Down-Regulation of c-Met Inhibits Growth in the Liver of Human Colorectal Carcinoma Cells

Matthew H. Herynk, Oliver Stoeltzing, Niels Reinmuth, Nila U. Parikh, Roger Abounader, John Laterra, Robert Radinsky, Lee M. Ellis, and Gary E. Gallick

Department of Cancer Biology, University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 [M. H. H., O. S., N. R., N. U. P., R. R., L. M. E., G. E. G.]; The Program in Cancer Biology, University of Texas Graduate School of Biomedical Sciences at Houston, Houston, Texas 77030 [M. H. H., R. R., L. M. E., G. E. G.]; and The Johns Hopkins University School of Medicine, Kennedy Krieger Research Institute, Baltimore, Maryland 21205 [R. A., J. L.]

ABSTRACT

Overexpression of c-Met, the protein tyrosine kinase receptor for the hepatocyte growth factor/scatter factor, has been implicated in the progression and metastasis of human colorectal carcinoma. To examine the role of c-Met on in vitro and in vivo growth of human colon tumor cell lines, stable subclones of the high metastatic human colorectal carcinoma cell line, KM20, isolated from a Dukes’ D patient, with reduced c-Met expression were obtained after transfection with a c-Met-specific targeting ribozyme. The subclones were only modestly reduced in c-Met expression because of c-Met playing an important role in cellular survival. However, a 60–90% reduction in basal c-Met autophosphorylation and kinase activity were observed. Correlating with the reduction in c-Met kinase activity, subclones with reduced c-Met expression had significantly reduced in vitro growth rates and soft-agar colony-forming abilities. The in vivo growth of these cells was examined at both the ectopic SQ site and the orthotopic site of metastatic growth, the liver. SQ growth was delayed significantly in the c-Met down-regulated clones compared with controls, with tumors growing on loss of the ribozyme construct. In contrast, tumor incidence was significantly reduced when the c-Met down-regulated cells were grown in the orthotopic liver site. Thus, c-Met activation may be important in metastatic growth of colon tumor cells in the liver. Collectively these data demonstrate that a small reduction in c-Met protein levels leads to profound biological effects, and potential c-Met inhibitors may be of therapeutic value in treatment of colon cancer.

INTRODUCTION

Fifty-seven thousand people are expected to die of colon cancer in the United States this year alone, second only to lung cancer (1). Many patients undergoing “curative” surgery to remove all of the visible tumor will succumb to metastatic disease that was not detectable at the time of surgery. Because strategies for treating metastatic lesions are largely ineffective, understanding the molecular mechanisms involved in CRC progression and metastasis is important for the development of novel and potentially effective therapies for the treatment of advanced-stage CRC. Signal transduction inhibitors targeting RPTKs are showing clinical promise (reviewed in Ref. 2). Because overexpression of several RPTKs is common in many malignancies including colon cancer, an understanding of RPTK contributions to tumor growth and progression is important in identifying potential therapeutic targets. Toward that end, we have been investigating the role of c-Met overexpression in the biology of colon tumor progression.

c-Met is a transmembrane RPTK that is primarily expressed in epithelial tissues (3). HGF/SF is the primary ligand for the c-Met proto-oncogene (3–5). The binding of HGF to c-Met activates multiple signaling pathways resulting in mitogenesis, motility, morphogenesis, survival, or a combination of these events depending on the cell type stimulated.

Numerous studies have implicated aberrant c-Met function in the progression and metastasis of human tumors including CRC (6), pancreatic carcinoma (7), melanoma (8), and osteosarcoma (9). Analysis of c-Met expression during CRC progression showed that 50% of the carcinoma specimens analyzed expressed 5–50-fold higher levels of c-Met mRNA transcripts and protein versus the adjacent normal colonic mucosa (6). The same study demonstrated c-Met overexpression in 70% of CRC liver metastases relative to the primary tumor. Additionally, comparison of the steady-state mRNA levels of CRC grown in the spleen and livers of nude mice revealed a 3-fold increase in c-Met mRNA expression in the liver metastases versus residual spleen tumors (10). Collectively, these data provide correlative evidence for a role of c-Met in CRC progression.

To examine the role of c-Met in other tumor types, the strategy of ribozyme-mediated c-Met down-regulation has been used (11, 12). In the glioblastoma cell lines U-373 (expressing c-Met only) and U-87 (expressing c-Met and HGF), Abounader et al. (11) demonstrated that stable ribozyme-expressing clones were reduced in c-Met expression by 95%, c-Met down-regulation in U-373 did not affect tumor growth or incidence. Reduction in tumor incidence and growth in the brain resulted only when an autocrine HGF/c-Met loop was present (U-87; Ref. 11). Therapeutic treatment of orthotopically implanted U-87 tumors with adenovirus and/or liposomes containing the c-Met or HGF-specific ribozyme increased the median survival of treated animals compared with untreated animals (13). These data suggest that in glioblastoma cells, c-Met down-regulation will reduce tumorigenicity only when c-Met is activated by autocrine production of HGF. In two human breast cancer cell lines, MDA-MB 231 and MCF-7, ribozyme-mediated c-Met down-regulation significantly reduced HGF-induced migration and invasion (12), relative to a control (PU1) construct.

Thus, whereas previous studies have demonstrated the efficacy of c-Met down-regulation in inhibiting tumor incidence and growth, none have examined the effects of growth at the metastatic site. We report that in colon tumor cells with intrinsically high expression of activated c-Met, down-regulation of the receptor is sufficient to reduce both incidence and growth of CRC cells in the liver, the primary site of metastases. Furthermore, we demonstrate that only modest down-regulation of c-Met is sufficient to have profound biological effects in colon tumor cells.

MATERIALS AND METHODS

Cell Lines and Cell Culture Conditions. The KM20 cell line was derived from a resected surgical specimen of a Dukes’ D primary colon cancer and produces liver metastases when injected into the cecum of nude mice (14, 15). Cells were maintained in MEM with 10% FBS, 1 mM sodium pyruvate, 0.1 mM

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2 To whom requests for reprints should be addressed, at Department of Cancer Biology, M. D. Anderson Cancer Center, 1515 Holcombe, Houston, TX 77401. Phone: (713) 792-3657; Fax: (713) 745-1927; E-mail: ggallick@mdanderson.org.

3 The abbreviations used are: CRC, colorectal carcinoma; HGF, hepatocyte growth factor; SF, scatter factor; RPTK, receptor protein tyrosine kinase; IP, immunoprecipitation; MOI, multiplicity of infection; ECA, immune complex kinase assay; MT3, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SQ, subcutaneous; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyltransferase-mediated nick end labeling; CMEM, complete minimum essential medium.
nonsenseal amino acids, 2 mM l-glutamine, and 2X-vitamin solution (Life Technologies, Inc., Grand Island, NY), at 37°C with 5% CO₂.

Ribozyme Plasmid Constructs. The construct (designated ribozyme) was designed to inhibit c-Met expression and cloned into a modified pZeo plasmid (developed by Dr. Hal Dietz, Howard Hughes Medical Institute, Baltimore, MD) and contains a Zeocin selection marker (16). Constructs contain a U1snRNA, c-Met antisense, and a hammerhead ribozyme that targets c-Met mRNA at residue 560 (designated 560). The ribozyme-containing and control plasmids (lacking the ribozyme and the c-Met targeting sequence but containing the U1snRNA and other plasmid sequences) have been described previously (11).

Calcium Phosphate Transfection and Zeocin Selection. One × 10⁶ subconfluently growing cells were transfected with a plasmid containing a c-Met-specific ribozyme or a control plasmid. Ten μg of DNA were precipitated with 0.25 mM CaCl₂ in 2X HEPES-buffered saline. The cells were overlayed with the precipitated DNA followed by glycerol (10% v/v FBS) shock. Cells were rinsed and subjected to medium containing 1000 μg/ml of Zeocin (Invitrogen, Bedford, MA) for selection of stable transfectants. Single colonies were isolated and expanded for further analyses.

IP and Western Blot Analysis. Cells were plated with MEM supplemented with 10% FBS, and 24 h later cells were serum-starved for 18 h followed by treatment with or without HGF (40 ng/ml) for 10 min before lysis [20 mM Tris-Cl (pH 8.0), 137 mM NaCl, 1% Triton X-100, 1 mM Na₃VO₄, 2 mM EDTA, and 1 complete Mini Protease Inhibitor Cocktail Tablet (Roche Diagnostics, Basel, Switzerland)]. c-Met was IPed from 200 μg of cell lysate with 1 μg of anti-c-Met antisera (Santa Cruz Biotechnology, Santa Cruz, CA) and protein A/G. IPed proteins or whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane (Amersham Corp., Arlington Heights, IL), and probed with anti-c-Met (Santa Cruz Biotechnology) or anti-phosphotyrosine (Upstate Biotechnology Inc., Lake Placid, NY). Antibodies were diluted in Tris-buffered saline-0.1% Tween 20 (v/v) with 5% dried milk and antiphosphotyrosine was diluted in Tris-buffered saline-0.1% Tween-20 with 3% BSA. Peroxidase-conjugated secondary antibodies, goat antimouse (Bio-Rad, Hercules, CA), or goat antirabbit (Bio-Rad) were used to detect the respective primary antibodies. Immuno-reactive proteins were visualized with Chemiluminescence Reagent Plus detection system (NEN, Boston, MA).

Ribozyme-expressing Adenovirus Constructs. The adenovirus expressing the 560 ribozyme (ad-c-Met) and control adenovirus (ad-PU1) were constructed, and viruses were harvested and purified as described previously (13). To determine c-Met expression, cells at 80% confluence were infected with adenoviruses at the indicated MOIs. After 48 h, cells were lysed and subjected to Western blotting as described above. For survival assays, cells were infected with ad-c-Met or ad-PU1 at increasing MOIs (as indicated) for 8 h. The cells were trypsinized, and 100 cells were plated in a 10-cm dish. Nine days later colonies were fixed and stained with crystal violet. For survival assays, cells were infected with ad-c-Met or ad-PU1 at increasing MOIs (as indicated) for 8 h. The cells were trypsinized, and 100 cells were plated in a 10-cm dish. Nine days later colonies were fixed and stained with crystal violet.

c-Met ICKAs. ICKA assay was performed as described previously (17). Briefly, c-Met was IPed from 300 μg of total cellular protein with 1 μg of anti-c-Met antisera (Santa Cruz Biotechnology) and 50 μl of 10% (w/v) Pansorbin cells (Calbiochem, San Diego, CA) with rotation at 4°C for 12 h. Acid-denatured rabbit muscle enolase (Sigma) was added as an exogenous phosphorylation substrate. Samples were subjected to 8% SDS-PAGE. The gel was stained with Coomassie blue and destained in 20% ethanol.

Cell Proliferation as Measured by MTT Analyses. MTT analysis was performed as described previously (18). Briefly, 3000 cells were plated into each well of a 96-well microtiter plate in CMEM containing 10% FBS. Twenty-four h later, medium was replaced with CMEM with or without HGF (20 ng/ml) and 50 μl of MTT was added to each well and allowed to incubate at 37°C for 4 h. The size and number of colonies were quantitated.

Boyden Chamber Migration Assay. Fifty-thousand cells were placed in the top of a control Matrigel migration chamber, with 8-μm pores (Fisher Scientific, Houston, TX). To the bottom chamber was added 0.5 ml of 5% FBS/CMEM with or without HGF (20 ng/ml). After 48 h cells were fixed and stained using the HEMA 3 stain set (Curtis Matheson Scientific, Houston, TX). To quantify migrating cells, 10 random fields were counted at ×200 magnification.

Animals and Cell Preparation for in Vivo Implantation. Male athymic nude mice purchased from the animal production area of the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD) were maintained under specific pathogen-free conditions and used for experiments at 6–8 weeks of age. All of the animal experiments were approved by The University of Texas-M. D. Anderson Cancer Center Animal Care and Use Committee pursuant to NIH guidelines.

Cells at 80% confluence were rinsed in Ca²⁺⁻ and Mg²⁺⁻-free HBSS and overlaid for 1 min with 0.05% trypsin and 0.02% EDTA solution. Cells were removed with a rubber cell scraper and pipetted in CMEM with 10% FBS to produce a single cell suspension. Cells were washed with HBSS, centrifuged, and resuspended in HBSS at a concentration of 20 × 10⁶ viable cells per ml (as determined by trypan blue exclusion).

SQ and Intrahepatic Tumor Cell Implantation in Nude Mice. Tumor cells (5 × 10⁵) were injected SQ into the right flank of nude mice in 0.05 ml of HBSS. Ten days after injection and every third day thereafter, length and width of tumors were measured. Volume was calculated as length × (width)². For intrahepatic growth of tumors, mice were anesthetized with nembutal. An abdominal midline incision was made and the liver was exteriorized, and 1 × 10⁵ cells were injected into the right liver lobe under the capsule in a volume of 0.05 ml.

PCNA. Thin sections (6–8 μm) of formalin-fixed, paraffin-embedded tissues were incubated with anti-PCNA antiserum (PC10; Carpinetia, CA) followed by secondary antimouse IgG2a (SeroClone/Harlan Bioproducts, Raleigh, NC) and incubated with Diaminobenzidine tetrahydrochloride (Invitrogen). PCNA-positive and total cells were counted in four high-power fields per tumor to give a percentage of proliferating cells.

Analysis of Apoptosis in Tissue Sections. To analyze the percentage of apoptosis in tumors, thin sections were analyzed using the Fluorescein Apoptosis Detection System (Promega Corp., Madison, WI) according to the manufacturer’s protocol. Briefly, fragmented DNA was fluorescein labeled using terminal transferase enzyme and visualized under fluorescent microscopy. Total nuclei were counterstained with Hoechst staining solution. Apoptotic and total number of cells were counted in four high-power fields from each tumor to give a percentage of apoptotic cells.

Statistical Analyses. Statistical analyses was performed using InStat 2.01 statistical software (GraphPad Software, San Diego, CA) using the Mann-Whitney U test, Student’s t test, or Fisher’s Exact test, where appropriate. Significance was determined with 95% confidence.

RESULTS

c-Met Down-Regulation in High Metastatic KM20 CRC Cells. To examine the effects of c-Met down-regulation, the 560 ribozyme-containing plasmid and control PU1 plasmid were transfected into KM20 cells. KM20 cells were chosen because of their high level of c-Met expression, a wild-type c-Met as determined by direct sequence analysis, and lack of detectable HGF expression as determined by reverse transcription-PCR analysis (data not shown). Thus, these cells are typical of many late-stage colon tumors wherein high expression of the c-Met receptor leads to constitutive activation. After selection in Zeocin containing medium, clones with reduced c-Met expression were identified by Western blotting analysis. Fig. 1A shows c-Met protein levels in KM20 parental, three c-Met down-regulated clones (360 clones), and two clones transfected with the control plasmid (PU1 clones). As compared with parental cells, clone 360–26 demonstrated the greatest reduction (32%) in c-Met protein. Clones 360–90 and 360–108 were reduced in c-Met expression 28% and 26%, respectively. In contrast, no c-Met reduction was observed in the clonal PU1 transfectants.

Adenoviral-mediated c-Met Down-Regulation. Because of the modest reduction in c-Met expression from stable transfectants, KM20 cells were infected with a ribozyme containing adenovirus
(ad-c-Met; Ref. 13). Fig. 1B shows the c-Met protein levels at the indicated MOI. A maximum of 45% reduction in c-Met expression was observed in cells infected with adenovirus containing the ribozyme when compared with control adenovirus-infected cells. Re-infection of ad-c-Met into growing cultures led to no greater inhibition of c-Met protein levels (data not shown). Thus, expression of the ribozyme from either plasmid or adenovirus reduced c-Met protein levels <2-fold.

**c-Met Tyrosine Phosphorylation.** To determine the effect of c-Met down-regulation on c-Met activation, c-Met was immunoprecipitated and Western blotted for phosphorytrosine and c-Met. KM20 parental cells had a high basal level of c-Met tyrosine phosphorylation (Fig. 2A, Lane 1). c-Met down-regulated clones demonstrated significantly reduced c-Met tyrosine phosphorylation. Tyrosine phosphorylation of clone 560–26 was reduced 80% with respect to KM20 parental cells (Fig. 2A, Lane 3). Clone 560–90 also had significantly reduced tyrosine phosphorylation to only 44% of parental cells (Fig. 2A, Lane 5). PU1 control clones demonstrated modestly increased basal levels of tyrosine phosphorylation compared with parental KM20 cells (Fig. 2A, Lane 9). All of the cells responded to exogenous HGF treatment with increased c-Met tyrosine phosphorylation (Fig. 2A, Lanes 2, 4, 6, 8, and 10). These data indicate that the high receptor density of c-Met on KM20 cells leads to partial RPTK activation and that a small reduction in c-Met protein levels results in a significant decrease in c-Met tyrosine phosphorylation.

**c-Met Kinase Activity.** To determine the intrinsic c-Met kinase activity, c-Met tyrosine phosphorylation and tyrosine phosphorylation of the exogenous substrate enolase were examined by an ICKA. KM20 parental cells and PU1 control transfectants demonstrated a high basal level of tyrosine kinase activity (Fig. 2B, Lanes 1, 9). Corresponding with reduced tyrosine phosphorylation, basal c-Met kinase activity in clones with down-regulated c-Met was reduced to 15–45% of parental cells (Fig. 2B, Lanes 3, 5, and 7). After 1 min of HGF treatment these c-Met down-regulated clones increased c-Met kinase activity between 1.3- and 2.6-fold. Clone 560–26, with the lowest c-Met levels, demonstrated the greatest response to HGF (Fig. 2B, Lanes 4, 6, and 8). In contrast, parental cells and control clones only had a modest response to HGF (Fig. 2B, Lanes 2 and 10). These data demonstrate that a modest reduction in c-Met expression levels leads to substantially reduced c-Met function.

**Reduction of c-Met Affects Cellular Survival.** The modest down-regulation of c-Met expression achieved in either stable transfectants with the ribozyme-expressing plasmid or by ad-c-Met-mediated ribozyme expression contrasted with results observed by Abou-nader et al. (11) in glioblastoma cells as well as our own results in prostate tumor cell lines (19). We observed similar results in another colon tumor cell line, KM12, where a maximum decrease in c-Met expression of 30% was observed (data not shown). Therefore, to determine whether c-Met might be important to colon tumor cell survival, KM20 cells were infected with increasing MOIs of ad-c-Met, and a colony-forming assay was performed. A dose-dependent decrease in colony number was observed in cells infected with ad-Pu1, consistent with the adenovirus inducing some toxicity (Fig. 3). However, much lower MOIs of ad-c-Met significantly reduced colony formation relative to the control virus, with few surviving colonies observed at MOIs as low as 10 (Fig. 3) suggesting that reduction of c-Met beyond a threshold level is incompatible with cell survival.

**In Vitro Growth.** Despite the role of c-Met in promoting cellular survival, the ability to decrease enzymatic activity of c-Met by only modest reduction in expression suggested that these small reductions in c-Met expression might have important biological consequences. To determine the in vitro growth rates of c-Met-down-regulated cells, MTT analysis was performed. Growth rates were analyzed in 0%, 5%, or 10% serum containing medium, and each clone was similar relative to controls for each serum concentration. Control clones behaved similarly to parental cells in all of the in vitro studies. As shown in Fig. 4A, c-Met down-regulated clones were reduced in proliferation relative to control clones (P < 0.01 versus Pu1–14 or Pu1–21 at 96 h). Clone 560–26, with the most reduced c-Met activity, demon-
C-MET DOWN-REGULATION INHIBITS TUMOR GROWTH

Fig. 3. Survival of KM20 cells after infection with ad-c-Met and ad-PU1. KM20 cells were infected with the adenoviruses at the indicated MOIs for 8 h. Cells were then trypsinized and replated at a density of 100 cells/10 cm plate in triplicate. Colonies that formed after 9 days were counted as described in “Materials and Methods.” bars, ±SD.

strated the most reduced growth rate with an in vitro doubling time of 288 h. Clones with intermediate down-regulation of c-Met were reduced in proliferation to a lesser extent with doubling times of 104 (560–90) and 80 (560–108) h. Treatment with HGF did not affect the growth rates as determined by MTT analysis. The in vitro doubling times correlate with the c-Met activity in these clones, suggesting that down-regulation of c-Met signaling reduced the in vitro growth rates significantly.

Soft Agar Colony-forming Ability. To determine the effect of c-Met down-regulation on anchorage-independent growth, soft agar colony-forming ability is shown in Fig. 4B. PU1–14 and PU1–21 clones formed high numbers of large colonies, 34 +/− 8 and 32 +/− 8 colonies, respectively (Fig. 4B). In contrast, c-Met down-regulated clones formed very few colonies, and the colonies that formed were small (inset, Fig. 4B). Clone 560–26 formed no colonies under any conditions, 560–90 and 560–108 formed 8 +/− 1 and 7 +/− 5 colonies, respectively (Fig. 4B). HGF treatment did not enhance colony formation of any clones (Fig. 4B). To determine whether reduced soft agar colony formation resulted from slower proliferation, a second set of experiments was performed in which colonies were counted after 36 days of incubation. No increase in number and size of colonies was observed at this longer incubation period (data not shown). These data demonstrate that c-Met down-regulation significantly reduced anchorage-independent growth and survival, independent of doubling time.

Analysis of Migration. Cells were seeded in the upper chamber of a standard two-chamber migration assay to analyze cell motility. Although all of the c-Met down-regulated clones had reduced migration compared with controls, basal migration rates were not statistically significant between PU1 control transfectants versus c-Met down-regulated clones as shown in Fig. 4C. However, the HGF-induced migration was significantly increased between PU1 control transfectants versus c-Met down-regulated clones (Fig. 4C; P < 0.01 for c-Met down-regulated clones versus either PU1–14 or PU1–21; Student’s t test). These data demonstrate that down-regulation of c-Met results in a reduced ability to migrate in response to HGF, correlating with c-Met activity. This reduced in vitro growth, migration, and soft agar colony-forming ability is consistent with observed reduction in c-Met kinase activity after ribozyme-mediated down-regulation.

SQ Growth of Tumors in Nude Mice. As c-Met down-regulation decreased the in vitro growth rates, in vivo growth rates were analyzed by injecting cells into the subcutis of nude mice. At 24 days after injection, KM20 and PU1 control transfectants were sacrificed because of morbidity. Comparison of the volume of KM20, PU1, and 560 tumors is shown in Fig. 5. All of the c-Met down-regulated tumors were statistically significantly smaller than PU1 control transfectants at 24 days after injection (P < 0.01 versus PU1–21; Mann-Whitney). These data demonstrate that down-regulation of c-Met reduces SQ growth rates significantly.

Although c-Met down-regulated clones produced significantly smaller tumors at 24 days after injection, they did eventually produce large tumors as shown in Fig. 5. Clone 560–26 demonstrated the greatest reduction in c-Met protein levels and kinase activity, and had the most significant growth delay (Fig. 5). To examine a mechanism...
and tumor volume was calculated as described in “Materials and Methods;” bars, ±SD.

for delayed growth, tumors were analyzed for maintenance of plasmid expression, PCNA, and apoptosis.

Analysis of SQ Tumors. As SQ tumors eventually grew to large sizes in the c-Met down-regulated cells, RNA analysis was performed to determine whether the plasmid was still present, or had been reduced or lost when large tumors developed in the 560 clones. All of the c-Met down-regulated clones demonstrated a direct correlation between reduced ribozyme expression and the delay in tumor growth rates (data not shown). These results suggest that loss of the plasmid, hence, ribozyme expression, is selected for and the increased tumor growth rates observed likely result from an outgrowth of cells that have lost the construct.

H&E staining of tumors revealed that PU1 and control tumors were characteristic of invasive colon tumors. In 560–26 cells, only small foci of tumors surrounded by stroma were observed (data not shown). SQ tumors were analyzed for PCNA to determine whether c-Met down-regulated clones had reduced in vivo proliferation. KM20 parental tumors and the PU1–14 control transfectant had >65% of the cells staining positive for PCNA (Fig. 6A). In contrast, clone 560–26, which produced the smallest tumors and had the greatest c-Met reduction, had only 33% PCNA-positive cells, and clone 560–90 had only 47% PCNA-positive cells (Fig. 6A). The Student’s t test demonstrated that c-Met down-regulated clones had statistically significantly fewer PCNA-positive cells than either KM20 (P < 0.02) or PU1–14 (P < 0.01).

In Vivo Tumor Cell Apoptosis. Apoptosis was estimated by TUNEL analysis of serial sections. Although all of the large tumors had areas of necrosis, cell death outside of regions of necrosis was relatively low. Tumors from KM20 parental and PU1–14 cells had in vivo apoptotic rates of <1% (Fig. 6B). Although c-Met down-regulated clones still had very low numbers of apoptotic cells (1.9%), this small increase was significantly increased relative to KM20 or PU1–14 (P < 0.05; Student’s t test; Fig. 6B).

Because c-Met down-regulated cells demonstrated changes in both proliferation and apoptosis, the ratio of proliferation to apoptosis was estimated, as shown in bar graph of Fig. 6C. This ratio corresponds well with tumor volume (scatter graph, Fig. 6C), suggesting that c-Met reduction may be inhibiting cell survival and increasing apoptosis.

Intrahepatic Growth. Whereas previous studies in other cell types using the same strategy of reducing c-Met have demonstrated the ability to decrease tumorigenicity at the primary site in orthotopic nude mouse models (11, 13), none have assessed the effects of growth at the metastatic site. Because the liver is the most common site of colon cancer metastases, we examined the effects of c-Met down-regulation on growth of colon tumor cells in the liver of nude mice. The results are presented in Table 1. KM20 parental cells and PU1 control transfectants formed tumors with an incidence of 90–100% (Table 1). In contrast, the incidence of tumor formation in c-Met down-regulated cells was substantially reduced, with 0 of 10 mice developing tumors in the clone with the highest c-Met reduction, 560–26. Thus, in contrast to implantation in the subcutis, in which all of the mice developed tumors, the incidence of tumor growth in the liver was significantly reduced by c-Met down-regulation. No significant differences in tumor volume were observed among KM20, PU1–14, and PU1–21 clones. In contrast, the tumors forming from ribozyme clones were significantly reduced in tumor size (Table 1).
These data demonstrate that c-Met down-regulation inhibits both the incidence and the growth of hepatic tumors.

**Analysis of Liver Tumors.** RNA analysis of ribozyme expression in the few liver tumors that formed also verified reduced ribozyme expression (data not shown). In addition, these tumors demonstrated reduced proliferation and increased apoptosis (data not shown). As with the SQ tumors, the ratio of proliferation to apoptosis rates were reduced significantly (bar graph, Fig. 7), and directly correlated with tumor size (scatter graph, Fig. 7). As the tumor incidence in the liver was reduced significantly after c-Met down-regulation, these tumors do not represent the major phenotype observed after c-Met reduction. Thus, these data suggest that tumors result from a loss of ribozyme expression, and c-Met down-regulation may be reducing cell survival at the liver metastatic site.

**DISCUSSION**

Increased c-Met signaling is a common occurrence in early stage CRC, with even greater expression occurring in advanced and metastatic disease. In the KM20 cell line, the result of overexpression is constitutive tyrosine phosphorylation of the receptor and reduced response to HGF. Studies in other tumor types have demonstrated several mechanisms for c-Met activation, including HGF/c-Met autocrine loop, activating point mutations, TPR-Met fusion protein, and failure to cleave c-Met into the α and β chains (20–28). Sequence analysis of several colon tumor cell lines, including KM20, have revealed a wild-type receptor (data not shown). Furthermore, reverse transcription-PCR analysis did not reveal the presence of steady-state mRNA transcripts specific to HGF, suggesting that activation is not the result of an autocrine loop. Therefore, activation of c-Met in KM20 cells is likely to occur by receptor clustering, as observed in other tumor systems (8, 29, 30).

Although HGF activation of c-Met has been demonstrated to induce various biological phenotypes, no previous studies have examined the role of constitutively activated c-Met in tumor growth of CRC models. Therefore, this study examined the effect of reducing c-Met expression by a ribozyme strategy (11). Interestingly, unlike glioblastoma (11, 13), breast tumor cells (12), and prostate tumor cells (19), where expression of the ribozyme resulted in c-Met reduction by 95%, the maximum reduction of c-Met steady-state protein levels that could be achieved in KM20 cells was only 25–32%. A similar result was also observed in the KM12 colon tumor cell line, derived from a Dukes’ B patient (data not shown). Thus, the results from this study suggest that colon tumor cells may be relatively unique in requiring a threshold c-Met level for growth and/or survival.

The low incidence of tumor growth in the liver is one of the most provocative findings of this study. Down-regulation of c-Met must lead to a reduction of signal transduction pathways important for survival of colon tumor cells in the liver. In hepatic metastases, Src activity is increased relative to primary colon tumors (17, 31). Furthermore, Src is involved in both proliferation (reviewed in Ref. 32) and survival pathways, (reviewed in Ref. 33), including specific survival pathways in colon tumor cells (34). Reduced Src activity decreases in vitro, the soft agar colony formation and proliferation rates, and in vivo, tumorigenicity of colon tumor cells (35). Thus, reduced Src may be one factor responsible for the phenotype of reduced c-Met activity. However, the multifunctional role of c-Met in activating signal transduction pathways suggests that other pathways may be down-regulated as well. For instance, activation of c-Met is

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**Table 1** Intrahepatic growth of c-Met down-regulated KM20 clones

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<tr>
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<th>Incidence</th>
<th>Average tumor volume in mm³ (Range)</th>
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<tr>
<td>KM20</td>
<td>10/11</td>
<td>334 (0–1015)</td>
</tr>
<tr>
<td>PU1-14</td>
<td>10/10</td>
<td>546 (0–1920)</td>
</tr>
<tr>
<td>PU1-21</td>
<td>9/10</td>
<td>134 (0–500)</td>
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<td>5/9³</td>
<td>7³ (0–27)</td>
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<tr>
<td>S60-108</td>
<td>4/9³</td>
<td>19³ (0–152)</td>
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³ P < 0.03 versus PU1-14, Fisher’s exact.
³ P < 0.02 versus PU1-14 or PU1-21, Mann-Whitney.

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**Fig. 7.** Quantitation of proliferation and apoptosis rates in liver tumors derived from c-Met-down-regulated clones. Serial sections of tumors were stained with PCNA or TUNEL and quantitated as described in “Materials and Methods.” A minimum of 3 tumors were quantitated for each group. Percentage of proliferating cells was divided by the percentage of apoptotic cells to obtain the proliferation:apoptosis ratio and is depicted on the left axis. Average tumor volume from tumors growing in the liver is depicted on the right axis. Individual tumors are represented by , and the average tumor volume is represented by the horizontal line.
associated with increased tyrosine phosphorylation of β-catenin, another important growth-regulatory protein (18, 36, 37). Thus, considerable additional work will be required to elucidate the pathways activated by c-Met overexpression.

Other studies down-regulating c-Met have demonstrated a >90% reduction in c-Met protein levels. In a clinical setting, chemotherapeutic reduction of c-Met signaling by 90% would be a difficult task. This raises the question, “is 90% reduction in c-Met signaling necessary, or is a smaller, more clinically attainable reduction sufficient to alter the biological phenotype of a metastatic cell resulting in a clinically effective therapy?” A 30% reduction in c-Met protein levels resulted in an 80% reduction in kinase activity. This suggests that therapies targeting c-Met protein would not be required to have a significant impact on total c-Met expression to result in a major effect on c-Met signaling and potentially on CRC liver metastatic growth and survival. Future work on the c-Met pathways may also provide insights into combinations of signal transduction-directed therapies that might be of benefit in the treatment of advanced-stage colon cancer.

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Down-Regulation of c-Met Inhibits Growth in the Liver of Human Colorectal Carcinoma Cells

Matthew H. Herynk, Oliver Stoeltzing, Niels Reinmuth, et al.


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