c-MET Expression in Myofibroblasts

Role in Autocrine Activation and Prognostic Significance in Lung Adenocarcinoma

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Hepatocyte growth factor (HGF) plays important roles in tumor development and progression. It is currently thought that the main action of HGF is of a paracrine nature: HGF produced by mesenchymal cells acts on epithelial cells that express its receptor c-MET. In this investigation, we explored the significance of c-MET expression in myofibroblasts, both in culture and in patients with lung adenocarcinoma. We first showed that human myofibroblasts derived from primary lung cancer expressed c-MET mRNA and protein by reverse transcription-polymerase chain reaction and Western blot analysis. Proliferation of myofibroblasts was stimulated in a dose-dependent manner by exogenously added recombinant human HGF whereas it was inhibited in a dose-dependent manner by neutralizing antibody to HGF. The addition of HGF in the culture medium stimulated tyrosine phosphorylation of c-MET. The c-MET protein was immunohistochemically detected in myofibroblasts in the invasive area of lung adenocarcinoma. Finally, the prognostic significance of c-MET expression in stromal myofibroblasts was explored in patients with small-sized lung adenocarcinomas. c-MET-positive myofibroblasts were observed in 69 of 131 cases (53%). A significant relationship between myofibroblast c-MET expression and shortened patient survival was observed in a whole cohort of patients including all pathological stages (two-sided $P = 0.0089$ by log-rank test) and in patients with stage IA disease (two-sided $P = 0.0019$ by log-rank test). These data suggest that the HGF/c-MET system constitutes an autocrine activation loop in cancer-stromal myofibroblasts. This autocrine system may play a role in inva-

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Hepatocyte growth factor (HGF) is a heterodimeric polypeptide exhibiting pleiotropic biological functions as a mitogen, motogen, and morphogen.¹ HGF is secreted as an inactive single-chain glycoprotein, and inactive HGF is converted to its active form (mature HGF) by a serine protease HGF activator.²⁻⁴ The mature HGF molecule is a heterodimer consisting of a 69-kd α -chain and a 34-kd β -chain.^{1,5} The mRNA expression and protein synthesis/secretion of HGF have been detected primarily in a variety of mesenchymal cells including fibroblasts, smooth muscle cells, and endothelial cells.⁴ Its receptor c-MET proto-oncogene product consists of an α -chain of 50 kd and a β -chain of 145 kd. The α -chain is exposed at the cell surface, whereas the β -chain spans the cell membrane and possesses an intracellular tyrosine kinase domain.⁶⁻⁹ The c-MET protein is expressed in epithelial cells of a variety of organs,¹⁰ melanocytes,¹¹ and non-epithelial cells including endothelial cells,¹² microglial cells,^{13,14} neurons,¹⁵ hepatic stellate cells,¹⁶ and muscle cells.¹⁷

Studies suggest a possible role of HGF/c-MET in tumor development and progression.¹⁸⁻²⁰ HGF stimulates the proliferation and migration of tumor cells *in vitro*. Transgenic mice that overexpress c-MET develop tumors.²¹ HGF is expressed mainly by stromal myofibroblasts, whereas c-MET is expressed by cancer cells.⁴ Although an HGF/c-MET autocrine loop has recently been reported in nonepithelial malignancies,²²⁻²⁶ the HGF/c-MET system is currently thought to function mainly in a paracrine manner.

Of the various malignant tumors, lung carcinoma is the most common cause of cancer death in the world. The prognosis of the patients is primarily dependent on

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the stage of the disease. However, even in patients with pathological stage I non-small-cell lung cancer, the overall survival is 64.6% (range, 55 to 72%).²⁷ Approximately 30 to 40% of pathological stage I patients have disease recurrence and die after curative resection.²⁸

It has been reported that tumor cells with thick fibrovascular stroma are more likely to metastasize in lung adenocarcinoma.²⁹ Similarly, in small-sized lung adenocarcinoma (maximum dimension 2 cm or less) the presence of active fibroblast proliferation correlates with poor prognosis.³⁰ Active fibroblasts are also commonly referred to as myofibroblasts because they show morphological characteristics of both fibroblasts and smooth muscle cells.^{31,32} The presence of myofibroblasts has been documented in several types of cancers, such as those of the uterine cervix,³³ colon,³⁴ ovary,³⁵ and breast.^{36,37} Myofibroblasts occur in a variety of other conditions including hypertrophic scars,³⁸ healing wound,³⁹ granulation tissue,³⁸ idiopathic pulmonary fibrosis,^{40,41} pulmonary hypertension,⁴² scleroderma lesions,⁴³ anterior capsular cataract,⁴⁴ heart pressure overload,⁴⁵ bleomycin-injured lung,⁴⁶ and postradiation fibrosis of the breast.⁴⁷ By secreting various growth factors and cytokines including HGF, these myofibroblasts may modulate the behavior of adjacent cancer cells.

In the present study, we demonstrate that stromal myofibroblasts of lung adenocarcinoma express c-MET both *in vitro* and *in vivo*. We then present evidence that the HGF/c-MET system functions as an autocrine stimulatory loop in cultured myofibroblasts derived from primary lung cancer. Finally, we show that c-MET expression in stromal fibroblasts is correlated with poor prognosis in small-sized lung adenocarcinoma.

Materials and Methods

Cells and Cell Culture

Human myofibroblast cell lines (MRC5, WI38) and human gastric cancer cell lines (MKN28, MKN45) were purchased from the Human Science Research Resources Bank. Human lung myofibroblasts (N421, T421, N425, T425, T501, N515, T5062, N5162, T5162) were obtained by subculturing fibroblastic cells that grew from enzymatically digested lung specimens. T421, T425, T501, T5062, and T5162 were grown from lung carcinoma tissues, whereas N421, N425, N515, and N5162 were from nontumor lung tissues. A human umbilical vein endothelial cell line (HUVEC.SV) was established by immortalization of human umbilical vein endothelial cells with SV40 large T antigen.

Human myofibroblast cell lines, lung myofibroblasts, and HUVEC.SV were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μ g/ml streptomycin. Human gastric cancer cell lines were cultured in RPMI1640 containing 10% FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were cultured at 37°C in humidified atmosphere of 5% CO₂ and 95% air. The culture was maintained by subculturing every 3 or 4 days.

Reagents

Recombinant human HGF, transforming growth factor β (TGF- β), epidermal growth factor (EGF), interleukin 1 β (IL-1 β), acidic fibroblast growth factor (aFGF), basic FGF (bFGF), FGF-4, keratinocyte growth factor (KGF), platelet-derived growth factor (PDGF)-AB, PDGF-BB, monoclonal anti-human HGF antibody (neutralizing antibody to HGF, clone no. 24612.111), and mouse IgG1 isotype control (clone no. 11711.11) were purchased from R & D Systems (Minneapolis, MN). The ND50 of anti-human HGF antibody is ~0.1 to 0.3 μ g/ml in the presence of 100 ng/ml of recombinant human HGF, according to the manufacturer. Polyclonal rabbit anti-c-MET antibody (no. 18321) was purchased from IBL Laboratories (Gumma, Japan). Rabbit immunoglobulin fraction (for negative control, no. X0936), mouse monoclonal anti-human α -smooth muscle actin (no. M0851), mouse monoclonal anti-human desmin (no. M0760), and mouse monoclonal anti-human cytokeratin AE1/AE3 (no. M3515) were purchased from DAKO. The anti-human cytokeratin AE1/AE3 is a mixture of AE1 and AE3 at a ratio of 4:1. Monoclonal antibody AE1 recognizes the 56.5-, 50-, 48-, and 40-kd keratins of the acidic subfamily, whereas monoclonal antibody AE3 reacts with the basic keratins of molecular weights 65 to 67, 64, 59, 58, 56, and 52 kd.

Patients and Samples

Formalin-fixed, paraffin-embedded tumor specimens were obtained from a series of 131 patients with small-sized lung adenocarcinomas (maximum tumor dimension 2 cm or less; median age, 59 years; range, 26 to 80 years) who underwent complete resection of tumors and mediastinal lymphadenectomy at the National Cancer Center Hospital, Japan, between 1984 and 1993. The pathological stage was determined by the new TNM staging system that had been recognized by the Union Internationale Contre le Cancer and the American Joint Committee on Cancer.⁴⁸ The median duration of follow-up was 5.7 years (range, 0.6 to 12.0 years). Patient and tumor characteristics are listed in Table 1.

Primers and Probes

The primers and probes used for reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time RT-PCR were as follows: c-MET primer, forward 5'-CATGCCGACAA-GTGCAGTA-3', reverse 5'-TCTTGCCATCATTGTCCAAC-3'; c-MET probe, 5'-TCCAGGCAGTGCAGCATGTAGTGAT-3'; HGF primer, forward 5'-GAGTTATCGAGGTCTCATGGATC-3', reverse 5'-CCAACGCTGACATGGAATT-3'; HGF probe, 5'-TCAGACACCACCCGGCACAAT-3'; E-cadherin primer, forward 5'-CTTCTCTCACGCTGTGTCATC-3', reverse 5'-CTC-CTGTGTTCTGTTAATGGT-3'; E-cadherin probe, 5'-TACAA-TGCCGCCATCGCTTACAC-3'; CD31 primer, forward 5'-GCA-GATAATTGCCATTCCCAGT-3', reverse 5'-CTTCATTACAGCAGCACATTGCAG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer, forward 5'-GAAGGTGAAG-

Table 1. Characteristics of Small-Sized Lung Adenocarcinoma

Clinical feature	No. of cases	Clinical feature	No. of cases
All cases	131	Nodal involvement (pN)	
Age (years)		Negative (pN0)	107
Range	26–80	Positive (pN1, 2)	24
Median	59	Pleural invasion	
Sex		Negative	95
Male	73	Positive	36
Female	58	Pathological stage	
Tumor size (mm)		IA	96
Range	7.0–20.0	IB	7
Mean	16.9	IIA	7
Vascular invasion		IIB	1
Negative	83	IIIA	11
Positive	48	IIIB	2
Lymphatic invasion		IV	7
Negative	77	Years follow-up	
Positive	43	Range	0.591–11.992
Equivocal	11	Median	5.744

GTCGGAGTC-3', reverse 5'-GAAGATGGTGTATGGGATTC-3'; GAPDH probe 5'-CAAGCTTCCCCTTCAGCC-3'.

The probes for c-MET, HGF, and E-cadherin were labeled with the fluorescent dyes 6-carboxyfluorescein (FAM) at the 5' end and *N,N,N',N'*-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3' end. The GAPDH probe was labeled with the fluorescent dyes 6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein (JOE) at the 5' end and *N,N,N',N'*-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3' end. The primers and probes were synthesized at Greiner Labortechnik Co. Ltd. and Applied Biosystems/Perkin Elmer, respectively.

RNA Isolation

Total RNA was isolated from frozen tissues and cultured cells using an RNeasy mini kit (Qiagen, Basel, Switzerland) following the manufacturer's protocol. All RNA samples were treated with deoxyribonuclease I, amplification grade (Gibco/BRL), followed by a clean-up with an RNeasy Mini Kit (Qiagen).

RT-PCR Analysis and Real-Time Quantitative RT-PCR Analysis

We performed a real-time quantitative one-step RT-PCR assay^{49,50} to quantify c-MET, HGF, E-cadherin, and GAPDH mRNAs in microdissected samples following the manufacturer's protocol (Applied Biosystems/Perkin Elmer). The real-time one-step RT-PCR reactions were performed using the TaqMan EZ RT-PCR core reagents (no. N808-0236, Applied Biosystems/Perkin Elmer) in an ABI PRISM 7700 sequence detection system (Applied Biosystems/Perkin Elmer). The RT-PCR reactions were performed using 96-well optical tubes and caps in a 50- μ l final reaction volume consisting of 200 nmol/L each primer, 100 nmol/L probe, 1 $\times$ TaqMan EZ buffer, 3 mmol/L manganese acetate, 300 μ mol/L each deoxyATP, deoxyCTP, deoxyGTP, 600 μ mol/L deoxyUTP, 0.1 U/ μ l rTth DNA polymerase, 0.01 U/ μ l AmpErase uracil-*N*-gly-

cosylase, 21.5 μ l RNase-free water, and 1 μ l RNA samples. The thermal cycling conditions consisted of an initial denaturation step with one cycle of 50°C for 2 minutes, one cycle of 60°C for 30 minutes, one cycle of 95°C for 5 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Each RT-PCR run included fivefold serial dilutions of a standard RNA, a no-template control, and test samples. We quantified transcripts of GAPDH as endogenous RNA controls, and each value was normalized on the basis of the GAPDH mRNA content. Experiments were performed in triplicate and the results were expressed as mean values. For ordinary RT-PCR analysis, we used a GeneAmp PCR System 9600 (Applied Biosystems/Perkin Elmer). All RT-PCR products were separated by 3% Nusieve 3:1 agarose gel (FMC BioProducts) and visualized by ethidium-bromide staining. Contamination was routinely checked by RT-PCR assay of RNA-free samples (water control).

Laser Beam-Based Microdissection

Tumor tissues were snap-frozen in OCT Compound (Sakura Finetech Co., Ltd., Japan). They were sliced into 15- μ m thick sections, mounted on glass slides 0.17 mm thick (very thin slides were needed to prevent laser energy from being dispersed before reaching the tissue section), and stained with hematoxylin and eosin. We microdissected the specimens with an UV-laser microscope system (PALM UV-laser microbeam; Wolfratshausen, Germany) to separate cancer and stromal cells.^{51,52} Total RNA was extracted from these microdissected specimens for real-time quantitative RT-PCR analysis.

Western Blot Analysis

Cells were lysed in a lysis buffer containing 1% Triton X-100 (Sigma Chemical Co.) and 1% Nonidet P-40 (Sigma Chemical Co.) and a cocktail of proteinase inhibitors. Equal amounts of protein samples were size-separated on discontinuous 6% polyacrylamide gels and trans-

ferred to nitrocellulose membranes. The membranes were immersed for 20 minutes in a blocking solution containing 5% skim milk and 0.1% Tween, and then incubated for 2 hours at room temperature with rabbit anti-cMET antibody or rabbit immunoglobulin IgG as a negative control. After washing, the membranes were incubated for 1 hour at room temperature with peroxidase-linked secondary antibody. The antigen was detected using enhanced chemiluminescence Western blotting detection reagents (Amersham, Arlington Heights, IL) following the instructions of the manufacturer.

Immunoprecipitation-Western Blot Analysis

For immunoprecipitation, cells were lysed in a buffer containing 1% deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, and a cocktail of proteinase inhibitors. Equal amounts of samples (500 μ g) were diluted with lysis buffer, and precleared with 90 μ l of protein-A Sepharose (Pharmacia, Uppsala, Sweden) (50% slurry) for 1 hour. After the addition of anti-c-MET antibody (5 μ g), samples were incubated overnight at 4°C. Immune complexes were precipitated by incubating the samples for 1 hour with 90 μ l of protein-A Sepharose. Sepharose beads were then washed three times with lysis buffer and once with water. After boiling in loading buffer, samples were subjected to Western blot analysis using anti-phosphotyrosine antibody (clone 4G10, dilution $\times$ 1/1000; Upstate Biotechnology). Western blot was performed essentially the same as described above except that 2% bovine serum albumin was used as a blocking solution.

Measurement of HGF in Culture Media

Subconfluent myofibroblasts were washed twice with serum-free DMEM and incubated for 24 hours with DMEM containing 1% FCS and 2 μ g heparin. The latter was included in the media to facilitate the release of matrix-bound HGF from the cell layer. The conditioned media were then subjected to the enzyme-linked immunosorbent assay (ELISA) analysis using Quatikine human HGF (R & D systems) following the manufacturer's protocol. The results were corrected for the protein content of the cell layer.

Cell Proliferation Assay

We analyzed the effect of HGF/c-MET signaling in cultured myofibroblasts by 5-bromo-2'-deoxyuridine (BrdU) incorporation into DNA, using a Cell Proliferation ELISA BrdU kit (catalog no. 1647229, Boehringer Mannheim, Mannheim, Germany).^{53,54} The human lung myofibroblasts (N421, N515, T5062, T5162, MRC5) were seeded at a density of 1×10^4 cells into collagen-coated 96-well tissue culture microplates (no. 4860-010, Iwaki Glass) in DMEM containing 10% FCS. Subsequently, cells were allowed to spread for 24 hours, cultured for an additional 24 hours in fresh DMEM containing 2% FCS, and then further incubated for 24 hours in fresh DMEM with 2%

FCS in the absence or presence of various concentrations of recombinant human HGF, TGF- β , EGF, IL-1 β , acidic FGF, basic FGF, FGF-4, KGF, PDGF-AB, PDGF-BB, neutralizing antibody to HGF, and mouse IgG1 isotype control. Next, the cells were incubated for 6 hours in fresh DMEM containing 10 μ mol/L BrdU. After BrdU labeling, the culture medium was removed, and the cells were fixed at room temperature for 30 minutes with a FixDenat solution (ethanol). Cells were then reacted for 90 minutes with 1:100-diluted peroxidase-conjugated monoclonal anti-BrdU antibody (clone BMG 6H8, Fab fragments), and after washing, samples were incubated at room temperature in a substrate solution containing tetramethylbenzidine. The enzymatic reaction was stopped after 15 minutes by adding 25 μ l of 1 mol/L H₂SO₄. The intensity of the color reaction was measured with an automatic ELISA plate reader using a 450-nm filter (model 3550, UV Microplate Reader; Bio-Rad Laboratories, Richmond, CA). All experiments were performed in triplicate and the data were expressed as means $\pm$ SD of triplicate samples.

Immunohistochemical Analysis

The resected specimens were fixed in formalin, embedded in paraffin, and prepared into 3- μ m-thick sections. Immunoperoxidase staining by the avidin-biotin-peroxidase complex method was performed with a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). The sections were deparaffinized with xylene, washed in phosphate-buffered saline (pH 7.4), heated by autoclave treatment, and incubated for 10 minutes with normal swine serum to block nonspecific binding of the antibody. The sections were exposed to rabbit anti-c-MET IgG (no. 18321, IBL Laboratories) at a dilution of 1:50, mouse monoclonal anti-human α -smooth muscle actin antibody (no. M0851, DAKO) at 1:200, mouse monoclonal anti-human desmin antibody (no. M0760, DAKO) at 1:100, or rabbit immunoglobulin as a negative control (no. X0936, DAKO) at 4°C overnight. Sections were then incubated with biotinylated secondary antibody for 30 minutes at room temperature, and with the Vectastain ABC reagent for 30 minutes. For visualization of the antigen, sections were immersed in 0.05% diaminobenzidine tetrahydrochloride solution containing 0.01% H₂O₂, and counterstained lightly with hematoxylin. The results were evaluated as positive when bundles of myofibroblasts were stained for c-MET in more than one microscopic area: occasional scattered c-MET-positive cells were considered negative.

Statistical Analysis

All statistical analyses were performed using the computer software StatView-J 5.0 (Abacus Concepts, Berkeley, CA). The results of the cell proliferation assay were analyzed by one-way analysis of variance followed by Scheffé's test. The chi-square test was used to examine the association between increased c-MET expression and various clinicopathological characteristics. The

Kaplan-Meier method was used to estimate survival as a function of time, and survival differences were analyzed by the log-rank test. To assess the correlation between survival time and multiple clinicopathological characteristics, multivariate analyses were performed using the Cox proportional hazards model. Differences were considered significant when the *P* value was less than 0.05.

Results

Laser-Beam Microdissection and Real-Time Quantitative RT-PCR Analysis

We have used the laser-beam microdissection method to identify genes that are selectively expressed in cancer or cancer-stromal cells of lung adenocarcinoma. After laser-beam microdissection, total RNA was isolated from dissected cancer and stromal cells, separately. Expression levels of the genes of interest were measured by real-time quantitative RT-PCR analysis. We microdissected, on average, ~10 sections per tumor sample. The amount of extracted RNA varied greatly from sample to sample. In one specimen, we were able to extract ~80 and 37 ng of total RNA from the tumor and stromal fraction, respectively, whereas in another sample only 1 to 2 ng of total RNA was extractable from both fractions. These differences probably reflected the different tumor-stromal cell ratio and cellularity in the various tumor specimens. In the course of these experiments, we encountered cases in which the stromal compartment contained substantial c-MET mRNA even when microdissections were performed very carefully. Three different tumor samples were analyzed, and the results are shown in Figure 1. As expected, HGF mRNA expression was found exclusively in the stromal compartment in all of the three cases. E-cadherin mRNA was found only in the cancer cell compartment in tumors 1 and 3. In tumor 2, there was a very low level of E-cadherin mRNA in the stromal compartment, suggesting a minor contamination of epithelial cells. Unexpectedly, c-MET mRNA was found both in cancer and stromal cell compartments in tumors 1 and 2. Although the c-MET signal in the stromal fraction of tumor 2 was weak, it could not be accounted for by the very low level of epithelial cell contamination. Thus, these preliminary experiments suggested that in a subset of lung adenocarcinomas c-MET mRNA may be expressed by stromal cells.

c-MET mRNA and Protein Expression in Cultured Myfibroblasts

In the microdissected tissue, myfibroblasts appeared to be the major cell type within the cancer stroma. Therefore, we next investigated whether cultured myfibroblasts express c-MET mRNA and protein. For this purpose, we analyzed c-MET mRNA expression in two established myfibroblast cell lines (MRC5 and WI38) and cultured myfibroblasts obtained from primary lung cancers and nontumor lung tissues. As shown in Figure

Figure 1. Laser-beam microdissection and real-time quantitative RT-PCR analysis. Expression levels of HGF, c-MET, and E-cadherin mRNAs were analyzed by real-time quantitative RT-PCR using total RNA extracted from lung cancer and stromal cells separated by the laser-beam microdissection method. The results were corrected for GAPDH. The laser-beam microdissection and the real-time quantitative RT-PCR analysis were performed as described in Materials and Methods. HGF mRNA expression was found exclusively in the stromal compartment in all of the three cases. E-cadherin mRNA was found only in the cancer cell compartment in tumors 1 and 3. In tumor 2, there was a very low level of E-cadherin mRNA in the stromal compartment, suggesting a minor contamination of epithelial cells. c-MET mRNA was found both in cancer and stromal cell compartments in tumors 1 and 2. Although the c-MET signal in the stromal fraction of tumor 2 was weak, it could not be accounted for by the very low level of epithelial cell contamination.

2A, c-MET mRNA was detected in all myfibroblasts, MKN28, and HUVEC.SV. To rule out the possibility that the c-MET message was derived from contaminating epithelial or endothelial cells, we examined the expression of E-cadherin and CD31 mRNAs in each culture. As expected, E-cadherin mRNA was found exclusively in MKN 28, and CD31 mRNA in HUVEC.SV. HGF mRNA was expressed in all myfibroblasts. GAPDH mRNA was amplified in all samples except the RNA-free sample (water control). These results showed that both HGF and c-MET mRNAs were co-expressed in cultured myfibroblasts.

The expression of c-MET protein was analyzed in cultured myfibroblasts by Western blot analysis. As shown in Figure 2B, two bands corresponding to a 145-kd c-MET β subunit and a 170-kd single-chain precursor of c-MET were detected in all myfibroblasts.

Expression Levels of HGF and c-MET mRNA in Cultured Myfibroblasts

Next we quantified by real-time RT-PCR analysis the relative abundance of HGF and c-MET mRNAs in myfibroblasts cultured from lung carcinoma (T5062, T5162, T421, and T501) and nontumor lung tissues (N5161,

Figure 2. A: RT-PCR analysis of HGF, c-MET, E-cadherin, CD31, and GAPDH mRNA expression in four cultured myofibroblasts derived from primary lung cancer and nontumor lung tissue, and two myofibroblast cell lines. RT-PCR analysis was performed as described in Materials and Methods. Representative results are shown. **Lane 1**, molecular weight marker; **lane 2**, N421; **lane 3**, N515; **lane 4**, T5062; **lane 5**, T5162; **lane 6**, WI38 (myofibroblast cell line); **lane 7**, MRC5 (myofibroblast cell line); **lane 8**, MKN28 (positive control for c-MET and E-cadherin); **lane 9**, HUVEC.SV (positive control for CD31); **lane 10**, RNA-free sample (negative control for RT-PCR). **B:** Western blot analysis of c-MET protein in cultured myofibroblasts. **Lane 1**, MKN45 (positive control for c-MET); **lane 2**, MRC5 (myofibroblast cell line); **lane 3**, WI38 (myofibroblast cell line); **lane 4**, T5162; **lane 5**, T501; **lane 6**, T5062; **lane 7**, N421; **lane 8**, N425; **lane 9**, N5162.

N5162, N421, N425, and N515). The results are shown in Figure 3. Overall, c-MET mRNA levels tended to be higher in tumor-derived myofibroblasts than in myofibroblasts derived from nontumor lung tissues. There seemed to be no clear differences in HGF mRNA levels between the two groups of myofibroblasts, and no clear correlation was observed between HGF and c-MET mRNA levels.

Figure 3. Scattergram showing expression levels of HGF and c-MET mRNAs in cultured myofibroblasts. HGF and c-MET mRNA levels were determined by real-time quantitative RT-PCR as stated in Material and Methods. The results were corrected for GAPDH and expressed in arbitrary units. Myofibroblasts from lung cancer (T5162, T5062, T421, T425) and from nontumor lung tissue (N421, N425, N515, N5162, N5161) were analyzed.

Figure 4. Secretion of HGF in the culture media of myofibroblasts. Cells were incubated for 24 hours in DMEM with 1% FCS and 2 μ g of heparin. The concentrations of HGF in the conditioned media were determined by ELISA. The results were corrected for the protein content of the cell layer and expressed as pg/ μ g protein. The data represent duplicate measurements for each conditioned media.

Secretion of HGF Protein by Cultured Myofibroblasts

Production of the HGF protein by myofibroblasts was confirmed by ELISA. After 24 hours incubation, significant amounts of HGF accumulated in the conditioned media (Figure 4). At the protein level, tumor-derived myofibroblasts appeared to secrete a higher amount of HGF than those from nontumor lung tissue.

Enhanced Tyrosine Phosphorylation of c-MET by HGF

We then examined whether addition of HGF could induce tyrosine phosphorylation of the c-MET protein in myofibroblasts. As shown in Figure 5, tyrosine phosphorylation of c-MET was rapidly enhanced in N421 myofibroblasts 15 minutes after the addition of HGF. Induction of c-MET phosphorylation was also observed in T425 myofibroblasts, but the magnitude of induction was modest. Interestingly, the basal levels of phosphorylation were relatively high in T425. This seemed to be because of endogenously produced HGF, because treatment with neutralizing antibody reduced the level of c-MET tyrosine phosphorylation.

Figure 5. Tyrosine phosphorylation of c-MET in myofibroblasts. Tyrosine phosphorylation of c-MET was examined as stated in Material and Methods by immunoprecipitation with anti-c-MET antibody followed by immunoblotting with anti-phosphotyrosine antibody. Treatment with HGF (25 ng/ml) rapidly induced tyrosine phosphorylation of c-MET. In T425, incubation with neutralizing anti-HGF antibody for 24 hours reduced basal levels of c-MET tyrosine phosphorylation. **Lane 1**, control; **lane 2**, 15 minutes after addition of HGF; **lane 3**, incubation with mouse immunoglobulin (negative control for **lane 4**); **lane 4**, 24 hours of incubation with neutralizing antibody against HGF.

Figure 6. The effect of HGF/c-MET and other growth factor signaling on proliferation of cultured myfibroblasts. Cell proliferation was measured by the BrdU incorporation assay. **A:** Proliferation of N515, T5162, and MRC5 myfibroblasts was stimulated by exogenous recombinant HGF. **B:** Proliferation of N421 myfibroblasts was stimulated by HGF and other growth factors (TGF- β , EGF, aFGF, bFGF, FGF4, PDGF-AB, PDGF-BB) and cytokine (IL-1 β). Values represent means $\pm$ SD of triplicate measurements. *, $P < 0.05$ versus control; results were evaluated by one-way analysis of variance followed by Scheffé's test.

Cell Proliferation Assay

Next, we analyzed the effect of HGF/c-MET signaling on the proliferation of myfibroblasts by the BrdU incorporation assay. Figure 6A shows the results for N515, T5162, and MRC5 myfibroblasts. The proliferation of these myfibroblasts was stimulated by exogenously added recombinant HGF in a dose-dependent manner. Similar results were obtained for other myfibroblasts as well. To compare the stimulatory effect of HGF with those of other growth factors, myfibroblasts were exposed to different concentrations of recombinant HGF and other growth factors (TGF- β 1, EGF, aFGF, bFGF, FGF4, PDGF-AB, PDGF-BB) and cytokine (IL-1 β). Figure 6B shows the results for N421 myfibroblasts. Again, treatment with HGF stimulated proliferation of myfibroblasts in a dose-dependent manner ($P < 0.05$). Maximum stimulation was observed at a concentration of 25 ng/ml. Other growth factors and cytokines, with the exception of KGF, also enhanced cell proliferation ($P < 0.05$). Comparison between these growth factors revealed that the maximum stimulation by HGF (1.71-fold) was comparable to the

Figure 7. Inhibition of myfibroblast growth by neutralizing antibody against HGF. Cell growth was inhibited by addition of anti-HGF antibody in a dose-dependent manner in N421 and T5062 myfibroblasts (**A** and **C**), but not by addition of control IgG1 (**B** and **D**). Values represent means $\pm$ SD of triplicate measurements. *, $P < 0.05$ versus control; results were evaluated by one-way analysis of variance followed by Scheffé's test.

augmentation by TGF- β (1.68-fold), EGF (1.45-fold), aFGF (1.50-fold), and IL-1 β (1.42-fold), and somewhat less than the stimulation by bFGF (2.18-fold), FGF-4 (3.14-fold), PDGF-AB (2.56-fold), and PDGF-BB (2.70-fold). These results suggest that HGF is a potent growth factor for myfibroblasts in culture.

Because myfibroblasts express HGF mRNA and protein, we reasoned that endogenously produced HGF may function as an autocrine growth factor in myfibroblasts. To test this possibility, we investigated whether proliferation of myfibroblasts can be inhibited by neutralizing antibody against HGF. As shown in Figure 7, A and C, proliferation of myfibroblasts was inhibited by addition of neutralizing antibody against HGF in a dose-dependent manner in N421 and T5062, but not by addition of control IgG1 (Figure 7, B and D). Anti-HGF antibody at a concentration of 2.5 μ g/ml inhibited cell proliferation of N421 and T5062 myfibroblasts by 53.2 and 37.1%, respectively. Cell growth of N515, T5162, and MRC5 was also inhibited by the addition of anti-HGF antibody (data not shown). These results thus demonstrate that endogenously produced HGF functions as an autocrine growth factor in myfibroblast cultures.

Immunohistochemical Analysis of the c-MET Protein Expression by Human Myfibroblasts in Lung Adenocarcinoma

Next, we investigated by immunohistochemistry whether stromal myfibroblasts in lung adenocarcinoma express the c-MET protein. To clearly discriminate c-MET myofi-

Figure 8. Immunohistochemical analysis of c-MET protein in cancer-stromal myofibroblasts. The c-MET protein expression in cancer-stromal myofibroblasts was evaluated by immunohistochemistry in 131 small-sized lung adenocarcinomas (maximum tumor dimension 2.0 cm or less). **A:** α -Smooth muscle actin was strongly expressed by almost all cancer stromal cells in the invasive area of a lung adenocarcinoma. **C** is a higher magnification of **A**. **B:** Some of the myofibroblasts that were positive for α -smooth muscle actin were also positive for c-MET. **D** is a higher magnification of **B**. Lung adenocarcinoma consisting of c-MET-negative tumor cells is shown here to clearly demonstrate stromal c-MET expression. **E:** α -Smooth muscle actin was strongly expressed by almost all cancer stromal cells in noninvasive lung adenocarcinoma. **F:** Only isolated c-MET-positive cells were occasionally observed in the thickened alveolar septa. Most of these cells represented pericytes, endothelial cells, and alveolar myofibroblasts. Original magnifications: $\times 12.5$ (**A** and **B**) and $\times 100$ (**C-F**).

broblasts and other cells that may stain positive for c-MET, serial sections were stained for c-MET, α -smooth muscle actin, desmin, and keratin. In invasive areas of lung adenocarcinoma, α -smooth muscle actin was strongly expressed by virtually all cancer-stromal myofibroblasts (Figure 8, A and C). The c-MET protein was also expressed by stromal myofibroblasts. However, c-

MET expression in stromal cells was much more restricted; only a subset of myofibroblasts was stained positive for the c-MET protein. Typically, these c-MET-positive cells formed interlacing bundles (Figure 8, B and D). Most cancer cells expressed the c-MET protein to a varying degree, but there were tumors in which c-MET expression was observed only in stromal myofibroblasts.

In noninvasive bronchiolo-alveolar carcinoma, α -smooth muscle actin was strongly expressed by almost all of the cancer-stromal cells, which represented pericytes, endothelial cells, and alveolar myofibroblasts⁵⁵ (Figure 8E). In contrast, only a small proportion of the cells that were positive for α -smooth muscle actin stained for c-MET (Figure 8F). No immunoreaction was observed in these c-MET-positive myofibroblasts when serial sections were stained for desmin and keratin (data not shown). Thus, these c-MET-positive cells were not smooth muscle cells or spindle-shaped cancer cells.

Prognostic Significance of c-MET Expression in Stromal Myofibroblasts of Lung Adenocarcinoma

To investigate the significance of stromal c-MET expression in lung adenocarcinoma, we studied c-MET expression by immunohistochemistry in a series of 131 patients with small-sized lung adenocarcinomas. c-MET expression in cancer-stromal myofibroblasts occurred in 69 of 131 patients (53%). Kaplan-Meier survival curves demonstrated an association between c-MET expression in stromal myofibroblasts and poor prognosis of patients when all pathological stages ($P = 0.0089$ by log-rank test, Figure 9A) were considered, or when pathological stage IA disease only was considered ($P = 0.0019$ by log-rank test, Figure 9B). In contrast, there was no such association in patients with stage IB ~ IV disease ($P = 0.9009$ by log-rank test, Figure 9C).

Univariate analysis showed a significant association between patient prognosis and the presence of c-MET expression in stromal myofibroblasts, as well as conventional prognostic factors, such as pathological stage, nodal involvement, pleural invasion, vascular invasion, and lymphatic invasion. Multivariate analysis revealed that pathological stage, nodal involvement, vascular invasion, lymphatic invasion, and the presence of c-MET-positive myofibroblasts are all independent prognostic factors (Table 2). These findings suggest that the induction of c-MET occurs during progression of lung adenocarcinoma, and that c-MET-positive myofibroblasts play a role in tumor invasion and metastasis.

We also analyzed the possibility of an association between c-MET expression in cancer-stromal myofibroblasts and various clinicopathological parameters. However, no significant association was found between c-MET expression in stromal myofibroblasts and various clinicopathological characteristics, including age ($P = 0.6515$), sex ($P = 0.6094$), pathological stage ($P = 0.3104$), nodal involvement ($P = 0.8711$), pleural invasion ($P = 0.6840$), vascular invasion ($P = 0.3237$), and lymphatic invasion ($P = 0.8265$) (Table 3).

Discussion

It has generally been assumed that HGF functions as a stromal cell-derived paracrine mediator that regulates the morphogenesis of epithelial cells.^{1,56,57} In cancer, HGF is

Figure 9. Kaplan-Meier survival curves according to the presence or absence of c-MET expression in cancer stromal myofibroblasts for all pathological stage patients with small-sized lung adenocarcinoma (A) as well as for patients with stage IA (B) or stage IB~IV (C) disease. The survival differences between the two curves were analyzed using the log-rank test.

produced mainly by stromal fibroblasts, whereas its receptor c-MET is expressed in carcinoma (epithelial) cells.^{4,20} It has been shown that stroma-derived HGF enhances cell growth and invasion of tumor cells. In this study, we have shown that both c-MET and HGF are expressed in cultured human myofibroblasts. Expression of the c-MET protein by stromal myofibroblasts was immunohistochemically confirmed in lung adenocarcinomas. *In vitro*, proliferation of myofibroblasts was stimulated by exogenously added recombinant HGF, whereas neutralizing antibody to HGF inhibited myofibroblast proliferation. Secretion of HGF by cultured myofibroblasts was confirmed by ELISA. Exogenously added recombinant human HGF enhanced tyrosine phosphorylation of

Table 2. Relationships between Clinicopathological Variables and Adverse Outcome in Small-Sized Lung Adenocarcinoma

Clinical feature	No. of cases	Univariate analysis			Multivariate analysis		
		P value	RR*	95% CI†	P value	RR	95% CI
All cases	131						
Age (years)							
(≥59 <i>versus</i> <59)	67 vs 64	0.3824	1.353	(0.687–2.665)	–	–	–
Sex							
(Male <i>versus</i> female)	73 vs 58	0.3295	1.411	(0.706–2.822)	–	–	–
Pathological stage							
(IB ~ IV <i>versus</i> IA)	35 vs 96	<0.0001	11.548	(5.475–24.356)	–	–	–
Nodal involvement (pN)							
(pN1, 2 <i>versus</i> pN0)	24 vs 107	<0.0001	7.589	(3.715–15.504)	<0.0001	7.104	(2.836–17.796)
Pleural invasion							
(+ <i>versus</i> –)	36 vs 95	0.0007	3.252	(1.650–6.411)	0.0577	2.079	(0.976–4.427)
Vascular invasion							
(+ <i>versus</i> –)	48 vs 83	0.0003	3.635	(1.816–7.273)	0.0316	2.402	(1.080–5.342)
Lymphatic invasion							
(+ <i>versus</i> –)	43 vs 77	<0.0001	9.963	(4.192–23.677)	0.0008	4.833	(1.916–12.191)
equivocal	11						
Immunohistochemistry							
(c-MET + <i>versus</i> –)	69 vs 62	0.0118	2.666	(1.242–5.721)	0.0057	3.087	(1.387–6.869)

*RR, relative risk.

†CI, confidence interval.

c-MET. Previously, we demonstrated HGF expression in stromal myofibroblasts of lung adenocarcinoma.⁵⁸ Thus the HGF/c-MET system may constitute an autocrine stimulatory loop in stromal myofibroblasts during tumor cell invasion. This autocrine system will further stimulate the myofibroblast activation that was initiated by paracrine factors released from adjacent cells.

With regard to HGF/c-MET signaling in cancer, co-expression of HGF and c-MET in cancer cells,^{59–61} including lung cancer,²³ has been reported previously. Secretion of HGF has also been demonstrated in some

cancer cell lines.^{62,63} Tsao and colleagues⁶² have shown that several lung adenocarcinoma cells express c-MET mRNA and secrete biologically active HGF. The conditioned media of these cells induced scattering of MDCK cells, and this scatter activity was inhibited by neutralizing antibody against HGF.⁶² The c-MET receptors on these cancer cells were constitutively phosphorylated by endogenously produced HGF, and neutralizing antibody against HGF reduced the level of c-MET tyrosine phosphorylation.⁶² Thus, the HGF/c-MET system may constitute an autocrine stimulatory loop in a subset of cancer cells, as well as in stromal myofibroblasts. The mechanisms by which cancer cells acquire the ability to synthesize and secrete HGF are unknown, but it has been speculated that this might represent epithelial-mesenchymal transition of cancer,⁶⁴ a phenomenon occasionally observed in aggressive cancer cells.

The expression of HGF and/or c-MET in lung cancer has been documented in several studies.^{23,65–67} However, c-MET immunoreactivity in stromal myofibroblasts was not described in these reports. The reason for this discrepancy is not clear, but it is possible that stromal c-MET immunoreactivity was ignored in previous studies, because they were focused mainly on c-MET expression in cancer cells. With regard to the prognostic value of HGF/c-MET expression, one study⁶⁵ showed that high levels of immunoreactive HGF were associated with poor survival in patients with non-small-cell lung cancer. It would be necessary to see whether there is any correlation between high levels of immunoreactive HGF in tumor extracts and c-MET expression in stromal myofibroblasts.

With regard to the role of stromal myofibroblasts in cancer progression, previous work has shown that myofibroblasts are particularly numerous within the stroma of primary invasive and metastatic carcinomas.^{68–71} In lung adenocarcinoma, active fibroblast proliferation³⁰ correlates with poor prognosis. In this study, we investigated the expression of c-MET in stromal myofibroblasts in a

Table 3. Relationships between Elevated c-MET Expression and Clinical Characteristics

Clinical feature	No. of cases	Elevated c-MET expression		P value*
		Positive	Negative	
All cases	131	69	62	
Age (years)				
<59	64	35	29	0.6515
≥59	67	34	33	
Sex				
Male	73	37	36	0.6094
Female	58	32	26	
Pathological stage				
IA	96	48	48	0.3104
IB ~ IV	35	21	14	
Nodal involvement (pN)				
Negative (pN0)	107	56	51	0.8711
Positive (pN1, 2)	24	13	11	
Pleural invasion				
Negative	95	49	46	0.6840
Positive	36	20	16	
Vascular invasion				
Negative	83	41	42	0.3237
Positive	48	28	20	
Lymphatic invasion				
Negative	77	41	36	0.8265
Positive	43	22	21	
Equivocal	11			

*P values were determined by the χ^2 test.

series of 131 patients with small-sized lung adenocarcinomas. The presence of c-MET expression in cancer-stromal myofibroblasts correlated with a reduced survival both for the whole cohort of patients including all pathological stages and for those with stage IA disease alone. These observations collectively suggest that myofibroblasts, especially those that express c-MET, play a role in the invasion and metastasis of lung adenocarcinoma.

We still do not know the mechanisms by which stromal expression of c-MET is induced in a subset of lung cancer. Stromal c-MET expression was observed mainly in invasive area of lung adenocarcinomas. Moreover, c-MET expression was restricted to a subset of myofibroblasts within the invasive area of the tumors, whereas α -smooth muscle actin was expressed in almost all stromal myofibroblasts both in noninvasive and invasive areas. This suggests that c-MET is induced by factors derived from invading tumor cells. The phenotype of myofibroblasts can be modulated by a number of factors, such as TGF- β , acidic FGF, basic FGF, and PDGF.⁶⁸⁻⁷¹ Nakamura and colleagues²⁰ recently demonstrated that IL-1, basic FGF, and PDGF released from tumor cells induced HGF expression in stromal fibroblasts. Our preliminary data show that c-MET expression in cultured myofibroblasts is regulated by a number of growth factors, such as TGF- β and PDGF. The factors that induce expression of c-MET in cancer-stromal myofibroblasts need to be clarified.

Another issue that needs to be addressed in the future is the possible biological differences among myofibroblasts cultured from normal tissues, myofibroblasts from granulation tissues, and/or myofibroblasts from tumor stroma. In the present study, we found no clear differences between myofibroblasts cultured from normal tissues and those from tumor stroma. However, myofibroblasts from normal tissues are activated by serum-derived factors during the cultivation process. Therefore, one must be cautious about interpreting these results. Nevertheless, it is interesting that there was a tendency for higher levels of c-MET mRNA in tumor-derived myofibroblasts, which also appeared to secrete higher amounts of HGF. It will be necessary to see if other differences exist between myofibroblasts from normal tissue and those from cancer stroma. With regard to myofibroblasts from granulation tissues and those from tumor stroma, both similarities and differences are apparent. Analogous relationships between tumor stroma generation and wound healing have been discussed by Dvorak.⁷² The most important difference would be that, whereas wounds and granulation tissue heal by themselves, tumors fail to do so. This implies that in tumor stroma, stromal cells are under constant stimuli from cancer cells, whereas they are under negative feedback control during wound healing. Obviously, genetic alterations of cancer cells are responsible for the aberrant release of stimulatory factors. Elucidation of this mechanism may provide valuable insight into the process of cancer invasion.

From a therapeutic viewpoint, HGF represents a possible target for the treatment of lung adenocarcinoma. Date and colleagues^{73,74} recently showed that HGF/NK4,

which is composed of an N-terminal hairpin domain and four kringle domains, is a potent antagonist that abrogates the mitogenic, motogenic, and morphogenic activities of HGF. NK4 inhibited invasion of tumors both *in vivo* and *in vitro*. More recently, Abounader and colleagues⁷⁵ used U1 small nuclear RNA/ribozyme to reduce HGF and c-MET expression in glioblastoma cells. Introduction of the transgene inhibited tumorigenicity and tumor growth *in vivo*. An alternative could be to target stromal myofibroblasts *per se* and inhibit their activation and secretion of HGF. Theoretically, this approach has an advantage because stromal myofibroblasts secrete other tumor-promoting soluble factors, such as vascular endothelial growth factor, TGF- β , matrix metalloproteinase-2, and IL-6.^{69-71,76,77} Moreover, unlike cancer cells, myofibroblasts are genetically stable, and thus less likely to develop drug resistance. This possibility for stroma-targeted cancer therapy should be explored by further investigations.

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References

1. Nakamura T: Structure and function of hepatocyte growth factor. *Prog Growth Factor Res* 1991, 3:67-85
2. Gak E, Taylor WG, Chan AM, Rubin JS: Processing of hepatocyte growth factor to the heterodimeric form is required for biological activity. *FEBS Lett* 1992, 311:17-21
3. Mizuno K, Tanoue Y, Okano I, Harano T, Takada K, Nakamura T: Purification and characterization of hepatocyte growth factor (HGF)-converting enzyme: activation of pro-HGF. *Biochem Biophys Res Commun* 1994, 198:1161-1169
4. Jiang W, Hiscox S, Matsumoto K, Nakamura T: Hepatocyte growth factor/scatter factor, its molecular, cellular and clinical implications in cancer. *Crit Rev Oncol Hematol* 1999, 29:209-248
5. Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, Tashiro K, Shimizu S: Molecular cloning and expression of human hepatocyte growth factor. *Nature* 1989, 342:440-443
6. Park M, Dean M, Kaul K, Braun MJ, Gonda MA, Vande Woude G: Sequence of MET protooncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors. *Proc Natl Acad Sci USA* 1987, 84:6379-6383
7. Naldini L, Weidner KM, Vigna E, Gaudino G, Bardelli A, Ponzetto C, Narsimhan RP, Hartmann G, Zarnegar R, Michalopoulos GK: Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor. *EMBO J* 1991, 10:2867-2878
8. Bottaro DP, Rubin JS, Faletto DL, Chan AM, Kmieciak TE, Vande Woude GF, Aaronson SA: Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science* 1991, 251:802-804
9. Giordano S, Zhen Z, Medico E, Gaudino G, Galimi F, Comoglio PM: Transfer of motogenic and invasive response to scatter factor/hepatocyte growth factor by transfection of human MET protooncogene. *Proc Natl Acad Sci USA* 1993, 90:649-653
10. Sonnenberg E, Meyer D, Weidner KM, Birchmeier C: Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. *J Cell Biol* 1993, 123:223-235
11. Halaban R, Rubin JS, White W: Met and HGF-SF in normal melanocytes and melanoma cells. *EXS* 1993, 65:329-339
12. Bussolino F, Di Renzo MF, Ziche M, Bocchietto E, Olivero M, Naldini L, Gaudino G, Tamagnone L, Coffey A, Comoglio PM: Hepatocyte

- growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. *J Cell Biol* 1992, 119:629-641
13. Di Renzo MF, Bertolotto A, Olivero M, Putzolu P, Crepaldi T, Schiffer D, Pagni CA, Comoglio PM: Selective expression of the Met/HGF receptor in human central nervous system microglia. *Oncogene* 1993, 8:219-222
14. Yamada T, Tsubouchi H, Daikuhara Y, Prat M, Comoglio PM, McGeer PL, McGeer EG: Immunohistochemistry with antibodies to hepatocyte growth factor and its receptor protein (c-MET) in human brain tissues. *Brain Res* 1994, 637:308-312
15. Honda S, Kagoshima M, Wanaka A, Tohyama M, Matsumoto K, Nakamura T: Localization and functional coupling of HGF and c-Met/HGF receptor in rat brain: implication as neurotrophic factor. *Brain Res Mol Brain Res* 1995, 32:197-210
16. Ikeda H, Nagoshi S, Ohno A, Yanase M, Maekawa H, Fujiwara K: Activated rat stellate cells express c-met and respond to hepatocyte growth factor to enhance transforming growth factor beta1 expression and DNA synthesis. *Biochem Biophys Res Commun* 1998, 250:769-775
17. Epstein JA, Shapiro DN, Cheng J, Lam PY, Maas RL: Pax3 modulates expression of the c-Met receptor during limb muscle development. *Proc Natl Acad Sci USA* 1996, 93:4213-4218
18. Weidner KM, Behrens J, Vandekerckhove J, Birchmeier W: Scatter factor: molecular characteristics and effect on the invasiveness of epithelial cells. *J Cell Biol* 1990, 111:2097-2108
19. Rong S, Bodescot M, Blair D, Dunn J, Nakamura T, Mizuno K, Park M, Chan A, Aaronson S, Vande Woude GF: Tumorigenicity of the met proto-oncogene and the gene for hepatocyte growth factor. *Mol Cell Biol* 1992, 12:5152-5158
20. Nakamura T, Matsumoto K, Kiritoshi A, Tano Y: Induction of hepatocyte growth factor in fibroblasts by tumor-derived factors affects invasive growth of tumor cells: in vitro analysis of tumor-stromal interactions. *Cancer Res* 1997, 57:3305-3313
21. Liang TJ, Reid AE, Xavier R, Cardiff RD, Wang TC: Transgenic expression of tpr-met oncogene leads to development of mammary hyperplasia and tumors. *J Clin Invest* 1996, 97:2872-2877
22. Ferracini R, Di Renzo MF, Scotlandi K, Baldini N, Olivero M, Lollini P, Cremona O, Campanacci M, Comoglio PM: The Met/HGF receptor is over-expressed in human osteosarcomas and is activated by either a paracrine or an autocrine circuit. *Oncogene* 1995, 10:739-749
23. Harvey P, Warn A, Newman P, Perry LJ, Ball RY, Warn RM: Immunoreactivity for hepatocyte growth factor/scatter factor and its receptor, met, in human lung carcinomas and malignant mesotheliomas. *J Pathol* 1996, 180:389-394
24. Borset M, Hjorth-Hansen H, Seidel C, Sundan A, Waaga A: Hepatocyte growth factor and its receptor c-met in multiple myeloma. *Blood* 1996, 88:3998-4004
25. Rao UN, Sonmez-Alpan E, Michalopoulos GK: Hepatocyte growth factor and c-MET in benign and malignant peripheral nerve sheath tumors. *Hum Pathol* 1997, 28:1066-1070
26. Laterra J, Nam M, Rosen E, Rao JS, Lamszus K, Goldberg ID, Johnston P: Scatter factor/hepatocyte growth factor gene transfer enhances glioma growth and angiogenesis in vivo. *Lab Invest* 1997, 76:565-577
27. Nesbitt JC, Putnam Jr JB, Walsh GL, Roth JA, Mountain CF: Survival in early-stage non-small cell lung cancer. *Ann Thorac Surg* 1995, 60:466-472
28. Strauss GM, Kwiatkowski DJ, Harpole DH, Lynch TJ, Skarin AT, Sugarbaker DJ: Molecular and pathologic markers in stage I non-small-cell carcinoma of the lung. *J Clin Oncol* 1995, 13:1265-1279
29. Nomori H, Hirohashi S, Noguchi M, Matsuno Y, Shimosato Y: Tumor cell heterogeneity and subpopulations with metastatic ability in differentiated adenocarcinoma of the lung. Histologic and cytofluorometric DNA analyses. *Chest* 1991, 99:934-940
30. Noguchi M, Morikawa A, Kawasaki M, Matsuno Y, Yamada T, Hirohashi S, Kondo H, Shimosato Y: Small adenocarcinoma of the lung. Histologic characteristics and prognosis. *Cancer* 1995, 75:2844-2852
31. Gabbiani G, Ryan GB, Majne G: Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia* 1971, 27:549-550
32. Gabbiani G, Hirschel BJ, Ryan GB, Statkov PR, Majno G: Granulation tissue as a contractile organ. A study of structure and function. *J Exp Med* 1972, 135:719-734
33. Cintonaro M, Bellizzi de Marco E, Leoncini P, Tripodi SA, Xu LJ, Sappino AP, Schmitt-Graff A, Gabbiani G: Expression of alpha-smooth-muscle actin in stromal cells of the uterine cervix during epithelial neoplastic changes. *Int J Cancer* 1991, 47:843-846
34. Sappino AP, Dietrich PY, Skalli O, Widge S, Gabbiani G: Colonic pericryptal fibroblasts. Differentiation pattern in embryogenesis and phenotypic modulation in epithelial proliferative lesions. *Virchows Arch A Pathol Anat Histopathol* 1989, 415:551-557
35. Czernobilsky B, Shezen E, Lifschitz-Mercer B, Fogel M, Luzon A, Jacob N, Skalli O, Gabbiani G: Alpha smooth muscle actin (alpha-SM actin) in normal human ovaries, in ovarian stromal hyperplasia and in ovarian neoplasms. *Virchows Arch B Cell Pathol Incl Mol Pathol* 1989, 57:55-61
36. Tsukada T, McNutt MA, Ross R, Gown AM: HHF35, a muscle actin-specific monoclonal antibody. II. Reactivity in normal, reactive, and neoplastic human tissues. *Am J Pathol* 1987, 127:389-402
37. Sappino AP, Skalli O, Jackson B, Schurch W, Gabbiani G: Smooth-muscle differentiation in stromal cells of malignant and non-malignant breast tissues. *Int J Cancer* 1988, 41:707-712
38. Eddy RJ, Petro JA, Tomasek JJ: Evidence for the nonmuscle nature of the "myofibroblast" of granulation tissue and hypertrophic scar. An immunofluorescence study. *Am J Pathol* 1988, 130:252-260
39. Doillon CJ, Hembry RM, Ehrlich HP, Burke JF: Actin filaments in normal dermis and during wound healing. *Am J Pathol* 1987, 126:164-170
40. Kuhn C, McDonald JA: The roles of the myofibroblast in idiopathic pulmonary fibrosis. Ultrastructural and immunohistochemical features of sites of active extracellular matrix synthesis. *Am J Pathol* 1991, 138:1257-1265
41. Broekelmann TJ, Limper AH, Colby TV, McDonald JA: Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. *Proc Natl Acad Sci USA* 1991, 88:6642-6646
42. Kapanci Y, Burgan S, Pietra GG, Conne B, Gabbiani G: Modulation of actin isoform expression in alveolar myofibroblasts (contractile interstitial cells) during pulmonary hypertension. *Am J Pathol* 1990, 136:881-889
43. Sappino AP, Masouye I, Saurat JH, Gabbiani G: Smooth muscle differentiation in scleroderma fibroblastic cells. *Am J Pathol* 1990, 137:585-591
44. Schmitt-Graff A, Pau H, Spahr R, Piper HM, Skalli O, Gabbiani G: Appearance of alpha-smooth muscle actin in human eye lens cells of anterior capsular cataract and in cultured bovine lens-forming cells. *Differentiation* 1990, 43:115-122
45. Leslie KO, Taatjes DJ, Schwarz J, vonTurkovich M, Low RB: Cardiac myofibroblasts express alpha smooth muscle actin during right ventricular pressure overload in the rabbit. *Am J Pathol* 1991, 139:207-216
46. Mitchell J, Woodcock-Mitchell J, Reynolds S, Low R, Leslie K, Adler K, Gabbiani G, Skalli O: Alpha-smooth muscle actin in parenchymal cells of bleomycin-injured rat lung. *Lab Invest* 1989, 60:643-650
47. Brouty-Boye D, Raux H, Azzarone B, Tamboise A, Tamboise E, Beranger S, Magnien V, Pihan L, Zardi L, Israel L: Fetal myofibroblast-like cells isolated from post-radiation fibrosis in human breast cancer. *Int J Cancer* 1991, 47:697-702
48. Mountain CF: Revisions in the International System for Staging Lung Cancer [see comments]. *Chest* 1997, 111:1710-1717
49. Heid CA, Stevens J, Livak KJ, Williams PM: Real time quantitative PCR. *Genome Res* 1996, 6:986-994
50. Gibson UE, Heid CA, Williams PM: A novel method for real time quantitative RT-PCR. *Genome Res* 1996, 6:995-1001
51. Cerroni L, Minkus G, Putz B, Hoffer H, Kerl H: Laser beam microdissection in the diagnosis of cutaneous B-cell lymphoma. *Br J Dermatol* 1997, 136:743-746
52. Schutze K, Posl H, Lahr G: Laser micromanipulation systems as universal tools in cellular and molecular biology and in medicine. *Cell Mol Biol* 1998, 44:735-746
53. Williamson K, Halliday I, Hamilton P, Ruddell J, Varma M, Maxwell P, Crookard A, Rowlands B: In vitro BrdUrd incorporation of colorectal tumour tissue. *Cell Prolif* 1993, 26:115-124
54. Huang S, Stupack D, Mathias P, Wang Y, Nemerow G: Growth arrest of Epstein-Barr virus immortalized B lymphocytes by adenovirus-delivered ribozymes. *Proc Natl Acad Sci USA* 1997, 94:8156-8161
55. Kapanci Y, Ribaux C, Chaponnier C, Gabbiani G: Cytoskeletal fea-

- tures of alveolar myofibroblasts and pericytes in normal human and rat lung. *J Histochem Cytochem* 1992, 40:1955–1963
56. Montesano R, Matsumoto K, Nakamura T, Orci L: Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell* 1991, 67:901–908
 57. Yang Y, Spitzer E, Meyer D, Sachs M, Niemann C, Hartmann G, Weidner KM, Birchmeier C, Birchmeier W: Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. *J Cell Biol* 1995, 131:215–226
 58. Yoshinaga Y, Matsuno Y, Fujita S, Nakamura T, Kikuchi M, Shimosato Y, Hirohashi S: Immunohistochemical detection of hepatocyte growth factor/scatter factor in human cancerous and inflammatory lesions of various organs. *Jpn J Cancer Res* 1993, 84:1150–1158
 59. Tuck AB, Park M, Sterns EE, Boag A, Elliott BE: Coexpression of hepatocyte growth factor and receptor (Met) in human breast carcinoma. *Am J Pathol* 1996, 148:225–232
 60. Jin L, Fuchs A, Schnitt SJ, Yao Y, Joseph A, Lamszus K, Park M, Goldberg ID, Rosen EM: Expression of scatter factor and c-met receptor in benign and malignant breast tissue. *Cancer* 1997, 79:749–760
 61. Joseph A, Weiss GH, Jin L, Fuchs A, Chowdhury S, O'Shaughnessy P, Goldberg ID, Rosen EM: Expression of scatter factor in human bladder carcinoma. *J Natl Cancer Inst* 1995, 87:372–377
 62. Tsao MS, Zhu H, Giaid A, Viallet J, Nakamura T, Park M: Hepatocyte growth factor/scatter factor is an autocrine factor for human normal bronchial epithelial and lung carcinoma cells. *Cell Growth Differ* 1993, 4:571–579
 63. Yoshinaga Y, Fujita S, Gotoh M, Nakamura T, Kikuchi M, Hirohashi S: Human lung cancer cell line producing hepatocyte growth factor/scatter factor. *Jpn J Cancer Res* 1992, 83:1257–1261
 64. Birchmeier W, Birchmeier C: Epithelial-mesenchymal transitions in development and tumor progression. *EXS* 1995, 74:1–15
 65. Siegfried JM, Weissfeld LA, Singh-Kaw P, Weyant RJ, Testa JR, Landreneau RJ: Association of immunoreactive hepatocyte growth factor with poor survival in resectable non-small cell lung cancer. *Cancer Res* 1997, 57:433–439
 66. Tsao MS, Liu N, Chen JR, Pappas J, Ho J, To C, Viallet J, Park M, Zhu H: Differential expression of Met/hepatocyte growth factor receptor in subtypes of non-small cell lung cancers. *Lung Cancer* 1998, 20:1–16
 67. Olivero M, Rizzo M, Madeddu R, Casadio C, Pennacchietti S, Nicotra MR, Prat M, Maggi G, Natali PG, Comoglio PM, Di Renzo MF: Overexpression and activation of hepatocyte growth factor/scatter factor in human non-small-cell lung carcinomas. *Br J Cancer* 1996, 74:1862–1868
 68. Wernert N: The multiple roles of tumour stroma. *Virchows Arch* 1997, 430:433–443
 69. Gregoire M, Lieubeau B: The role of fibroblasts in tumor behavior. *Cancer Metastasis Rev* 1995, 14:339–350
 70. Seemayer TA, Schurch W, Lagace R: Myofibroblasts in human pathology. *Hum Pathol* 1981, 12:491–492
 71. Ronnov-Jessen L, Petersen OW, Bissell MJ: Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. *Physiol Rev* 1996, 76:69–125
 72. Dvorak HF: Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 1986, 315:1650–1659
 73. Date K, Matsumoto K, Shimura H, Tanaka M, Nakamura T: HGF/NK4 is a specific antagonist for pleiotrophic actions of hepatocyte growth factor. *FEBS Lett* 1997, 420:1–6
 74. Date K, Matsumoto K, Kuba K, Shimura H, Tanaka M, Nakamura T: Inhibition of tumor growth and invasion by a four-kringle antagonist (HGF/NK4) for hepatocyte growth factor. *Oncogene* 1998, 17:3045–3054
 75. Abounader R, Ranganathan S, Lal B, Fielding K, Book A, Dietz H, Burger P, Laterra J: Reversion of human glioblastoma malignancy by U1 small nuclear RNA/ribozyme targeting of scatter factor/hepatocyte growth factor and c-met expression. *J Natl Cancer Inst* 1999, 91:1548–1556
 76. Hlatky L, Tsionou C, Hahnfeldt P, Coleman CN: Mammary fibroblasts may influence breast tumor angiogenesis via hypoxia-induced vascular endothelial growth factor up-regulation and protein expression. *Cancer Res* 1994, 54:6083–6086
 77. Fukumura D, Xavier R, Sugiura T, Chen Y, Park EC, Lu N, Selig M, Nielsen G, Taksir T, Jain RK, Seed B: Tumor induction of VEGF promoter activity in stromal cells. *Cell* 1998, 94:715–725