

c-Met Is a Potentially New Therapeutic Target for Treatment of Human Melanoma

Neelu Puri, Salman Ahmed, Varalakshmi Janamanchi, Maria Tretiakova, Osvaldo Zumba, Thomas Krausz, Ramasamy Jagadeeswaran, and Ravi Salgia

Abstract Purpose: c-Met is a receptor tyrosine kinase involved in cell growth, invasion, metastases, and angiogenesis. In this study, we investigated the role of c-Met in melanoma biology using a novel small-molecule tyrosine kinase inhibitor SU11274 and small interfering (si) RNA against the receptor.

Experimental Design: The effects of SU11274 and c-Met siRNA were studied on proliferation, apoptosis, differentiation, reactive oxygen species, and intracellular signaling. c-Met mutations were examined, and the expression of c-Met and activated c-Met was studied in nevi, primary, and metastatic melanoma.

Results: c-Met was expressed in 6:7 melanoma cell lines by immunoblotting. SU11274 inhibited cell growth in all melanoma cell lines by 85% to 98% with an IC_{50} between 1 and 2.5 $\mu\text{mol/L}$ and caused apoptosis (12-58%) in five out of six cell lines. siRNA against c-Met inhibited proliferation of melanoma cells by 60%. This is the first study that shows that SU11274 and siRNA induced microphthalmia-associated transcription factor (MITF) and several other melanoma differentiation proteins and a morphologically differentiated phenotype. SU11274 also inhibited reactive oxygen species formation and phosphorylation of c-Met receptor, AKT and S-6 kinase by the hepatocyte growth factor. A new missense c-Met mutation N948S was identified in cell lines and R988C in tumor tissue in the juxtamembrane domain of c-Met. It was found that c-Met was expressed in 88% of melanomas and 15% of nevi, and that c-Met (pY1003) was activated in 21% of human melanomas.

Conclusion: These results support the role of c-Met in proliferation, apoptosis, differentiation, and tumor progression of melanoma. SU11274 could be used in the therapeutic inhibition of melanoma.

The incidence of malignant melanoma has increased rapidly during the past several decades. A study published in 2006 estimated there would be 62,190 new cases of melanoma and 7,910 deaths that year, making it the most common of all skin malignancies (1). The majority of these deaths are due to distant metastases from the primary site because melanoma is notorious for its propensity to metastasize. Melanoma is poorly responsive to cytotoxic chemotherapy (2), and the survival of

patients is based on screening, early detection, and wide resection of the primary lesion. The overall survival for patients with metastatic melanoma ranges from only 4.7 to 11 months, with a median survival of 8.5 months (3). c-Met is overexpressed (4) and associated with the metastatic potential of melanoma and patient survival (5-7).

c-Met is a receptor tyrosine kinase (RTK) that has been known to stimulate the invasive growth of cancer cells, increase their metastatic potential, and is also known to be expressed and mutated in a variety of solid tumors (8). The structure of c-Met consists of a disulfide-linked α - β heterodimer with a molecular weight of 190 kDa (9). The 140-kDa β chain spans the membrane; however, the α chain is only located extracellularly, and both these forms are cleaved from a 170-kDa precursor form. The intracellular part of the receptor can be divided into (a) a juxtamembrane region, followed by (b) a tyrosine kinase catalytic domain, and (c) a COOH-terminal sequence, which is responsible for coupling the receptor to intracellular cell signaling molecules (10). All of these three domains contain tyrosine residues that are phosphorylated upon ligand binding. The major phosphorylation site of c-Met is in the catalytic domain, where three tyrosines (Tyr^{1230/1234/1235}) are located, and is the region in which ATP is bound. The phosphorylated tyrosines in the COOH-terminal sequence (Tyr¹³⁴⁹ and Tyr¹³⁵⁶) act as docking sites for

Authors' Affiliation: Departments of Hematology/Oncology and Pathology, University of Chicago Medical Center, Chicago, Illinois
Received 3/30/06; revised 11/3/06; accepted 11/30/06.

Grant support: This work was supported by NIH/National Cancer Institute grants CA100750-04 and CA125541-01 and Institutional Cancer Research Awards from the University of Chicago Cancer Centre with the American Cancer Society and the V-Foundation (R. Salgia).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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

Requests for reprints: Ravi Salgia, Department of Medicine, Section of Hematology/Oncology, University of Chicago Medical Center, 5841 S. Maryland Avenue, MC 2115, Chicago, IL 60607. Phone: 773-702-4399; Fax: 773-784-1698; E-mail: rsalgia@medicine.bsd.uchicago.edu.

©2007 American Association for Cancer Research.
doi:10.1158/1078-0432.CCR-06-0776

downstream signaling molecules (10). The juxtamembrane Tyr¹⁰⁰³ of the cytoplasmic part of c-Met is required for ubiquitination and degradation by the proteasome pathway, making it a part of a negative feedback loop for c-Met signaling (10). The natural ligand for c-Met is the hepatocyte growth factor (HGF), which is also known as scatter factor. HGF is a multifunctional cytokine acting as a mitogen, motogen, and morphogen for many epithelial cells. HGF is physiologically secreted by cells of mesenchymal origin and acts on neighboring epithelial cells through a paracrine loop (2). Many missense mutations of c-Met have been reported in a variety of cancers, with the majority of them identified in the cytoplasmic activation loop tyrosine kinase domain (10). c-Met activating point mutations in the kinase domain are implicated as the cause of hereditary papillary renal carcinoma (11). In addition, kinase domain mutations have been observed in sporadic papillary renal carcinoma, ovarian cancer, childhood hepatocellular carcinoma, and gastric cancer (11–13). Juxtamembrane domains of RTKs are thought to be key regulators of catalytic functions. We identified unique activating mutations of c-Met in the juxtamembrane domain (R988C and T1010I) and semaphorin domain (E168D), as well as insertions and alternative splice forms involving the juxtamembrane in small cell lung cancer and non-small cell lung cancer (10, 14).

In normal skin, c-Met is present on epithelial cells and melanocytes, whereas HGF is produced mainly by mesenchymal cells and, consequently, interacts with its receptor in a paracrine manner (15). HGF is a mitogen of human melanocytes, and overexpression of c-Met correlates with the invasive growth phase of melanoma cells (4). Recent studies by Herlyn's group have shown that most of melanoma cells, but not normal melanocytes, produce HGF, which can induce sustained activation of its receptor (2). Hence, an autocrine HGF/c-Met signaling loop may be involved in the development of melanomas. Consistent with this, prolonged HGF stimulation induces the down-regulation of the intercellular adhesive molecule E-cadherin that is implicated in the control of melanocyte proliferation (2). Finally, in transgenic mice that ubiquitously expressed HGF, ectopic localization of melanocytes and hyperpigmentation in skin were observed, and melanoma arose spontaneously. In these mice, UV radiation-induced carcinogenesis was accelerated (16). It is suggested that c-Met autocrine activation induced the development of malignant melanoma and the acquisition of the metastatic phenotype (17).

Here, we show that a novel small-molecule tyrosine kinase inhibitor of the c-Met inhibits growth of melanoma by causing apoptosis and induces differentiation. We further show that HGF induced signaling, and the generation of reactive oxygen species (ROS) could be inhibited by this drug. We also illustrate that c-Met can also be inhibited by using small interfering (si) RNA, and siRNA also induces differentiation in melanoma similar to SU11274. We also analyzed the functional expression and activation of c-Met in melanoma tumor sections and finally identified unique mutations in the juxtamembrane domain in melanoma cell lines and tumor tissue.

Materials and Methods

Reagents and antibodies. SU11274: [(3Z)-N-(3-chlorophenyl)-3-({3,5-dimethyl-4-[(4-methylpiperazin-1-yl)carbonyl]-1H-pyrrol-2-

yl)methylene)-N-methyl-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide] (obtained from Pfizer Inc., San Diego, CA) was suspended in DMSO and kept in small aliquots at -20°C . Phosphospecific antibody to pY1230/1234/1235 (autophosphorylation site) was obtained from Biosource International (Camarillo, CA). Phosphospecific antibodies for pS473 on AKT and pT421/pS424 on p70 S-6-kinase were obtained from Cell Signaling Technology (Beverly, MA). Total c-Met and BCL_{XL} antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and β -actin from Sigma-Aldrich (St. Louis, MO). For detecting melanoma differentiation proteins, the following antibodies were used: anti-tyrosinase monoclonal antibody (clone T311; Nova-Castra, Newcastle-upon-Tyne, United Kingdom), anti-gp-100 monoclonal antibody (MO 634, DAKO, Carpinteria, CA) and anti-MART-1/Melan-A monoclonal antibody (clone M2-7C10, Signet, Dedham, MA).

Cell lines and cell culture and cell cycle analysis. MM-AN, MU, PM-WK, MM-RU, MM-MC, MM-LH, and RPM-EP melanoma cells were grown in MEM (Cellgro, Herndon, VA) with 10% fetal bovine serum (Gemini Bioproducts, Woodland, CA). Triplicate melanoma cell cultures were grown in MEM with 10% fetal bovine serum and treated with a final concentration of 5 to 10 $\mu\text{mol/L}$ of SU11274 or an equal volume of diluent (DMSO) as a control for 96 h. Cells were collected, stained with propidium iodide, and analyzed with a Becton Dickinson (San Jose, CA) FACS Scan and Cell Quest software.

Preparation of cell lysates and immunoblotting. Cells were lysed in lysis buffer containing 20 mmol/L Tris (pH, 8.0), 150 mmol/L NaCl, 10% glycerol, 1% NP40, 0.42% NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na_3VO_4 , and 10 $\mu\text{L/mL}$ protease inhibitor cocktail (Sigma-Aldrich) as described previously (18). Cell lysates were separated by 7.5% or 10% SDS-PAGE under reducing conditions. Proteins were transferred to an immobilization membrane (Bio-Rad Laboratories, Hercules, CA) and immunoblotted using the enhanced chemiluminescence technique (PerkinElmer Life and Analytical Sciences, Torrance, CA).

Cell growth analysis. For measuring the effect of SU11274 on the growth of melanoma cells, MM-AN, MU, PM-WK, MM-RU, MM-MC, and RPM-EP cells were plated at 6×10^4 cells in 60-mm dishes in MEM with 10% serum. After 24 h, different concentrations of SU11274 were added, and after 96 h, cells were trypsinized and counted in a cell counter (Beckman Coulter, Fullerton, CA). Each data point was repeated in triplicate.

Specific phosphorylation of c-Met and other signaling molecules via HGF and inhibition with SU11274. MU and MC cells that expressed c-Met were deprived of growth factors by incubation in serum-free medium containing 0.5% bovine serum albumin for 24 h with or without SU11274 (5 $\mu\text{mol/L}$). After treatment with or without SU11274 in serum-free medium, cells were stimulated with or without HGF (EMD Biosciences, San Diego, CA) at 40 ng/mL for 7.5 min at 37°C . After harvesting, the cells were subjected to the standard procedures of immunoblotting. Antibodies specifically against phosphorylated c-Met and other proteins are as described above.

ROS measurement by immunofluorescence. About 5×10^4 MU melanoma cells were plated in 35-mm Petri dishes. After 24 h, MU melanoma cells were kept in serum-free media containing 0.5% bovine serum albumin with and without 5 $\mu\text{mol/L}$ SU11274 for 12 h. Cells were treated with 40 ng/mL HGF for 7.5 min, then treated with 10 $\mu\text{mol/L}$ dihydroethidine (Molecular Probes Inc., Eugene, OR) for 30 min, after which they were visualized under a fluorescent Olympus microscope IX81, 20 \times objective, ND3 and rhodamine filter sets. Fluorescence was then quantified using Image J and graphed as shown.

siRNA and transfection. siRNA against c-Met was obtained from Dharmacon (Lafayette, CO). Briefly, four pooled siRNA duplexes were transiently transfected into MU melanoma cells by the Dharmacon protocol using OligofectAMINE obtained from Invitrogen (Carlsbad, CA). Mock transfection was done in parallel using SignalSilence control siRNA (Cell Signaling Technology) as negative control. For studies on the effect of siRNA on differentiation proteins, MU melanoma cells were transfected with siRNA as described above, cell lysates were prepared after 12 h, and immunoblotting was done as described.

Immunohistochemistry. For immunohistochemistry, we analyzed paraffin-embedded, formalin-fixed tissues from patients with 20 nevi (4 intradermal, 2 junctional, 14 compound), 16 primary melanomas (5 superficial-spreading type, 9 nodular, 2 spindle), and 24 metastatic melanomas. All cases have been retrieved from pathology archives at the University of Chicago Hospital with institutional approved Institutional Review Board protocol. Immunostaining procedures were as described by Ma et al. (19). For total c-Met immunostaining, 3D4 mouse monoclonal antibody from Zymed was used, and phospho-Met [pY1003] from Biosource was used. Appropriate negative controls for the immunostaining were prepared by omitting the primary antibody step and substituting it with nonimmune rabbit serum.

All of the slides were reviewed and scored by two pathologists independently. For each case, the most representative tumor section was examined at 200× magnification to identify the percentage of tumor cells with c-Met expression.

Cell lines and DNA sequence analysis. Genomic DNA was isolated from five melanoma cell lines using a QIAamp DNA mini kit (Qiagen, Valencia, CA). Melanoma tumors were obtained from pathology

archives at the University of Chicago Hospital with institutional approved Institutional Review Board protocol, and tumor DNA was isolated using proteinase K (0.03 mAU) from Qiagen overnight at 56°C. The semaphorin domain, juxtamembrane domain, and tyrosine kinase domain in c-Met genomic DNA were sequenced using standard PCR and sequencing techniques. Each PCR reaction contained 50 ng/mL of DNA, 1× Platinum Taq buffer, 1 mmol/L deoxynucleotide triphosphates, 2.5 mmol/L MgCl₂, 0.5 U Platinum Taq enzyme (Invitrogen), and 0.2 μmol/L forward and reverse primers in a 20-μL reaction volume. The PCR primer sequences and PCR conditions used for the c-Met mutational analyses (semaphorin domain, juxtamembrane domain, and tyrosine kinase domain) are available upon request. The resulting PCR products were purified using a Qiaquick PCR purification kit (Qiagen). Sequencing fragments were detected using ABI Prism DNA Analyzer 3730XL (Applied Biosystems, Foster City, CA), and chemistry used was big dye version 3. The nucleotide position numbering was relative to the first base of the translational initiation codon according to full-length human c-Met cDNA (14, 19).

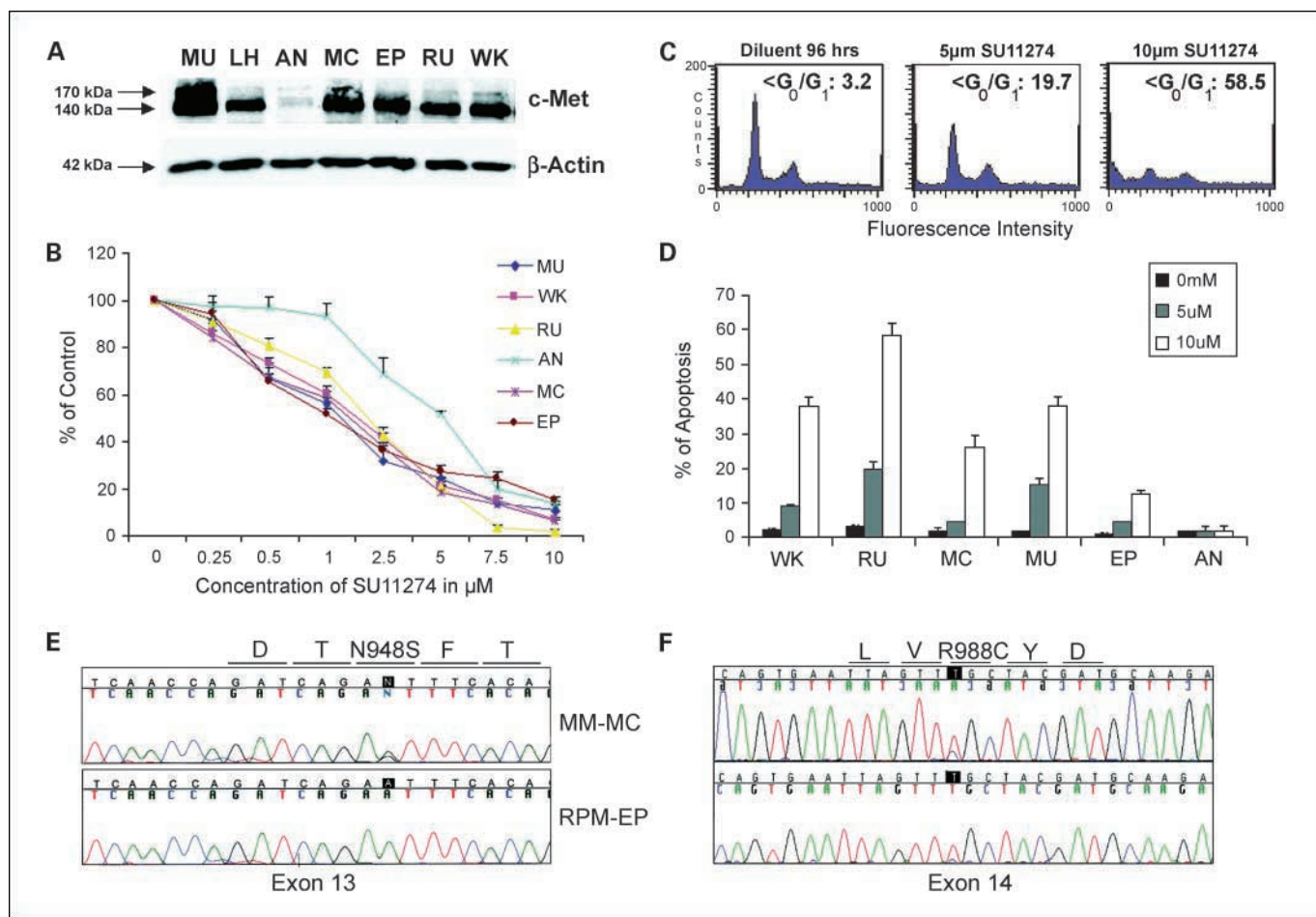
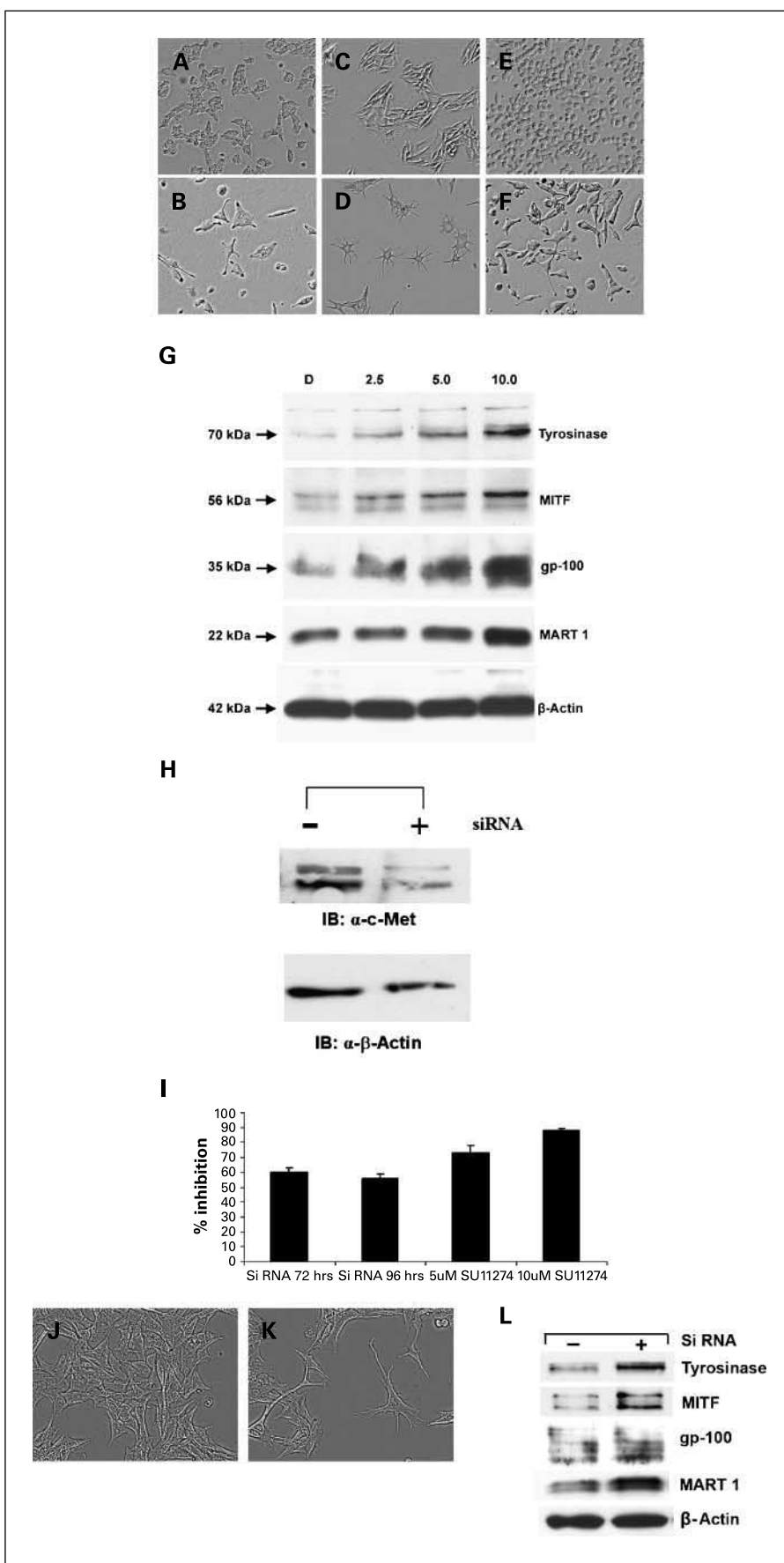


Fig. 1. A, c-Met is expressed in six out of the seven melanoma cell lines as seen by immunoblotting. Cells were harvested, and Western blotting was done using c-Met polyclonal antibody (C-12). The 170-kDa protein band represents the precursor form of the glycosylated c-Met, and the 140-kDa band is the biologically active transmembrane β subunit of c-Met. β-Actin was used as an internal loading control. B, inhibition of c-Met with the specific small-molecule inhibitor SU11274 inhibits growth of melanoma cells. Cells from six melanoma cell lines were plated in Petri dishes and allowed to adhere overnight followed by treatment with the indicated concentrations of SU11274. Cells were counted in triplicate after 96 h. C, SU11274 induces apoptosis in melanoma cells. MU melanoma cells were treated with diluent, 5 or 10 μmol/L SU11274, both diluted in media containing 10% fetal bovine serum. Cells were collected after 96 h and stained with propidium iodide and analyzed by FACS. About 19% to 58% of the cells were apoptotic after treatment with 5 or 10 μmol/L SU11274 as indicated by the sub-G₀-G₁ population of the cells. D, Melanoma cells were cultured and treated as described above. Apoptotic cells were determined by FACS scan, and it was found that SU11274 induced apoptosis in all cell lines except MM-AN cells. E, new juxtamembrane missense heterozygous mutation of c-Met identified in melanoma cell line MM-MC. Point mutation 2843 A > G resulted in a missense substitution from asparagine to serine at codon 948 (N948S). F, new juxtamembrane missense mutation R988C identified in melanoma tumor tissue. Point mutation 2962 C > T resulted in missense substitution from arginine to cysteine.

Fig. 2. *A* and *B*, MM-MC melanoma cells without and with SU11274 showing a change of cell morphology to that resembling normal human melanocytes. Cells were treated for 72 h with 5 $\mu\text{mol/L}$ SU11274, after which cells were photographed. *C-F*, MU and MM-RU treated without and with SU11274. Similar differentiation changes were also seen in these cell lines. *G*, c-Met inhibitor induced markers of melanocyte differentiation. Cells were treated with different concentrations of SU11274, and after 12 h, cells were harvested, and immunoblotting was done. SU11274 induced up-regulation of tyrosinase, MITF, gp-100, and Melan-A/MART-1. *H*, the immunoblot shows the inhibition of c-Met expression by siRNA gene silencing targeting c-Met mRNA. MU melanoma cells were transfected with c-Met specific siRNA duplexes or mock as described in Materials and Methods for 72 h and analyzed using an immunoblot. Down-regulation of c-Met protein expression by c-Met siRNA can be clearly seen. *I*, a comparison between the inhibition of c-Met siRNA and that of SU11274 on the growth of MU melanoma cells. MU melanoma cells were plated and allowed to attach overnight. They were then transfected with c-Met siRNA or mock, and the number of cells were counted on a Coulter counter at 72 and 96 h. Cells were also treated with 5 and 10 $\mu\text{mol/L}$ SU11274, and cells were counted with a Coulter counter after 96 h. As can be seen, SU11274 inhibited the growth of MU melanoma cells more effectively at 5 and 10 $\mu\text{mol/L}$, and there was 72% and 88% inhibition of growth in comparison to 56%, which was seen with c-Met siRNA. *J*, MU melanoma cells untreated and (*K*) treated cells with c-Met siRNA for 72 h and photographed. As can be seen, transfection with c-Met siRNA induces a melanocyte like phenotype, and melanoma cells seem differentiated. *L*, MU melanoma cells were plated and allowed to attach overnight. They were then transfected with c-Met siRNA or mock. After 12 h, cells were harvested, and immunoblotting was done. Similar to SU11274, c-Met siRNA induced up-regulation of tyrosinase, MITF, gp-100, and Melan-A/MART-1.



Downloaded from <http://aacrjournals.org/clinccancerres/article-pdf/13/7/2249/1974495/2249.pdf> by guest on 08 October 2024

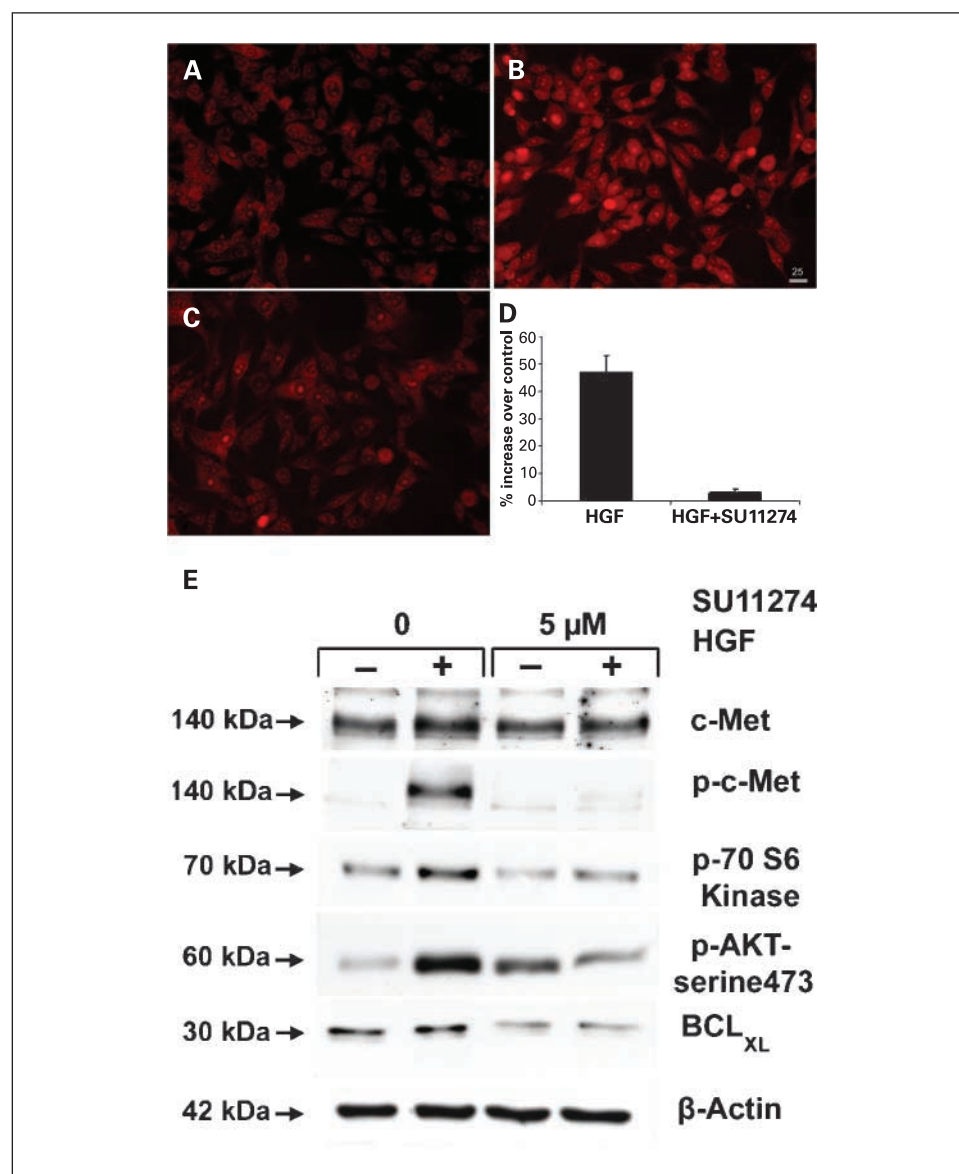


Fig. 3. SU11274 decreases ROS. *A*, photograph of untreated MU melanoma cells treated with dihydroethidine and visualized by fluorescence microscopy. *B*, MU melanoma cells treated with 40 ng/mL of HGF for 7.5 min and then visualized by fluorescence microscopy showed a significant increase in ROS and fluorescence (47%). *C*, MU melanoma cells treated with SU11274 5 μ mol/L for 12 h followed with HGF as described above exhibited a decrease in the ROS response to HGF (3%). These results are depicted graphically (*D*). *E*, proteins modulated in MU melanoma after treatment with SU11274. MU melanoma cells were prestarved and stimulated with 40 ng/mL of HGF for 7.5 min. Cells were harvested and immunoblotted using a phosphospecific antibody against p-Met at the phospho-epitopes (pY1230/1234/1235). Immunoblots were also done using antibodies against p-S6K (T421/S424), p-AKT (S473), and BCL_{xL}. β -Actin served as a loading control.

Results

Expression of *c-Met* in melanoma cell lines. The expression of *c-Met* RTK protein was studied in seven melanoma cell lines (Fig. 1A). It was found that the 140-kDa β subunit of *c-Met* was expressed in all seven melanoma cell lines, and MM-AN melanoma cells expressed minimal levels of *c-Met* in comparison to other melanoma cell lines.

Effect of SU11274 on the proliferation and apoptosis of melanoma cell lines. SU11274, which is a highly specific inhibitor of *c-Met*, was tested on the six melanoma cell lines. In all melanoma cell lines that expressed *c-Met*, the IC₅₀ of SU11274 was between 1 and 2.5 μ mol/L (Fig. 1B). The IC₅₀ of MM-AN, which expresses minimal levels of *c-Met*, was found to be 5 μ mol/L, 2-fold higher than that of cells lines that expressed abundant levels of *c-Met* (Fig. 1B). Apoptosis was seen in all five melanoma cell lines that expressed *c-Met*. By 96 h, between 12% and 58% of cells in different melanoma cell lines appeared

in the sub-G₀-G₁ region of the fluorescence-activated cell sorting (FACS) profile indicating apoptosis (Fig. 1C and D). No apoptosis could be detected in MM-AN cells (Fig. 1D).

Novel *c-Met* mutations in melanoma. We have recently described *c-Met* mutations in lung cancer (14), and in this study, we evaluated whether similar mutations could also be found in melanoma. We analyzed melanoma cell lines MM-AN, PM-WK, MM-RU, MM-MC, and RPM-EP for mutations in the "hotspots" of *c-Met*. A new missense heterozygous mutation N948S was found in exon 13 in the juxtamembrane domain in PM-WK, MM-RU, and MM-MC (Fig. 1E).

We analyzed 14 melanoma tumors and discovered a novel mutation R988C in exon 14 in the juxtamembrane domain (Fig. 1F). This mutation in the juxtamembrane domain has also been seen in non-small cell and small cell lung cancer tumors (14, 19); however, this is the first report of a mutation in *c-Met* in human melanoma tumors. Clustering of mutations in the juxtamembrane domain of *c-Met* has been linked to induced

cytoskeletal changes and increase in tumorigenicity (14). There were no mutations found in the semaphorin or catalytic tyrosine kinase domain.

Effect of SU11274 on differentiation of melanoma cell lines. After treatment of MM-RU, MU, and MM-MC melanoma cell lines for 72 h with 5 $\mu\text{mol/L}$ SU11274, it was evident that all three cell lines exhibited a marked change in morphology, with a dendritic and differentiated appearance (Fig. 2A-F) similar to normal human melanocytes (20). MU melanoma cells, which are melanized, spindle-shaped, or tripolar cells, became very dendritic and differentiated on exposure to SU11274 (Fig. 2C and D). MM-RU and MM-MC, which are less differentiated, amelanotic, round, or polygonal cells, became bipolar, tripolar, or multipolar and phenotypically resembled the normal human melanocyte (Fig. 2A, B, E, and F). Thus, SU11274 could induce a differentiated phenotype in a wide variety of melanoma cells melanized, amelanotic, less and more differentiated melanoma cells.

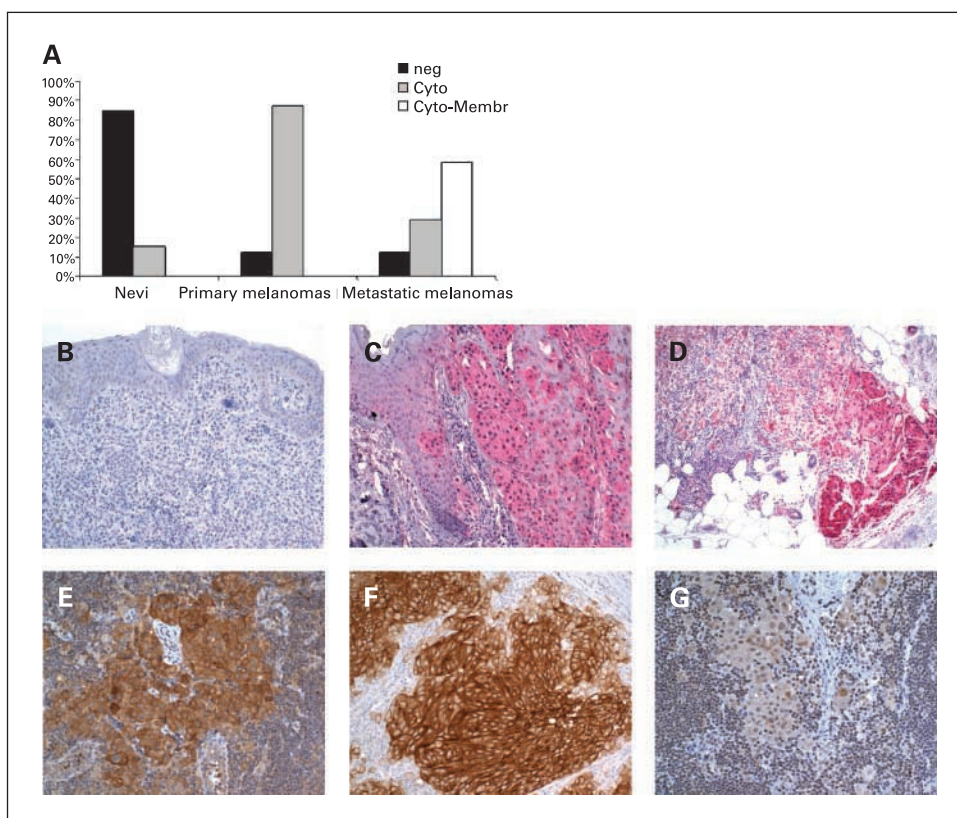
It was hypothesized that SU11274 could not only induce a differentiated phenotype, but also the microphthalmia-associated transcription factor (MITF), tyrosinase, and other melanocyte-associated differentiation markers. By immunoblotting 12 h after treatment with SU11274, an increase in levels of MITF (3-fold) was seen with a concomitant increase in the levels of melanocyte-associated antigens tyrosinase (12-fold), gp-100 (6-fold), and MART-1 (2-fold; Fig. 2G). The increase in the level of various differentiation proteins was determined by densitometric analysis after treatment with 10 $\mu\text{mol/L}$ SU11274.

siRNA against c-Met inhibits growth and induces differentiation of melanoma cells. The effect of siRNA against c-Met was

also studied on the proliferation and differentiation of MU melanoma cells. It was found that c-Met protein expression was down-regulated (47%) 72 h after treatment with siRNA (Fig. 2H). Furthermore, it was found that 72 and 96 h after transfection with Oligofectamine, the proliferation of MU melanoma cells was reduced by 60% and 56%, respectively (Fig. 2I). SU11274 at 5 and 10 $\mu\text{mol/L}$ inhibited the growth of melanoma cells by 72% and 88%, respectively (Fig. 2I). The inhibitory effect of SU11274 was more pronounced than siRNA because SU11274 is a potent and selective inhibitor of c-Met activity and function (21, 22). SU11274 also inhibits c-Met signal transduction, particularly AKT, which is necessary for cell survival and growth (21, 22). It was found that siRNA could mimic the effect of SU11274 on differentiation and also induce a differentiated and dendritic phenotype similar to normal human melanocytes in MU melanoma cells (Fig. 2J and K). Similar to SU11274, c-Met siRNA 12 h after transfection induced an increase in MITF (4-fold) and several of the melanocyte differentiation antigens, tyrosinase (4-fold), gp-100 (2-fold), and MART-1 (2-fold), which have been targets for immunotherapy (Fig. 2L). The increase in the level of various differentiation proteins was determined by densitometric analysis after treatment with c-Met siRNA.

Stimulation by HGF leads to an increase in ROS; however, treatment with SU11274 decreases ROS in MU melanoma cells. We analyzed the endogenous levels of ROS in MU melanoma cells with and without HGF treatment using dihydroethidine by fluorescence microscopy. Dihydroethidine is a redox-sensitive probe that has widely been used to detect intracellular superoxide anion (23). The relative levels of ROS

Fig. 4. A, analysis of the frequency and localization of c-Met expression by immunohistochemical staining in nevi and melanomas. c-Met was negative in 85% of nevi, focally positive in three compound nevi (15% of cases). In primary melanomas, the vast majority of cases (87.5%) showed cytoplasmic staining. In metastatic melanomas, 29% cases exhibited cytoplasmic positivity, and 58.5% exhibited both cytoplasmic and membranous staining. B, negative c-Met staining of compound nevus, expression of c-Met (C), and overexpression of c-Met (D) in the invasive front of primary human melanoma. E, metastatic melanoma showing cytoplasmic c-Met expression. F, metastatic melanoma in lymph node showing cytoplasmic and membranous c-Met expression. G, metastatic melanoma showing expression of activated c-Met as seen by staining with antibody against phospho-c-Met epitope pY1003.



were increased in response to HGF by 47%, and SU11274 decreased the ROS response to HGF to 3% (Fig. 3A-D). Because a ROS response is necessary for the survival of cancer cells, these results indicate that lowering ROS responses considerably by SU11274 could be used as a potential chemotherapeutic agent.

SU11274 prevented tyrosine phosphorylation of c-Met in MU melanoma cells and blocked c-Met-dependent signaling events. The ability of SU11274 to inhibit activation of c-Met was examined by immunoblotting. Treatment with HGF increased the autophosphorylation of c-Met at the activation loop site phospho-epitope (pY1230/1234/1235). SU11274 completely abolished the phosphorylation of the above tyrosine residues at the activation site (Fig. 3E). HGF binding to c-Met activated its tyrosine kinase, AKT (S-473), and S-6 kinase (T-421/S424; Fig. 3E). At 5 $\mu\text{mol/L}$, SU11274 completely inhibited HGF-induced phosphorylation of c-Met, AKT, and S-6 kinase (Fig. 3E). Additionally, SU11274 down-regulated BCL_{XL}, an inhibitor of apoptosis (Fig. 3E), indicating that SU11274 stimulates apoptosis as was seen earlier (Fig. 1D). As seen in Supplementary Fig. S1, we found no effect of the c-Met mutation in MM-MC on the intrinsic c-Met kinase activity or downstream signaling events in comparison to MU, which does not have a c-Met mutation. In addition, SU11274 inhibited phosphorylation of c-Met and AKT in both MM-MC and MU (Fig. 3E and Supplementary Fig. S1), indicating that c-Met inhibitors could also possibly be therapeutically effective in individuals with melanoma with c-Met mutations.

Expression of c-Met and activated phospho-Met in melanoma. Finally, we analyzed paraffin-embedded, formalin-fixed tissues from 20 patients with nevi and 40 with malignant melanomas. Both total c-Met and activated phospho-Met (pY1003) were shown in paraffin-embedded, formalin-fixed melanoma tissues using immunohistochemistry techniques (Fig. 4A-G). The phosphospecific antibody against the phospho-epitope (pY1230/1234/1235) reacted very weakly in paraffin sections and, hence, was not used. c-Met was negative in 85% of nevi (Fig. 4A and B) and positive in 88% of melanomas examined (Fig. 4A and C). Primary melanomas exhibited cytoplasmic positivity (Fig. 4C and D), whereas metastatic melanomas showed both cytoplasmic and membranous pattern of c-Met staining (Fig. 4E and F). Interestingly, preferential expression of phospho-Met was located in the invasive front of the melanoma (Fig. 4D). In addition to the expression of c-Met, expression of phospho-Met (pY1003) showed that c-Met was activated at the phospho-epitope (pY1003; Fig. 4G) in 21% of human melanoma, indicating that the activation of c-Met can occur in melanoma. Expression of activated c-Met at phospho-epitopes (pY1003) was not detected in normal epidermis or nevi.

Discussion

The studies of RTKs in solid tumors have come to fruition as molecularly targeted therapy (24). We show that the HGF/c-Met pathway is functional in melanoma, and this may be a useful target for therapeutic intervention. c-Met was functionally expressed in all melanoma cell lines, and most tumor tissues had unique preferential expression at the tumor invasive front. Targeted inhibition of c-Met either via SU11274 or specific siRNA led to decreased cell growth and viability. SU11274 inhibition of c-Met abrogated tyrosine phosphoryla-

tion of cellular proteins, including c-Met itself, as well as its downstream signaling proteins. SU11274 and c-Met siRNA were also able to induce MITF, several melanoma differentiation proteins, and a phenotype similar to normal human melanocytes. Stimulation of ROS by HGF led to increased ROS formation, which was completely inhibited by SU11274. Lastly, unique c-Met alterations in the juxtamembrane domain were identified in melanoma cell lines and tumor tissue. It has been shown that the juxtamembrane domain has a novel role in c-Met signaling, motility, tumorigenicity, and migration (14). The novel c-Met mutations reported in this study are the first to be reported in melanoma.

This is the first report of the effect of a small-molecule inhibitor SU11274 on human melanoma. Small-molecule inhibitors specifically targeting c-Met represent an attractive novel targeted therapeutic approach. Met kinase autophosphorylation was reduced on sites that have been shown to be important for the activation of pathways involved in cell growth and survival, especially the phosphoinositide-3-kinase pathway, which is responsible for events such as proliferation, reduced apoptosis, and anchorage independence (25, 26). SU11274 decreases the phosphorylation of AKT, which is downstream of phosphoinositide-3-kinase, and inhibits an antiapoptotic protein BCL_{XL}, which is downstream of AKT.

Other strategies to inhibit c-Met reported are NK4 HGF (truncated form of HGF), peptide inhibition, antibody inhibition, siRNA, and ribozymes (27–31). Inhibition of c-Met by siRNA represents a novel and powerful strategy. Here, we found that treatment with siRNA lowered c-Met protein expression and inhibited the viability of melanoma cells considerably. Both c-Met siRNA and SU11274 caused differentiation of melanoma cells and induced the transcription factor MITF, which regulates the expression of major melanoma-associated differentiation proteins, such as tyrosinase, TRP-1, and TRP-2 (32, 33). Melanoma differentiation is also associated with slower cell proliferation (34). Induced differentiation might be especially helpful in the immunotherapy of melanoma, when SU11274- or siRNA-induced increase in the expression of several differentiation antigens, such as tyrosinase, gp-100, and MART-1, may potentially permit targeting of melanoma by sensitized T lymphocytes (35–37).

In this study we also found that c-Met is expressed in cell lines and tumor tissues. This is the first study where activated c-Met has been detected in tumor tissue in melanoma. c-Met activation could happen as a result of overexpression, activating mutations, or gene amplification (10). Interestingly, a 2-fold increase in the expression of phosphorylated c-Met was seen in melanoma in comparison to nevi, indicating that perhaps c-Met has an important potential role in the development of melanoma. A new missense N948S mutation was identified in melanoma cell lines, and a novel mutation R988C was detected in melanoma tumor tissues in the juxtamembrane domain. This is the first report of a mutation in c-Met in melanoma. Mutations in the juxtamembrane domain in small cell lung cancer are activating, influencing cell transformation, anchorage-dependent proliferation, cytoskeletal functions, and cell motility and migration (14). More tumor specimens need to be analyzed to detect further mutations in c-Met and their role in melanoma tumorigenesis.

In summary, this study illustrates the potential of targeting the c-Met receptor in melanoma. SU11274 and

siRNA against c-Met are promising molecules toward this end. Additionally, both siRNA and SU11274 can give us important insights into the biological roles of c-Met and HGF.

Acknowledgments

We cordially thank Can Gong for her excellent technical assistance with immunostaining.

References

- Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2006. *CA Cancer J Clin* 2006;56:106–30.
- Li G, Schaidler H, Satyamoorthy K, Hanakawa Y, Hashimoto K, Herlyn M. Downregulation of E-cadherin and Desmoglein 1 by autocrine hepatocyte growth factor during melanoma development. *Oncogene* 2001;20:8125–35.
- Sun W, Schuchter LM. Metastatic melanoma. *Curr Treat Options Oncol* 2001;2:193–202.
- Natali PG, Nicotra MR, Di Renzo MF, et al. Expression of the c-Met/HGF receptor in human melanocytic neoplasms: demonstration of the relationship to malignant melanoma tumour progression. *Br J Cancer* 1993;68:746–50.
- Slominski A, Wortsman J, Carlson AJ, Matsuoka LY, Balch CM, Mihm MC. Malignant melanoma. *Arch Pathol Lab Med* 2001;125:1295–306.
- Barnhill RL, Mihm MC, Jr. The histopathology of cutaneous malignant melanoma. *Semin Diagn Pathol* 1993;10:47–75.
- Cruz J, Reis-Filho JS, Silva P, Lopes JM. Expression of c-met tyrosine kinase receptor is biologically and prognostically relevant for primary cutaneous malignant melanomas. *Oncology* 2003;65:72–82.
- To CT, Tsao MS. The roles of hepatocyte growth factor/scatter factor and met receptor in human cancers [review]. *Oncol Rep* 1998;5:1013–24.
- Faletto DL, Tsarfaty I, Kmiecik TE, Gonzatti M, Suzuki T, Vande Woude GF. Evidence for non-covalent clusters of the c-met proto-oncogene product. *Oncogene* 1992;7:1149–57.
- Ma PC, Maulik G, Christensen J, Salgia R. c-Met: structure, functions and potential for therapeutic inhibition. *Cancer Metastasis Rev* 2003;22:309–25.
- Schmidt L, Duh FM, Chen F, 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.
- Lee JH, Han SU, Cho H, et al. A novel germ line juxtamembrane Met mutation in human gastric cancer. *Oncogene* 2000;19:4947–53.
- Park WS, Dong SM, Kim SY, et al. Somatic mutations in the kinase domain of the Met/hepatocyte growth factor receptor gene in childhood hepatocellular carcinomas. *Cancer Res* 1999;59:307–10.
- Ma PC, Kijima T, Maulik G, et al. c-MET mutational analysis in small cell lung cancer: novel juxtamembrane domain mutations regulating cytoskeletal functions. *Cancer Res* 2003;63:6272–81.
- Hsu MY, Meier F, Herlyn M. Melanoma development and progression: a conspiracy between tumor and host. *Differentiation* 2002;70:522–36.
- Noonan FP, Otsuka T, Bang S, Anver MR, Merlino G. Accelerated ultraviolet radiation-induced carcinogenesis in hepatocyte growth factor/scatter factor transgenic mice. *Cancer Res* 2000;60:3738–43.
- Otsuka T, Takayama H, Sharp R, et al. c-Met autocrine activation induces development of malignant melanoma and acquisition of the metastatic phenotype. *Cancer Res* 1998;58:5157–67.
- Maulik G, Kijima T, Ma PC, et al. Modulation of the c-Met/hepatocyte growth factor pathway in small cell lung cancer. *Clin Cancer Res* 2002;8:620–7.
- Ma PC, Jagadeeswaran R, Jagadeesh S, et al. Functional expression and mutations of c-Met and its therapeutic inhibition with SU11274 and small interfering RNA in non-small cell lung cancer. *Cancer Res* 2005;65:1479–88.
- Puri N, Mojamdar M, Ramaiah A. *In vitro* growth characteristics of melanocytes obtained from adult normal and vitiligo subjects. *J Invest Dermatol* 1987;88:434–8.
- Sattler M, Pride YB, Ma P, et al. A novel small molecule met inhibitor induces apoptosis in cells transformed by the oncogenic TPR-MET tyrosine kinase. *Cancer Res* 2003;63:5462–9.
- Berthou S, Aebersold DM, Schmidt LS, et al. The Met kinase inhibitor SU11274 exhibits a selective inhibition pattern toward different receptor mutated variants. *Oncogene* 2004;23:5387–93.
- Zhao H, Kalivendi S, Zhang H, et al. Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. *Free Radic Biol Med* 2003;34:1359–68.
- Lydon NB, Druker BJ. Lessons learned from the development of imatinib. *Leuk Res* 2004;28 Suppl 1: S29–38.
- Rameh LE, Cantley LC. The role of phosphoinositide 3-kinase lipid products in cell function. *J Biol Chem* 1999;274:8347–50.
- Toker A. Protein kinases as mediators of phosphoinositide 3-kinase signaling. *Mol Pharmacol* 2000;57:652–8.
- Abounader R, Ranganathan S, Lal B, 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;9:1548–56.
- Michieli P, Basilico C, Pennacchietti S, et al. Mutant Met-mediated transformation is ligand-dependent and can be inhibited by HGF antagonists. *Oncogene* 1999;18:5221–31.
- Michieli P, Mazzone M, Basilico C, et al. Targeting the tumor and its microenvironment by a dual-function decoy Met receptor. *Cancer Cell* 2004;6:61–73.
- Shinomiya N, Gao CF, Xie Q, et al. RNA interference reveals that ligand-independent met activity is required for tumor cell signaling and survival. *Cancer Res* 2004;64:7962–70.
- Wickramasinghe D, Kong-Beltran M. Met activation and receptor dimerization in cancer: a role for the Sema domain. *Cell Cycle* 2005;4:683–5.
- Goding CR. Mitf from neural crest to melanoma: signal transduction and transcription in the melanocyte lineage. *Genes Dev* 2000;14:1712–28.
- Park HY, Gilchrist BA. More on MITF. *J Invest Dermatol* 2002;119:1218–9.
- Puri N, Eller MS, Byers HR, Dykstra S, Kubera J, Gilchrist BA. Telomere-based DNA damage responses: a new approach to melanoma. *FASEB J* 2004;18:1373–81.
- Bakker AB, Schreurs MW, de Boer AJ, et al. Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes. *J Exp Med* 1994;179:1005–9.
- Dudley ME, Wunderlich JR, Robbins PF, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 2002;298:850–4.
- Rivoltini L, Kawakami Y, Sakaguchi K, et al. Induction of tumor-reactive CTL from peripheral blood and tumor-infiltrating lymphocytes of melanoma patients by *in vitro* stimulation with an immunodominant peptide of the human melanoma antigen MART-1. *J Immunol* 1995;154:2257–65.