

RESEARCH ARTICLE

Inhibition of human non-small cell lung tumors by a *c-Met* antisense/U6 expression plasmid strategy

LP Stabile^{1,2}, JS Lyker¹, L Huang³ and JM Siegfried^{1,2}

¹Department of Pharmacology, University of Pittsburgh, Pittsburgh, PA, USA; ²Lung Cancer Program, University of Pittsburgh Cancer Institute, Pittsburgh, PA, USA; and ³Center for Pharmacogenetics, University of Pittsburgh, Pittsburgh, PA, USA

c-Met is a receptor tyrosine kinase whose activation by hepatocyte growth factor (HGF) can lead to transformation and tumorigenicity in a variety of tumors. We investigated the effects of suppressing *c-Met* protein expression in human non-small cell lung tumors. Expression plasmids containing either sense or antisense sequences of the human *c-met* gene were constructed under control of the U6 snRNA promoter. A U6 control plasmid was also constructed that did not contain any *c-met* sequence. These constructs have been examined both in vitro and in an in vivo tumor xenograft model. The *c-Met* protein was downregulated by 50–60% in two lung cancer cell lines that were transiently transfected with the *c-Met* antisense versus U6 control. Tumor cells

treated with the *c-Met* antisense construct also show decreased phosphorylation of *c-Met* and MAP kinase when exposed to exogenous HGF. Lung cancer cells were grown as xenografts in mice and treated by intratumoral liposome-mediated transfer of the *c-Met* sense, antisense or U6 control plasmids. The treatment of lung tumors with *c-Met* antisense versus U6 control plasmid resulted in the downregulation of the *c-Met* protein expression, a 50% decrease in tumor growth over a 5-week treatment period and an increased rate of apoptosis. These results suggest that targeting the HGF/*c-Met* pathway may be an effective novel strategy to treat lung cancer patients.

Gene Therapy (2004) 11, 325–335. doi:10.1038/sj.gt.3302169

Keywords: *c-Met*; lung cancer; antisense; liposomes; growth factors

Introduction

Lung cancer is currently the leading cause of cancer mortality in US men and women. The 5-year survival rate for all stages of lung cancer combined is only 15%. The survival rate is 48% for cases detected when the disease is localized; however, only 15% of the cases are diagnosed at this early stage. Surgical resection for cure is often only applicable to early-stage disease. Lung cancer patients currently have few therapeutic options and new approaches are needed to improve long-term survival. Growth factors and their receptors play important roles in the growth and progression of lung tumors and are potential therapeutic targets for lung cancer treatment.

c-Met was originally identified as the protein product of the *c-met* proto-oncogene and is a member of the tyrosine kinase family of receptors whose activation can lead to transformation and tumorigenicity.^{1,2} *c-Met* has since been found to be the receptor for hepatocyte growth factor (HGF), also known as scatter factor.^{3–6} As the name implies, HGF was independently isolated as a hepatic regeneration factor⁴ and as a fibroblast-derived mitogen for epithelial cells.^{6,7} HGF is primarily produced by mesenchymal cells, particularly fibroblasts, and acts on *c-Met*-expressing epithelial and endothelial cells in a paracrine manner.⁸ However, there is now growing

evidence of autocrine HGF production in lung tumor cells as well as other tumor types.^{9–12} Thus, the HGF/*c-Met* signaling pathway seems to be mediated by both paracrine and autocrine mechanisms.

The mature *c-Met* protein consists of a 50 kDa α -chain disulfide linked to a 145 kDa β -chain. The α -chain is exposed at the cell surface, whereas the β -chain spans the cell membrane and possesses an intracellular tyrosine kinase domain.^{2,3,5} The α and β chains are derived from the proteolytic cleavage of a single-chain 170 kDa precursor protein. Binding of the HGF ligand to *c-Met* activates its tyrosine kinase activity and leads to multiple cellular responses, such as proliferation,⁴ cell scattering,⁷ and branching morphogenesis.¹³ These cellular responses to *c-Met* activation by HGF then mediate a plethora of biological activities, which include embryological development, wound healing, tissue regeneration, angiogenesis, growth, invasion and morphogenic differentiation.^{8,14} Given that several of these processes are known to be involved in tumorigenesis, the HGF/*c-Met* signaling pathway also plays a significant role in the pathogenesis and biology of many human cancers.

Several studies have suggested a possible role of the HGF/*c-Met* system in tumor development and progression.^{4,6,15} In normal cells, *c-Met* activation is a ligand-dependent transient event, whereas in tumor cells *c-Met* activity is usually constitutively upregulated. The molecular mechanisms that are thought to activate *c-Met* constitutively and thus contribute to tumorigenesis, including mutations in *c-Met* as well as overexpression of *c-Met* and/or HGF. Several studies support these

mechanisms of activation. First, many somatic and germline mutations, mainly in the tyrosine kinase domain, have been identified in human cancers, including lung metastases, which cause constitutive activation of the receptor.^{14,16–18} In some non-small cell lung cancer cell lines, constitutive activation of c-Met has been correlated with anchorage-independent, phosphatidylinositol 3-kinase-dependent cell survival.¹⁹ Second, HGF stimulates the proliferation and migration of tumor cells *in vitro*. Third, transgenic mice that overexpress c-Met develop tumors;²⁰ HGF-expressing transgenic mice also have been reported to form tumors in various organs.²¹ Fourth, several studies have shown that c-Met is overexpressed in many types of human tumors and cell lines from tissues of breast,²² ovary,²³ prostate,^{24,25} thyroid,^{26,27} pancreas,²⁸ colorectal,²⁹ gastric carcinomas,^{30,31} brain,³² oral squamous cell carcinomas³³ and lung.³⁴ Similarly, elevated levels of HGF have been reported in most of the above-mentioned human cancers as well as in inflammatory lung diseases.³⁵ Finally, the coexpression of c-Met and HGF has been reported in many types of tumors setting up an autocrine loop in which secreted HGF binds to c-Met and causes constitutive activation of the receptor and downstream signaling pathways.³⁶ Recent reports suggest that c-Met can also be transactivated through other membrane receptors independent of HGF.³⁷

Increased c-Met and/or HGF expression by human tumor cells has been associated with high tumor grade and poor prognosis. For example, c-Met has been found to be a strong independent negative prognostic factor in breast cancer,²² biliary tract carcinoma,³⁸ gastric carcinoma³⁹ and nasopharyngeal carcinoma.⁴⁰ In non-small cell lung carcinoma cell lines and primary tumors, c-Met overexpression correlated with a higher tumor stage and worse outcome than those without overexpression.³⁴ The strong correlation between c-Met expression and patient survival clearly supports the hypothesis that the HGF/c-Met pathway plays an important role in the pathogenesis of human cancers. Like c-Met, HGF has also been shown to be a negative prognostic indicator of survival and recurrence in many cancers, including breast⁴¹ and non-small cell lung cancer.⁴² Interestingly, in the 56 primary non-small cell lung cancer cases examined for HGF by quantitative Western blotting by Siegfried *et al*, the HGF content of tumors defined a subset of stage I patients who had relapsed and had shortened survival times. In addition, patients whose T status was greater than 1 and had elevated HGF rapidly relapsed and died from their disease. Thus, in non-small cell lung cancer patients, HGF could be a useful prognostic indicator for both early and advanced stage patients.⁴² Coexpression of HGF and c-Met has been reported in approximately 50% of lung adenocarcinomas and this coexpression has been reported to correlate with poor prognosis.^{43,44} Since this pathway is important in disease progression, it indicates a potential target for tumor control by inhibiting the c-Met protein.

In this study, we have constructed U6 expression plasmids containing either the sense or antisense sequences targeting the translation start site of the human *c-met* gene for delivery into lung tumor cells as a potential therapeutic strategy to inhibit lung tumor growth. Such a strategy is expected to block the translation of mRNA into protein. We have tested these

constructs both *in vitro* and in an *in vivo* tumor xenograft model and have shown downregulation of the c-Met protein and decreased tumor growth *in vivo*. The tumors transfected with the antisense construct also show increased apoptosis *versus* controls. This method may be a novel approach to treat lung cancer patients alone or in combination with other agents.

Materials and methods

U6 expression constructs

A modified U6 expression vector, pGEM2U6, containing *XhoI* and *NsiI* sites for convenient cloning was generated as described.⁴⁵ Oligonucleotides specific for the human *c-met* sense or antisense sequences, targeting the translation start site, were synthesized by Invitrogen (Carlsbad, CA, USA). The oligonucleotides were 40 nt long and contained asymmetrical tails to allow them to be annealed and ligated into the *XhoI* and *NsiI* sites of the U6 vector. These constructs were subcloned, sequenced and verified to contain the *c-met* sense or antisense sequences in the correct orientation (Figure 1). Short RNAs that begin at the translation start codon have been reported to be the most efficient in binding the target RNA.⁴⁶

Cell lines and culture conditions

Non-small cell lung tumor cell lines, 201T and 128-88T, were established in our laboratory from primary tissue specimens.⁴⁷ These lung tumors were verified to be primaries to the lung by pathological assessment. These cells were maintained *in vitro* in Basal Medium Eagle (BME) (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum and antibiotics.

Transient transfection

201T and 128-88T cells were plated in BME medium containing 10% fetal bovine serum and antibiotics at 1×10^6 cells/plate in 100 mm plates. The following day, the cells were transfected in serum-free conditions. The transfections were performed using LipofectACE (Life Technologies, Grand Island, NY, USA) according to the manufacturers' instructions. The conditions for transfection in these two cell lines had been optimized using cotransfection of green fluorescent protein and the U6 constructs (data not shown). Transfection efficiency was determined to be 60–70%. All plasmids used for transfection were purified using the Qiagen EndoFree kit to remove harmful endotoxins. Cells were washed

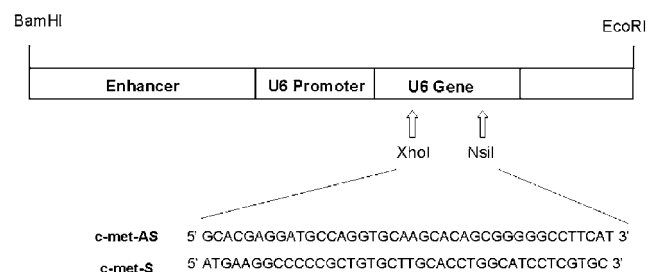


Figure 1 Schematic representation of the U6 expression cassette in the pGEM vector with the c-Met antisense (AS) and sense (S) oligonucleotide sequence shown.

twice with $1 \times$ phosphate-buffered saline (PBS) and transiently transfected with the c-Met sense, c-Met antisense or U6 empty vector expression constructs. At 48 and 72 h after transfection, total RNA and protein extracts, respectively, were prepared.

Protein extraction and Western analysis

At 72 h following transient transfection of the U6 constructs in 201T and 128-88T cells, the medium was removed from the cells and the cells were washed two times with ice-cold PBS. A measure of 2 ml of homogenization buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 5 mM $MgCl_2$, 330 mM sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.01% trypsin inhibitor, 1 μ g/ml leupeptin) was added to each plate and scraped into a tube. The cells were homogenized two times, 15 s each, with a polytron. The homogenate was centrifuged at 4°C for 8 min at 1500 r.p.m. The supernatant was removed and centrifuged at 100 000 g for 30 min at 4°C. The resulting pellet was dissolved in sample buffer (2% SDS, 10% sucrose, 10 mM DTT, 60 mM Tris, pH 6.8). The protein concentration was measured using the Pierce BCA-200 Protein Assay Kit.

For experiments in Figures 4 and 5, 10 ng/ml recombinant HGF (rHGF) protein (R&D systems, Minneapolis, MN, USA) was added for 10 min to half of the plates 72 h following transient transfection of the c-Met antisense, c-Met sense or U6 empty vector constructs. Cells were washed one time with ice-cold PBS. The protein was extracted by adding 600 μ l of ice-cold RIPA Buffer ($1 \times$ PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml PMSF, 0.045 mg/ml aprotinin (Sigma Chemical Co., St Louis, MO, USA), and 1 mM sodium orthovanadate) per 100 mm plate and scraped into a microfuge tube. The cell lysate was passed through a 21-gauge needle to shear genomic DNA. A measure of 10 μ l of 10 mg/ml PMSF stock was added and incubated 30–60 min on ice. The cell lysate was microcentrifuged at 10 000 g for 10 min at 4°C. The protein concentration in the supernatant was measured.

For tumor xenografts (Figure 7), tumors were removed after the 5-week treatment period and a section for protein extraction was frozen in liquid nitrogen. Tumor tissue was weighed and diced into very small pieces using a clean razor blade. The protein was extracted by adding 3 ml of ice-cold RIPA buffer per gram of tumor and homogenized using a polytron. A measure of 20 μ l of 10 mg/ml PMSF stock were added to each lysate and incubated on ice for 30–60 min. The cell lysate was centrifuged at 10 000 g for 10 min at 4°C. The protein concentration in the supernatant was measured using the Pierce BCA-200 Protein Assay Kit.

For the detection of c-Met (Figures 4 and 7), equal amounts of protein (25 μ g) were separated by size on a 6% SDS-Tris-Glycine gel (Invitrogen) and transferred onto a nitrocellulose membrane. Nonspecific binding sites were blocked by incubation in $1 \times$ TBS-T (0.2 M Tris, 0.14 M NaCl, 0.1% Tween 20) containing 5% dry milk for 1 h at room temperature, followed by incubation overnight at 4°C with a 1:250 dilution of rabbit polyclonal anti-c-Met antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in $1 \times$ TBS-T containing 1% dry milk. After the blots were washed three times in $1 \times$ TBS-T (10 min each at room temperature), horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham,

Piscataway, NJ, USA) was added at a 1:1000 dilution and incubated for 2 h at room temperature. After three more washes with $1 \times$ TBS-T, the immunoreactive peptide was detected by SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL, USA), followed by exposure to autoradiography film. SeeBlue Plus 2 prestained protein standards (Invitrogen) were also included in each gel. The blots were stripped using Restore™ Western Blot Stripping Buffer (Pierce). Blots were reprobbed using a mouse anti-actin monoclonal primary antibody (Santa Cruz Biotechnology) at a 1:1000 dilution and a horseradish peroxidase-conjugated sheep anti-mouse IgG secondary antibody (Amersham) at a 1:1000 dilution.

For the detection of phospho-c-Met (Figure 4), equal amounts of protein (25 μ g) were separated by size on a 6% SDS-Tris-Glycine gel and transferred onto a nitrocellulose membrane. Nonspecific binding sites were blocked the same as for c-Met as described above. The primary antibody was a 1:1000 dilution of rabbit polyclonal anti-c-Met (pYpYp^{1230/1234/1235}) phosphospecific antibody (BioSource International, Camarillo, CA, USA) and secondary was a 1:1000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG. Washes and detection were performed as described above.

For the detection of phospho-p44/42 MAP Kinase (Figures 5 and 7), equal amounts of protein (25 μ g) were separated by size onto a 10% SDS-Tricine gel and transferred to nitrocellulose membrane. Nonspecific binding sites were blocked the same as for c-Met as described above. The primary antibody was a 1:1000 dilution of rabbit polyclonal anti-phospho-p44/42 MAP kinase antibody (Cell Signaling Technology, Beverly, MA, USA) and secondary antibody was a 1:2000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG. Washes and detection were performed as described above. Blots were stripped and reprobbed with a 1:1000 dilution of rabbit polyclonal anti-total p44/42 MAP Kinase antibody (Cell Signaling Technology). All blots were scanned by densitometry and quantitation was carried out using ImageQuaNT analysis.

RNA isolation and ribonuclease protection assay

RNA was isolated from the cells or tumors by the guanidinium thiocyanate method.⁴⁸ For cells, 201T cells were transiently transfected with c-Met sense, c-Met antisense or U6 empty vector constructs as described in the transient transfection section. The total RNA was isolated from the cells 48 h after transfection. For tumor xenografts, tumors were harvested after the 5-week treatment period and stored in RNAlater™ (Ambion, Austin, TX, USA) until RNA isolation. Ribonuclease protection assays were performed using the RPAIII™ kit (Ambion) according to the manufacturer's instructions. Total RNA (20 μ g) from each transiently transfected treatment or 50 μ g of total RNA from tumor xenografts was hybridized with 2×10^4 c.p.m. of ³²P-labeled RNA probe specific for each treatment. The solution was treated with a mixture of RNase A and T₁. The resulting hybridized products protected from RNase digestion were separated on a 5% denaturing polyacrylamide gel. The gel was dried and exposed to a phosphor screen. The exposure times were 2 days for the *in vitro* experiment and 7 days for the *in vivo* experiment.

The endogenous c-Met probe was derived from PCR amplification of a plasmid containing c-Met cDNA. Primer 1, 5'CGGAATTCCTTTGCAGCGCGTTGACTTATT contained an *EcoRI* site at its 5' end (underline) for subcloning purposes and the remaining sequence starts 1267 nt past the translation start site. Primer 2, 5'CGCGGATCCTCCTGATCGAGAAACCACAAC contained a *BamHI* site at its 5' end (underline) and the remaining sequence starts 1412 nt past the translation start site. This amplified region of the *c-met* gene was not part of the 40 bp of the transgene, thus it should only recognize the *c-met* endogenous gene. The PCR-amplified fragment was subcloned into the *EcoRI* and *BamHI* sites of pSPORT1 (Life Technologies) and linearized with *EcoRI* for use in an *in vitro* transcription reaction using SP6 RNA polymerase. This probe has been verified to only recognize a 145 nt endogenous *c-met* product, not the transgenes (data not shown). For the probes that recognize the transgenes, the plasmids that were used for transfection were digested with *DraI*, which is 5' of the expected transgene product. A 1.5 kb product was gel isolated using the Qiagen gel extraction kit. These products were used for *in vitro* transcription reactions using SP6 RNA polymerase to make antisense probes specific for U6, *c-met* antisense or *c-met* sense transgenes. These probes should recognize an 82 nt transgene fragment representing the 40 bp *c-met* sense, antisense or U6 sequences and a portion of the U6 gene on either side, as well as smaller fragments representing the endogenous U6 or *c-met* genes.

Tumor xenograft model

In all, 25 female SCID mice (5 weeks of age; Harlan Sprague-Dawley, Indianapolis, IN, USA) were used for each experiment. 201T lung tumor cells were injected s.c. at two sites per mouse (4×10^6 cells/site). The mice were divided into four treatment groups (five animals per group): (a) no treatment (none); (b) liposomes alone; (c) liposomes plus the U6 empty vector; (d) liposomes plus *c-met*-sense vector; and (e) liposomes plus *c-met*-antisense vector. Each plasmid (50 μ g) was complexed with 50 nmol DC-chol/dioleoylphosphatidyl-ethanolamine (DOPE) liposomes⁴⁹ in a 50 μ l volume and injected directly into the tumor three times a week for 5 weeks. Tumor size was measured before each weekly injection and reported as an average tumor volume, calculated as: $(l \times w \times h \times \delta) / 2$ (mm³), where *l* is the length, *w* is the width and *h* is the height of the tumor measured with calipers. At the end of the 5-week period, the animals were sacrificed, and the tumors were removed. One-third of the tumor was harvested for protein analysis, one-third for RNA analysis and the other third was fixed in 10% formalin for immunohistochemical analysis. Animal care was in strict compliance with the institutional guidelines established by the University of Pittsburgh.

Apoptosis assay

The percentage of apoptotic cells in 201T tumor xenografts treated with the *c-met* antisense construct or U6 empty vector was determined using the ApopTag[®] Peroxidase *In situ* Apoptosis Detection Kit (Intergen Company, Purchase, NY, USA). Tumors were harvested after the 5-week treatment period, fixed in formalin and paraffin embedded. Sections were deparaffinized accord-

ing to standard procedures, incubated with proteinase K for 15 min and washed two times with water. Slides were incubated in 3% H₂O₂ in PBS for 5 min at room temperature and washed two times with PBS. The slides were then incubated for 15 min at 37°C with a terminal transferase enzyme that catalyzes the addition of digoxigenin-labeled nucleotides to the 3'-OH ends of the fragmented DNA, followed by three washes in PBS. After color development and counterstain, the specimens were mounted. Slides were read and scored for the number of positive cells per five high-power fields.

Results

c-Met protein expression is downregulated in lung tumor cell lines following transient transfection with the *c-Met* antisense construct

Antisense gene therapy relies on the disruption of target gene expression by binding to the mRNA and preventing translation. The pGEM2U6 RNA expression cassette has been used previously for antisense therapy and is an innovative vector for high expression of antisense RNAs.^{50,51} Plasmid constructs such as the U6 expression cassette has several advantages over synthetic antisense oligonucleotide techniques. First, it has been engineered to express short RNA antisense sequences and almost all of the U6 gene can be replaced with any other sequence since there is no internal control region in the sequence encoding the structural component of the RNA. Second, the U6 promoter has very strong activity in all types of cells. Third, it is transcribed by RNA polymerase III, resulting in high expression levels within the cell. U6 snRNA is constitutively expressed in all mammalian cells at about 0.5 million copies per cell.⁵² We tested the ability of the *c-Met* antisense U6 expression construct to inhibit *c-Met* protein expression *in vitro* in two non-small cell lung cancer cell lines that we have shown previously to express *c-met*, but not HGF⁵³ (Figure 2). A 50–60% decrease in *c-Met* protein expression was observed in cells treated with the antisense plasmid versus cells treated with the U6 empty vector. A 100% decrease in *c-Met* protein would not be expected since only 60–70% of the cells take up the plasmid. This suggests that in the cells that do express the antisense plasmid, approximately 90% protein inhibition is observed. A slight decrease (<20%) was observed in *c-Met* protein expression in cells treated with the *c-Met* sense construct versus the U6 empty vector. These small changes observed with sense vectors have been reported by others.⁵¹ This decrease was not considered to be significant. Actin protein expression did not change with these treatments. This experiment was repeated three times in each cell line with two samples per treatment. These cell lines represent both adenocarcinoma (201T) and squamous cell lung tumors (128-88T). There appears to be no difference in the effect of the *c-Met* antisense plasmid between these two types of tumors.

RNA is expressed from the transfected plasmids

In order to determine if the transfected plasmids could be transcribed to RNA, a ribonuclease protection assay was used to distinguish between the endogenous *c-met* RNA and the transgenes. We were able to detect the transgenes in cells that were transiently transfected with

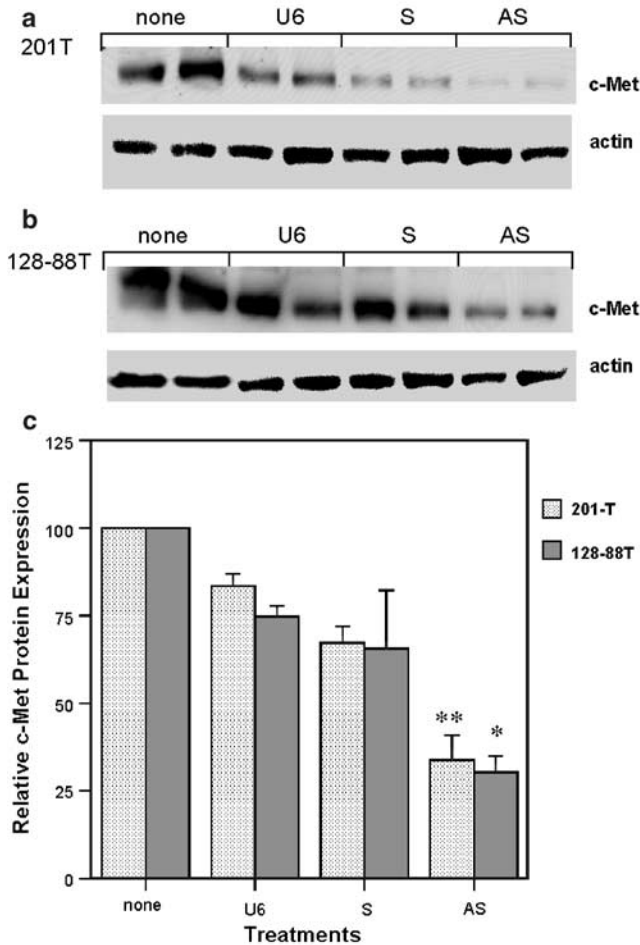


Figure 2 Immunoblots of whole-cell lysates from lung tumor cell lines. (a) Cell extracts were prepared from 201T cells or (b) 128-88T cells that were transiently transfected with the U6 empty vector control, the c-Met sense plasmid (S) the c-Met antisense plasmid (AS) or no transfection (none) and analyzed by Western blotting using a rabbit polyclonal anti-Met antibody or a mouse monoclonal anti-actin antibody (c) Quantitation of three independent experiments each of which had two samples per treatment using ImageQuant analysis. The results are expressed relative to the no treatment control. * $P < 0.05$ compared to U6 control, ** $P < 0.005$ compared to U6 control. Data were analyzed by a two-tailed Student's *t*-test.

the U6 empty vector, c-Met sense or c-Met antisense (Figure 3a). The endogenous *c-met* gene was detected in all of the samples. The level of expression of the transgenes was higher than the endogenous gene probably due to the high transfection efficiency in this cell line. The transfection efficiency in this cell line as monitored by cotransfection with green fluorescent protein was 60–70% (data not shown). It is worth noting that the conditions optimized for *in vitro* gene transfer are not the same as those for *in vivo* gene transfer.

Biological effects of the antisense construct *in vitro*

To monitor the biological effects of the antisense construct *in vitro*, phosphorylation levels of the c-Met protein and also the activation of the MAP kinase pathway were examined. Previous studies have shown that HGF stimulates phosphorylation of c-Met and activation of the MAP kinase pathway (data not shown).

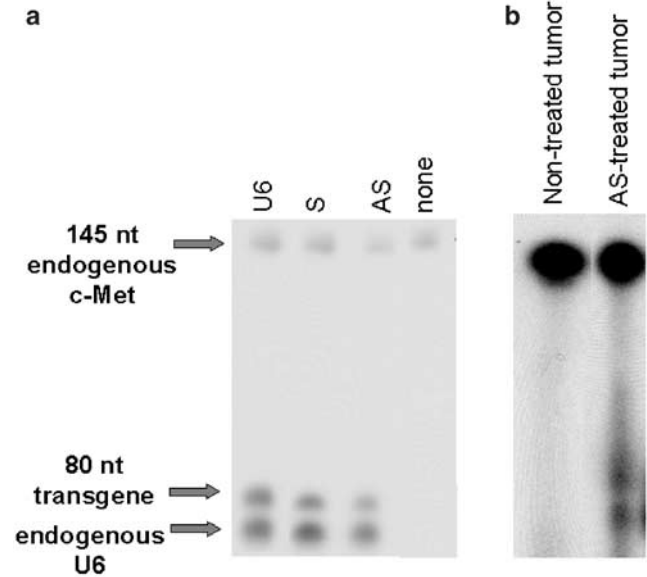


Figure 3 RNA is expressed from the transfected plasmids. (a) Total RNA was isolated from 201T lung cancer cells 48 h after transient transfection of either the U6 empty vector control, the c-Met sense plasmid (S), the c-Met antisense plasmid (AS) or no treatment (none). RNA (20 μ g) was analyzed using a ribonuclease protection assay and probes specific for the endogenous *c-met* gene or the U6, sense or antisense transgene. (b) Total RNA was isolated from tumor xenografts either not treated or treated with the c-Met antisense plasmid. 50 μ g of RNA was analyzed using a ribonuclease protection assay and probes specific for the endogenous *c-met* gene or the antisense transgene.

In 201T cells that were transiently transfected with the c-Met antisense construct and then treated with exogenous recombinant HGF protein, the ability of these cells to phosphorylate c-Met was approximately six times lower than in cells transfected with the c-Met sense construct, U6 empty vector or not transfected at all (Figure 4). In this regard, cells treated with c-Met antisense followed by stimulation with HGF showed only a 2.6-fold increase in phospho-c-Met over c-Met antisense-treated cells that were not exposed to HGF, whereas cells transfected with the c-Met sense, U6 empty vector or not transfected at all show a 13.2-, 12.3- and 21-fold increase with HGF stimulation, respectively. These blots were stripped and reprobed with an antibody for total c-Met protein. Since these cells were transiently transfected with either the c-Met antisense, sense or U6 empty vector, similar amounts of c-Met in all samples are not expected. As expected, the total c-Met levels were approximately 60% lower in the cells that were transfected with c-Met antisense construct. An actin blot was performed as a loading control and the average protein expression of actin between these treatments did not change.

We next examined the ability of lung tumor cells treated with c-Met antisense to phosphorylate p44/42 MAP kinase. In 201T cells that were transiently transfected with the c-Met antisense construct and then treated with exogenous HGF, the ability of the cells to activate the MAP kinase pathway was dramatically lower (1.7-fold over basal levels) than in cells transfected with the U6 empty vector (8.5-fold over basal levels) or not transfected at all (164-fold over basal levels) (Figure 5). Cells transfected with the c-Met sense

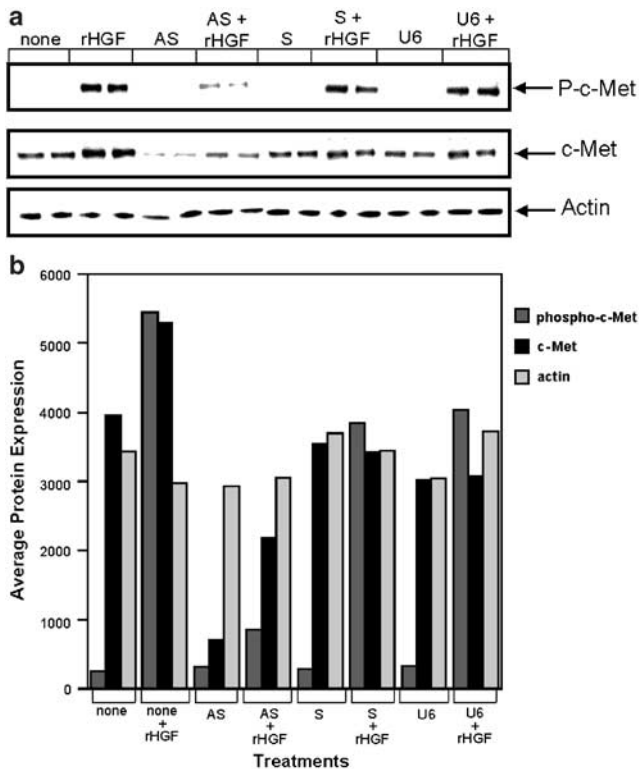


Figure 4 Detection of phosphorylated c-Met in cells transfected with the antisense plasmid. (a) 201T lung cancer cells were transiently transfected with the c-Met antisense plasmid (AS), the c-Met sense plasmid (S), the U6 control plasmid (U6) or no treatment (none). At 72 h after transfection, half of the samples were treated with 10 ng/ml rHGF for 10 min and the others received no treatment. Total cell lysates were prepared and 25 μ g of total protein was separated by size on a 6% SDS-Tris-Glycine gel and immunoblotted with a rabbit polyclonal anti-c-Met (pYpYpY^{1230/1234/1235}) phosphospecific antibody. The blots were stripped and reprobed with a rabbit polyclonal anti-c-Met antibody (not phosphospecific) and once again with a mouse monoclonal anti-actin antibody. (b) Quantitation of the blots by densitometric scanning and ImageQuant analysis. Graph represents the average value of two samples per treatment.

construct show similar results in this assay as the cells transfected with the U6 empty vector (data not shown). The large effect observed in the nontreated cells appears to be due to the fact that the transfection alone gives higher background levels of phospho-MAPK as compared to nontransfected samples. A similar pattern of phosphorylation of MAP kinase was observed in the transfected cells as with the c-Met phosphorylation experiments. These results suggest that the antisense plasmid is affecting downstream signaling pathways. The total MAP kinase protein expression was similar in all samples. Similar results were obtained using the 128-88T cell line (data not shown).

In vivo tumor growth in immunocompromised mice

We first wanted to verify that the chimeric RNA from the transgenes was expressed in the tumor xenografts. The results using the ribonuclease protection assay show that the RNA from the c-met antisense transgene is expressed in the tumors from the injected plasmid, however, at a much lower level than that observed *in vitro* (Figure 3b). This could be because the transfection efficiency is not as high *in vivo* as we had observed in the *in vitro* system

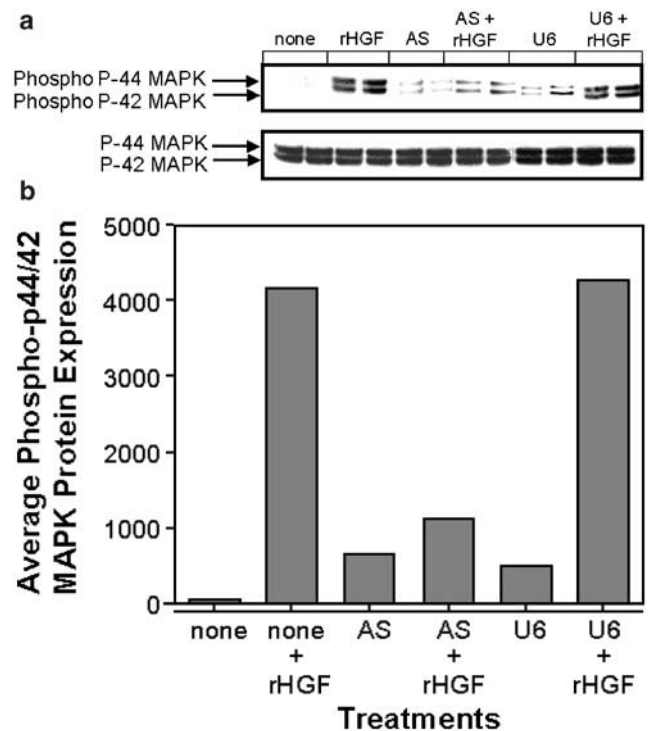


Figure 5 Activation of the MAP Kinase pathway in cells transfected with the antisense plasmid. (a) 201T lung cancer cells were transiently transfected with either the c-Met antisense plasmid (AS) or the U6 control plasmid (U6) or no treatment (none). At 72 h after transfection, half of the samples were treated with 10 ng/ml rHGF for 10 min and the others received no rHGF treatment. Total cell lysates were prepared and 25 μ g of total protein was separated by size on a 10% SDS-tricine gel and immunoblotted with a mouse monoclonal anti-phospho-p44/42 MAPK antibody. The blots were stripped and reprobed with a mouse monoclonal anti-p44/42 MAPK antibody. (b) Quantitation of the blots by densitometric scanning and ImageQuant analysis. Graph represents the average values for phospho-p44/42 protein expression from two samples per treatment.

(20–30% as determined by intratumoral injection of a HA-epitope-tagged plasmid versus 60–70% as determined by cotransfection with green fluorescent protein, data not shown). In addition, U6 RNA is probably quickly degraded *in vivo*.

Growth experiments were conducted *in vivo* to assess whether the c-Met antisense construct could inhibit tumor growth. The 201T cell line that was used in this experiment showed decreased c-Met protein expression when transiently transfected with the c-Met antisense plasmid *in vitro* (Figure 2) and cell growth was reduced by approximately 30% (data not shown). Figure 6 shows a representative experiment with five animals per treatment group each bearing two tumors per mouse. As shown in Figure 6, c-Met antisense treatment decreased tumor growth by 51% compared to U6-treated controls (average tumor volume of 863.6 ± 215 mm³ for antisense versus 1767.1 ± 280.5 mm³ for U6). This was statistically significant with a *P*-value of < 0.05 . Tumor growth in sense-treated tumors was inhibited by 15% compared to U6-treated controls (average tumor volume of 1519.0 ± 484 versus 1767.1 ± 280.5 mm³). Tumors that received no treatment (none) grew the fastest with an average tumor volume of 2081.7 ± 514 mm³. Tumors treated with DC-Chol liposomes alone grew similarly

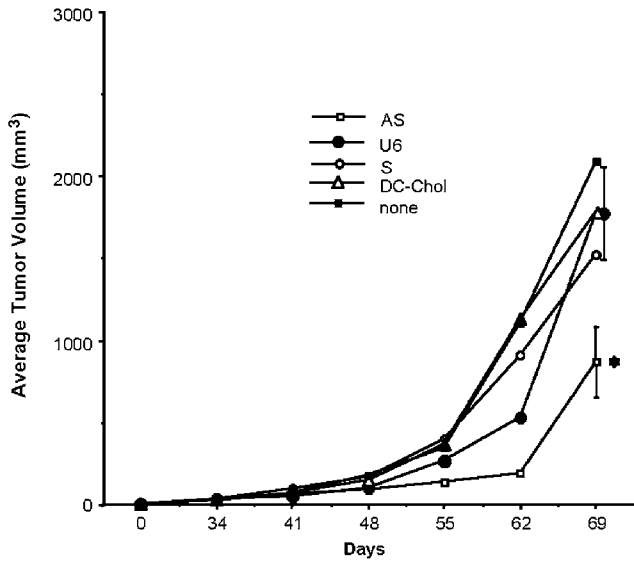


Figure 6 201T tumor growth in SCID mice. Five groups of mice received the following treatments three times per week for 5 weeks: c-Met antisense (□), U6 empty vector control (●), c-Met sense (○), DC-Chol liposomes alone (△) and no treatment (■). Each point represents the mean value for nine tumors per group. The standard error is reported for U6 and AS treatments only. This is a representative experiment that was repeated two additional times. Tumor volume was plotted over time. * $P < 0.05$ (versus U6 empty vector control). Data were analyzed by a two-tailed Student's *t*-test.

to the U6-treated tumors (1775.2 ± 283.0 versus 1767.1 ± 280.5 mm³). This experiment was repeated two additional times with similar results. Thus, the use of c-Met antisense plasmid gene therapy may be a valuable treatment for lung cancer. Although we achieved <50% tumor growth inhibition, c-Met antisense may be useful in combination with other available treatments to achieve further inhibition of tumor growth.

Frozen tumor sections from this experiment were harvested for protein and used in Western analysis (Figure 7). c-Met protein expression was decreased an average of 35–40% in the tumors treated with c-Met antisense plasmid versus U6 empty vector control. Phospho-MAP kinase protein expression was decreased by 48% in these tumors versus U6 control. These changes were statistically significant ($P < 0.0001$). The decreases in both c-Met and phospho-MAP kinase protein expression in c-Met antisense-treated tumors versus DC-Chol or nontreated tumors also showed statistically significant changes ($P < 0.0001$ in each case except c-Met levels in nontreated versus antisense is $P < 0.005$). Actin and total MAP kinase showed no detectable differences among all of the treatment groups. In some cases there was a correlation between phospho-MAP kinase and c-Met protein expression; however, this was not uniformly observed. There appeared to be a correlation between individual tumor growth and c-Met protein expression. The tumors that grew the slowest also had the lowest amounts of c-Met protein (data not shown). Vice versa, the fastest growing tumors had the highest amounts of c-Met protein. For example, the antisense-treated tumor sample in lane 1 of Figure 7b shows the lowest c-Met expression and also the smallest change in tumor growth among that treatment group.

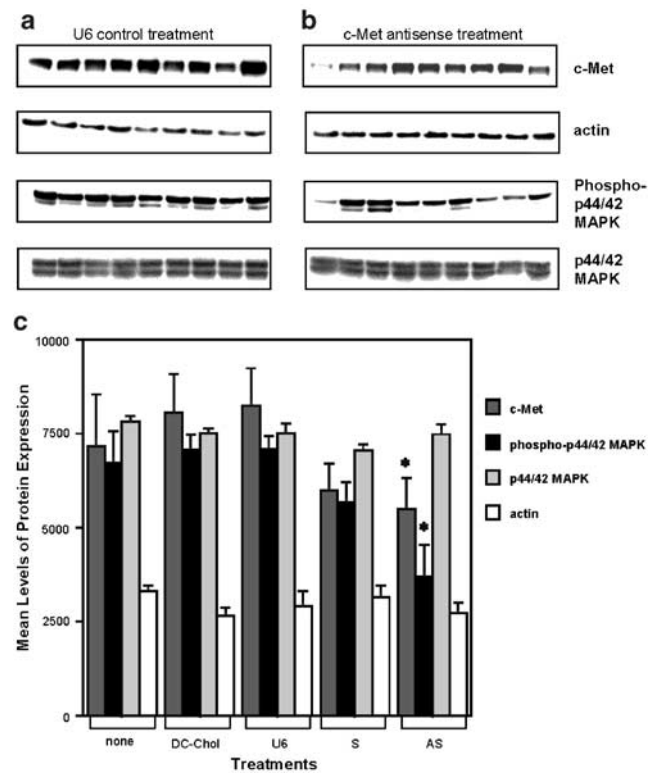


Figure 7 Immunoblots of whole-cell lysates prepared from nine individual 201T xenografts grown in SCID mice and treated with either (a) the U6 empty vector or (b) c-Met antisense plasmid for a 5-week period. Each lane represents an individual tumor extract that was either treated with the U6 control or the c-Met antisense plasmid. An equal amount of protein (50 µg) for each sample was analyzed by Western blotting using antibodies for c-Met, phospho-p44/42 MAPK, p44/42 MAPK, or actin. (c) Represents the mean value \pm s.e. of the chemiluminescent signals using ImageQuant analysis. * $P < 0.0001$ versus U6 control, none or DC-Chol (with one exception in c-Met levels with no treatment versus AS, $P < 0.005$). Data were analyzed using a two-tailed Student's *t*-test.

Apoptosis is increased in tumors treated with c-Met antisense construct

We next examined whether or not apoptosis is increased in the tumors treated with the antisense construct. The possible mechanisms for tumor growth inhibition include necrosis or apoptosis. Slides stained with hematoxylin and eosin revealed no distinguishable differences among the various treatment groups, indicating that necrosis is probably not involved. A section of each tumor was fixed in formalin and paraffin embedded. An ApopTag[®] *in situ* hybridization kit was used to detect single- or double-stranded breaks associated with apoptotic cells. This system can also detect early-stage apoptosis involving chromatin condensation, before any morphological changes are noticeable. There was a four-fold difference in the amount of staining in antisense- versus U6-treated tumors (Figure 8). Quantitation was performed by counting the number of positive cells per five high-powered fields in five U6 control-treated tumors and five antisense-treated tumors. A positive control included slides of tissue from a normal female rodent mammary gland and a negative control was performed without active terminal deoxynucleotidyl transferase mix (data not shown). The proteinase K digestion was included in the negative control to account

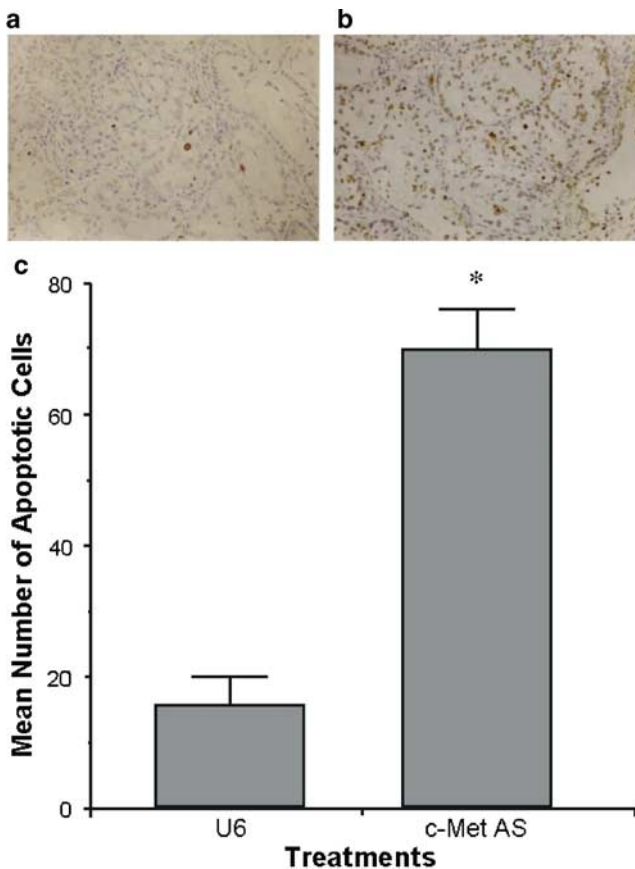


Figure 8 Apoptosis is increased in tumors treated with the c-Met antisense construct. ApoptTag[®] staining of (a) a representative tumor treated with the U6 empty vector control plasmid and (b) a representative tumor treated with the c-Met antisense plasmid. (c) Quantitation of ApoptTag[®] stained slides from five U6 control treated tumors and five c-Met antisense-treated tumors.

for nonspecific incorporation of nucleotides or for nonspecific binding of enzyme conjugate. A similar difference (three- to four-fold) in the amount of staining was observed in antisense- versus DC-Chol- or non-treated tumors (data not shown).

Discussion

c-Met is a receptor tyrosine kinase that plays an important role in the pathogenesis of many human cancers. Several lines of evidence suggest that the HGF/c-Met signaling pathway is a good target for cancer therapy, including the identification of a number of activating mutations as well as cancers identified with overexpression of the receptor. In this study, we report that lung tumor xenograft volume can be inhibited by 50% when treated with intratumoral cationic injection of the c-Met antisense in the U6 expression cassette. This antitumor effect was accompanied by downregulation of c-Met and phospho-MAP kinase protein expression and increased apoptosis.

Small nuclear RNAs (snRNAs) are a special class of RNA molecules that play a central role in splicing of introns from pre-messenger RNAs. The U6 snRNA, which the expression cassette used in this study is derived from, has several unique properties among this

class of molecules. First, in contrast to the other snRNA genes that are transcribed by RNA polymerase II and do not contain introns or TATA boxes, U6 genes are transcribed by RNA polymerase III and contain TATA boxes. Second, it is highly unusual because almost all of the structural U6 core can be replaced with almost any other sequence without affecting transcription because the U6 promoter does not have any internal control regions. In fact, only the first 6 nt and last 18 nt of the U6 transcript are necessary for the synthesis of the U6 chimeric RNA.⁵⁴ The human U6 promoter is composed of a proximal region containing a TATA box and a proximal sequence element and a distal region containing an octamer motif.

The U6 expression cassette used in this study was developed specifically to generate sequence-specific oligonucleotides for antisense gene regulation.⁴⁵ This strategy has been used in preclinical studies previously to inhibit the growth of various tumors *in vivo*. For instance, intratumoral liposome-mediated transfer of either a U6-epidermal growth factor receptor antisense plasmid⁵¹ or a U6-transforming growth factor alpha plasmid⁵⁵ were shown to inhibit tumor growth in a head and neck cancer xenograft model and this was correlated with increased apoptosis in the antisense-treated tumors. Similarly, in this model system, *in vivo* liposome-mediated therapy with an U6-Stat3 antisense plasmid has been shown to inhibit tumor growth and Stat3 activation, increase apoptosis and decrease Bcl-xL expression.⁵⁶ In a cervical tumor model, antisense sequences from the E6 and E7 oncogenes of human papillomavirus type 16, which are involved in the malignant phenotype of cervical carcinoma, were inserted into the U6 expression construct and complexed with DC-Chol liposomes. Intratumoral injection of these plasmids has been shown to inhibit the *in vivo* growth of cervical tumors in mice.⁵⁰

Nonviral gene delivery methods such as cationic liposomes have distinct advantages over adenoviral or retroviral vectors. DC-Chol is a novel cationic cholesterol derivative that contains a tertiary amine head and a cholesterol linked by a carbamoyl bond.⁴⁹ When mixed with the lipid DOPE in the presence of chloroform, followed by drying, rehydration and sonication, DC-Chol produces stable liposomes that are efficient in transferring DNA. DC-Chol/DOPE liposomes have been used in several clinical trials. For example, cystic fibrosis patients received multiple doses of the cystic fibrosis transmembrane conductance regulator gene complexed with DC-Chol/DOPE cationic liposomes delivered to the nasal epithelium with negligible toxicity, inflammation or immune response reported.⁵⁷ In a separate trial, patients with advanced breast or ovarian cancer received intracavitary injection of DC-Chol complexed with the E1A anticancer gene once a week with only slight treatment-related effects observed.⁵⁸ Recently, a phase II trial has demonstrated no toxicity as well as modest tumor response of intratumoral injections of the E1A gene complexed with DC-Chol in head and neck squamous cell carcinomas.⁵⁹ The development of aerosolized preparations of these liposome formulations may be clinically useful in the further development of these agents in lung cancer.

Owing to the overwhelming evidence for the role of the HGF/c-Met pathway in the pathogenesis of human

cancers, the development of therapeutic inhibitors of c-Met is not without precedence. First, the geldanamycin family of anisamycin antibiotics has been shown to inhibit HGF/c-Met signaling. For example, at nanomolar concentrations, the geldanamycins have been shown to downregulate c-Met protein expression and inhibit HGF-mediated cell motility and invasion in MDCK-2 cells.⁶⁰ In small cell lung cancer cell lines, geldanamycin has been demonstrated to decrease cell growth and viability and to increase apoptosis.⁶¹ Second, peptides from the c-Met receptor tail have been shown to bind to the receptor and inhibit c-Met kinase activity *in vitro* and inhibit HGF-mediated invasive growth of A549 cells by 50%.⁶² Third, HGF and c-Met expression have been targeted using an U1/snRNA/ribozyme approach to inhibit human glioma growth.^{63,64} Similarly, the use of a hammerhead ribozyme encoding c-Met antisense sequences has been shown to reduce both migration and invasiveness of breast cancer cell lines *in vitro*.⁶⁵ There are many other inhibitors of this pathway, including antagonists of Grb2 Src homology 2 domain interactions,⁶⁶ modulation of Gab1 phosphorylation with okadaic acid,^{67,68} and dominant-negative Src.⁶⁹ Recently, it has been shown that an ATP analog, K252a, can inhibit c-Met autophosphorylation as well as other oncogenic properties of c-Met-mediated tumors.⁷⁰ The cyclooxygenase-2 inhibitor, celecoxib, has also been recently suggested to downregulate the HGF/c-Met signaling pathway.^{71,72} To our knowledge, there is no tyrosine kinase inhibitor specific for c-Met currently available. Collectively, these data show that c-Met is an attractive target for therapy in a variety of solid tumor types and c-Met inhibitors could be clinically useful for lung cancer.

There are many populations of lung cancer patients who would be candidates for delivery of antitumor agents by intratumoral injection. For example, neoadjuvant therapy of lung tumors is being increasingly performed to reduce tumor burden prior to surgery. This therapy is usually a combination of irradiation of the lung and chemotherapy. Since irradiation is known to induce the expression of c-Met,⁷³ inclusion of an agent targeting the c-Met protein could be of benefit for lung cancer patients undergoing neoadjuvant therapy. Another group of lung cancer patients is those with bronchioloalveolar carcinoma. This type of lung cancer progresses almost entirely within the lung and is often unresectable because of extensive involvement of pulmonary tissue. A third group of lung cancer patients who could benefit from intratumoral injection of an anticancer agent is the patient group with locally advanced lung cancer. Lung cancer often recurs locally within the lung, and such patients have usually failed other types of therapy. Many of these patients are not eligible for repeat surgery due to poor lung function. Patients falling into this category have been candidates for p53 gene replacement therapy, which has been given by intratumoral injection in phase I and II trials.⁷⁴ In some cases, access to the tumors was accomplished by bronchoscopy. A fourth group of patients who are candidates are those with metastases to distant organs. Intratumoral injection of p53 gene vectors has been accomplished in these patients by CT-guided needle injection into the metastatic lesion.⁷⁴ Since the role of HGF in the adult is to initiate response to injury, substantial side effects are not expected. Likewise,

targeting of the epidermal growth factor receptor, a ubiquitously expressed protein, has resulted in few clinical side effects in cancer therapy.

We have also tested this strategy in a breast cancer model and have shown similar results (data not shown), suggesting that this may be an effective therapy in other c-Met overexpressing tumors. We are currently testing these constructs in an orthotopic lung model involving intratracheal injection of the c-Met antisense construct, and also in combination with HGF inhibitors to try to inhibit the entire pathway. Our goal is to continue the preclinical development of therapies to target this pathway specifically in lung cancer patients who currently have few therapeutic options.

Acknowledgements

We gratefully acknowledge the technical assistance of John Dileo in the Huang laboratory for supplying the DC-Chol and UPMC Developmental Pathology Lab for their technical assistance with the ApopTag[®] assay. This work was supported by Grant CA 79882 awarded to JMS and by CA74918 to LH from the National Cancer Institute. LPS was supported by a fellowship from the American Lung Association.

References

- 1 Cooper CS *et al.* Amplification and overexpression of the met gene in spontaneously transformed NIH3T3 mouse fibroblasts. *EMBO J* 1986; **10**: 2623–2628.
- 2 Park M *et al.* Sequence of MET proto-oncogene cDNA has features characteristic of the tyrosine kinase family of growth factor receptors. *Proc Natl Acad Sci USA* 1987; **84**: 379–6383.
- 3 Bottaro DP *et al.* Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science* 1991; **251**: 802–804.
- 4 Nakamura T, Nawa K, Ichihara A. Partial purification and characterization of hepatocyte growth factor from serum of hepatotomized rats. *Biochem Biophys Res Commun* 1984; **122**: 1450–1459.
- 5 Naldini L *et al.* Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor. *EMBO J* 1991; **10**: 2867–2878.
- 6 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.
- 7 Stoker M, Gherardi E, Peryman M, Gray J. Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. *Nature* 1987; **327**: 239–242.
- 8 Rosen E *et al.* Smooth muscle-derived factor stimulates mobility of human tumor cells. *Invasion Metas* 1990; **10**: 49–64.
- 9 Tsao MS *et al.* 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.
- 10 Yoshinaga Y *et al.* Human lung cancer cell line producing hepatocyte growth factor/scatter factor. *Jpn J Cancer Res* 1992; **83**: 1257–1259.
- 11 Tokunou M *et al.* c-MET expression in myofibroblasts: role in autocrine activation and prognostic significance in lung adenocarcinoma. *Am J Pathol* 2001; **158**: 1451–1463.
- 12 Yi S, Tsao MS. Activation of hepatocyte growth factor-met autocrine loop enhances tumorigenicity in a human lung adenocarcinoma cell line. *Neoplasia* 2000; **2**: 226–234.

- 13 Montesano R, Matsumoto K, Nakamura T, Orci L. Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell* 1991; **67**: 901–908.
- 14 Jeffers M et al. Activating mutations for the met tyrosine kinase receptor in human cancer. *Proc Natl Acad Sci USA* 1997; **94**: 11445–11450.
- 15 Rong S et al. Tumorigenicity of the met proto-oncogene and the gene for hepatocyte growth factor. *Mol Cell Biol* 1992; **12**: 5152–5158.
- 16 Di Renzo MF et al. Somatic mutations of the MET oncogene are selected during metastatic spread of human HNSC carcinomas. *Oncogene* 2000; **19**: 1547–1555.
- 17 Lorenzato A et al. Novel somatic mutations of the MET oncogene in human carcinoma metastases activating cell motility and invasion. *Cancer Res* 2002; **62**: 7025–7030.
- 18 Schmidt L et al. Germline and somatic mutations in the tyrosine kinase domain of the met proto-oncogene in papillary renal carcinomas. *Nat Genet* 1997; **16**: 68–73.
- 19 Qiao H et al. Constitutive activation of met kinase in non-small-cell lung carcinomas correlates with anchorage-independent cell survival. *J Cell Biochem* 2002; **86**: 665–677.
- 20 Liang TJ et al. Transgenic expression of tpr-met oncogene leads to development of mammary hyperplasia and tumors. *J Clin Invest* 1996; **97**: 2872–2877.
- 21 Takayama H et al. Diverse tumorigenesis associated with aberrant development in mice overexpressing hepatocyte growth factor/scatter factor. *Proc Natl Acad Sci USA* 1997; **94**: 701–706.
- 22 Ghossoub RA et al. Expression of c-met is a strong independent prognostic factor in breast carcinoma. *Cancer* 1998; **82**: 1513–1520.
- 23 Di Renzo MF et al. Overexpression of the Met/HGF receptor in ovarian cancer. *Int J Cancer* 1994; **58**: 658–662.
- 24 Humphrey PA et al. Hepatocyte growth factor and its receptor (c-met) in prostatic carcinoma. *Am J Pathol* 1995; **147**: 386–396.
- 25 Pisters LL et al. C-met proto-oncogene expression in benign and malignant human prostate tissues. *J Urol* 1995; **154**: 293–298.
- 26 Di Renzo MF et al. Overexpression of the c-met/HGF receptor gene in human thyroid carcinomas. *Oncogene* 1992; **7**: 2549–2553.
- 27 Trovato M et al. Expression of the hepatocyte growth factor and c-met in normal thyroid, non-neoplastic, and neoplastic nodules. *Thyroid* 1998; **8**: 125–131.
- 28 Di Renzo MF et al. Expression of the Met/hepatocyte growth factor receptor in human pancreatic cancer. *Cancer Res* 1995; **55**: 1129–1138.
- 29 Di Renzo MF et al. Overexpression and amplification of the Met/HGF receptor gene during the progression of colorectal cancer. *Clin Cancer Res* 1995; **1**: 147–154.
- 30 Kaji M et al. Participation of c-met in the progression of human gastric cancers: anti-c-met oligonucleotides inhibit proliferation or invasiveness of gastric cancer cells. *Cancer Gene Ther* 1996; **3**: 393–404.
- 31 Nakajima M et al. The prognostic significance of amplification and overexpression of c-met and c-erb B-2 in human gastric carcinomas. *Cancer* 1999; **85**: 1894–1902.
- 32 Koochekpour S et al. Met and hepatocyte growth factor/scatter factor expression in human gliomas. *Cancer Res* 1997; **57**: 5391–5398.
- 33 Morello S et al. MET receptor is overexpressed but not mutated in oral squamous cell carcinomas. *J Cell Physiol* 2001; **189**: 285–290.
- 34 Ichimura E, Maeshima A, Nakajima T, Nakamura T. Expression of c-met/HGF receptor in human non-small cell lung carcinomas *in vitro* and *in vivo* and its prognostic significance. *Jpn J Cancer Res* 1996; **87**: 1063–1069.
- 35 Yanagita K et al. Hepatocyte growth factor may act as a pumotropic factor on lung regeneration after acute lung injury. *J Biol Chem* 1993; **269**: 21212–21217.
- 36 To CT, Tsao MS. The roles of hepatocyte growth factor/scatter factor and met receptor in human cancers (Review). *Oncol Rep* 1998; **5**: 1013–1024.
- 37 Danilkovitch-Miagkova A, Zbar B. Dysregulation of met receptor tyrosine kinase activity in invasive tumors. *J Clin Invest* 2002; **109**: 863–867.
- 38 Terada T, Nakanuma Y, Sirica AE. Immunohistochemical demonstration of MET overexpression in human intrahepatic cholangiocarcinoma and in hepatolithiasis. *Hum Pathol* 1998; **29**: 75–80.
- 39 Huang TJ et al. Overexpression of the c-met protooncogene in human gastric carcinoma – correlation to clinical features. *Acta Oncol* 2001; **40**: 638–643.
- 40 Qian CN et al. Met protein expression level correlates with survival in patients with late-stage nasopharyngeal carcinoma. *Cancer Res* 2002; **62**: 589–596.
- 41 Yamashita J et al. Immunoreactive HGF is a strong and independent predictor of recurrence and survival in human breast cancer. *Cancer Res* 1994; **54**: 1630–1633.
- 42 Siegfried JM et al. Association of immunoreactive hepatocyte growth factor with poor survival in resectable non-small cell lung cancer. *Cancer Res* 1997; **57**: 433–439.
- 43 Harvey P et al. Immunoreactivity for hepatocyte growth factor and its receptor, met, in human lung carcinomas and malignant mesothelioma. *J Pathol* 1996; **180**: 389–394.
- 44 Takanami I et al. Hepatocyte growth factor and c-Met/hepatocyte growth factor receptor in pulmonary adenocarcinomas: an evaluation of their expression as prognostic markers. *Oncology* 1996; **53**: 392–397.
- 45 Noonberg SB et al. *In vivo* generation of highly abundant sequence-specific oligonucleotides for antisense and triplex gene regulation. *Nucleic Acids Res* 1994; **22**: 2830–2836.
- 46 Noonberg SB, Rodeck U, Weinmann R, Herlyn M. Stable transfection of human malignant melanoma cells with basic fibroblast growth factor antisense cDNA. *Ann NY Acad Sci* 1992; **660**: 293–294.
- 47 Siegfried JM et al. Evidence for autocrine actions of neuromedin B and gastrin-releasing peptide in non-small cell lung cancer. *Pulm Pharmacol Ther* 1999; **12**: 291–302.
- 48 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 1987; **162**: 156–159.
- 49 Gao X, Huang L. A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochem Biophys Res Commun* 1991; **179**: 280–285.
- 50 He Y, Huang L. Growth inhibition of human papillomavirus 16 DNA-positive mouse tumor by antisense RNA transcribed from U6 promoter. *Cancer Res* 1997; **57**: 3993–3999.
- 51 He Y et al. Inhibition of human squamous cell carcinoma growth *in vivo* by epidermal growth factor receptor antisense RNA transcribed from the U6 promoter. *J Natl Cancer Inst* 1998; **90**: 1080–1087.
- 52 Sauterer RA, Goyal A, Zieve GW. Cytoplasmic assembly of small nuclear ribonucleoprotein particles from 6 S and 20 S RNA-free intermediates in L929 mouse fibroblasts. *J Biol Chem* 1990; **265**: 1048–1058.
- 53 Singh-Kaw P, Zarnegar R, Siegfried JM. Stimulatory effects of hepatocyte growth factor on normal and neoplastic human bronchial epithelial cells. *Am J Physiol* 1995; **268**: L1012–L1020.
- 54 Kunkel GR, Pederson T. Transcription of a human U6 small nuclear RNA gene *in vivo* withstands deletion of intragenic sequences but not of an upstream TATATA box. *Nucleic Acids Res* 1989; **17**: 7371–7379.
- 55 Endo S et al. TGF-alpha antisense gene therapy inhibits head and neck squamous cell carcinoma growth *in vivo*. *Gene Ther* 2000; **7**: 1906–1914.
- 56 Grandis JR et al. Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis *in vivo*. *Proc Natl Acad Sci USA* 2000; **97**: 4227–4232.

- 57 Hyde SC *et al*. Repeat administration of DNA/liposomes to the nasal epithelium of patients with cystic fibrosis. *Gene Ther* 2000; **7**: 1156–1165.
- 58 Hortobagyl GN *et al*. Cationic liposome-mediated E1A gene transfer to human breast and ovarian cancer cells and its biologic effects: a phase I clinical trial. *J Clin Oncol* 2001; **19**: 4183–4184.
- 59 Villaret D *et al*. A multicenter phase II study of tgDCC-E1A for the intratumoral treatment of patients with recurrent head and neck squamous cell carcinoma. *Head Neck* 2002; **24**: 661–669.
- 60 Webb CP *et al*. The geldanamycins are potent inhibitors of the hepatocyte growth factor/scatter factor-met-urokinase plasminogen activator-plasmin proteolytic network. *Cancer Res* 2000; **60**: 342–349.
- 61 Maulik G *et al*. Modulation of the c-Met/hepatocyte growth factor pathway in small cell lung cancer. *Clin Cancer Res* 2002; **8**: 620–627.
- 62 Bardelli A *et al*. A peptide representing the carboxyl-terminal tail of the met receptor inhibits kinase activity and invasive growth. *J Biol Chem* 1999; **274**: 29274–29281.
- 63 Abounader R *et al*. 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.
- 64 Abounader R *et al*. *In vivo* targeting of SF/HGF and c-met expression via U1snRNA/ribozymes inhibits glioma growth and angiogenesis and promotes apoptosis. *FASEB J* 2002; **16**: 108–110.
- 65 Jiang WG *et al*. A hammerhead ribozyme suppresses expression of hepatocyte growth factor/scatter factor receptor c-Met and reduces migration and invasiveness of breast cancer cells. *Clin Cancer Res* 2001; **7**: 2555–2562.
- 66 Atabey N *et al*. Potent blockade of hepatocyte growth factor-stimulated cell motility, matrix invasion and branching morphogenesis by antagonists of Grb2 Src homology 2 domain interactions. *J Biol Chem* 2001; **276**: 14308–14314.
- 67 Sachs M *et al*. Essential role of Gab1 for signaling by the c-Met receptor *in vivo*. *J Cell Biol* 2000; **150**: 1375–1384.
- 68 Gual P *et al*. Sustained recruitment of phospholipase C-gamma to Gab1 is required for HGF-induced branching tubulogenesis. *Oncogene* 2000; **19**: 1509–1518.
- 69 Nakaigawa N, Weirich G, Schmidt L, Zbar B. Tumorigenesis mediated by MET mutant M1268T is inhibited by dominant-negative Src. *Oncogene* 2000; **19**: 2996–3002.
- 70 Morotti A *et al*. K252a inhibits the oncogenic properties of Met, the HGF receptor. *Oncogene* 2002; **21**: 4885–4893.
- 71 Baatar D *et al*. Selective cyclooxygenase-2 blocker delays healing of esophageal ulcers in rats and inhibits ulceration-triggered c-Met/hepatocyte growth factor receptor induction and extracellular signal-regulated kinase 2 activation. *Am J Pathol* 2002; **160**: 963–972.
- 72 Chen JH, Liu TY, Wu CW, Chi CW. Nonsteroidal anti-inflammatory drugs for treatment of advanced gastric cancer: cyclooxygenase-2 is involved in hepatocyte growth factor mediated tumor development and progression. *Med Hypothesis* 2001; **57**: 503–505.
- 73 Qian LW *et al*. Radiation stimulates HGF receptor/c-Met expression that leads to amplifying cellular response to HGF stimulation via upregulated receptor tyrosine phosphorylation and MAP kinase activity in pancreatic cancer cells. *Int J Cancer* 2003; **104**: 542–549.
- 74 Nemunaitis J *et al*. Adenovirus-mediated p53 gene transfer in sequence with cisplatin to tumors of patients with non-small cell lung cancer. *J Clin Oncol* 2000; **18**: 609–622.