Published OnlineFirst February 3, 2009; DOI: 10.1158/1535-7163.MCT-08-0627

Interactions between PTEN and the c-Met pathway in glioblastoma and implications for therapy

Yunqing Li,[1] Fadila Guessous,[1] Charles DiPierro,[2] Ying Zhang,[1] Tucker Mudrick,[1] Lauren Fuller,[1] Elizabeth Johnson,[1] Lukasz Marcinkiewicz,[1] Matthew Engelhardt,[1] Benjamin Kefas,[1] David Schiff,[1] Jin Kim,[4] and Roger Abounader[1,3]

Departments of [1]Neurology, [2]Pathology, and [3]Microbiology, University of Virginia, Charlottesville, Virginia; and [4]Galaxy Biotech LLC, Cupertino, California

Abstract

The tyrosine kinase receptor c-Met and its ligand hepatocyte growth factor (HGF) are frequently overexpressed and the tumor suppressor PTEN is often mutated in glioblastoma. Because PTEN can interact with c-Met-dependent signaling, we studied the effects of PTEN on c-Met-induced malignancy and associated molecular events and assessed the potential therapeutic value of combining PTEN restoration approaches with HGF/c-Met inhibition. We studied the effects of c-Met activation on cell proliferation, cell cycle progression, cell migration, cell invasion, and associated molecular events in the settings of restored or inhibited PTEN expression in glioblastoma cells. We also assessed the experimental therapeutic effects of combining anti-HGF/c-Met approaches with PTEN restoration or mTOR inhibition. PTEN significantly inhibited HGF-induced proliferation, cell cycle progression, migration, and invasion of glioblastoma cells. PTEN attenuated HGF-induced changes of signal transduction proteins Akt, GSK-3, JNK, and mTOR as well as cell cycle regulatory proteins p27, cyclin E, and E2F-1. Combining PTEN restoration to PTEN-null glioblastoma cells with c-Met and HGF inhibition additively inhibited tumor cell proliferation and cell cycle progression. Similarly, combining a monoclonal anti-HGF antibody (L2G7) with the mTOR inhibitor rapamycin had additive inhibitory effects on glioblastoma cell proliferation. Systemic *in vivo* deliveries of L2G7 and PTEN restoration as well as systemic *in vivo* deliveries of L2G7 and rapamycin additively inhibited intracranial glioma xenograft growth. These preclinical studies show for the first time that PTEN loss amplifies c-Met-induced glioblastoma malignancy and suggest that combining anti-HGF/c-Met approaches with PTEN restoration or mTOR inhibition is worth testing in a clinical setting. [Mol Cancer Ther 2009;8(2):376–85]

Introduction

Glioblastoma is the most common and most deadly human primary brain tumor. Patients with glioblastoma usually die within 1 year of diagnosis. Glioblastoma formation and growth has been associated with multiple molecular dysfunctions. Among these, overexpression of the tyrosine kinase receptor c-Met and its ligand hepatocyte growth factor (HGF) and loss of the tumor suppressor PTEN are thought to play important roles. HGF and c-Met are overexpressed in human gliomas, and expression levels correlate with glioma malignancy and grade (1–5). Inhibition of HGF or c-Met expression leads to inhibition of *in vivo* brain tumor formation and growth (6, 7). The loss or mutation of the tumor suppressor PTEN is one of the most common genetic occurrences in glioblastoma. Somatic mutations of PTEN are detected in >40% of glioblastomas and PTEN protein expression is very low or absent in two-thirds of these tumors (8–10). PTEN can interact with c-Met-dependent signaling at various levels. Such interactions include the antagonistic effect of PTEN on phosphatidylinositol 3-kinase, which plays an essential role in c-Met-dependent cell signaling. Another possible interaction consists in PTEN's ability to bind and dephosphorylate the p52 isoform of Shc, thus inhibiting the recruitment of the Grb2 adaptor and the subsequent activation of the mitogen-activated protein kinase (MAPK) cascade, which is another key mediator of HGF malignant effects (11, 12). Using expression microarrays, we showed previously that PTEN restoration to glioblastoma cells changed c-Met-dependent gene expression in a complex and heterogeneous manner (13). However, the effects of PTEN expression on c-Met-dependent malignancy have not been investigated to date. Understanding these effects is important as it would determine if combining HGF/c-Met inhibition with PTEN restoration strategies or strategies that counteract the effects of PTEN loss such as mTOR inhibition could have therapeutic advantage. It would also help determine if the PTEN status affects the efficacy of emerging clinically applicable anti-HGF/c-Met therapies.

In this study, we investigated the molecular and functional interactions between HGF/c-Met and PTEN in glioblastoma. More specifically, we addressed two major questions: (*a*) How does PTEN expression or loss alter c-Met-dependent malignancy and associated signaling?

Received 7/7/08; revised 11/18/08; accepted 12/4/08;

published OnlineFirst 02/03/2009.

Grant support: NIH grant RO1 NS045209 (R. Abounader) and University of Virginia Cancer Center.

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.

Requests for reprints: Roger Abounader, University of Virginia Health System, Old Medical School, 4819, P.O. Box 800168, Charlottesville, VA 22908. Phone: 434-982-6634; Fax: 434-243-6843.
E-mail: ra6u@virginia.edu

doi:10.1158/1535-7163.MCT-08-0627

Published OnlineFirst February 3, 2009; DOI: 10.1158/1535-7163.MCT-08-0627

and (*b*) Does combining HGF/c-Met inhibition with PTEN restoration or mTOR inhibition have experimental therapeutic advantage? We found that PTEN expression inhibits c-Met-induced cell proliferation, cell cycle progression, cell migration, and cell invasion. PTEN expression attenuated c-Met-induced protein tyrosine phosphorylation and activation or inactivation of multiple signaling molecules and molecular events known to mediate c-Met functions. Moreover, we found that combining HGF/c-Met inhibition with PTEN restoration or mTOR inhibition, which counteracts some effects of PTEN loss, additively inhibits cell proliferation, cell cycle progression, and *in vivo* tumor growth. These data show that PTEN loss and c-Met activation additively affect glioma malignancy. The findings also provide a rationale for testing combinations of clinically applicable HGF/c-Met inhibitors with PTEN restoration or clinically applicable mTOR inhibitors in clinical trials.

Materials and Methods

Cell Culture and Reagents

Human U87 glioblastoma cells were grown in Eagle's MEM (Cellgro Mediatech) containing 10% fetal bovine serum (FBS), 0.15% sodium bicarbonate, 1 mmol/L sodium pyruvate, and 0.1 mol/L nonessential amino acids (Life Technologies). Human A172 glioblastoma cells were grown in DMEM (Cellgro Mediatech) supplemented with 10% FBS and 4.5 g/L glucose. Human SF767 glioblastoma cells (a kind gift from Dr. Russel Pieper, University of California at San Francisco) were grown in MEM (Invitrogen) supplemented with 10% FCS. All cells were grown at 37°C in 5% CO_2-95% O_2. The mTOR inhibitor rapamycin was purchased from Sigma-Aldrich. The anti-HGF monoclonal antibody L2G7 is a kind gift from Galaxy Biotech. PTEN and control small interfering RNA (siRNA) were purchased from Santa Cruz Biotechnology. Human recombinant HGF is a kind gift from Genentech.

Vectors and Transfections

Adenoviruses encoding wild-type PTEN (Ad-PTEN), anti-HGF U1/ribozymes (Ad-U1/HGF), anti-c-Met U1/ribozymes (Ad-U1/Met), and control adenovirus (Ad-control) were constructed as described previously (7, 13). The cells were infected with adenovirus vectors (multiplicity of infection = 10) for 24 h before treatment with 50 ng/mL HGF. Under these conditions, restored PTEN protein levels are of comparable magnitude with PTEN protein levels in normal human astrocytes (13, 14). For stable PTEN expression, the full-length human PTEN cDNA was subcloned into the mammalian expression vector pcDNA3.1-Zeo (Invitrogen) to generate pcDNA/PTEN. Cells were transfected with pcDNA/PTEN using Fugene transfection reagent (Roche Applied Science) according to the manufacturer's instructions. Transfected cells were selected in zeocin and PTEN expression was verified by immunoblotting.

siRNA-Mediated PTEN Knockdown

PTEN expression was inhibited in SF767 cells expressing wild-type PTEN with PTEN siRNA (Santa Cruz Biotechnology). The cells were transfected with 10 nmol/L PTEN siRNA or scrambled control siRNA using Oligofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions. PTEN protein inhibition was verified by immunoblotting.

Growth Curves

U87 cells (30,000 per well) or A172 cells (40,000 per well) were seeded in 10% FBS medium, transferred to 0.1% FBS medium (for c-Met activation experiments), or 3% FBS medium (for c-Met inhibition experiments) and infected with adenoviruses for 24 h before treatment with 50 ng/mL HGF. The cells were harvested by trypsinization every day for 5 days and counted with a hemocytometer. To assess effects of combining the mTOR inhibitor rapamycin with anti-HGF antibody (L2G7) on cell proliferation, U87 cells (30,000 per well) or A172 cells (40,000 per well) were grown in 10% FBS medium, exposed to 0.1% FBS medium, and treated with 40 nmol/L rapamycin and/or 20 μg/mL L2G7. The cells were counted as described above.

Cell Cycle Analysis

The effects of c-Met and PTEN on cell cycle progression were assessed using propidium iodide flow cytometry as described previously (15). Briefly, U87, A172, or SF767 cells were plated in 10% FBS medium overnight and subsequently exposed to 0.1% FBS medium (for c-Met activation experiments) or 3% FBS medium (for c-Met inhibition experiments) and infected with adenoviruses or transfected with siRNA for 24 h before treatment with or without 50 ng/mL HGF for additional 24 h. The cells were washed with PBS, harvested, and fixed in 70% (v/v) ethanol. The cells were then treated with 20 μg DNase-free RNase and stained with propidium iodide. Cell samples were analyzed on a FACScan (Becton Dickinson).

Invasion Assay

The effects of c-Met and PTEN on cell invasion were assessed using a Transwell invasion assay (BD Biosciences). A cell culture insert was coated with 30 μL collagen type IV (250 μg/mL). U87 or A172 cells (1×10^5) were infected with adenoviruses for 24 h, resuspended in 300 μL of 0.1% FBS medium with or without 50 ng/mL HGF, and placed in the top chamber, and 600 μL of 10% FBS medium were placed in the bottom chamber. After incubation for 6 h at 37°C in 5% CO_2, the cells on the top membrane surface were mechanically removed. Cells that migrated to the bottom side of the membrane were fixed and stained with 0.1% crystal violet (Sigma-Aldrich). Photographs were taken and stained cells were counted under a microscope in five randomly chosen fields.

Migration Assay

The effects of c-Met and PTEN on cell migration were assessed by a scratch wound assay. Briefly, cells grown in 6-well plates were infected with adenoviruses for 48 h and the medium was changed to 0.1% FBS overnight. The near-confluent cell monolayers were then carefully scratched using 20 μL sterile pipette tips. Cell debris was removed by washing with PBS. The wounded monolayers were kept in 0.1% FBS medium with or without 50 ng/mL HGF for additional 24 h and photographs were taken under a light microscope.

Published OnlineFirst February 3, 2009; DOI: 10.1158/1535-7163.MCT-08-0627

Immunoprecipitation and Immunoblotting

Immunoprecipitation was used to determine if PTEN binds to c-Met. Briefly, U87 and A172 cells were transfected with the PTEN trapping mutant D92 (a kind gift of Dr. Kenneth Yamada, NIH) or GFP (control). D92 irreversibly binds to its target and has been previously successfully used to determine molecules that bind to PTEN (16). Twenty-four hours post-transfection, the cells were treated with 50 ng/mL HGF for 10 min and subsequently lysed with radioimmunoprecipitation assay buffer (1% Igepal, 0.5% sodium deoxycholate, and 0.1% SDS in PBS). Protein (1 mg) was incubated with 4 μg c-Met antibody (Upstate Biotechnology) or PTEN antibody (Cell Signaling Technologies) for 2 h before incubation with protein A plus G beads (Santa Cruz Biotechnology) overnight at 4°C. The beads were collected by centrifugation, washed five times with lysis buffer, heated to 100°C in Laemmli buffer, and subjected to immunoblotting for PTEN or c-Met, respectively, as described below. Immunoblotting was done as described previously using antibodies specific for phosphotyrosine, phospho-c-Met, phospho-MAPK, MAPK, phospho-Akt, Akt, phospho-GSK-3α/β, and GSK-3α/β (Cell Signaling Technologies), Cdk2, cyclin E, E2F-1, PTEN, and β-actin (Santa Cruz Biotechnology), and p27 (BD Biosciences; refs. 15, 17).

Tumor Formation *In vivo*

The effects of combining L2G7-mediated HGF inhibition with either PTEN restoration or rapamycin-mediated mTOR inhibition on *in vivo* tumor growth were tested in an intracranial glioblastoma xenograft model. For combinations of PTEN restoration and L2G7 treatment, stable clones of U87 cells expressing wild-type PTEN or control were generated as described above. The clones (3×10^5 cells) were stereotactically implanted into the brains of immunodeficient mice ($n = 10$). One week post-tumor implantation, the animals were treated with i.p. injections of 100 μg L2G7 or control twice per week for 3 weeks. For combinations of L2G7 and rapamycin treatments, wild-type U87 cells (3×10^5) were stereotactically implanted into the brains of immunodeficient mice ($n = 10$). One week post-tumor implantation, the animals were treated with i.p. injections of L2G7 (100 μg twice per week), rapamycin (40 μg three times per week), L2G7 + rapamycin (same doses and schedule as single treatments), or vehicle control for 2 weeks. All animals were euthanized 1 week after the last treatment. The brains were removed, sectioned, and stained with H&E. Maximal tumor cross-sectional area was measured by computer-assisted image analysis.

Statistics

All immunoblots show representative results from at least two experiments. All other experiments were repeated at least three times and the results were expressed as mean ± SE. When appropriate, two group comparisons were analyzed with a t test and multiple group comparisons were analyzed with a Dunnett's test. P values were calculated. $P < 0.05$ was considered significant.

Results

PTEN Status Affects c-Met-Dependent Glioblastoma Cell Proliferation and Cell Cycle Progression

We found previously that c-Met activation contributes to glioblastoma malignancy by inducing cell proliferation and cell cycle progression (6, 7). To determine how PTEN expression affects these c-Met-induced malignancy variables, we restored PTEN to PTEN-null U87 and A172 glioblastoma cells and inhibited PTEN in wild-type PTEN SF767 glioblastoma cells and studied the effects of c-Met activation on cell proliferation and cell cycle progression in these settings. PTEN was restored to U87 and A172 cells by infection with Ad-PTEN or Ad-Control for 24 h (multiplicity of infection = 10). The cells were subsequently treated with or without 50 ng/mL HGF. Cell proliferation and cell cycle were analyzed as described in Materials and Methods. PTEN expression significantly inhibited basal and HGF-induced cell proliferation in both cell lines ($n = 6$; $P < 0.05$, PTEN relative to control and PTEN + HGF relative to control + HGF; Fig. 1A). PTEN expression also significantly and completely inhibited HGF-induced cell cycle progression in U87 cells ($n = 3$; $P < 0.05$) and partially inhibited HGF-induced cell cycle progression in A172 cells ($n = 4$; $P < 0.05$; Fig. 1B). To assess the effects of endogenous PTEN on c-Met-dependent cell proliferation and cell cycle progression, we inhibited PTEN in wild-type PTEN SF767 cells by transfection with 10 nmol/L PTEN siRNA for 48 h before treatment with or without 50 ng/mL HGF. Inhibition of PTEN expression in SF767 cells amplified HGF-induced cell proliferation and cell cycle progression ($n = 3$; Fig. 1C). These data show that the PTEN status significantly affects c-Met-dependent glioblastoma cell proliferation and cell cycle progression.

PTEN Expression Inhibits c-Met-Dependent Glioblastoma Cell Invasion and Cell Migration

c-Met activation contributes to glioblastoma malignancy by inducing tumor cell migration and invasion (18–20). To determine if PTEN expression affects c-Met-dependent glioblastoma cell migration and invasion, we restored PTEN to PTEN-null U87 and A172 cells and assessed the effects of c-Met activation on these malignancy variables. PTEN was restored to U87 and A172 cells by infection with Ad-PTEN or Ad-Control for 24 h (multiplicity of infection = 10). The cells were subsequently treated with or without 50 ng/mL HGF and cell invasion and migration were analyzed as described in Materials and Methods. PTEN expression significantly inhibited HGF-induced Transwell cell invasion in U87 cells ($n = 3$; $P < 0.05$, PTEN + HGF relative to control + HGF; Fig. 2A). PTEN expression significantly inhibited basal as well as HGF-induced Transwell cell invasion in A172 cells ($n = 3$; $P < 0.05$, PTEN relative to control and PTEN + HGF relative to control + HGF; Fig. 2A). PTEN expression also inhibited basal and HGF-induced cell migration in U87 cells (Fig. 2B). These data show that the PTEN status strongly affects c-Met-dependent glioblastoma cell invasion and cell migration.

Published OnlineFirst February 3, 2009; DOI: 10.1158/1535-7163.MCT-08-0627

PTEN Attenuates c-Met-Dependent Signal Transduction

To determine how PTEN expression affects c-Met-dependent signal transduction, we restored PTEN to PTEN-null U87 and A172 cells and assessed the effects of c-Met activation on overall protein tyrosine phosphorylation and on specific signal transduction molecules shown previously to mediate c-Met functions (6, 7, 17, 21, 22). PTEN was restored to U87 and A172 cells by infection with

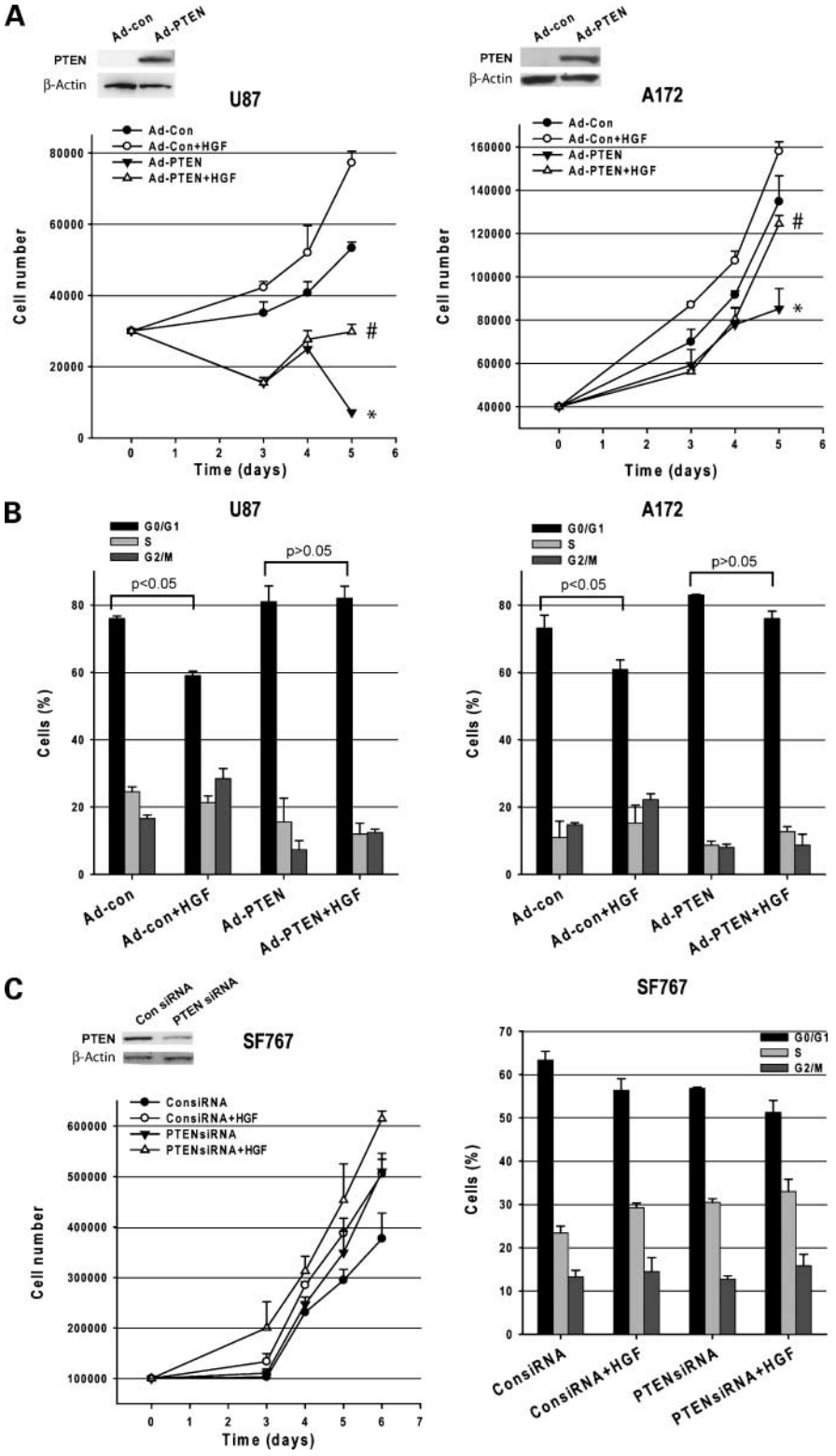

Figure 1. PTEN status affects c-Met-dependent glioblastoma cell proliferation and cell cycle progression. **A** and **B,** PTEN was expressed in PTEN-null U87 and A172 glioblastoma cells via adenovirus (Ad-PTEN) infections before treatment with HGF. The effects of PTEN expression on cell proliferation and G_1-S cell cycle progression were assessed by cell counting and by propidium iodide flow cytometry, respectively. The results show that PTEN restoration significantly inhibits basal and HGF-induced cell proliferation in U87 and A172 cells (**A**). Similarly, PTEN expression inhibited basal and HGF-induced cell cycle progression in U87 and A172 cells (**B**). *, $P < 0.05$, relative to Ad-Control; #, $P < 0.05$, relative to Ad-Control + HGF. *Bars,* SE. **C,** PTEN expression was inhibited in wild-type PTEN SF767 cells by siRNA transfection before treatment with HGF. Cell proliferation and cell cycle progression were analyzed as described above. The results show that inhibition of PTEN expression and activation of c-Met additively induce cell proliferation and cell cycle progression. The immunoblots confirm PTEN protein restoration in U87 and A172 and PTEN protein inhibition in SF767 cells. *Ad-con,* control adenovirus. *Bars,* SE.

Published OnlineFirst February 3, 2009; DOI: 10.1158/1535-7163.MCT-08-0627

Ad-PTEN or Ad-Control for 24 h (multiplicity of infection = 10). The cells were subsequently treated with or without 50 ng/mL HGF for different times (5 min-24 h). Cell lysates were extracted and immunoblotted with anti-phosphotyrosine or with antibodies specific to the proteins being investigated. HGF treatment induced tyrosine phosphorylation of various proteins in both cell lines. PTEN restoration inhibited basal as well as HGF-induced tyrosine phosphorylation of some of these proteins in U87 cells and HGF-induced tyrosine phosphorylation in A172 cells (Fig. 3A). This suggests that PTEN inhibits c-Met-dependent signaling by inhibiting various molecules that are activated by HGF. To determine if PTEN directly affects c-Met phosphorylation, we used immunoblotting and coimmunoprecipitation and found that PTEN does not alter c-Met phosphorylation relative to total c-Met and does not coimmunoprecipitate with c-Met (data not shown). Therefore, PTEN appears to act on molecules downstream of c-Met. To identify some of the c-Met-activated molecules that are inhibited by PTEN, the effects of PTEN and HGF on selected proteins known to mediate c-Met functions were investigated. The cells were treated as described above and analyzed for protein levels and activation with immunoblotting using specific antibodies and phospho-antibodies. Treatment of the cells with HGF markedly increased the phosphorylation of Akt, p42/44 MAPK, JNK, GSK-3α/β, and mTOR, induced protein levels of Cdk2 and E2F-1, and inhibited protein levels of p27. PTEN expression

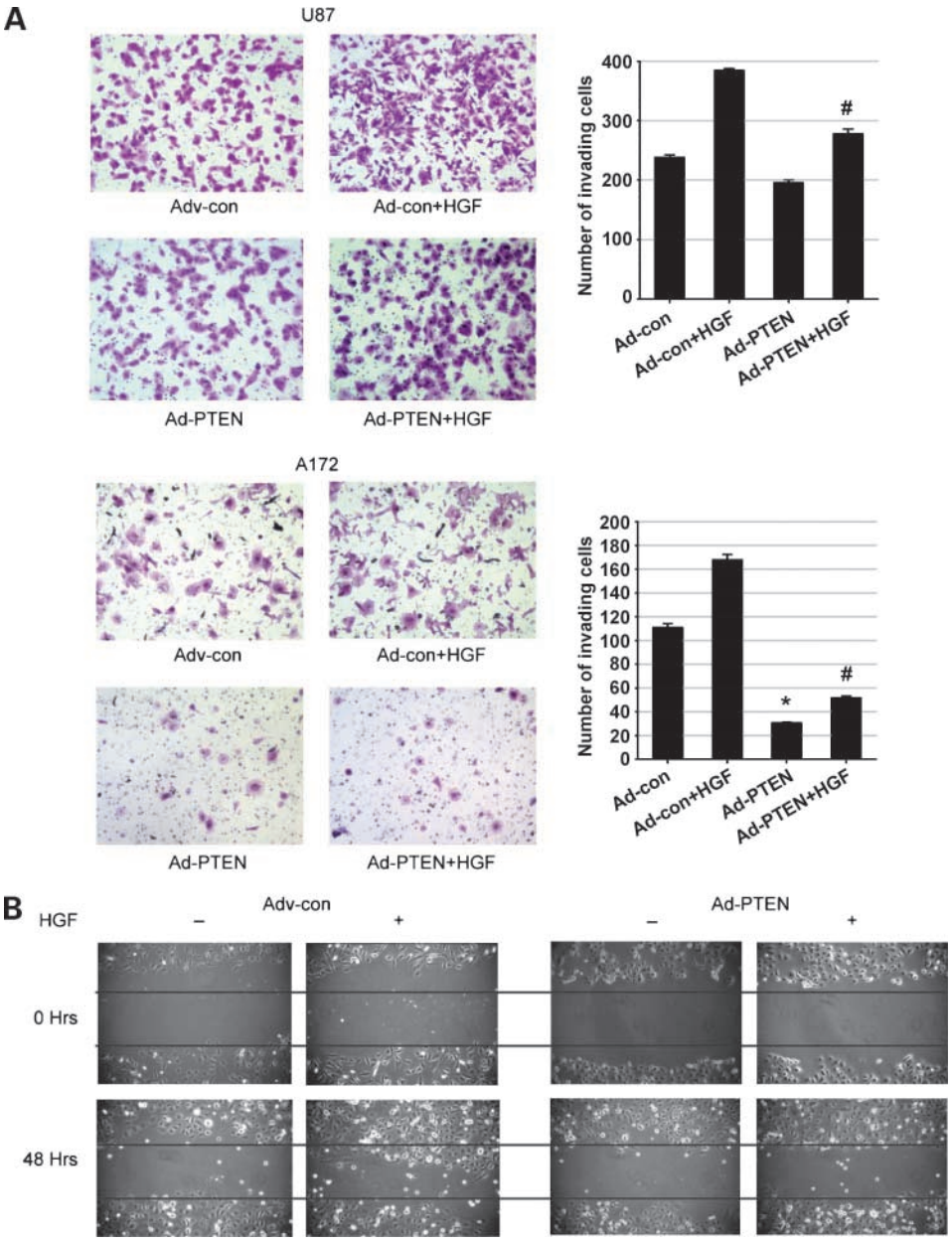

Figure 2. PTEN expression inhibits c-Met-dependent glioblastoma cell invasion and cell migration. PTEN was expressed in PTEN-null U87 and A172 glioblastoma cells via adenovirus (Ad-PTEN) infections before treatment with HGF. The effects of PTEN expression on HGF-induced cell invasion and cell migration were analyzed using a Transwell invasion assay and a migration assay. **A,** PTEN expression inhibited basal and HGF-induced cell invasion in U87 and A172 cells. Photographs show representative stained membranes. Graphs show quantification of invading cells. *, $P < 0.05$, relative to control; #, $P < 0.05$, relative to control + HGF. *Bars,* SE. **B,** migrating U87 cells show that PTEN expression inhibits basal and HGF-induced tumor cell migration.

Published OnlineFirst February 3, 2009; DOI: 10.1158/1535-7163.MCT-08-0627

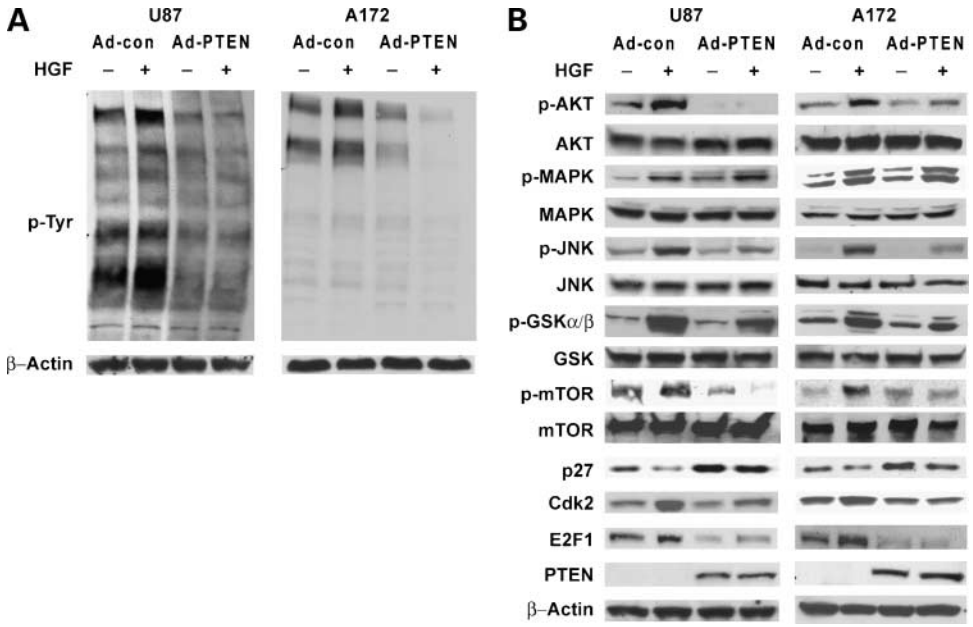

Figure 3. PTEN attenuates c-Met-dependent signal transduction. PTEN-null U87 and A172 cells were infected with adenoviruses encoding PTEN (Ad-PTEN) or control (Ad-control) for 24 h before treatment with HGF for either 5 min (Akt), 15 min (MAPK, JNK, GSK, mTOR, and phospho-Tyr), or 24 h (p27, Cdk2, and E2F-1). **A,** total protein was electrophoretically separated and immunoblotted for phosphotyrosine to determine the effects of PTEN on HGF-induced overall protein tyrosine phosphorylation. The results show that PTEN alters basal and HGF-induced total tyrosine phosphorylation. **B,** cell lysates were immunoblotted for selected signal transduction proteins know to mediate c-Met functions. The results show that PTEN inhibits HGF-induced phosphorylation of Akt, JNK, GSK, and mTOR and the HGF-mediated changes in p27, Cdk2, and E2F-1 expression but does not affect of the phosphorylation of p42/p44 MAPK.

inhibited HGF-induced phosphorylation of Akt, JNK, GSK-3α/β, and mTOR but did not affect HGF-induced phosphorylation of p42/44 MAPK. Consistent with its effects on the cell cycle, PTEN restoration inhibited HGF-induced E2F-1, partially inhibited HGF-induced Cdk2, and blocked HGF-induced inhibition of p27 (Fig. 3B). These data show that PTEN strongly but selectively affects c-Met-dependent signal transduction.

Combination of HGF/c-Met Inhibition and PTEN Restoration or mTOR Inhibition Additively Inhibit Glioblastoma Cell Proliferation and Cell Cycle Progression

Because c-Met activation has greater malignant effects in the setting of PTEN loss, we hypothesized that combining PTEN restoration with HGF/c-Met inhibition might lead to greater antiproliferative effects in glioblastoma. To test this hypothesis, we restored PTEN and inhibited HGF/c-Met singly and in combination and assessed the effects on cell proliferation and cell cycle progression. PTEN was restored by infection of U87 cells with Ad-PTEN, and HGF and c-Met were inhibited by infection of the cells with Ad-U1/HGF and Ad-U1/Met, respectively, as described previously (6, 7). PTEN expression and HGF and c-Met knockdown were verified by immunoblotting. Cell proliferation and cell cycle were assessed as described above. Single inhibition of HGF/c-Met as well as single PTEN restoration led to inhibition of cell proliferation and cell cycle progression (Fig. 4A). Combined inhibition of HGF/c-Met and PTEN restoration led to significantly greater inhibitory effects on cell cycle progression ($n = 9$; $P < 0.05$ for G_0-G_1 fraction) and cell proliferation ($n = 3$; $P < 0.05$ at day 5) compared with single treatment (Fig. 4A).

Because PTEN gene restoration and U1/ribozyme-mediated HGF/c-Met inhibition are presently not clinically applicable approaches, we also tested the combined effects of monoclonal anti-HGF antibody-mediated inhibition of HGF in combination with mTOR inhibition with rapamycin. A humanized version of the monoclonal anti-HGF antibody (L2G7) has been developed and will enter clinical trials in the near future. mTOR inhibition was chosen as the closest clinically useful approach to counteracting some effects of PTEN loss. U87 and A172 cells were treated with L2G7, rapamycin, a combination of both, or control and assessed for cell proliferation. L2G7 or rapamycin single treatment significantly inhibited cell proliferation relative to control in both cell lines. Combination treatment of L2G7 and rapamycin had significantly greater inhibitory effects on tumor cell proliferation than each therapy mode alone ($P < 0.05$, relative to single treatment at day 7 for U87 and day 5 for A172). These data provide for the first time a rationale for testing combinations of anti-HGF/c-Met therapies and anti-mTOR therapies in a clinical setting.

Combinations of HGF Inhibition and PTEN Restoration or mTOR Inhibition Additively Inhibit *In vivo* Glioblastoma Xenograft Growth

To determine if combining PTEN restoration and HGF inhibition have *in vivo* experimental therapeutic advantage, we generated PTEN-expressing U87 clones by stable transfection with plasmids encoding PTEN and control clones by transfection with plasmids encoding empty vector. PTEN expression was verified by immunoblotting. Two PTEN-expressing clones and two control clones were implanted in immunodeficient mice 1 week before treatment with i.p. L2G7 or control for 3 weeks. One week after the last treatment, the animals were euthanized and tumor maximal cross-sectional areas were measured. Both PTEN restoration and L2G7 treatment led to a significant reduction of tumor cross-sectional areas relative to control ($n = 10$; $P < 0.05$, relative to control; Fig. 5A). The combination of PTEN restoration and L2G7 treatment

Published OnlineFirst February 3, 2009; DOI: 10.1158/1535-7163.MCT-08-0627

displayed a significantly greater inhibition of tumor growth than each single treatment ($n = 10$; $P < 0.05$, relative to single treatment). Notably, in the combination group, except for one animal, which developed a very small tumor, no animal showed signs of tumor growth but only scar tissue around the needle track. Therefore, combination therapy led to an additive inhibitory effect on *in vivo* tumor growth and tumorigenicity over single therapies. These data show for the first time that combining PTEN restoration with HGF/c-Met inhibition might have therapeutic value.

Because PTEN expression restoration is currently not clinically feasible, we used mTOR inhibition as an alternative approach to counteracting the effects of PTEN loss in combination with L2G7-mediated HGF inhibition. mTOR is activated by PTEN loss and mediates some of the malignant effects of PTEN loss. Also, it was recently shown that mTOR inhibition leads to Akt activation via a feedback mechanism involving receptor tyrosine kinases, providing an additional rationale for combining HGF/c-Met inhibition with mTOR inhibition. We treated established wild-type U87 glioblastoma xenograft-bearing mice ($n = 10$) with systemic injections of L2G7, the mTOR inhibitor rapamycin, a combination of both, or control for 2 weeks. The animals were euthanized 1 week after the last treatment and tumor sizes were measured. Systemic L2G7 and rapamycin single treatments significantly inhibited tumor growth relative to control ($n = 10$; $P < 0.05$ relative to control). Combined systemic delivery of L2G7 and rapamycin led to significantly greater inhibition of xenograft

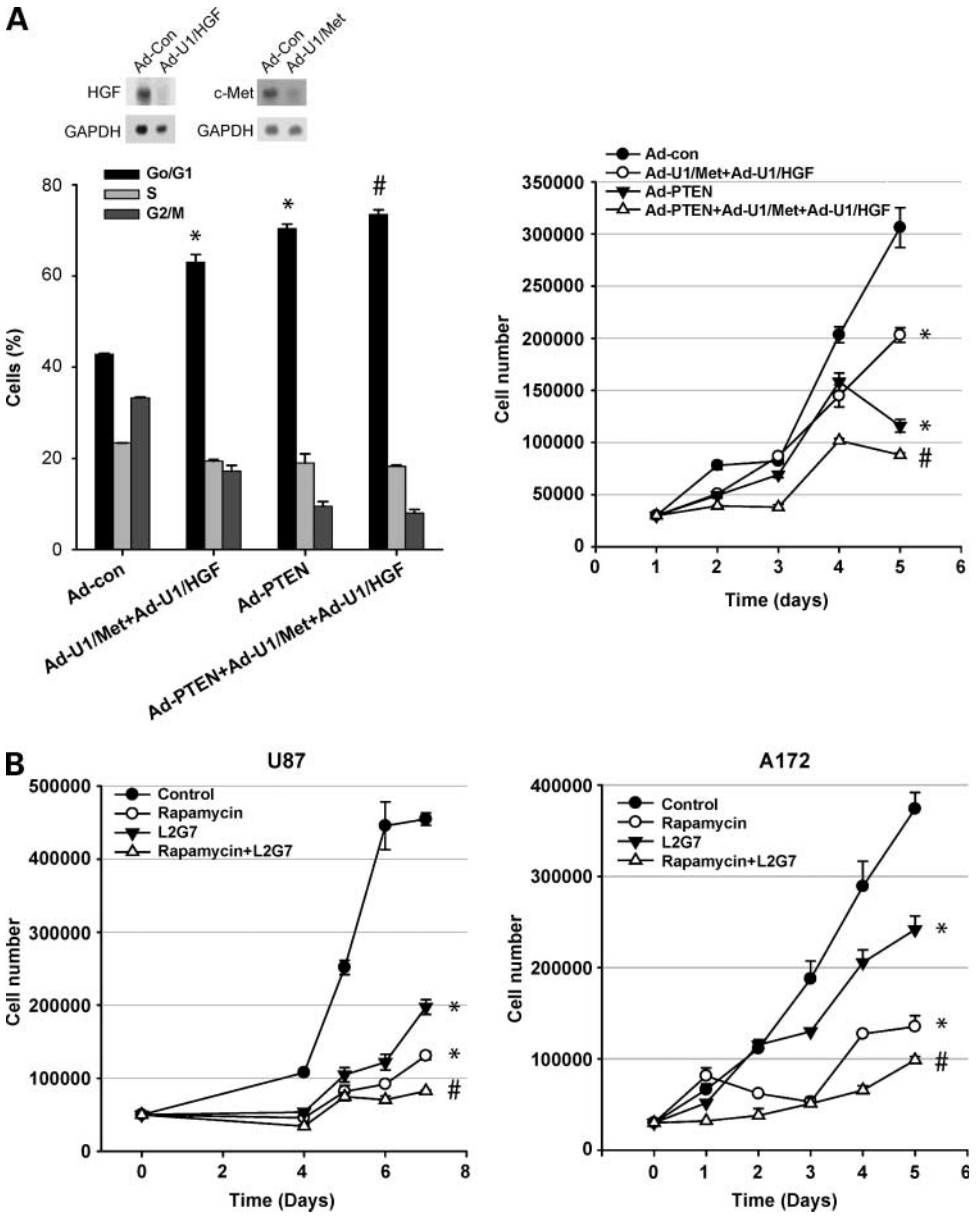

Figure 4. Combined HGF/c-met inhibition and PTEN restoration or mTOR inhibition additively inhibit glioblastoma cell proliferation and cell cycle progression. **A,** U87 cells were infected with adenoviruses encoding anti-HGF and anti-c-Met U1/ribozymes (Ad-U1/ribozymes), PTEN (Ad-PTEN), a combination of both, or control (Ad-Control). Cell proliferation and cell cycle were assessed by cell counting and by propidium iodide flow cytometry, respectively. The results show that combining PTEN restoration with HGF/c-Met expression inhibition additively inhibits glioblastoma cell proliferation and cell cycle progression. The blots confirm HGF and c-Met knockdown in the cells. *, $P < 0.05$, relative to control; #, $P < 0.05$, relative to single treatment. *Bars,* SE. **B,** U87 and A172 cells were treated with anti-HGF monoclonal antibody (L2G7), the mTOR inhibitor rapamycin, a combination of both, or solvent control and assessed for cell proliferation as described above. The results show that combining clinically applicable L2G7-mediated inhibition of HGF with clinically applicable rapamycin-mediated inhibition of mTOR additively inhibits glioblastoma cell proliferation. *, $P < 0.05$, relative to control; #, $P < 0.05$, relative to single treatment. *Bars,* SE.

Published OnlineFirst February 3, 2009; DOI: 10.1158/1535-7163.MCT-08-0627

growth than single treatments ($n = 10$; $P < 0.05$, relative to single treatment; Fig. 5B). Therefore, combination therapy of L2G7 and rapamycin led to an additive inhibitory effect on *in vivo* tumor growth over single therapies. These data show for the first time that combining HGF/c-Met inhibition with mTOR inhibition is worth testing in a clinical setting.

Discussion

Based on its multifunctional and multilevel involvement in human cancer in general and brain tumors in particular, the HGF/c-Met pathway has emerged as a compelling molecular target for therapy. Molecular inhibitors of HGF and c-Met pathway, including neutralizing monoclonal antibodies to HGF and small-molecule inhibitors of c-Met kinase, have been developed (23–25). These agents are under active investigation in preparation for future clinical trials. Because PTEN could interact with c-Met-dependent signaling, the success of anti-c-Met/HGF therapies could depend on the PTEN status of the targeted tumors. Such a dependency was shown for EGFR proangiogenic effects and for anti-EGFR clinical therapies where coexpression of PTEN and EGFRvIII in tumors of human giloblastoma patients was significantly associated with clinical response to EGFR kinase inhibitors (26, 27). The present study sought to investigate the effects of PTEN on c-Met-dependent malignancy and associated molecular events and to determine if combining anti-HGF/c-Met therapies with PTEN functional restoration can lead to improved glioblastoma therapies. We show functional and molecular interactions between PTEN and HGF/c-Met in glioblastoma malignancy. We show that PTEN expression/loss strongly affects c-Met-dependent cell cycle progression, cell proliferation, cell invasion, and cell migration. We find that PTEN strongly but selectively attenuates c-Met-dependent signaling. We also show that combining PTEN restoration or counteracting PTEN loss with mTOR inhibitors with HGF/c-Met inhibition additively inhibits the growth of experimental glioblastoma xenografts.

The effects of PTEN on c-Met-dependent malignancy likely occur at multiple levels. PTEN counteracts the effects of phosphatidylinositol 3-kinase on Akt that is activated by c-Met. This is evidenced by the almost complete inhibition by PTEN of c-Met-induced phosphorylation of Akt and downstream molecules including mTOR. PTEN could also theoretically dephosphorylate proteins that are activated by c-Met as has been reported for Shc, a c-Met docking molecule. PTEN also affects c-Met-dependent expression of genes that regulate malignancy as we have reported previously (13). A combination of the above factors is consistent with the strong effects of PTEN on c-Met-dependent malignancy variables that we describe in the present study. Importantly, this study shows for the first time that combining anti-HGF/c-Met therapies and PTEN restoration or mTOR inhibition has additive antitumor effects in glioblastoma. This finding has preclinical significance as clinically applicable anti-HGF/c-Met agents and

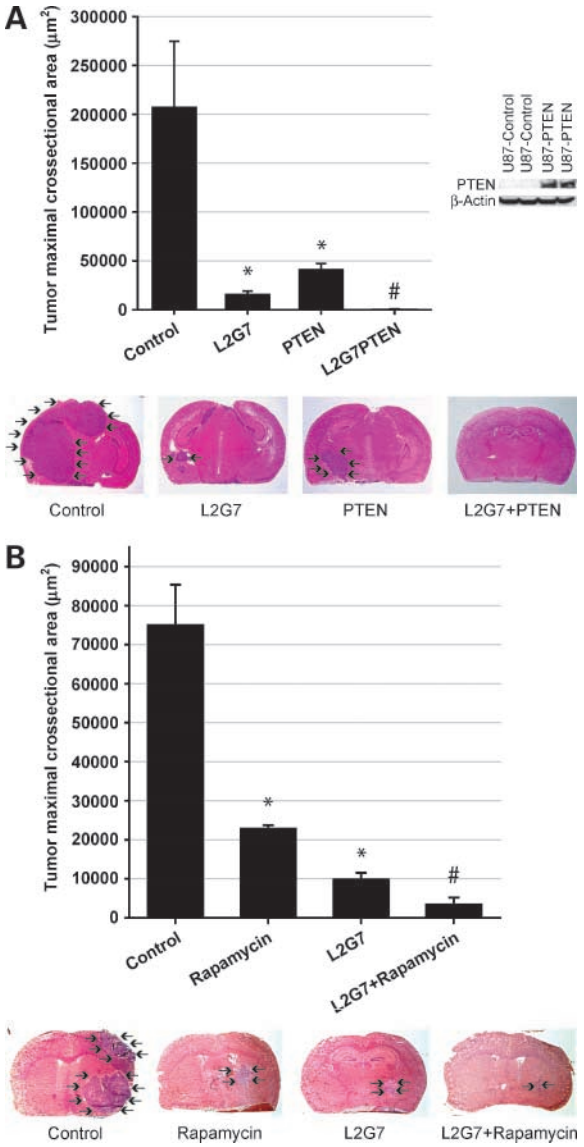

Figure 5. Combined HGF inhibition and PTEN restoration or mTOR inhibition additively inhibit *in vivo* glioblastoma xenograft growth. **A,** PTEN-expressing U87 clones were generated by stable transfection with plasmids encoding PTEN. Two PTEN-expressing clones and two control clones (3×10^5 cells) were implanted in immunodeficient mice brains ($n = 10$) 1 wk before systemic treatment with anti-HGF monoclonal antibody L2G7 for 3 wk. The animals were euthanized 1 wk after the last treatment and tumor cross-sectional areas were measured on H&E-stained brain cross-sections with computer-assisted image analysis. The results show that PTEN restoration and HGF inhibition additively inhibit *in vivo* glioblastoma tumor growth. Immunoblots show PTEN levels in control and PTEN-expressing clones. *Top,* quantification of tumor cross-sectional areas; *bottom,* representative brain tumor cross-sections. *, $P < 0.05$, relative to control; #, $P < 0.05$, relative to L2G7 and PTEN. *Bars,* SE. **B,** wild-type U87 glioblastoma cells (3×10^5) were implanted in immunodeficient mice brains ($n = 10$). One wk post-tumor implantation, the animals were treated with i.p. injections of L2G7, the mTOR inhibitor rapamycin, L2G7 + rapamycin, or control for 2 wk. The animals were euthanized 1 wk after the last treatment and tumor cross-sectional areas were measured on H&E-stained brain cross-sections with computer-assisted image analysis. The results show that HGF and mTOR inhibitions additively inhibit *in vivo* glioblastoma tumor growth. *, $P < 0.05$, relative to control; #, $P < 0.05$, relative to single treatment. *Bars,* SE.

Published OnlineFirst February 3, 2009; DOI: 10.1158/1535-7163.MCT-08-0627

clinically applicable mTOR inhibitors have been developed. mTOR is a downstream target and important mediator of phosphatidylinositol 3-kinase/Akt and is activated after PTEN loss (28). Inhibition of mTOR reverses some of the effects of PTEN loss (29–31). Inhibition of mTOR was shown to synergize with the antiglioma effects of EGFR inhibition (32–34). Because genetic restoration of PTEN is not clinically feasible, we selected mTOR inhibition as a clinically applicable approach to counteracting the effects of PTEN loss. mTOR inhibitors are already in clinical trials of glioma patients (35). Additionally, mTOR inhibition has been shown to induce upstream receptor tyrosine kinase signaling leading to Akt activation, providing additional rationale for combining mTOR inhibitors with anti-HGF/c-Met therapies (36). The monoclonal anti-HGF antibody used in the present study was developed by Galaxy Biotech and shown previously to potently inhibit HGF binding to c-Met as well as glioblastoma cell and xenograft growth (23). In the present study, the antibody was used at a dose that results in maximal antitumor effects against orthotopic U87 glioma xenografts as determined by Galaxy Biotech and by us in pilot experiments (23). This indicates that combining an mTOR inhibitor with an anti-HGF antibody might improve the latter's maximal efficacy against gliomas. This antibody has been humanized and is planned for clinical trials in the near future and a functionally similar anti-HGF mAb (AMG102) is currently in clinical trial for recurrent glioblastoma. Our work provides a rationale for the clinical testing of combinations of anti-HGF/c-Met agents and mTOR inhibitors to achieve better therapeutic outcomes. Our studies also suggest that the PTEN status is likely to affect anti-HGF and anti-c-Met therapies.

Because cancer in general and brain tumors in particular are extremely heterogeneous with multiple molecular dysfunctions and numerous histopathologically distinct entities, therapies that target a single pathway such as HGF/c-Met will at best benefit only a subset of patients. It is likely that HGF/c-Met pathway targeting will be most efficacious in combination with other existing and emerging therapies. Basic and translational research has provided a rationale for combining anti-HGF/c-Met therapies with traditional cytotoxic therapies such as radiotherapy and chemotherapy (37). More recent research showed the redundancy of receptor tyrosine kinase pathways in human cancer and suggested that combining multiple receptor tyrosine kinase inhibitors might be an advantageous therapeutic approach (38). This study extends beyond and complements the above studies by investigating the role a tumor suppressor and its potential targeting in combination with receptor tyrosine kinase pathway inhibition.

In conclusion, we show for the first time in human cancer that PTEN expression affects c-Met-dependent malignancy and associated cell signaling and molecular events and that combining anti-HGF/c-Met approaches with PTEN restoration approaches or mTOR inhibition might have therapeutic value in glioblastoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Lamszus K, Laterra J, Westphol M, Rosen EM. Scatter factor/hepatocyte growth factor content and function in human gliomas. Int J Dev Neurooosci 1999;17:517–30.

2. Rosen EM, Laterra J, Joseph A, et al. Scatter factor expression and regulation in human glial tumors. Int J Cancer 1996;67:248–55.

3. Koochekpour S, Jeffers M, Rulong S, et al. Met and hepatocyte growth factor/scatter factor expression in human gliomas. Cancer Res 1997;57:5391–8.

4. Moriyama T, Kataoka H, Kawano Y, et al. Comparative analysis of expression of hepatocyte growth factor and its receptor, c-met, in gliomas, meningiomas and schwannomas. Cancer Lett 1998;124:149–55.

5. Schmidt MO, Westphal M, Hagel C, et al. Levels of vascular endothelial growth factor, hepatocyte growth factor/scatter factor and basic fibroblast growth factor in human gliomas and their relationship to angiogenesis. Int J Cancer 1999;84:10–8.

6. 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;91:1548–56.

7. Abounader R, Lal B, Luddy C, et al. *In vivo* targeting of SF/HGF and c-met expression via U1snRNA/ribozymes inhibits glioma growth and angiogenesis and promotes apoptosis. FASEB J 2002;16:108–10.

8. Teng DH, Hu R, Lin H, et al. MMAC1/PTEN mutations in primary tumor specimens and tumor cell lines. Cancer Res 1997;57:5221–5.

9. Wang SI, Puc J, Li J, et al. Somatic mutations of PTEN in glioblastoma multiforme. Cancer Res 1997;57:4183–6.

10. Sano T, Lin H, Chen X, et al. Differential expression of MMAC/PTEN in glioblastoma multiforme: relationship to localization and prognosis. Cancer Res 1999;59:1820–4.

11. Tanimura S, Chantani Y, Hoshino R, et al. Activation of the 41/43 kDa mitogen-activated protein kinase signaling pathway is required for hepatocyte growth factor-induced cell scattering. Oncogene 1998;17:57–65.

12. Xiao GH, Jeffers M, Bellacosa A, Mitsuuchi Y, Vande Woude GF, Testa JR. Anti-apoptotic signaling by hepatocyte growth factor/Met via the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase pathways. Proc Natl Acad Sci U S A 2001;98:247–52.

13. Abounader R, Reznik T, Colantuoni C, Martinez-Murillo F, Rosen EM, Laterra J. Regulation of c-Met-dependent gene expression by PTEN. Oncogene 2004;23:9173–82.

14. Li Y, Guessous F, Kwon S, et al. PTEN has tumor-promoting properties in the setting of gain-of-function p53 mutations. Cancer Res 2008;68:1723–31.

15. Li Y, Lal B, Kwon S, et al. The scatter factor/hepatocyte growth factor: c-met pathway in human embryonal central nervous system tumor malignancy. Cancer Res 2005;65:9355–62.

16. Gu J, Tamura M, Pankov R, et al. Shc and FAK differentially regulate cell motility and directionality modulated by PTEN. J Cell Biol 1999;146:389–403.

17. Li Y, Guessous F, Johnson EB, et al. Functional and molecular interactions between the HGF/c-Met pathway and c-Myc in large-cell medulloblastoma. Lab Invest 2008;88:98–111.

18. Brockmann MA, Ulbricht U, Gruner K, Fillbrandt R, Westphal M, Lamszus K. Glioblastoma and cerebral microvascular endothelial cell migration in response to tumor-associated growth factors. Neurosurgery 2003;52:1391–9; discussion 9.

19. Hamasuna R, Kataoka H, Meng JY, et al. Reduced expression of hepatocyte growth factor activator inhibitor type-2/placental bikunin (HAI-2/PB) in human glioblastomas: implication for anti-invasive role of HAI-2/PB in glioblastoma cells. Int J Cancer 2001;93:339–45.

20. Hamasuna R, Kataoka H, Moriyama T, Itoh H, Seiki M, Koono M. Regulation of matrix metalloproteinase-2 (MMP-2) by hepatocyte growth factor/scatter factor (HGF/SF) in human glioma cells: HGF/SF enhances

Published OnlineFirst February 3, 2009; DOI: 10.1158/1535-7163.MCT-08-0627

MMP-2 expression and activation accompanying up-regulation of membrane type-1 MMP. Int J Cancer 1999;82:274 – 81.

21. Abounader R, Ranganathan S, Kim BY, Nichols C, Laterra J. Signaling pathways in the induction of c-met receptor expression by its ligand scatter factor/hepatocyte growth factor in human glioblastoma. J Neurochem 2001;76:1497 – 508.

22. Bowers DC, Fan S, Walter K, et al. Scatter factor/hepatocyte growth factor activates AKT and protects against cytotoxic death in human glioblastoma via PI3-kinase and AKT-dependent pathways. Cancer Res 2000;60:4277 – 83.

23. Kim KJ, Wang L, Su YC, et al. Systemic anti-hepatocyte growth factor monoclonal antibody therapy induces the regression of intracranial glioma xenografts. Clin Cancer Res 2006;12:1292 – 8.

24. Burgess T, Coxon A, Meyer S, et al. Fully human monoclonal antibodies to hepatocyte growth factor with therapeutic potential against hepatocyte growth factor/c-Met-dependent human tumors. Cancer Res 2006;66:1721 – 9.

25. Christensen JG, Schreck R, Burrows J, et al. A selective small molecule inhibitor of c-Met kinase inhibits c-Met-dependent phenotypes *in vitro* and exhibits cytoreductive antitumor activity *in vivo*. Cancer Res 2003;63:7345 – 55.

26. Abe T, Terada K, Wakimoto H, et al. PTEN decreases *in vivo* vascularization of experimental gliomas in spite of proangiogenic stimuli. Cancer Res 2003;63:2300 – 5.

27. Mellinghoff IK, Wang MY, Vivanco I, et al. Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. N Engl J Med 2005;353:2012 – 24.

28. Ozes ON, Akca H, Mayo LD, et al. A phosphatidylinositol 3-kinase/Akt/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling through insulin receptor substrate-1. Proc Natl Acad Sci U S A 2001;98:4640 – 5.

29. Podsypanina K, Lee RT, Politis C, et al. An inhibitor of mTOR reduces neoplasia and normalizes p70/S6 kinase activity in Pten + /- mice. Proc Natl Acad Sci U S A 2001;98:10320 – 5.

30. Grunwald V, DeGraffenried L, Russel D, Friedrichs WE, Ray RB, Hidalgo M. Inhibitors of mTOR reverse doxorubicin resistance conferred by PTEN status in prostate cancer cells. Cancer Res 2002;62:6141 – 5.

31. Hu X, Pandolfi PP, Li Y, Koutcher JA, Rosenblum M, Holland EC. mTOR promotes survival and astrocytic characteristics induced by Pten/AKT signaling in glioblastoma. Neoplasia 2005;7:356 – 68.

32. Goudar RK, Shi Q, Hjelmeland MD, et al. Combination therapy of inhibitors of epidermal growth factor receptor/vascular endothelial growth factor receptor 2 (AEE788) and the mammalian target of rapamycin (RAD001) offers improved glioblastoma tumor growth inhibition. Mol Cancer Ther 2005;4:101 – 12.

33. Rao RD, Mladek AC, Lamont JD, et al. Disruption of parallel and converging signaling pathways contributes to the synergistic antitumor effects of simultaneous mTOR and EGFR inhibition in GBM cells. Neoplasia 2005;7:921 – 9.

34. Reardon DA, Quinn JA, Vredenburgh JJ, et al. Phase 1 trial of gefitinib plus sirolimus in adults with recurrent malignant glioma. Clin Cancer Res 2006;12:860 – 8.

35. Cloughesy TF, Yoshimoto K, Nghiemphu P, et al. Antitumor activity of rapamycin in a Phase I trial for patients with recurrent PTEN-deficient glioblastoma. PLoS Med 2008;5:e8.

36. O'Reilly KE, Rojo F, She QB, et al. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res 2006;66:1500 – 8.

37. Lal B, Xia S, Abounader R, Laterra J. Targeting the c-Met pathway potentiates glioblastoma responses to γ-radiation. Clin Cancer Res 2005;11:4479 – 86.

38. Stommel JM, Kimmelman AC, Ying H, et al. Coactivation of receptor tyrosine kinases affects the response of tumor cells to targeted therapies. Science 2007;318:287 – 90.

Published OnlineFirst February 3, 2009; DOI: 10.1158/1535-7163.MCT-08-0627

Molecular Cancer Therapeutics

Interactions between PTEN and the c-Met pathway in glioblastoma and implications for therapy

Yunqing Li, Fadila Guessous, Charles DiPierro, et al.

Mol Cancer Ther 2009;8:376-385. Published OnlineFirst February 3, 2009.