Reversion of Human Glioblastoma Malignancy by U1 Small Nuclear RNA/Ribozyme Targeting of Scatter Factor/Hepatocyte Growth Factor and c-met Expression

Roger Abounader, Srikanth Ranganathan, Bachchu Lal, Kevin Fielding, Adam Book, Hal Dietz, Peter Burger, John Laterra

Background: Expression of scatter factor (SF), also known as hepatocyte growth factor (HGF), and its receptor, c-met, is often associated with malignant progression of human tumors, including gliomas. Overexpression of SF/HGF in experimental gliomas enhances tumorigenicity and tumor-associated angiogenesis (i.e., growth of new blood vessels). However, the role of endogenous SF/HGF or c-met expression in the malignant progression of gliomas has not been examined directly. In this study, we tested the hypothesis that human glioblastomas can be SF/HGF–c-met dependent and that a reduction in endogenous SF/HGF or c-met expression can lead to inhibition of tumor growth and tumorigenicity. Methods: Expression of the SF/HGF and c-met genes was inhibited by transfecting glioblastoma cells with chimeric transgenes consisting of U1 small nuclear RNA, a hammerhead ribozyme, and antisense sequences. The effects of reduced SF/HGF and c-met expression on 1) SF/HGF-dependent induction of immediate early genes (c-fos and c-jun), indicative of signal transduction; 2) anchorage-independent colony formation (clonogenicity), an in vitro correlate of solid tumor malignancy; and 3) intracranial tumor formation in immunodeficient mice were quantified. Statistical tests were two-sided. Results: Introduction of the transgenes into glioblastoma cells reduced expression of the SF/HGF and c-met genes to as little as 2% of control cell levels. Reduction in c-met expression specifically inhibited SF/HGF-dependent signal transduction (P<.01). Inhibition of SF/HGF or c-met expression in glioblastoma cells possessing an SF/HGF–c-met autocrine loop reduced tumor cell clonogenicity (P = .005 for SF/HGF and P = .009 for c-met) and substantially inhibited tumorigenicity (P<.0001) and tumor growth in vivo (P<.0001). Conclusions: To our knowledge, this is the first successful inhibition of SF/HGF and c-met expression in a tumor model directly demonstrating a role for endogenous SF/HGF and c-met in human glioblastoma. Our results suggest that targeting the SF/HGF–c-met signaling pathway may be an important approach in controlling tumor progression. [J Natl Cancer Inst 1999;91:1548–56]
Scatter factor (SF), also known as hepatocyte growth factor (HGF), is a multifunctional growth factor that plays a role in the regulation of cell growth, cell motility, morphogenesis, and angiogenesis (1–6). The only known receptor for SF/HGF is the c-met proto-oncogene product, a transmembrane tyrosine kinase receptor (7,8). SF/HGF and c-met are found in a wide variety of normal human tissues. SF/HGF is mainly expressed and secreted by a multitude of mesenchymally derived cells, whereas c-met messenger RNA (mRNA) and protein are detected in the epithelium of almost all tissues (9,10). These expression patterns are consistent with the paracrine role of SF/HGF and c-met in developmental mesenchymal–epithelial interactions, such as in branching morphogenesis of liver and breast (10–12). In addition, normal to high levels of SF/HGF and c-met are found in several neoplastic human tissues (9,13), where the SF/HGF–c-met signaling pathway is thought to play a role in oncogenesis and malignant tumor progression (13–15).

Glioblastoma multiforme is the most common and most malignant glial neoplasm. Despite very aggressive treatment, these malignant gliomas are associated with an average life expectancy of only 9 months. The formation and malignant progression of human gliomas are complex processes and involve genetic mutations, chromosomal multiploidy, and aberrant epigenetic influences of multiple mitogens and angiogenic factors. Several studies (14,16–18) have shown that human gliomas express SF/HGF and c-met and that expression levels are associated with malignant progression. In addition, SF/HGF gene transfer to glioblastoma cells enhances tumorigenicity, tumor growth, and tumor-associated angiogenesis in vivo (19,20). While these correlative and gain-of-function findings are consistent with a role for SF/HGF–c-met signaling in the malignancy of gliomas and other tumors, these findings do not definitely demonstrate a dependency of tumor growth on this signaling pathway. Specifically, the effects of reducing the expression of endogenous SF/HGF or c-met on the malignancy of human glioblastomas, indeed on the malignancy of any tumor, have not been explored.

In this article, we have used a novel chimeric U1 small nuclear RNA (U1snRNA), ribozyme, and antisense construct to inhibit SF/HGF and c-met gene expression in human glioblastoma cells possessing an autocrine SF/HGF–c-met loop (U-87 MG) and in human glioblastoma cells expressing c-met but not SF/HGF (U-373 MG). Then we studied the effects of this inhibition on the malignant phenotype of these human glioblastoma cell lines in vitro and in vivo.

**MATERIALS AND METHODS**

**Constructs.** The parent vector pZeoU1EcoSpe used in this study and referred to as pU1 was derived from wild-type U1snRNA as previously described (21). The U1snRNA, which constitutes the framework of the construct, is an essential component of the spliceosome complex and is stable and abundant in the nucleus of mammalian cells. Ribozymes are autocatalytic RNA structures that cleave their targets in a site-specific manner. The hammerhead ribozyme used in this construct cleaves the RNA at the GUC consensus sequence. It is flanked by two antisense sequences that determine the specificity of the targeted RNA by binding to their complementary sequences. Four complementary pairs of oligonucleotides that encode SF/HGF and c-met antisense, as well as the 22 nucleotides of the hammerhead ribozyme, were synthesized and annealed at 40 °C. The antisense/ribozyme sequences were chosen so that the ribozyme cleaves the targeted mRNA immediately 3' of the GUC ribozyme cleavage consensus sequence, at positions 547 and 701 of the human SF/HGF mRNA and at positions 292 and 560 of the human c-met mRNA (Fig. 1, A). The antisense/ribozyme
duplexes were ligated into pU1 at the EcoRI and SpeI sites to create pU1/SF and pU1/met vectors (Fig. 1, A). All ligation junctions were sequenced to verify the orientation of the inserts. The sequence of the resulting chimeric RNA was analyzed with the use of a program that predicts RNA structure (22) so that we could ensure a maximal preservation of the U1snRNA stem loops, the ribozyme secondary structure, and the accessibility of the antisense sequence to the target mRNA (Fig. 1, B). The U1snRNA/ribozyme/antisense construct is designated as U1snRNA/ribozyme throughout the article.

Transfection and screening of U-373 MG/U-87 MG human glioblastoma cells. Wild-type U-373 MG and U-87 MG glioblastoma cells were transfected with either pU1/met, pU1/SF, or pU1 (as a control), with the use of the poly-cationic reagent Lipofectamine (15 μg/mL; Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD). Control-transfected and U1snRNA/ribozyme-transfected cell lines were treated identically with regard to transfection conditions and maintenance in selection medium. Transfected cell lines were selected in the presence of 100 μg/mL Zeocin (Invitrogen Corp., Carlsbad, CA). The cell lines transfected with pU1 (control), pU1/SF, or pU1/met were screened for SF/HGF or c-met mRNA and protein levels by northern blotting and immunoblotting as described below. The screening was repeated two to four times, and the mean values of SF/HGF or c-met mRNA relative to glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA were calculated for each cell line. Cell lines with 89% or more injection of targeted mRNA were designated knockdown (suffix “KD”) to describe the nearly complete inhibition of targeted gene expression. For this study, we selected two U-373 MG control-transfected (suffix “CT”) cell lines designated U373-CT (C1 and C2), three U-373 MG c-met knockdown cell lines designated U373-MET-KD (292-1, 560-1, and 560-2), two U-87 MG control-transfected cell lines designated U87-CT (C3 and C4), three SF/HGF knockdown cell lines designated U87-SF-KD (547-1, 547-2, and 701-1), and three c-met knockdown cell lines designated U87-MET-KD (292-2, 292-3, and 560-3).

Induction of expression of c-met, c-fos, and c-jun. U373-CT and U373-MET-KD cells were grown to 70%–75% confluence on 10-cm dishes and prepared for in vitro induction of gene expression. For this study, we selected two U-373 MG control-transfected (suffix “CT”) cell lines designated U373-CT (C1 and C2), three U-373 MG c-met knockdown cell lines designated U373-MET-KD (292-1, 560-1, and 560-2), two U-87 MG control-transfected cell lines designated U87-CT (C3 and C4), three SF/HGF knockdown cell lines designated U87-SF-KD (547-1, 547-2, and 701-1), and three c-met knockdown cell lines designated U87-MET-KD (292-2, 292-3, and 560-3).

Northern blot hybridization. RNA was isolated with the use of the RNAeasy™ Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer’s directions. Northern blot analysis was performed with modifications of a previously reported procedure (23). In brief, 10 μg of total RNA was subjected to electrophoresis on a 1% agarose gel and transferred to a Nytran membrane (Schleicher & Schuell, Dassel, Germany). Hybridization was performed with complementary DNA (cDNA) probes for the coding regions of human SF/HGF (2.2 kilobases [kb]), c-met (1.3 kb), c-fos (1.35 kb), and c-jun (1.5 kb), which were originally reported procedure and following the manufacturer's directions. The membranes were reacted with HRP-conjugated secondary antibody IgG (Jackson ImmunoResearch, West Grove, PA) at a 1: 1 000 dilution. Bound antibodies were then visualized with the use of an ECL western blotting detection kit (Amersham), and the digitized images were quantitatively analyzed by densitometry (Molecular Dynamics, Sunnyvale, CA).

Colonies formation in soft agar. Anchorage-independent tumor cell proliferation was assessed by use of colony formation in soft agar according to the method of Levine et al. (26). U-87 MG and U-373 MG knockdown or control-transfected cells were plated (10 000 cells per well) and incubated at 37 °C in 5% CO₂ at 95% O₂ in minimum essential medium containing 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 1% fetal bovine serum. After 2 weeks, the cells were stained blue with Wright’s solution, and the number of colonies larger than 100 μm in diameter (U-87 MG-derived cell lines) or 75 μm in diameter (U-373 MG-derived cell lines) were determined by use of computer-assisted image analysis (19).

Tumor formation in vivo. Confluent monolayers of control-transfected and SF/HGF or c-met knockdown cells were trypsinized and resuspended in serum-free medium at 5 × 10⁵ cells/μL. The cells (2 μL) were injected intracranially into the caudate/putamen by use of a 26-gauge beveled-tip syringe as previously described (19). At 3 weeks after implantation for U87-derived tumors and at 12 weeks after implantation for U373-derived tumors, the mice were killed by decapitation, and their brains were dissected and rapidly frozen on dry ice. Cryostat sections (30 μm thick) were fixed in 4% paraformaldehyde and stained with hematoxylin-eosin. The maximal tumor cross-sectional area was determined by computer-assisted image analysis, and the tumor volume was estimated with the use of the following formula: volume = (square root of maximal tumor cross-sectional area)²/27. Mice received injections of either one of the following cell lines: two control-transfected U-373 MG, three c-met knockdown U-373 MG, two control-transfected U-87 MG, three c-met knockdown U-87 MG, or three SF/HGF knockdown U-87 cell lines. Each group contained six mice, for a total of 78 mice receiving injections. The animals were anesthetized with a mixture of xylazine (0.01 mg/g body weight; Phoenix Pharmacueticals, St. Joseph, MO) and ketamine hydrochloride (0.1 mg/g body weight; Parke-Davis, Morris Plains NJ). All animal manipulations were done in accordance with The Johns Hopkins University Animal Care and Use Committee.

Determination of proliferation index. The proliferation index was assessed by immunocytochemistry with monoclonal Ki-67 antibody as described before (28). Briefly, 20-μm thin tissue sections were reacted with primary anti-Ki-67 antibody (DAKO A/S, Glostrup, Denmark) (1:100 dilution), washed, and then treated with HRP-conjugated secondary anti-rabbit IgG (Sigma Chemical Co.). After being washed, the sections were developed in 3,3'-diaminobenzidine (DAB) (Sigma Chemical Co.) and stained with hematoxylin-eosin. To quantify proliferative activity, we counted DAB-stained tumor cells relative to the total number of cells on photomicrographs.

Statistical methods. Data on tumor size and Ki-67 labeling of tumors in vivo were analyzed with the use of Bonferroni/Dunn multiple comparisons tests. Data on tumorigenicity were analyzed by two-sided Fisher’s exact tests. Data on the in vitro anchorage-independent colony formation of the U373-derived cell lines and U87-derived cell lines were analyzed by two-sided Student’s t test and by Bonferroni/Dunn multiple comparisons test, respectively. Data for immediate early response gene induction were analyzed by two-sided Student’s t test. All tests were performed with the use of the Statview 4.0 computer program. Numerical data are expressed as means ± standard deviation (SD) with degrees of freedom (df) calculated according to the statistical tests used.

RESULTS

Inhibition of SF/HGF and/or c-met gene expression in glioblastoma cells by chimeric U1snRNA/ribozyme gene transfer. We have previously shown that the U-87 MG human glioblastoma cell line expresses both c-met and SF/HGF and that the U-373 MG human glioblastoma cell line used in this study expresses c-met but not SF/HGF (14, 19, 24). U-373 MG and U-87 MG glioblastoma cells were stably transfected with chimeric U1snRNA/ribozyme constructs designed to specifically
target SF/HGF or c-met or with control plasmid (Fig. 1), and clonal cell lines were subsequently screened for SF/HGF or c-met mRNA by northern blot hybridization. SF/HGF and c-met mRNA levels in control-transfected cells were very similar and comparable to mRNA levels in wild-type cells (not shown). The mRNA levels of the U1snRNA/ribozyme-targeted genes (c-met or SF/HGF) in most knockdown clones were less than 50% of the mean mRNA levels of control-transfected cell lines with several clones expressing less than 10% of control levels. No differences were seen in the efficiency of gene expression knockdown between the different gene sequences targeted (not shown). SF/HGF and c-met protein levels were assessed in conditioned media and total cell lysates, respectively, with the use of immunoblotting. The extent of SF/HGF and c-met protein reduction generally reflected the extent of mRNA inhibition. The control and knockdown cell lines selected for subsequent experiments and their relative levels of c-met and SF/HGF mRNA and protein are shown in Table 1 and Fig. 2.

**Inhibition of c-met induction by U1snRNA/ribozyme.** Serum, phorbol esters, cytokines, and various growth factors that may influence c-met expression levels in vivo induce c-met gene expression in cultured cells. Thus, an effective knockdown strategy should inhibit inducible as well as basal c-met expression levels. To assess the efficiency of chimeric U1snRNA/ribozyme, we examined the effects of two potent inducers of c-met, PMA and serum, on c-met expression in U373 MG c-met knockdown (U373-MET-KD) and U373 MG control-transfected (U373-CT) cells. The c-met mRNA levels in U373-CT cells were increased after treatment of the cells with 10 ng/mL PMA and 10% serum by approximately 10-fold and sevenfold, respectively, relative to untreated U373-CT cells (Fig. 3). The mRNA levels of c-met after induction by PMA and serum remained approximately 10-fold lower in U373-MET-KD relative to the treated U373-CT cells (Fig. 3). These data show that the chimeric U1snRNA/ribozyme used in this study efficiently diminishes target mRNA even under conditions that substantially induce c-met gene transcription.

**Inhibition of induction of immediate early response genes by SF/HGF after U1snRNA/ribozyme c-met knockdown.** Activation of c-met results in downstream signaling events that mediate cell responses to SF/HGF. One of these events is the induction of the immediate early genes c-fos and c-jun. To determine if U1snRNA/ribozyme c-met knockdown is sufficient to alter c-met-dependent signal transduction, we examined the induction of c-fos and c-jun by SF/HGF in U373-CT and U373-MET-KD cell lines. Induction of c-fos/c-jun by PMA and serum, which is not mediated by the c-met-receptor and, therefore, not expected to be altered in U373-MET-KD cells, was examined as controls. Treatment of U373-CT cells with PMA (10 ng/mL), SF/HGF (10 ng/mL), and serum (10%) for 30 minutes increased c-fos mRNA levels by 306% ± 126% (mean ± SD), 140% ± 60%, and 480% ± 82% (Fig. 4, A), respectively, and increased c-jun mRNA by 44% ± 22%, 42% ± 16%, and 82% ± 30% (Fig. 4, B), respectively, relative to basal levels. PMA and serum increased c-fos mRNA levels in U373-MET-KD cells, similar to results seen in the U373-CT cells, 236% ± 92% (P = .40 compared with induction in U373-CT cells, with df = 6) and 678% ± 292% (P = .24 compared with induction in U373-CT cells; df = 6), respectively. In contrast, the increase in c-fos mRNA levels in response to SF/HGF in U373-MET-KD cells was one fifth (25% ± 9.4%) that in U373-CT cells (P = .009 compared with induction in U373-CT cells; df = 6) (Fig. 4, A). PMA and serum induced c-jun mRNA levels in U373-MET-KD cells by 22% ± 10.8% (P = .13 compared with induction in U373-CT cells; df = 6) and 52% ± 16% (P = .12 compared with induction in U373-CT cells, df = 6), respectively. In contrast, the levels of c-jun mRNA remained unchanged after incubation of U373-MET-KD cells with SF/HGF (2% ± 8%; P = .004 compared with induction in U373-CT cells; df = 6) (Fig. 4, B). Thus, c-met knockdown statistically significantly reduced c-fos and c-jun induction by SF/HGF, whereas induction by PMA and serum was not statistically significantly altered. These data show that U1snRNA/ribozyme-mediated c-met knockdown specifically inhibits c-met-mediated downstream signal transduction.

**Inhibition of anchorage-independent colony formation by U1snRNA/ribozyme disruption of the SF/HGF–c-met autocrine loop.** Anchorage-independent colony formation, an in vitro correlate of solid tumor malignancy, was studied in soft agar. U1snRNA/ribozyme-mediated c-met knockdown had no effect on colony formation in U-373 MG cells that lack an SF/HGF–c-met autocrine loop (1.3 ± 1.1 colonies per field [mean ± SD] for U373-MET-CT as compared with 1.1 ± 0.8 colonies per field for U373-MET-KD; P = .87; df = 4). In contrast, colony formation in U-87 MG cells that normally express both SF/HGF and c-met was decreased 17-fold following SF/HGF knockdown (21.3 ± 8.1 colonies per field versus 1.2 ± 1.1 colonies per field; P = .005; df = 4). Anchorage-independent colony formation was also reduced 11-fold following c-met knockdown (21.3 ± 8.1 colonies per field versus 1.9 ± 0.9 colonies per field; P = .009; df = 3). These results indicate that U1snRNA/ribozyme-mediated SF/HGF knockdown and c-met knockdown reduce the malignant phenotype of glioblastomas in vitro by disrupting autocrine SF/HGF–c-met-dependent cell stimulation.

**Inhibition of glioblastoma tumorigenicity in vivo by**

---

**Table 1. Inhibition of scatter factor/hepatocyte growth factor (SF/HGF) and c-met gene expression in human glioblastoma cells by U1 small nuclear RNA (U1snRNA)/ribozyme gene transfer**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Messenger RNA (mRNA) levels, % of control levels</th>
<th>Protein levels, % of control levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>U373-MET-KD</td>
<td>292-1: 11</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>292-2: 8</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>292-3: 7</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>560-3: 8</td>
<td>13</td>
</tr>
<tr>
<td>547-1</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>547-2</td>
<td>7</td>
<td>23</td>
</tr>
<tr>
<td>701-1</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>U87-SF-KD</td>
<td>547-1: 14</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>547-2: 7</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>701-1: 9</td>
<td>2</td>
</tr>
<tr>
<td>U87-MET-KD</td>
<td>292-1: 11</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>292-2: 8</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>292-3: 7</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>560-3: 8</td>
<td>13</td>
</tr>
</tbody>
</table>

*SF/HGF and c-met mRNA and protein levels in U-373 MG and U-87 MG human glioblastoma clonal cell lines stably transfected with chimeric U1snRNA/ribozyme designed to inhibit SF/HGF (U87-SF-KD cells) or c-met (U373-MET-KD cells and U87-MET-KD cells) as measured by northern blot analysis or immunoblotting. The first three digits of each cell line numeric designation indicate the cleavage site on the targeted mRNA as shown in Fig. 1. Data are shown as percentage of mRNA and protein levels relative to control-transfected cell lines and represent the mean values of two to four independent analyses.
U1snRNA/ribozyme disruption of the SF/HGF–c-met autocrine loop. The roles of SF/HGF and c-met on tumor formation and growth in vivo were studied by implantation of control-transfected and SF/HGF- and c-met knockdown cell lines into the striatum of immunodeficient mice. In nude mice, both U373-CT and U373-MET-KD cell lines formed small tumors 12 weeks after implantation in all animals (0.15 mm³ ± 0.17 mm³ and 0.27 mm³ ± 0.16 mm³, respectively). Tumor sizes did not differ significantly between the two groups (P > .056; df = 28).

Control-transfected U-87 MG clones formed large tumors (10.0 mm³ ± 8.5 mm³) in all animals. For the U87-SF-KD cell lines, only seven of 18 mice formed tumors that were 83-fold smaller than those of the controls (P < .0001; df = 27) (Table 2, Fig. 5). Of 18 mice receiving injections of U87-MET-KD cells, 14 developed tumors that were one hundredth the size of controls (P < .0001; df = 27) (Table 2, Fig. 5). These results show that the tumorigenicity and growth in vivo of human glioblastoma xenografts that express an autocrine SF/HGF–c-met loop are substantially inhibited by U1snRNA/ribozyme targeting of SF/HGF or c-met expression.

Alteration of glioblastoma histology and cell proliferation index in vivo by U1snRNA/ribozyme targeting of SF/HGF or c-met. The histology of U87-SF-KD and U87-MET-KD cell lines differed from that of the controls, which is consistent with a more differentiated phenotype. Tumors derived from knockdown cell lines were less hypercellular and consisted of larger cells with lower nuclear-to-cytoplasmic ratios (Fig. 5). Tumor cell expression of the nuclear proliferation antigen Mib-1 was measured by immunocytochemistry with the use of monoclonal anti-Ki-67 antibody. The percentage of cells positive for Mib-1 defines the growth fraction of a given cell population and correlates with human glioma malignancy and poor prognosis. In both U87-SF-KD and U87-MET-KD, the fraction of tumor cells expressing Mib-1 was significantly less than in U87-CT (21.5% ± 5.9%, 21.6% ± 3.0%, and 40.3% ± 5.1%, respectively) (P < .0001; df = 10), which is consistent with the reduced tumor growth rates in the knockdown cell lines (Fig. 5).

U1snRNA/ribozyme disruption of the SF/HGF–c-met autocrine loop. The roles of SF/HGF and c-met on tumor formation and growth in vivo were studied by implantation of control-transfected and SF/HGF- and c-met knockdown cell lines into the striatum of immunodeficient mice. In nude mice, both U373-CT and U373-MET-KD cell lines formed small tumors 12 weeks after implantation in all animals (0.15 mm³ ± 0.17 mm³ and 0.27 mm³ ± 0.16 mm³, respectively). Tumor sizes did not differ significantly between the two groups (P > .056; df = 28).

Control-transfected U-87 MG clones formed large tumors (10.0 mm³ ± 8.5 mm³) in all animals. For the U87-SF-KD cell lines, only seven of 18 mice formed tumors that were 83-fold smaller than those of the controls (P < .0001; df = 27) (Table 2, Fig. 5). Of 18 mice receiving injections of U87-MET-KD cells, 14 developed tumors that were one hundredth the size of controls (P < .0001; df = 27) (Table 2, Fig. 5). These results show that the tumorigenicity and growth in vivo of human glioblastoma xenografts that express an autocrine SF/HGF–c-met loop are substantially inhibited by U1snRNA/ribozyme targeting of SF/HGF or c-met expression.

Alteration of glioblastoma histology and cell proliferation index in vivo by U1snRNA/ribozyme targeting of SF/HGF or c-met. The histology of U87-SF-KD and U87-MET-KD cell lines differed from that of the controls, which is consistent with a more differentiated phenotype. Tumors derived from knockdown cell lines were less hypercellular and consisted of larger cells with lower nuclear-to-cytoplasmic ratios (Fig. 5). Tumor cell expression of the nuclear proliferation antigen Mib-1 was measured by immunocytochemistry with the use of monoclonal anti-Ki-67 antibody. The percentage of cells positive for Mib-1 defines the growth fraction of a given cell population and correlates with human glioma malignancy and poor prognosis. In both U87-SF-KD and U87-MET-KD, the fraction of tumor cells expressing Mib-1 was significantly less than in U87-CT (21.5% ± 5.9%, 21.6% ± 3.0%, and 40.3% ± 5.1%, respectively) (P < .0001; df = 10), which is consistent with the reduced tumor growth rates in the knockdown cell lines (Fig. 5).

U1snRNA/ribozyme disruption of the SF/HGF–c-met autocrine loop. The roles of SF/HGF and c-met on tumor formation and growth in vivo were studied by implantation of control-transfected and SF/HGF- and c-met knockdown cell lines into the striatum of immunodeficient mice. In nude mice, both U373-CT and U373-MET-KD cell lines formed small tumors 12 weeks after implantation in all animals (0.15 mm³ ± 0.17 mm³ and 0.27 mm³ ± 0.16 mm³, respectively). Tumor sizes did not differ significantly between the two groups (P > .056; df = 28).

Control-transfected U-87 MG clones formed large tumors (10.0 mm³ ± 8.5 mm³) in all animals. For the U87-SF-KD cell lines, only seven of 18 mice formed tumors that were 83-fold smaller than those of the controls (P < .0001; df = 27) (Table 2, Fig. 5). Of 18 mice receiving injections of U87-MET-KD cells, 14 developed tumors that were one hundredth the size of controls (P < .0001; df = 27) (Table 2, Fig. 5). These results show that the tumorigenicity and growth in vivo of human glioblastoma xenografts that express an autocrine SF/HGF–c-met loop are substantially inhibited by U1snRNA/ribozyme targeting of SF/HGF or c-met expression.

Alteration of glioblastoma histology and cell proliferation index in vivo by U1snRNA/ribozyme targeting of SF/HGF or c-met. The histology of U87-SF-KD and U87-MET-KD cell lines differed from that of the controls, which is consistent with a more differentiated phenotype. Tumors derived from knockdown cell lines were less hypercellular and consisted of larger cells with lower nuclear-to-cytoplasmic ratios (Fig. 5). Tumor cell expression of the nuclear proliferation antigen Mib-1 was measured by immunocytochemistry with the use of monoclonal anti-Ki-67 antibody. The percentage of cells positive for Mib-1 defines the growth fraction of a given cell population and correlates with human glioma malignancy and poor prognosis. In both U87-SF-KD and U87-MET-KD, the fraction of tumor cells expressing Mib-1 was significantly less than in U87-CT (21.5% ± 5.9%, 21.6% ± 3.0%, and 40.3% ± 5.1%, respectively) (P < .0001; df = 10), which is consistent with the reduced tumor growth rates in the knockdown cell lines (Fig. 5).
DISCUSSION

To our knowledge, this is the first report of SF/HGF–c-met gene expression knockdown and its effects on SF/HGF-mediated cell signaling and tumorigenesis in glioblastoma cells, indeed in any tumor model. We have combined U1snRNA, ribozyme, and antisense technologies to achieve a high degree of inhibition of gene expression in the U-373 MG and U-87 MG glioblastoma cells used in this study. The U1snRNA, which constitutes the framework of the construct, is an essential component of the spliceosome complex and is stable and abundant in the nucleus of mammalian cells (29). Important attributes of U1snRNA are its potent and constitutively active promoter, stable stem loops with a high GC content, the ability of the unusual trimethylguanosine 5′ cap and Sm protein interactions to signal transport of U1snRNA to the nucleus (30), and the lack of polyadenylation of mature small nuclear RNAs, a characteristic that favorably influences transcript trafficking and localization (31). All of these factors theoretically contribute to the high expression and stability of construct-derived regulatory transcripts, especially in the nucleosome (21). Ribozymes are autocatalytic RNA structures that cleave their targets in a site-specific manner. The hammerhead ribozyme used in this construct cleaves the RNA at the GUC consensus sequence. It is flanked by two antisense sequences that determine the specificity of the targeted RNA by binding to their complementary sequences. Using a similar construct, Montgomery and Dietz (21) knocked-out fibrillin-1 expression in human osteosarcoma cells to levels undetectable by northern blot analysis. They also showed that U1/ribozyme RNA specifically accumulates in the cell nuclear compartment and that inhibition of targeted RNA required the construct’s U1 flanking sequences. The relative contributions of RNA–RNA duplex formation versus ribozyme action to the degradation of targeted RNA by this chimeric construct has not been established and is under active investigation. In our system, basal levels of SF/HGF and c-met mRNA and protein were lowered to as little as 2% of control

Fig. 4. Knockdown of c-met inhibits c-met-dependent signal transduction. This inhibition is shown by the substantially lower levels of c-fos (A) and c-jun (B) messenger RNA (mRNA) in the U-373 MG c-met knockdown glioblastoma cells (U373-MET-KD) as compared with control-transfected cells (U373-CT) after treatment with 10 ng/mL scatter factor/hepatocyte growth factor (SF/HGF) (shown as SF on fig.). Responses to 10 ng/mL phorbol 12-myristate 13-acetate (PMA) and 10% serum (SER), which induce c-fos and c-jun in a c-met-independent manner, were not affected by c-met knockdown (P>1). The mRNA levels were determined by northern blot analysis. Bars represent percent change in mRNA levels on the same blots (upper panels) and normalized to glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA levels on the same blots (lower panels). Quantitative data represent means ± SD of two different control and three different knockdown clonal cell lines from two experiments (n = 4). Blots shown are from a representative experiment. Unt. = untreated. P = .009 in A (c-fos expression) and P = .004 in B (c-jun expression) (comparing percent change in SF/HGF-treated U373-CT cells with the percent change in SF/HGF-treated C-MET-KD cells), two-sided unpaired Student’s t tests; degrees of freedom = 6.

Table 2. Inhibition of in vivo tumorigenicity and tumor growth by U1 small nuclear RNA/ribozyme-mediated knock-down of scatter factor/hepatocyte growth factor and c-met gene expression

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of mice with tumor/ No. of mice receiving injection</th>
<th>Tumor volume, mm³, mean ± standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>U87-CT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>6/6</td>
<td>4.0 ± 0.50</td>
</tr>
<tr>
<td>C4</td>
<td>5/5</td>
<td>15.1 ± 19.7</td>
</tr>
<tr>
<td>Pooled U87-CT</td>
<td>11/11</td>
<td>10.0 ± 8.5</td>
</tr>
<tr>
<td>U87-SF-KD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>547-1</td>
<td>2/6</td>
<td>0.01 ± 0.002</td>
</tr>
<tr>
<td>547-2</td>
<td>5/6</td>
<td>0.34 ± 0.29</td>
</tr>
<tr>
<td>701-1</td>
<td>0/6</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Pooled U87-SF-KD</td>
<td>7/18†</td>
<td>0.12 ± 0.25†</td>
</tr>
<tr>
<td>U87-MET-KD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>292-2</td>
<td>4/6</td>
<td>0.15 ± 0.14</td>
</tr>
<tr>
<td>292-3</td>
<td>5/6</td>
<td>0.08 ± 0.08</td>
</tr>
<tr>
<td>560-3</td>
<td>5/6</td>
<td>0.06 ± 0.07</td>
</tr>
<tr>
<td>Pooled U87-MET-KD</td>
<td>14/18‡</td>
<td>0.10 ± 0.08‡</td>
</tr>
</tbody>
</table>

*Control-transfected (U87-CT), scatter factor knockdown (U87-SF-KD), and c-met knockdown (U87-MET-KD) cells (10⁶) were stereotactically injected into the striatum of severe combined immunodeficiency (SCID)-Beige mice. Brains were removed 3 weeks after implantation. Cryostat sections (30 μm thick) were stained with hematoxylin–eosin, and tumor sizes were measured by computer-assisted image analysis.

†P<.0001 in comparison to pooled U87-CT tumors by two-sided Fisher’s exact test.

‡P<.0001 in comparison to pooled U87-CT tumors by Bonferroni/Dunn test, degrees of freedom = 27.
levels. The c-met mRNA levels remained significantly reduced under conditions of induced gene expression, demonstrating the effectiveness of the construct in degrading targeted mRNA. The incomplete knock-out of SF/HGF or c-met expression could be explained by the inaccessibility of a small pool of targeted mRNA to the chimeric U1snRNA/ribozyme transcript. Such a mechanism is suggested by our experimental finding of the same proportional reduction in basal and induced c-met mRNA in knockdown cells relative to basal and induced levels in control transfected cells. Another possible explanation is that glioblastoma cells completely lacking c-met receptors (or SF/HGF synthesis in cells with an autocrine SF/HGF–c-met loop) are not viable. The achieved knockdown did, however, lead to significant phenotypic changes, as evidenced by numerous criteria.

To determine if c-met knockdown affects downstream signal transduction, we examined c-fos and c-jun induction in U373-MET-KD and U373-CT cells after stimulation of the cells with SF/HGF. Activation of the c-met receptor was shown to induce expression of immediate early genes in several cell types and tissues (32) but not previously in glioblastoma cells. We showed that this is also true for U-373 MG glioblastoma cells and that this response is strongly reduced (c-fos) or abolished (c-jun) by the U1snRNA/ribozyme, indicating a significant inhibition of downstream signal transduction after c-met receptor gene knockdown. Importantly, c-met receptor-independent induction of c-fos and c-jun by PMA and serum was not affected in U373-MET-KD, demonstrating the specificity of the effects of U1snRNA/ribozyme-mediated knockdown to c-met-dependent pathways.

A number of previous studies support a role for the SF/HGF–c-met signaling pathway in the formation and malignant progression of various tumors, including gliomas. This conclusion is based on 1) the high expression levels of SF/HGF and/or c-met in various tumors and their correlation with malignancy and poor prognosis (9,14,17,24), 2) the enhanced tumorigenicity or malignant transformation following overexpression of SF/HGF or c-met in tumor cells (15,19,20,33,34), and 3) the demonstration that activating c-met mutations promote tumor formation (35). Recently, Date et al. (36) reported inhibition of growth of an experimental gallbladder carcinoma by the systemic administration of a synthetic SF/HGF N-terminal peptide (NK4) capable of functioning as a SF/HGF antagonist. To date, however, no previous study had examined the biologic effects of inhibiting endogenous SF/HGF or c-met gene expression in tumor cells. We hypothesized that the growth of human glioblastomas can be, in part, dependent on SF/HGF and/or c-met expression and that inhibiting SF/HGF or c-met would decrease their tumorigenicity and growth rates. We tested this hypothesis by assessing in vitro and in vivo malignancy of SF/HGF and/or c-met knockdown in glioblastoma cells that express only c-met (U-373 MG) or that express both SF/HGF and c-met (U-87 MG). For control U-373 MG cells, anchorage-independent clonogenicity was low and in vivo tumor formation was slow relative to control U-87 MG cells that possess an autocrine SF/HGF–c-met loop. Knockdown of c-met did not significantly alter the malignant phenotype of U-373 MG cells in vitro or in vivo, indicating that the malignancy of glioblastomas lacking an autocrine SF/HGF–c-met loop or other paracrine source of SF/HGF cannot be inhibited by targeting tumor cell c-met receptor expression or function alone. Thus, host-derived SF/HGF from brain or systemic organs appears to play little, if any, role in the growth of these experimental intracranial gliomas. In contrast, when the autocrine loop of U-87 MG cells was inhibited, anchorage-independent clonogenicity, tumorigenicity, and tumor growth rates in vivo were dramatically reduced. While targeting either SF/HGF or c-met led to a comparable inhibition in tumor growth rates, greater inhibition of tumorigenicity in vivo resulted from SF/HGF gene knockdown. This greater in vivo response might be explained by the inhibition of SF/HGF-stimulated paracrine processes, such as angiogenesis, in addition to the inhibition of autocrine processes as a result of the SF/HGF knockdown (Fig. 6).

Suppression of glioblastoma growth by inhibiting SF/HGF and/or c-met is likely to involve multiple mechanisms (Fig. 6). Studies show that the establishment of an SF/HGF–c-met autocrine loop can cause malignant transformation (15,34). In tumor cells that express both SF/HGF and c-met, SF/HGF can activate c-met receptors on the tumor cell itself and act on c-met receptors present on surrounding tissues, such as vascular endothelial cells. SF/HGF is a potent angiogenic factor (37), and we previously reported (20) that SF/HGF gene transfer enhances the growth of an experimental rat glioma probably by stimulating angiogenesis. SF/HGF can also activate tumor cell c-met receptors, stimulating various downstream events such as the induction of genes that affect tumorigenicity, angiogenic...
To our knowledge, this first report of SF/HGF–c-met gene expression, making it a potentially important tool for sequences is very effective in the targeted inhibition of glioblastoma malignancy, affecting by the specific U1/ribozyme constructs used in this study. Glioma cells release reciprocal interactions between tumor cells and their surroundings, and the vascular permeability factors, such as vascular endothelial growth factor (VEGF) that c-met activation may induce glioblastoma cell secretion of other angiogenic/ permeability factors through c-met-dependent and/or transcription-independent pathways. The SF/HGF–c-met knockdown in solid tumors shows that the malignancy of human gliomas can be SF/HGF–c-met dependent and that inhibiting SF/HGF or c-met expression or function might be of significant therapeutic value in these extremely aggressive central nervous system neoplasms. Practical approaches that overcome obstacles of delivering genetic and biological drugs across the blood-brain barrier to invasive glial neoplasms will need to be available before these and similar therapeutic strategies can be extrapolated to humans.

**Fig. 6.** Mechanisms by which scatter factor/hepatocyte growth factor (SF/HGF) contributes to glioblastoma malignancy. 1 = Glioblastoma-derived SF/HGF activates glioblastoma cell c-met receptors resulting in autocrine growth stimulation (19), tumor cell migration (14), and apoptosis inhibition (41), via transcription-dependent and/or transcription-independent pathways. 2 = Glioblastoma cell-derived SF/HGF enhances tumor-associated angiogenesis via paracrine stimulation of vascular endothelial cells that express c-met receptors (37,20). 3 = c-met activation may induce glioblastoma cell secretion of other angiogenic/ permeability factors, such as vascular endothelial growth factor (VEGF) that stimulate tumor angiogenesis and increase vessel permeability (38). 4 = Host-derived SF/HGF may also play an important role in stimulating tumor growth under certain conditions. While SF/HGF knockdown in glioblastoma cells inhibits mechanisms 1, 2, and 3, c-met knockdown inhibits 1 and 3. This model is based on our findings from the current article and from other published studies as referenced in the article.

**References**


NOTES

Supported by Public Health Service grants RO1NS32148 (J. Laterra) (National Institute of Neurological Disorders and Stroke) and RO1AR41135 (H. Dietz) (National Institute of Arthritis and Musculoskeletal and Skin Diseases), National Institutes of Health, Department of Health and Human Services; by the Smilow Foundation (H. Dietz); and by the Howard Hughes Medical Institute.

We thank Drs. Kirby Smith and Jyh-Feng Lu for their helpful discussions and Ms. Angela T. Williams for help in manuscript preparation.

Manuscript received February 1, 1999; revised June 29, 1999; accepted July 13, 1999.