell lines and reveals that the knockdown of Met results in a loss of aspects of EGFR signaling as well. Although previous work has suggested that activation of EGFR can result in the activation of Met phosphorylation, we did not observe phosphorylation of Met at Y1349 in the HCC827 cell line, which contains both EGFR amplification and mutation (data not shown). In addition to EBC-1 and H1993 cells, we also found EGFR basal phosphorylation in Met-amplified gastric cancer cell lines, and where tested (GTL-16 cells), EGFR phosphorylation was dependent on Met (data not shown). Thus, it seems that the relationship between amplified *Met* and EGFR activation may be common to *Met*-amplified cancer cell lines. However, the role of EGFR in the growth of Met-amplified cancer cell lines remains to be determined. Smolen et al. (14) reported that transient EGFR small interfering RNA did not affect the growth of Met-amplified SNU5 gastric cells. We observed minor growth inhibition in H1993 cells on transient shRNA knockdown of EGFR1, as well as in stable lines with EGFR knockdown, suggesting that EGFR is contributing to growth in H1993 cells (data not shown). Curiously, we were unable to significantly inhibit basal EGFR phosphorylation in *Met*-amplified cell lines with the EGFR inhibitor gefitinib (data not shown).

The multistep hypothesis of cancer development (27) proposes that multiple gain of function and loss of function alterations are required to transform a normal cell to malignancy. In contrast, we have shown that for a subset of *Met*-amplified cancer cells, cell proliferation is dependent on a single activated tyrosine kinase. However, in support of this hypothesis, we have shown that multiple oncogenic pathways are activated by Met kinase in *Met* amplified cell lines. Ras alleles are wild type in both EBC-1 and H1993 cells, but ras is activated similarly to the homozygous mutant K-ras in A549 cells. As described above, intense tyrosine phosphorylation of catenin family members was found only in *Met*-amplified cells. Finally, both EBC-1 and H1993 cell lines have a basal level of phosphorylation in EGFR1, which is dependent on Met, and which may be contributing to cell growth. Thus, amplified Met activates multiple signaling pathways that, in combination, may be sufficient for oncogenesis.

Our results and those of Smolen et al. (14) regarding Met, combined with previous work for EGFR (28–31) and Her2 (32, 33), show that cancer cell lines can be dependent on a single amplified receptor tyrosine kinase for viability and growth. This requirement for growth *in vitro* may correlate with *in vivo* growth in patients. In fact, recent evidence suggests that the amplification of EGFR in lung and colon cancer is significantly associated with survival and response rates after EGFR inhibitor therapy (2, 28, 34). It will be of interest to determine whether patients with amplified *Met* kinase have clinical responses to Met inhibitors similar to those observed for EGFR and Her2 inhibitors in cancers with respective *EGFR* and *Her2* amplification (2, 35).

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Correction: Met Dependence of Lung Cancer Cell Lines

In the article on Met dependence of lung cancer cell lines in the March 1, 2007 issue of *Cancer Research* (1), the sequence listed as M3 on page 2082 should be as follows:

GAGCTGTGAGAATATACACTTCAAGAGAGTGTATATTCTCACAGCTC.

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