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# Constitutive Activation of Met Kinase in Non-Small-Cell Lung Carcinomas Correlates With Anchorage-Independent Cell Survival

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**Abstract** Lung cancer is currently the most frequent cause of cancer death in North America. Hepatocyte growth factor (HGF) and its receptor Met are frequently over-expressed in non-small-cell lung carcinomas (NSCLC), but their potential role in tumor progression is not clearly known. To assess the role of HGF/Met signaling in lung carcinomas, we have examined the expression, activation status, and function of Met in NSCLC cell lines (n = 7), established from primary tumors or pleural fluids of cancer patients. We observed Met expression in three NSCLC cell lines, two of which exhibited constitutive tyrosine-phosphorylation of Met, and Met kinase activity. In addition, the observed constitutive activation of Met was sustained under anchorage-independent conditions, and correlated with phosphatidyl inositol 3-kinase-dependent cell survival. Immunoreactive HGF-like protein was secreted by two Met-positive and two Met-negative NSCLC cell lines. However HGF activity, as determined by the ability to induce cell scattering and tyrosine-phosphorylation of Met in reporter cell lines, was detected in conditioned medium from only one Met-negative NSCLC cell line: none of the conditioned media from Met-expressing NSCLC cell lines showed detectable HGF activity. Thus, constitutive activation of Met in NSCLC cell lines may occur at least in part through intracrine, or HGF-independent mechanisms. Interestingly, additional paracrine stimulation with exogenous recombinant HGF was required for DNA synthesis and correlated with increased activation of ERK1/2 in all Met-positive NSCLC cell lines, regardless of the basal activation status of Met. These findings indicate that a medium level of constitutive activation of Met occurs in some NSCLC cell lines, and correlates with survival of detached carcinoma cells; whereas additional paracrine stimulation by recombinant HGF is required for DNA synthesis. Thus constitutive and paracrine activation of Met may provide complementary signals that promote survival and proliferation, respectively, during tumor progression of NSCLC. J. Cell. Biochem. 86: 665–677, 2002. 

**Key words:** HGF; Met; cell adhesion; survival; cell proliferation; metastasis; lung cancer

Growth factors, which act in a paracrine or autocrine manner, are important regulators of stromal-epithelial interactions in both normal and malignant cell growth and development. Hepatocyte growth factor (HGF) is secreted primarily from mesenchymal/stromal cells of many different tissues [Nakamura et al., 1986; Zarnegar and Michalopoulos, 1989; Noji et al., 1990], whereas HGF receptor (Met) expression is detected in a variety of epithelial (and some

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non-epithelial) cells in a broad range of tissues [Tajima et al., 1992]. HGF is secreted as an inactive single-chain pro-HGF protein of 105 kDa [Mizuno and Nakamura, 1993]. Pro-HGF is cleaved at Arg494-Val495, and is converted to a heterodimeric active HGF form consisting of disulfide-linked α (65 kDa) and β (30 kDa) chains of 463 and 234 amino acid residues, respectively [Comoglio, 1993; Gherardi et al., 1993; Tsao et al., 1993; Mizuno et al., 1994; Mars et al., 1995; Shimomura et al., 1995]. The cleavage is mediated by serine proteases, such as HGF converting enzyme [Mizuno et al., 1994], blood-coagulation factor VIII [Shimomura et al., 1995], and urokinase-type plasminogen activator [Comoglio, 1993]. In addition, naturally-occurring isoforms of HGF consisting of the NK1 and NK2 domains have been identified [Schwall et al., 1996]; NK1 and NK2 isoforms have been shown to be both antagonistic and agonistic, and can modulate Met kinase activity [Schwall et al., 1996]. The *met* proto-oncogene product, a member of the tyrosine kinase receptor family, has been identified as the HGF receptor [Bottaro et al., 1991]. A two-tyrosine motif in the carboxyl terminus of Met acts as a multi-functional docking site for SH2 domain-containing transducer proteins, resulting in stimulation of HGF-induced functions [Ponzetto et al., 1994; Fixman et al., 1996]. In normal tissues, paracrine activation of Met by HGF is tightly regulated, and affects a variety of epithelial cell functions including cell proliferation [Rubin et al., 1991; Rahimi et al., 1994], survival [Amicone et al., 1997], and differentiation [Tsarfaty et al., 1994]. In contrast, over-expression of HGF and activation of Met promote formation of tumors and metastasis in many tissue types [Rong et al., 1994; Liang et al., 1996; Takayama et al., 1997].

Non-small cell lung carcinomas (NSCLC), including adenocarcinoma, squamous cell carcinoma, and large cell anaplastic carcinoma represent about 80% of human lung cancers [Sagawa et al., 1998]. Met is frequently over-expressed or amplified in NSCLC [Tsao et al., 1998], as well as breast [Tuck et al., 1996], ovarian [Di Renzo et al., 1994], and colorectal carcinomas [Di Renzo et al., 1995], melanomas [Natali et al., 1993], and osteosarcomas [Yamada et al., 1994]. In addition, HGF as well as the NK1 and NK2 isoforms are frequently over-expressed in NSCLC tissues [Olivero et al., 1996; Yamashita et al., 1998; Tsao et al., 2001], and in some NSCLC cell lines [Tsao et al., 1993; Yi et al., 1998]. This high level of HGF expression correlates with shortened survival of lung cancer patients [Siegfried et al., 1997]. In contrast, HGF expression in corresponding normal lung epithelium is low, although expression of the NK2 isoform by some bronchial epithelial cells in vitro has been shown [Tsao et al., 1993]. Experimentally, high ectopic expression of HGF has been shown to enhance HGF/Met signaling and tumorigenicity in one NSCLC cell line [Yi and Tsao, 2000]. However, over-expression of Met in some cancers can result in its own activation and does not depend on an autocrine mechanism [Rusciano et al., 1999; Wang et al., 2001]. Thus, the activity of endogenous secreted HGF, the status of HGF/Met activation, and its role in tumorigenesis in NSCLC is not clearly known.

To assess the role of autocrine HGF/Met signaling in NSCLCs, we have examined the expression, activity, and function of HGF and Met in a panel of NSCLC cell lines ($n = 7$), established from primary tumors or pleural fluids of cancer patients. We identified three Met-expressing NSCLC cell lines, two of which expressed constitutively activated Met. Constitutive activation of Met was sustained in detached NSCLC cell lines and correlated with phosphatidyl inositol 3-kinase (PI3K)-dependent cell survival. Immunoreactive HGF-like protein was secreted by two Met-positive, and two Met-negative NSCLC cell lines. Although, HGF activity was detected in one Met-negative NSCLC cell line, none of the Met-positive cell lines showed detectable activity of secreted HGF protein. Interestingly, additional paracrine stimulation with recombinant HGF was required for DNA synthesis. Thus constitutive and paracrine (or endocrine) activation of Met may provide important survival and mitogenic signals, respectively, during tumor progression in NSCLC.

## MATERIALS AND METHODS

### Antibodies

Mouse anti-phosphotyrosine (PY20) monoclonal antibody, mouse anti-human Met IgG, and rabbit anti-ERK 2 antibody were purchased from Santa Cruz Biotechnology (San Jose, CA). Mouse phospho-specific anti-ERK1/2 IgG was kindly provided by Erik Schaefer (QCB/Hop-

kinton Division of BioSource International (Hopkinton, MA). Sheep anti-human HGF IgG was provided by Genentech Inc. (San Francisco, CA). Rabbit antibody against a GST fusion protein corresponding to an N-terminal (N-ter) sequence of HGF (Q32-E120), which recognizes the HGF α chain, was produced at Queen's University.

### Human Lung Carcinoma Cell Lines

Human NSCLC cell lines SW-900 [Fogh et al., 1977], WT-E [Campling et al., 1995], SK-Luci-6 [Anger et al., 1999], QU-DB [Cole et al., 1986], LC-T [Campling et al., 1992], and BH-E [Campling et al., 1995] were established from primary tumors or pleural fluids from lung cancer patients (Table I). One NSCLC cell line, A549 [Giard et al., 1973], was obtained from ATCC (Clone CCL-185). A positive control consisted of a human fibroblast cell line HEL-299 (ATCC clone CCL-137), transfected with human HGF cDNA. The above cell lines were routinely grown in RPMI-1640 medium, supplemented with 7% FBS. In some experiments, a non-malignant human bronchial epithelial cell line (HBE4, provided by Dr. M. Tsao) was also used, and was cultured in DMEM-based medium, supplemented as described elsewhere [Viallet et al., 1994]. All cell lines were confirmed to be mycoplasma negative as previously described [Elliott et al., 1992].

### PCR Primers

The primers were designed according to the sequence of human HGF cDNA from Genbank using PC/GENE computer software. Primers were engineered to detect HGF cDNA in both human and mouse, but not cDNA of the homologous family member, macrophage-stimulating protein (MSP) [Donate et al., 1994]. The primers used for HGF amplification have been described in detail elsewhere [Hung and Elliott, 2001]. In addition, primers for the housekeeping gene glucuronidase B (GUS B) [Ivanchuk et al., 1997] were used as a control. The products of the PCR amplifications for HGF and GUS B were 560 and 192 bp, respectively.

### RT-PCR Analysis

Total RNA from various lung tumor cell lines was extracted using TriZol reagent (InVitrogen Canada, Burlington, ON), and used in a reverse transcriptase reaction to produce cDNA using a First-Strand Synthesis Kit (Amersham Biosciences, Baie d'Urfe, PQ). PCR reactions were carried out with, or without, direct labeling of primers with [$\alpha$-$^{32}$P]dATP (Mandel Scientific, Guelph, ON). cDNA (50 ng) was added to each 10 μl reaction, which also contained 20 mM Tris (pH 8.3), 50 mM KCl, 0.2 mM dNTPs, and 1.5 mM $MgCl_2$. Oligonucleotide primers specific for HGF and GUS B (internal control) were used. GUS B was chosen as a control, since its mRNA level was found empirically to correspond approximately to that of HGF. The reaction was initiated by the addition of 1 unit of Taq polymerase (InVitrogen Canada), and samples were then incubated at 95°C for 1 min (denaturing), 55°C for 1 min (annealing), 72°C for 1 min (elongation). Unlabeled and labeled PCR was carried out with 25 cycles. The reaction products were resolved on a 1.5% agarose gel. Unlabeled PCR products were stained with

**TABLE I. Expression of HGF and Met in Human Non-Small-Cell Lung Carcinoma Cell Lines**

| NSCLC carcinoma cell lines[a] | Histology | Origin | HGF | | | Met | |
|---|---|---|---|---|---|---|---|
| | | | mRNA | Protein | Activity | Protein | Activity[b] |
| SW-900 | Squamous cell carcinoma | Primary tumor | +[c] | + | − | + | Constitutive |
| WT-E | Squamous cell carcinoma | Pleural effusion | ± | + | − | + | Constitutive |
| SK-Luci-6 | Large cell anaplastic | Primary tumor | + | + | + | − | − |
| QU-DB | Large cell anaplastic | Primary tumor | − | − | − | − | − |
| BH-E | Adenocarcinoma | Pleural effusion | + | + | − | − | − |
| LC-T | Adenocarcinoma | Primary tumor | − | − | − | − | − |
| A549 | Adenocarcinoma | Primary tumor | ± | − | − | + | Paracrine |

[a]See Materials and Methods for designation of NSCLC cell lines. The following are the references for the cell lines listed: SW-900 [Fogh et al., 1977], WT-E [Campling et al., 1995], SK-Luci-6 [Anger et al., 1999], QU-DB [Cole et al., 1986], LC-T [Campling et al., 1992], BH-E [Campling et al., 1995], and A549 [Giard et al., 1973].
[b]Cell lysates were immunoprecipitated with anti-Met IgG, and subjected to SDS–PAGE and Western blotting with anti-PY IgG. In a parallel approach, immunoprecipitates were subjected to a Met kinase assay. See Materials and Methods for details. Constitutive versus paracrine Met kinase activity is indicated.
[c]+, positive; ±, trace amount; −, negative.

ethidium bromide and visualized under UV light illumination. Labeled PCR products were measured in gel slices using a liquid scintillation counter.

### Conditioned Media and Copper (II) Affinity Chromatography

For conditioned media (CM) production, cell lines were grown to 80% confluence, washed in pre-warmed serum-free RPMI, and incubated for 24 h at 37°C in serum-free RPMI. CM was harvested, centrifuged at 2,000*g* for 30 min, and frozen at −70°C in 5-ml aliquots. Purification of HGF from CM was carried out using Cu(II) affinity chromatography based on the Cu(II) binding ability of HGF [Rahimi et al., 1996]. The principal of separation of HGF from biological samples by Cu(II) affinity chromatography is based on the fact that the kringle domains of HGF have several cationic sequences (His-X-His) in the kringle domains of the HGF molecule. Five milliliters of each CM was loaded onto a 1 ml Cu(II) chelating column, which had been equilibrated with equilibration buffer (20 mM sodium phosphate, pH 7.2, 1 M NaCl, 1 mM imidazole). HGF protein bound specifically to the Cu(II) affinity column, and unbound proteins were washed away with 15 column volumes of the same buffer. HGF protein was eluted using elution buffer containing 80 mM imidazole, and the eluant was concentrated using an Amicon Microcon-10 concentrator before being analyzed on 8% SDS–PAGE under reducing conditions. Proteins were then transferred onto nitrocellulose membrane and probed with sheep anti-HGF IgG or rabbit anti-human HGF (N-ter) IgG. The bands were revealed using an ECL kit (Amersham Biosciences).

### Immunoprecipitation and Western Blot Analysis

Lung carcinoma cell lines were grown to 80% confluence and serum-starved for 24 h. Cell lines were then rinsed with phosphate-buffered saline and lysed in a lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EGTA, 1% Nonidet P-40, 1 mM $Na_3VO_4$, 50 mM NaF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged for 10 min at 14,000*g* in an IEC/Micromax centrifuge at 4°C. Protein concentration of supernatants was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL). Equal amounts of protein from cell lysates were immunoprecipitated with the indicated antibodies at 4°C for 2 h. Immunoprecipitates were collected on protein A-Sepharose (Amersham Biosciences), washed three times with lysis buffer, and subjected to 8% SDS–PAGE under reducing conditions, followed by western blotting with the indicated antibodies. Immunoreactive bands were revealed using an ECL kit.

### Met Kinase Assay

Cells were cultured to 80% confluence and serum-starved overnight. The cells were then treated with, or without, recombinant HGF (20 ng/ml) for 20 min at 37°C. The cells were lysed and extracts were normalized for protein concentration and immunoprecipitated with anti-Met IgG. The immunoprecipitates were washed twice with cold lysis buffer and once with cold kinase buffer containing 20 mM Pipes (pH 7.0), 10 mM $MnCl_2$, and 100 μM $Na_3VO_4$, and resuspended in cold kinase buffer. The reaction was initiated by addition of 10 μCi [$\gamma$-$^{32}$P]-dATP. The reaction was carried out at 30°C for 10 min and stopped by adding 2× SDS–PAGE sample buffer and boiling for 3 min. The samples were subjected to 8% SDS–PAGE and the gel was treated with 1 M KOH for 30 min at 45°C to hydrolyze Ser/Thr phosphorylation sites. The gel was then fixed in 45% MeOH and 10% acetic acid for 30 min at room temperature and dried. The autophosphorylation of Met was analyzed by a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

### Met Activation Assay for HGF Activity

The human NSCLC cell line, A549, which expresses Met, but not HGF protein in our system, was used as a read-out for Met activation. Serum-starved A549 cells were exposed for 30 min to CM from various NSCLC cell lines, HGF (20 ng/ml), or medium alone. The cells were lysed and subjected to immunoprecipitation with anti-Met antibody. Immunoprecipitates were subjected to 8% SDS–PAGE under reducing conditions, and immunoblotting was carried out with anti-phosphotyrosine or anti-Met (loading control) antibody. Immunoreactive bands were revealed using an ECL kit.

### MDCK Scatter Assay

Madin-Darby canine kidney (MDCK) epithelial cells were plated in 24-well plates (NUNC,

InVitrogen, Canada) at $3\times10^3$ cells/well and incubated overnight in DMEM plus 10% FBS at 37°C. Adherent cells were washed in serum-free medium, and undiluted CMs (0.5 ml/well) were added. After further incubation for 24 h, the degree of scattering was assessed visually. Photographs were taken using a Leica inverted microscope equipped with MCID-M4 imaging software (Imaging Research, Brock University, St. Catherines, ON).

### Cell Survival

Carcinoma cells were cultured to 80% confluence, serum-starved overnight, and harvested with PBS containing 0.5 mM EDTA and 0.5 mM EGTA. The cells were then seeded at a density of $2\times10^4$ cells in 1.5 ml of RPMI-1640 medium containing 0.5 mg/ml BSA in 0.6% agar-coated 35 mm Corning non-tissue culture plates [Qiao et al., 2000]. After 24 h incubation at 37°C with the indicated treatment, cells were collected and centrifuged in Eppendorf tubes (1,000*g* for 5 min), replated in a 96-well plate with 7% FBS/RPMI medium, and incubated at 37°C for 4 h. Cell survival was then measured using a colorimetric method based on the conversion of MTS tetrazolium to formazan (CellTiter aqueous Kit, Promega, Madison, WI).

### DNA Synthesis

Carcinoma cells ($1\times10^4$) were plated in triplicate in a 24-well plate and serum-starved overnight. The cells were then incubated with the indicated concentration of recombinant HGF at 37°C in 5% $CO_2$. After 24 h, 0.2 μCi of [$^3$H]-thymidine (Amersham Biosciences) was added, and cells were incubated for an additional 24 h. Cells were harvested and aliquots of cells (1,000/well) were placed in 96-well microtiter plates and transferred to filters using a Titertek cell harvester (ICN, Costa Mesa, CA), and DNA synthesis was measured as incorporation of [$^3$H]-thymidine in a scintillation counter (Beckman, Mississauga, ON, Canada).

## RESULTS

### Constitutive Activation of Met Occurs in NSCLC Cell Lines

As a first step to examine the role of HGF/Met signaling in lung carcinoma cells, we examined the expression and activation status of Met in a panel of seven NSCLC cell lines. The results showed high expression of Met in two NSCLC cell lines, SW-900 and WT-E, compared to A549 cells (Fig. 1A, Table I). SW-900 and WT-E cells exhibited a high level of spontaneous tyrosine-phosphorylation of Met, compared to A549 cells which showed only paracrine induction with recombinant HGF of Met tyrosine-phosphorylation (Fig. 1B). Analysis of in vitro Met kinase activity revealed a 2 to 3-fold constitutive activation of Met in SW-900 and WT-E cells, compared to A549 cells (Fig. 1C).

### Expression of HGF mRNA and Protein in NSCLC Cell Lines

The observed constitutive activation of Met in NSCLC cell lines could be due to co-expression of HGF and Met and activation of an HGF/Met autocrine loop. We therefore assessed the level of HGF mRNA and protein in our panel of NSCLC. First, we used an RT-PCR approach to achieve maximal sensitivity. The linearity of the PCR reaction was assessed to establish the optimal reaction conditions, and the reaction was performed with internal controls to detect GUS B mRNA with specific primers. Of the Met-expressing NSCLC cell lines tested, only SW-900 showed significant levels of HGF mRNA detected using unlabeled PCR (Fig. 2); whereas WT-E and A549 showed only trace levels detected using radiolabeled PCR (data not shown). Of four Met-negative cell lines tested, two (BH-E and SK-Luci-6) showed strong HGF mRNA expression. In comparison, a non-malignant human bronchial epithelial cell line, HBE4, showed no detectable expression of HGF mRNA.

To detect HGF secreted by carcinoma cell lines, we purified HGF protein from concentrated CM using Cu(II) affinity chromatography [Rahimi et al., 1996]. This technique is based on the ability of HGF to bind to Cu(II) [Rahimi et al., 1996]. Western blotting of eluted fractions was carried out, and putative HGF protein was detected with rabbit anti-human HGF IgG, which recognizes the α chain of the HGF molecule (Fig. 3, Table I). CMs from NSCLC cell lines SW-900, SK-Luci-6, and BH-E showed strong expression of immunoreactive bands corresponding to pro-HGF (100 kDa) and HGF α chain (65 kDa), similar to the pattern of HGF mRNA expression in Figure 2. In addition, CM from WT-E cells showed a low level of immunoreactivity corresponding to HGF α chain (65 kDa). The lower molecular weight immunoreactive band (55–60 kDa) may represent HGF degradation products. CMs from cell lines

QU-DB, LC-T (Fig. 3) and A549 (data not shown) showed no detectable HGF immunoreactivity. Controls consisted of recombinant HGF and CM from HEL-299 cells transfected with human HGF cDNA. Thus, immunoreactive HGF-like protein is secreted by two Met-positive, and two Met-negative, NSCLC cell lines tested.

### Limited HGF Activity in Conditioned Media of NSCLC Cell Lines

The activity of secreted HGF requires appropriate processing [Comoglio, 1993; Mizuno et al., 1994; Shimomura et al., 1995], and can be modulated by the extracellular environment, for example, proteolysis [Fehlner-Gardiner et al., 1999] or binding to ECM proteins [Rahimi et al., 1994]. We therefore examined the activity of putative HGF in CMs from the above carcinoma cell lines, as determined by the ability to activate Met in A549 cells, and to stimulate scattering of MDCK cells. Of all CMs tested, only SK-Luci-6 stimulated strong tyrosine phosphorylation of Met, similar to that of the recombinant HGF control (Fig. 4). No detectable Met phosphorylation above baseline was seen in CMs from any of the other cell lines tested. Similarly, CM from SK-Luci-6 stimulated scattering of MDCK cells, whereas that from the other NSCLC cell lines tested did not (Fig. 5).

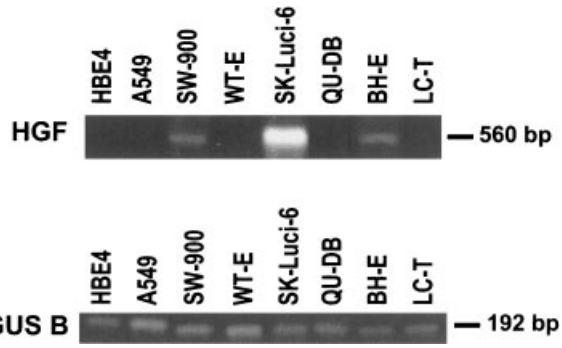


**Fig. 2.** Analysis of HGF mRNA in NSCLC cell lines. Total RNA from various NSCLC cell lines was extracted and used in a reverse transcription reaction to produce cDNA. A non-malignant lung bronchial cell line, HBE4 [Viallet et al., 1994], was used as non-malignant control cells. Primers specific for HGF and GUS B (as internal control) were added to the cDNA. The PCR reaction was carried out with 25 cycles, and the reaction products were visualized following staining with ethidium bromide as described in Materials and Methods. The results are representative of three experiments.

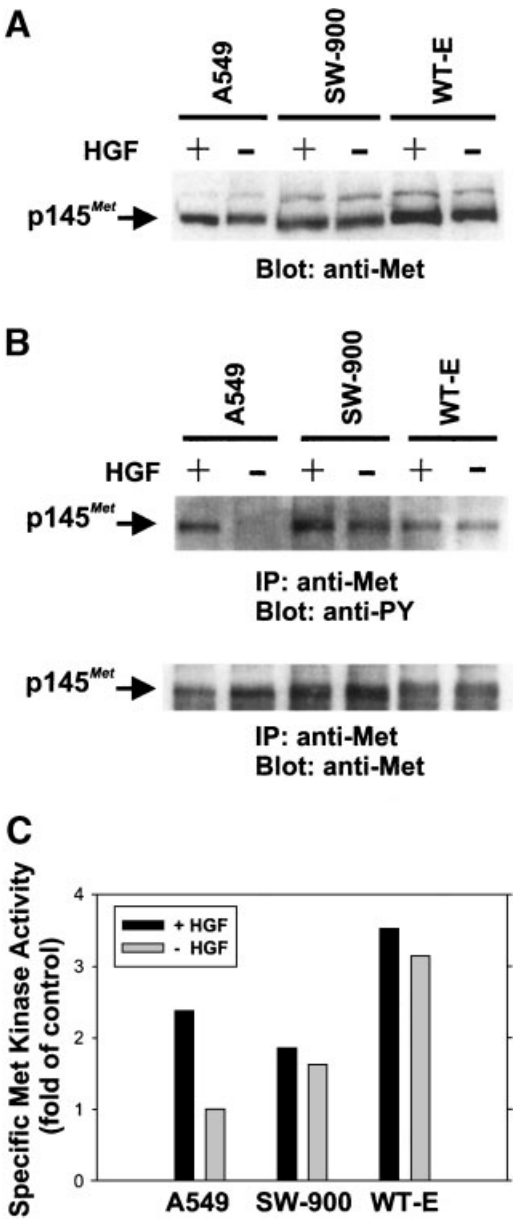


**Fig. 1.** Status of Met activation in NSCLC cell lines. NSCLC cell lines A549, SW-900, and WT-E were cultured to 80% confluence and serum-starved overnight. The cells were then treated with recombinant HGF (20 ng/ml) for 20 min at 37°C, and lysed. (**Panel A**) Clarified cell lysates were normalized for protein concentration, subjected to reducing SDS–PAGE and immunoblotted with anti-Met IgG. Bands were visualized with ECL reagents. Equal protein loading was confirmed with an unrelated anti-ERK 2 IgG (data not shown). (**Panel B**) In a separate experiment, normalized cell lysates were precipitated with anti-Met IgG. Half of each immunoprecipitate was subjected to reducing SDS–PAGE and western blotting. The blot was probed with anti-phosphotyrosine (PY) antibody, and the bands were visualized with ECL reagents. The same blot was stripped and re-probed with anti-Met IgG as a loading control. (**Panel C**) The other half of each immunoprecipate was assayed for Met kinase activity in vitro as described in Materials and Methods. The signal densities were measured with a PhosphoImager and plotted relative to the control A549 cells without HGF (lower panel). The results are representative of three experiments.

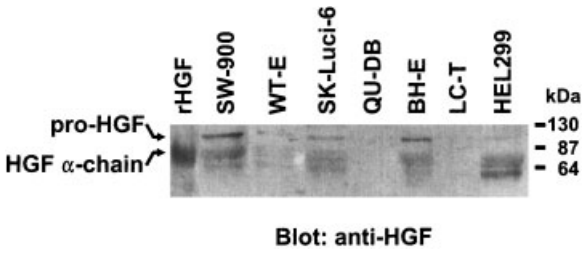


**Fig. 3.** Detection of HGF protein in conditioned media of NSCLC cell lines. CMs were collected from NSCLC cell lines SW-900, WT-E, SK-Luci-6, QU-DB, BH-E, and LC-T, and putative HGF was purified using $Cu^{2+}$ affinity chromatography. Fractions containing putative HGF were concentrated by Microcon concentrators, and were analyzed by reducing SDS–PAGE, followed by Western blotting with anti-HGF IgG. Controls consisted of recombinant HGF and CM from HEL-299 cells transfected with human HGF cDNA. Immunoreactive bands were revealed by ECL. Arrows corresponding to pro-HGF and mature HGF are shown. The results are representative of three experiments.

Thus, HGF secreted by SK-Luci-6 had strong activity, whereas immunoreactive HGF-like protein secreted by SW-900, WT-E, and BH-E showed no detectable activity in two independent assays. These results suggest that ligand-independent mechanisms of Met activation may be involved in SW-900 and WT-E cells.

### Constitutive Activation of Met in NSCLC Cell Lines Does Not Require Cell Anchorage

Recently, we [Qiao et al., 2000], and others [Rusciano et al., 1999; Wang et al., 2001], have

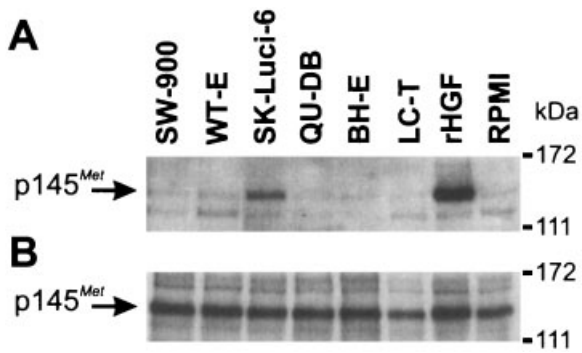


**Fig. 4.** Analysis of HGF activity of conditioned media from NSCLC cell lines. A549 cells (Met-positive, HGF-negative) were serum-starved overnight and incubated with CMs from the NSCLC cell lines listed in Figure 3. Controls consisted of cells incubated without, or with, recombinant HGF (40 ng/ml). After 30 min of incubation at 37°C, cells were washed with ice-cold PBS, lysed in lysis buffer, and immunoprecipitated with rabbit anti-Met IgG. Immunoprecipitates were washed three times with lysis buffer before analysis by reducing SDS–PAGE. Proteins were analyzed using western blotting with anti-PY IgG (**A**). The blot was stripped and reprobed with anti-Met IgG (**B**). The results are representative of three experiments.

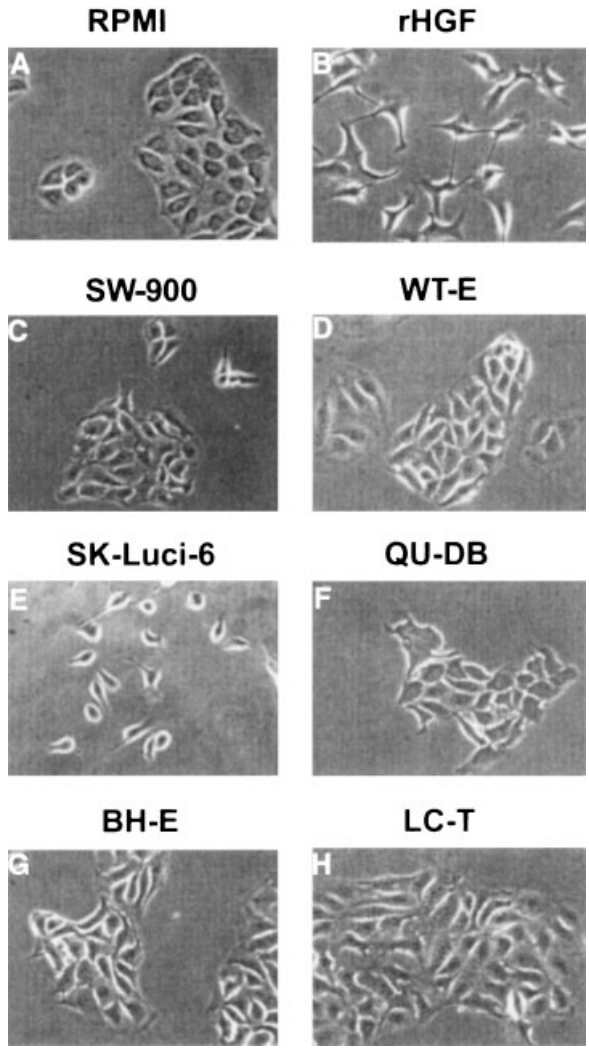


**Fig. 5.** Analysis of scattering activity of conditioned media from NSCLC cell lines. MDCK cells were incubated at 37°C in 5% $CO_2$ atmosphere for 24 h with CMs collected from the NSCLC cell lines in Figure 3, as described in Materials and Methods. Controls consisted of cells incubated with RPMI (**A**) or recombinant HGF (**B**). After 24 h, scattering was assessed visually, and representative images were photographed, using an inverted Leica microscope. The results are representative of three experiments.

shown that cell adhesion can promote ligand-independent activation of Met. Furthermore, Wang et al. [2001] reported recently that activation of Met by cell attachment induces and sustains hepatocellular carcinomas in transgenic mice. We therefore examined whether cell adhesion is required for constitutive activation of Met in NSCLC cell lines. The results showed that both SW-900 and WT-E cells sustained significant tyrosine-phosphorylation of Met, following 4 h incubation in suspension culture (Fig. 6A). In contrast, A549 cells (Met-positive,

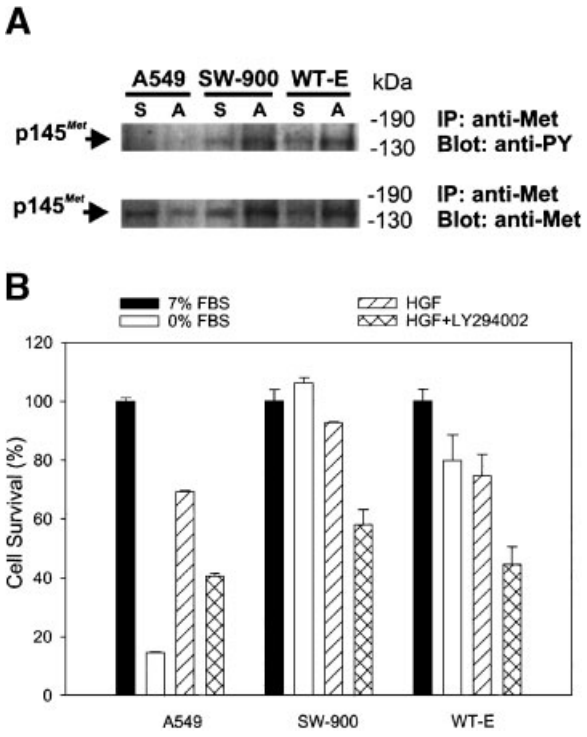


**Fig. 6.** Analysis of activation of Met and cell survival of NSCLC cell lines under anchorage-independent conditions. (**Panel A**) NSCLC cell lines A549, SW-900, and WT-E were serum-starved over night, and reseeded into suspension culture(s), or left on tissue culture plates (A) for 4 h at 37°C. The cells were then lysed and cell extracts were immunoprecipitated with anti-Met IgG and analyzed by Western blotting with anti-PY (upper) or anti-Met (lower) antibodies. The results are representative of two experiments. (**Panel B**) NSCLC cell lines A549, SW-900, and WT-E were serum-starved over night, and seeded in suspension cultures alone, or with 7% FBS, or with recombinant HGF (20 ng/ml). The PI3K inhibitor, LY294002 (25 μM), was added to a duplicate HGF-treated group for each cell line. After 24 h incubation at 37°C, cells were transferred to 96-well plates, and surviving cells were measured with the MTS colorimetric assay as described in Materials and Methods. The results are expressed as mean ± range of duplicates, and are representative of two experiments.

HGF-negative) required stimulation with recombinant HGF to maintain the same level of Met activation. Thus, constitutive activation of Met in NSCLC cell lines SW-900 and WT-E is sustained under anchorage-independent conditions.

### NSCLC Cell Lines With Constitutive Activation of Met Show Spontaneous PI3K-Dependent Cell Survival

We have previously shown that HGF/Met signaling induces a PI3K-dependent cell survival response in detached carcinoma cells [Qiao et al., 2000]. We therefore examined survival of SW-900 and WT-E cell lines, which express constitutively activated Met. Both cell lines showed spontaneous cell survival under anchorage-independent conditions (Fig. 6B). In contrast, A549 cells, which displayed paracrine activation of Met, required addition of exogenous recombinant HGF to elicit a cell survival response. Inhibition of PI3K activity with the LY294002 inhibitor [Qiao et al., 2000] abrogated both spontaneous, and HGF-induced, cell survival in NSCLC cell lines (Fig. 6B and data not shown). Thus, NSCLC cell lines expressing constitutively activated Met can sustain PI3K-dependent cell survival under anchorage-independent conditions.

### Paracrine HGF Requirement for Induction of DNA Synthesis in Non-Small Cell Lung Carcinoma Cell Lines

We also examined DNA synthesis in the various NSCLC cell lines under serum-starved conditions. The results showed a low basal level of DNA synthesis, and strong paracrine stimulation with recombinant HGF in A549, SW-900, and WT-E cell lines (Fig. 7). In addition, concomitant activation of extracellular signal-regulated kinases (ERK1/2), required for cell proliferation [Qiao et al., 2000], was observed in all three cell lines (Fig. 8). Thus, additional paracrine stimulation with HGF is required for an optimal DNA synthesis response regardless of the basal activation status of Met in these cells.

## DISCUSSION

A broad range of genetic markers have been associated with invasive lung cancer including amplification of EGF receptor [Rusch et al., 1995], *K-ras* point mutations [Wistuba et al., 2001], and dominant negative mutations of p53 [Rusch et al., 1995]. However, genetic changes similar to those found in lung cancers can often be detected in the non-malignant bronchial epithelium of current and former smokers, and can persist for many years after smoking cessation, thus rendering early diagnosis difficult [Mao et al., 1997]. Over-expression of HGF and Met has been observed in a large proportion of human NSCLC tissues [Olivero et al., 1996; Tsao et al., 1998, 2001], and has been suggested as a possible independent early predictor of poor survival of lung cancer patients [Olivero et al., 1996]. However, there are very few studies

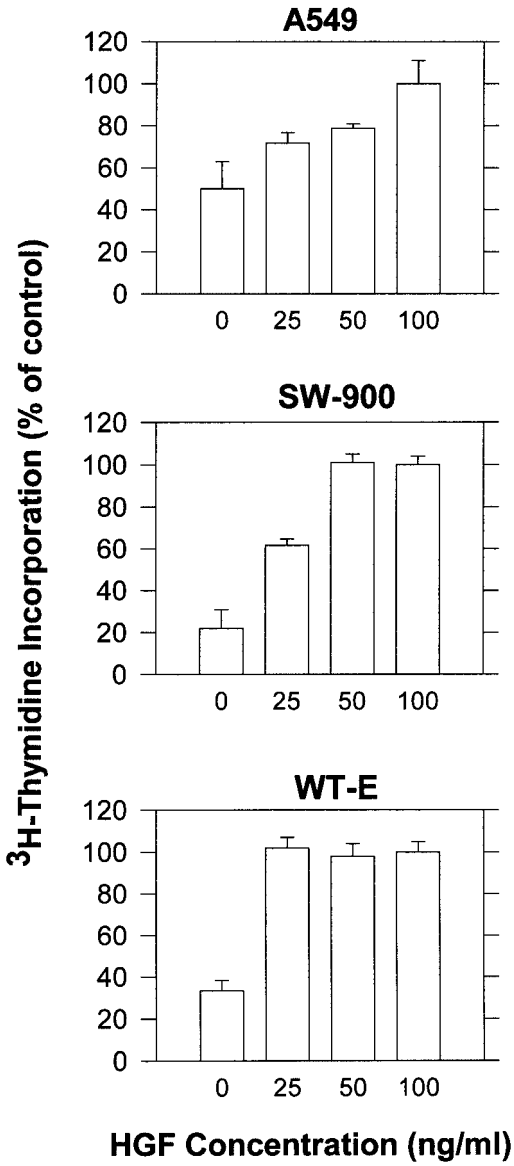


**Fig. 7.** HGF-induced DNA synthesis of NSCLC cell lines. NSCLC cell lines A549, SW-900, WT-E, and FR-E were serum-starved overnight, and subcultured in 24-well tissue culture plates ($10^4$ cells/well) without, or with, recombinant HGF at the concentrations indicated, as described in the Materials and Methods. After 24 h, 0.2 μCi of [$^3$H]-thymidine was added, and cells were incubated for an additional 24 h. Cells were then harvested, transferred to filters, and the incorporation of [$^3$H]-thymidine was measured using a scintillation counter. Results are expressed as the mean cpm of quadruplicate wells ± SD. The results are representative of three experiments.

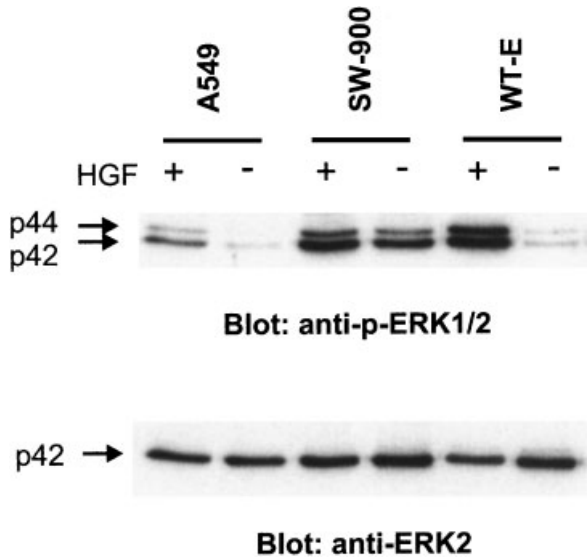


**Fig. 8.** Status of ERK1/2 activation in NSCLC cell lines. A549, SW-900, and WT-E were cultured to 80% confluence and serum-starved overnight. The cells were then treated with recombinant HGF (20 ng/ml) for 20 min at 37°C, and lysed. Clarified cell lysates were normalized for protein concentration, subjected to reducing SDS–PAGE and immunoblotted with anti-phospho-ERK1/2. Bands were visualized with ECL reagents. Equal protein loading was confirmed with anti-ERK 2 IgG.

correlating the activation status of Met with tumorigenic properties of NSCLC.

We have investigated the expression and activation status of Met, cell survival, and proliferation in a panel of seven NSCLC cell lines, derived from primary lung tumors and pleural effusions in lung cancer patients. Two NSCLC cell lines, SW-900 and WT-E, showed constitutive activation of Met compared to A549 cells, which required paracrine stimulation with recombinant HGF for Met activation. We further showed that SW-900 and WT-E cells maintained spontaneous cell survival in the absence of anchorage, an important step in malignant transformation [Dedhar, 1995]. These findings suggest that constitutive activation of Met may provide a selective survival advantage in the development of NSCLC. Future studies need to determine if the expression and activation of Met in NSCLC will be a useful early diagnostic or prognostic indicator, a monitor for chemoprevention, or a potential target for developing new therapies.

Over-expression of HGF has been shown to cause activation of an HGF/Met autocrine loop and enhanced tumorigenicity in a human lung adenocarcinoma cell line [Yi and Tsao, 2000]. To investigate the possible role of autocrine HGF expression in the constitutive activation of Met in NSCLC cells, we measured the level of expression of HGF mRNA and protein. HGF mRNA was readily detected in three NSCLC cell lines (SK-Luci-6, BH-E, and SW-900) using unlabeled RT-PCR; whereas trace levels of HGF

mRNA were detected also in WT-E and A549 NSCLC cell lines using a radiolabeled RT-PCR approach (data not shown). Purification of putative HGF protein by Cu(II) affinity chromatography and Western blot analysis showed immunoreactive bands corresponding to both pro-HGF (100 kDa) and mature HGF $\alpha$ chain (65 kDa) in two Met-positive, and two Met-negative, NSCLC cell lines tested. In addition, immunoreactive bands in the 55–60 kDa region were detected, suggesting the presence of a putative NK2 isoform [Schwall et al., 1996] or degradation products of HGF [Fehlner-Gardiner et al., 1999]. Despite the presence of putative HGF protein in CMs, only one of the HGF-producing cell lines (SK-Luci-6) showed significant biological activity of secreted HGF, as determined by scattering of MDCK cells and stimulation of tyrosine-phosphorylation of Met in A549 cells. The lack of detectable HGF activity in CM from the other cell lines could reflect low sensitivity of the assay, inactivation of HGF due to association with extracellular matrix proteins (e.g., proteoglycans such as heparin sulfate [Rahimi et al., 1994]), or proteolytic degradation [Fehlner-Gardiner et al., 1999]. It is also possible that the anti-HGF antibody cross-reacts with the related family member, MSP [Donate et al., 1994], or ligands for another receptor tyrosine kinase that can act co-operatively with Met. Thus, the secreted immunoreactive HGF protein is unlikely to contribute significantly to the observed constitutive activation of Met in SW-900 and WT-E cells, although intracrine activation of Met, as has been shown for epidermal growth factor receptor [Cao et al., 1995] and fibroblast growth factor receptor-2 [Peng et al., 2001], may be involved. Alternatively, ligand-independent activation could occur due to over-expression of Met protein on the cell surface [Rusciano et al., 1999].

Previous findings have indicated that cell attachment can promote ligand-independent activation of Met, correlating with its over-expression in some melanoma, colorectal, and hepatocellular carcinoma cell lines [Wang et al., 1996, 2001; Rusciano et al., 1999; Qiao et al., 2000]. We have also shown that cell adhesion to osteopontin promotes activation of Met and increased cell migration in mammary carcinoma cells [Tuck et al., 2000]. We therefore examined whether constitutive activation of Met in SW-900 and WT-E cells was dependent on cell attachment. Interestingly, our results show that constitutive activation of Met in SW-900 and WT-E cells is sustained even under anchorage-independent conditions. In contrast, one Met-positive cell line, A549, exhibited only paracrine stimulation by HGF of Met. Thus, constitutive activation of Met in SW-900 and WT-E cells occurs independent of anchorage, or exogenous HGF ligand. High density of Met expression, as was seen on SW-900 and WT-E cells (Fig. 1A), could contribute to ligand-independent activation of Met in these cells [Rusciano et al., 1999; Wang et al., 2001]. It is also possible that co-operative interaction with other receptor tyrosine kinases such as EGFR [Jo et al., 2000], or mutations in *met* [Di Renzo et al., 2000], may contribute to the strong constitutive activation of Met in these cells.

NSCLC cells expressing constitutively activated Met showed spontaneous cell survival under anchorage-independent conditions, compared to A549 cells, which required stimulation with recombinant HGF. PI3K activity was required for both HGF-induced, and spontaneous survival of NSCLC cell lines, consistent with the role of a PI3K-dependent survival pathway, as previously described [Qiao et al., 2000]. Interestingly, serum-starved SW-900 and WT-E cells, which exhibit a medium level of Met kinase activity, required additional paracrine stimulation with recombinant HGF for an optimal proliferation response. Our results suggest that a medium level of constitutive Met activation is associated with PI3K-dependent survival of NSCLC cells; whereas additional paracrine stimulation with recombinant HGF and high level of Met activation, concomitant with increased ERK1/2 activation, is required for DNA synthesis. The differential survival and proliferation responses in cells exhibiting constitutive versus paracrine levels of Met kinase activity could represent quantitative or qualitative differences in the signaling events involved.

In summary, our results show heterogeneity of HGF and Met expression and activation in NSCLC cell lines. Carcinoma cells expressing functional HGF protein but not Met, could mediate paracrine or endocrine stimulation of adjacent Met-expressing carcinoma cells leading to enhanced survival and growth in the primary tumor site. In addition, constitutive activation of Met in some NSCLC cells occurs in the absence of cell anchorage, and could enhance survival in conditions where adhesion to

surrounding basement membrane proteins is disrupted, such as invasion from the primary tumor and seeding of foreign tissue sites. Our findings further show that while a medium level of constitutive Met activation correlates with cell survival; a high level of Met activation is required for DNA synthesis in NSCLC cells. Future studies will examine the mechanisms of constitutive activation of Met in NSCLC cells, and the differential effects of constitutive versus paracrine HGF/Met signaling in the development of NSCLC.

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