Glioblastoma multiforme is a WHO Grade IV glial tumor that carries a universally poor prognosis, with a median patient survival of 1 year after diagnosis.5 Aggressive surgery combined with radiotherapy and chemotherapy modestly improves survival. Major therapeutic obstacles include glioma cell resistance to standard treatments, high proliferative capacity, and profound invasiveness of glioma cells.2,3 Despite decades of intense research that has uncovered several molecular mechanisms of glioma cell tumorigenicity, significant clinical improvement has not been realized.19 Therefore, a more complete characterization of the molecular mechanisms of tumorigenicity is necessary to uncover targets for novel therapies. In previous studies, we and others have investigated the expression of the WT1 gene in brain tumors in humans, including gliomas of all grades, and have found detectable expression in tumor cells but not in normal cells, suggesting an important role for WT1 in glioma biology.7,8,22,26 In particular, we have shown that 80% of analyzed human GBM specimens express WT1, whereas normal astrocytes do not.7 We have also shown that WT1 silencing caused significantly slower growth after the subcutaneous inoculation of tumor cells in the flanks of athymic nude mice and was associated with an increased latency period.

**Effect of WT1 gene silencing on the tumorigenicity of human glioblastoma multiforme cells**

**Laboratory investigation**

AARON J. CLARK, M.D., PH.D.,1,2 JOY L. WARRE, PH.D.,3,4 MIKE Y. CHEN, M.D., PH.D.,1,2 MARTIN R. GRAF, PH.D.,2 TIMOTHY E. VAN METER, PH.D.,2 WAGNER G. DOS SANTOS, PH.D.,2 HELEN L. FILLMORE, PH.D.,1,2 AND WILLIAM C. BROADDUS, M.D., PH.D.1,2

Departments of 1Anatomy and Neurobiology, 2Neurosurgery, 3Pathology, and 4Human Genetics, Virginia Commonwealth University, Medical College of Virginia Campus, Richmond, Virginia

Object. Wilms tumor 1 (WT1) is overexpressed in many human cancers, including glioblastoma multiforme (GBM). In another study, the authors showed that transient WT1 silencing increases the radiosensitivity of glioma cells. Studies of nonglioma cell lines have demonstrated that WT1 promotes cell proliferation and survival; however, this ability has not been rigorously analyzed in human GBM.

**Methods.** The authors tested the efficacy of 2 sequences of short hairpin RNA (shRNA) directed against WT1 in U251MG human GBM cells and found that 1 sequence was capable of stably silencing WT1 expression. They then evaluated the effect of WT1 silencing on cellular proliferation, invasion, and in vivo tumor formation.

**Results.** Stable WT1-shRNA expression significantly decreased the proliferation of U251MG cells in vitro as demonstrated by both an adenosine 5′-triphosphate–based viability assay and tritiated thymidine uptake. Furthermore, stable WT1 silencing caused significantly slower growth after the subcutaneous inoculation of U251MG cells in the flanks of athymic nude mice and was associated with an increased latency period.

**Conclusions.** Data in this study provide proof of the principle that downregulation of WT1 causes decreased tumorigenicity of a GBM cell line in vitro and in vivo and suggest that WT1 is a promising target for novel molecular GBM therapies, perhaps in combination with standard treatment modalities. (DOI: 10.3171/2008.11.JNS08368)

**KEY WORDS** • glioblastoma multiforme • Wilms tumor 1 • tumorigenicity • short hairpin RNA • nude mice

**Abbreviations used in this paper:** ANOVA = analysis of variance; ATP = adenosine 5′-triphosphate; GBM = glioblastoma multiforme; GFP = green fluorescent protein; PBS = phosphate-buffered saline; SDS = sodium dodecyl sulfate; shRNA = short hairpin RNA; WT1 = Wilms tumor 1; [3H]Td = tritiated thymidine.
Furthermore, the mRNA is alternatively spliced to generate 4 main isoforms, designated +/+, −/−, −/+ or +/−, depending on the inclusion or exclusion of exon 5 and 9 nucleotides in exon 9 known as KTS.\textsuperscript{11} We have shown that gliomas of all grades, including GBM, and glioma cell lines express the WT1(+/KTS) isoforms.\textsuperscript{2} These isoforms may be involved in RNA processing in addition to having both overlapping and unique transcriptional properties relative to the WT1(−/KTS) isoforms.\textsuperscript{16,21} The functional significance of exon 5 is less well understood but may affect its interaction with other proteins.\textsuperscript{28}

Although originally classified as a tumor suppressor, WT1 was later found to be overexpressed in many types of cancer, including breast carcinoma and acute leukemia, suggesting an oncogenic function for the gene.\textsuperscript{14,24} The overexpression of WT1 causes an increase in cancer cell proliferation,\textsuperscript{33} whereas WT1 suppression in human breast cancer and leukemia cell lines by using antisense modalities leads to differentiation, decreased proliferation, and increased sensitivity to treatment.\textsuperscript{1,35} Of note, in vitro studies of cell lines derived from solid tumors, including a glioma cell line, have shown that WT1 silencing with WT1-shRNA induced apoptosis.\textsuperscript{32} Especially relevant to gliomas, which cause death by the direct invasion and destruction of normal brain tissue, WT1 overexpression recently has been shown to increase the invasion of ovarian cancer cells.\textsuperscript{15} Based on the studies described above, we hypothesized that WT1 downregulation would decrease the tumorigenicity of glioma cells. To test this hypothesis, we used U251MG human GBM cells, which express moderate levels of endogenous WT1. We generated U251MG cells stably transduced with either empty vector or WT1-shRNA and examined the effect on proliferation, invasion, and in vivo tumor growth.

\section*{Methods}

\subsection*{Plasmid Construction}

Short interfering RNA (Dharmacon) was tested for efficacy in cell culture per the manufacturer’s protocol (data not shown). Forward and reverse oligos were generated using the provided sequence. Plasmid WT1-shRNA expression vectors were constructed according to the manufacturer’s instructions (pSUPER.retro.neo+gfp, OligoEngine). Briefly, the pSUPER vector was linearized using HindIII and BclIII restriction enzymes. The annealed forward and reverse oligos were ligated into the linearized vector with T4 DNA ligase (New England Biolabs, Inc.) to generate either empty pSUPER vector or pSUPER.shWT1 expression vector. The vectors were then used to transform DH5α bacteria (Invitrogen) selected with ampicillin. Plasmid purification was performed using a plasmid mini kit (Qiagen). The presence of positive clones was confirmed by EcoRI and HindIII digestion and sequencing. The packaging cell line 293T was then transfected with either empty pSUPER vector or pSUPER.shWT1 vector in addition to plC-ampho packaging vector and pME-VSVg envelope vector by using FuGENE 6 transfection reagent according to the manufacturer’s protocol (Roche). After a 2-day incubation at 37°C, medium containing the retrovirus was harvested, filtered, and stored in aliquots at −80°C.

\subsection*{Cell Culture, Viral Transduction, and Sample Preparation}

The U251MG human malignant glioma cells were obtained from the American Type Culture Collection and were grown and passaged in Dulbecco modified Eagle medium containing 10% fetal bovine serum, 1% glutamine, 1% nonessential amino acids, and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO\textsubscript{2}. The LN-18 human malignant glioma cell lines were kindly provided by Dr. Erwin Van Meir (Emory University) and grown in a similar fashion. For transduction, cells were plated in 6-well plates at a density of 0.25 × 10\textsuperscript{6} cells per well and allowed to attach overnight. Medium was then replaced with 0.5 ml of infection medium (without fetal bovine serum) containing virus. Cells were incubated with gentle agitation for 2 hours, at which time 1.5 ml of medium (with serum) was added to the cells. After viral transduction, cells were maintained in Dulbecco modified Eagle medium supplemented with 300 μg/ml of G418 (Geneticin), which was exchanged every 3 days with fresh selection medium. Total RNA was isolated from cell lines using the Trizol extraction protocol (Invitrogen). The concentration of RNA was determined using spectrophotometry. Protein was extracted from cell lines using SDS buffer (50 mM Tris-Cl, 1% SDS, and 10% glycerol) supplemented with protease inhibitors. The concentration of protein was determined by DC protein assay (detergent compatible protein assay, BioRad).

\subsection*{Western Blot Analysis}

Twenty to 40 μg of protein was separated on a 4–12% Bis-Tris NuPAGE gel and subjected to electrophoresis per the manufacturer’s instructions (Invitrogen). Protein was transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk solution for 1 hour at room temperature. Mouse anti–WT1 monoclonal antibody (1:200 dilution, DakoCytomation) was diluted in blocking buffer. The membranes were incubated with primary antibodies overnight at 4°C and were washed 6 times in Tris-buffered saline containing 0.05% Tween-20 before and after a 1-hour room-temperature incubation with horseradish peroxidase–conjugated anti–mouse secondary antibody (1:1000, Rockland Immunochemicals, Inc.). Blots were developed using an enhanced chemiluminescence detection system (Amersham Biosciences). As a control for protein loading we used anti–cyclophilin A monoclonal antibody (1:1000, Upstate Biotechnology). Densitometric analysis normalizing WT1 protein expression to that of a loading control, cyclophilin A, was performed using ImageJ software (National Institutes of Health).

\subsection*{Proliferation Assays}

Cells were plated in 5 wells of 96-well tissue culture plates at a density of 0.5 × 10\textsuperscript{4} cells per well. Cell viability was measured using the CellTiter-GLO luminescent cell viability assay (Promega) at designated time points after plating. Relative luminescence was detected on a
LUMistar luminescence plate reader (BMG Labtech). Tritiated thymidine uptake was analyzed by plating in quintuplicate 0.5 × 10^{3} cells per well in 96-well, flat-bottom tissue culture plates and pulsing with 1 μCi of [3H]TdR at 4 days after plating (Amersham Biosciences). Cells were cultured for an additional 18 hours and then stored at −80°C. The incorporation of [3H]TdR was used as a measure of proliferation and was analyzed using a 96-well plate harvester and a beta-plate reader (Packard Instrument). Data are expressed as the mean counts per minute of the quintuplicate experimental cultures.

Invasion Assay

A modified 96-well chemotaxis assay (Neuroprobe, Inc.) was used to assess tumor cell invasiveness. Assay chambers consisted of a fitted manifold with a porous polycarbonate membrane (8-μm pores), which had been precoated for 45 minutes at 37°C with 25 μg/ml of growth factor-reduced Matrigel (50 μl/well, BD Biosciences). Cells were plated in 5 wells at a density of 0.5 × 10^{4} cells per well. After 48 hours, cells that had not invaded the membrane were removed from the upper surface of the manifold by gently wiping with a sterile swab. An EDTA/PBS solution (0.05 M) was added to each well and incubated at 37°C for 15 minutes to detach cells that had invaded the membrane but remained attached. Cells were collected by centrifugation of the plate at 300 G. Cell growth medium was removed from the lower chamber, and fresh medium added to each well. Collected cells in the lower chamber were lysed for 10 minutes in CellTiter-GLO ATP viability assay reagents, following the manufacturer’s protocol (Promega). Cell lysates were transferred to opaque, white-walled 96-well plates, and relative luminescence was detected on a LUMistar luminescence plate reader (BMG Labtech). Two independent assays were replicated.

In Vivo Tumor Growth in Nude Mice

For inoculation, cells were first washed with sterile PBS and harvested using trypsin-EDTA treatment. Dispersed cells were resuspended in sterile PBS and adjusted to 3 × 10^{5} cells/ml, and 200 μl (6 × 10^{5}) cells of the cell suspension was injected subcutaneously in the dorsal region of 4- to 6-week-old male athymic BALB/c (nu/nu) mice (Harlan Sprague Dawley, Inc.). For each of the 3 cell lines, 5 mice were inoculated. Four days after inoculation, tumor size was measured every 2 days for 6 weeks by using a dial caliper, and tumor volumes were calculated as (length × width^{2})/2. Mice were killed when the tumor reached 800 mm^{3}. The tumors were dissected from the skin and underlying muscle, snap frozen, and stored at −80°C. Tissue was homogenized and protein extracted using SDS buffer and sonication. Two independent experiments were replicated for a total of 30 mice.

Histological Analysis

Tumor tissues were fixed in formalin, embedded in paraffin, and 5-μm serial sections were prepared. A representative section was stained with H & E for histopathology. All slides were read in a blinded manner. Microscopy was performed at the Virginia Commonwealth University Microscopy Facility.

Statistical Analysis

Differences between 2 groups were analyzed using the Student t-test. Differences among 3 groups were calculated using an ANOVA, followed by the Tukey-Kramer honestly significant difference (HSD) test as a post hoc analysis. Differences between in vivo growth rates were analyzed using repeated-measures ANOVA.

Results

Decreased Proliferation by WT1-shRNA

We have shown that LN-18 cells express WT1(+KTS) protein, but we were also interested in studying a glioma cell line that expressed high levels of WT1 and was capable of forming tumors in a mouse model. The U251MG cells expressed high levels of WT1 (Fig. 1). Sequencing data showed that, similar to other glioma cell lines, U251MG cells expressed +KTS isoforms of WT1 (data not shown). The doublet on Western blot represents the WT1(+/+) and WT1(−/−) isoforms, which include or exclude exon 5 based on an alternative splicing event. We tested 2 sequences of shRNA targeting WT1—referred to as 2N and 4N—to determine the efficacy of WT1 silencing in both U251MG and LN-18 cell lines. After 3 weeks of maintenance in selection medium, protein was extracted and analyzed by Western blot for WT1 expression. Viral transduction with 4N shRNA resulted in a marked decrease in WT1 protein expression in both cell lines relative to cells transduced with empty vector, whereas transduction with 2N did not (Fig. 2A). All subsequent experiments utilized 4N shRNA-transduced cells (now referred to as “U251.Sh” and “LN18.Sh”). Each treatment group was maintained as a pooled population of cells instead of selecting individual clones to eliminate the possibility of clonal differences in growth characteristics affecting the growth measurements. Viral transduction with either empty vector or WT1-shRNA did not cause an observable change in the morphological features of the cells relative to their respective parental cell line (Fig. 2B). In U251MG cells transduced with either empty vector (U251.VC) or WT1-shRNA–containing vector, the majority of cells expressed GFP, demonstrating stable expression of the plasmid vector (Fig. 2C). The effect of WT1 silencing on in vitro proliferation was examined using an ATP-based viability assay at Days 0, 1, 3, 5, and 7 after inoculation. The U251.Sh cells proliferated at a significantly slower rate than the U251.VC cells (p < 0.0003; Fig. 2D). Tritiated thymidine incorporation performed on Day 5 confirmed the decrease in proliferation (p = 0.0003; Fig. 2E). The WT1 knockdown was maintained throughout these experiments. The WT1 silencing in LN-18 cells caused a less dramatic but statistically significant decrease in proliferation over the course of 5 days (Fig. 2F).

Silencing of WT1 Decreased Invasion

Glioblastoma multiforme cell invasion into normal brain is a major contributor to the malignancy of these
Effect of WT1 silencing on GBM tumorigenicity

![Fig. 1. Gel blots demonstrating LN-18 and U251MG GBM cell expression of WT1 protein. Forty micrograms of protein extracted from the U251MG and PC3 cells was separated using SDS–polyacrylamide gel electrophoresis and examined for WT1 expression by Western blot. The PC3 prostate carcinoma cell extract was used as a positive control for WT1 expression. The U251MG cells expressed high levels of WT1 protein. The anti–cyclophilin A (CypA) antibody demonstrated equal protein loading in all lanes.](image)

tumors. We therefore analyzed the effect of WT1 silencing on GBM cell invasion by using an in vitro Matrigel invasion assay. After 48 hours, 63% fewer viable U251.Sh cells were present in the lower chamber of the in vitro invasion assay apparatus relative to the U251.VC cells (p = 0.01; Fig. 3).

**Silencing of WT1 Decreased Growth of Tumor Xenografts in Nude Mice**

The definitive test of tumorigenicity is in vivo tumor formation. To test tumorigenicity, the flanks of 5 nude mice were subcutaneously inoculated with U251.Sh cells, and these animals were compared with 5 mice inoculated with U251 parental cells and 5 inoculated with U251.VC cells. The experiment was replicated twice (Fig. 4B). The U251 parental cells and U251.VC cells formed tumors within a very short latency period. The U251.Sh tumors remained undetectable for a significantly longer period of time relative to either the U251 parental cells or U251.VC cells (9 vs 4 days, p = 0.01). Eventually tumors formed in 100% of the animals inoculated with any of the 3 cell lines. On all days, mice bearing the U251.Sh tumors had visibly smaller lesions than those with either U251 parental or U251.VC tumors (Fig. 4A). At 22 days postinoculation, when the first animal was killed with a tumor > 800 mm³, the U251.Sh tumors were significantly smaller than the U251 parental or U251.VC tumors (U251.Sh: 156.0 mm³, U251: 570.6 mm³, and U251.VC: 720.5 mm³; p = 0.0004). The U251 parental and U251.VC tumors were not significantly different (p > 0.05). An analysis of the growth curves revealed a significant decrease in the growth rate of the U251.Sh tumors relative to the U251 parental and U251.VC tumors (p < 0.0001; Fig. 4B left). Despite differences in tumor growth rates, 100% of the lesions reached 800 mm³ by 34 days postinoculation, and the animals were killed. Note, however, that the U251.Sh tumors reached 800 mm³ in 31.3 days, whereas the U251 parental and U251.VC tumors did so in 26.4 and 23.6 days, respectively (p = 0.0028). A second independent experiment was performed on the same number of animals with similar results (Fig. 4B right). Although U251.Sh tumors grew at a rate significantly different from the U251 parental and U251.VC tumors (p < 0.0001), in the second replicated experiment the U251 parental and U251.VC tumors grew at a significantly different rate (p < 0.0001). Histological analysis of a subset of tumors indicated that all examined lesions were densely cellular (Fig. 4C). However, Ki 67 and proliferating cell nuclear antigen immunohistochemistry failed to demonstrate a difference in proliferation between the groups, suggesting that all tumors were proliferating at an equivalent rate when the mice were killed (data not shown). Because the U251.Sh tumors eventually formed large tumors, Western blotting was performed on the excised tumors and probed for WT1 expression to determine if WT1 downregulation by shRNA expression had been lost. While WT1 silencing was maintained at the time of inoculation in cell lines growing in culture, all U251.Sh tumors regained WT1 expression by the time they had reached 800 mm³ (Fig. 4D). Interestingly, WT1-shRNA tumors appeared to re-express a higher level of the WT1(–exon 5) isoform, as demonstrated by the higher intensity of the lower molecular weight band. Consistent with this observation, WT1 immunohistochemistry demonstrated equivalent WT1 protein expression in tumor specimens representative of the 3 treatment groups (data not shown).

**Discussion**

To examine the effect of WT1 expression on GBM cell tumorigenicity, we generated U251MG and LN-18 human GBM cells stably expressing shRNA targeting WT1. Viral transduction with WT1-shRNA was capable of stably silencing the WT1 gene over the course of multiple passages. Silencing of WT1 significantly decreased the proliferation of U251MG and LN-18 cells in culture. Interestingly, we observed a decrease in viable cells that had migrated through a Matrigel-coated membrane, suggesting that WT1 silencing decreased the invasive potential of U251MG cells. Most importantly, WT1 silencing was associated with increased tumor latency and decreased growth rate in vivo after inoculation in athymic nude mice.

The role of WT1 in GBM has not been extensively characterized. The results of the present study suggest that one function of WT1 in GBM is to maintain the high proliferative rate characteristic of the tumor. One previous study has documented a decrease in viability 72 hours after treatment of U87MG, A172, and T98G GBM cells by using WT1 antisense oligodeoxynucleotides. Although these results correlate well with the cell proliferation findings presented here, we have shown in this study and others that U87MG cells do not express endogenous WT1. Moreover, this paper does not include a depiction of U87MG WT1 protein expression or the efficiency of WT1 knockdown in any of the cell lines ex-
More recently, a study by Tatsumi et al.\textsuperscript{32} has demonstrated that the transfection of A172 glioma cells with WT1-shRNA causes a decrease in WT1 expression, which is associated with an increase in mitochondrial damage and apoptosis. This finding suggests that, in addition to proliferation, the difference in observed viability could be due in part to apoptotic effects. Likewise, in models of neuronal differentiation, WT1 maintains cells in an undifferentiated state.\textsuperscript{17,34} Silencing of WT1 could promote a more differentiated phenotype of astrocytoma cells with a lower proliferative capacity. In addition, we have observed an increase in autophagy after WT1 silencing, which could contribute to the decreased growth rate observed (our unpublished data, 2007). Interestingly, we did not observe an in vitro or in vivo morphological change after stable silencing of WT1. More consistent with this finding would be a change in the cell cycle. Studies of other cancer cell types support a role for WT1 in increasing proliferation. The inhibition of WT1 expression by antisense oligodeoxynucleotides in K562 and MM6 leukemia cell lines causes decreased proliferation and viability. Zapata-Benavides et al.\textsuperscript{35} have shown that WT1 antisense oligodeoxynucleotides treatment of breast cancer cell lines causes decreased proliferation, which is associated with decreased cyclin D1; however, direct transactivation is not demonstrated. Furthermore, in MCF-7 and MDA486 breast cancer cells, WT1 directly upregulates the expression of the protooncogene c-myc, a known stimulator of proliferation.\textsuperscript{12} These in vitro functional studies are potentially clinically relevant as high WT1 expression in both leukemia and breast cancer correlates with a worse prognosis.\textsuperscript{14,24}

![Graph of the ATP-based cell viability assay performed on U251MG cells on Days 0, 1, 3, 5, and 7 after plating showing the decrease in proliferation of the U251.Sh cells relative to cells transduced with empty vector (U251.VC). *p < 0.0003.](image1.png)

**Fig. 2.** Silencing of WT1 decreased in vitro cell proliferation. A: Gel blots showing U251MG and LN-18 cells virally transduced with expression vectors containing GFP and 1 of 2 sequences (2N and 4N) of WT1-shRNA. After several weeks of selection pressure, protein was extracted from the pooled population and analyzed for WT1 silencing by Western blot. B: Phase-contrast microscopy images demonstrating the structure of parental and virally transduced cells. Original magnification × 10. C: Fluorescence microscopy images (upper) revealing that the majority of U251MG cells transduced with the expression vector stably expressed GFP. Light microscopy images (lower) for comparison. Original magnification × 40. D: Graph of the ATP-based cell viability assay performed on U251MG cells on Days 0, 1, 3, 5, and 7 after plating showing the decrease in proliferation of the U251.Sh cells relative to cells transduced with empty vector (U251.VC). *p < 0.0003. E: Bar graph of results of the [3H]Tdr uptake assay performed on Day 5 after plating confirming that the U251.Sh cells proliferated more slowly. *p = 0.0003. F: Graph of an ATP-based cell viability assay demonstrating the proliferation of LN18.Sh cells on Days 0, 1, 3, and 5 after plating relative to the LN18.VC cells. *p < 0.05.
Effect of WT1 silencing on GBM tumorigenicity

Utilizing a 2-chamber in vitro cellular invasion assay, we observed a significant decrease in the viable cells in the lower chamber. Our results suggested a role for WT1 in GBM cell invasion. However, given the strong antiproliferative effect of WT1 silencing observed in U251MG cells, we cannot discount the possibility that the difference in viable cells that had migrated through the Matrigel-coated membrane was due to a difference in proliferation rather than invasion. Although the relationship between WT1 and GBM cell invasion has not been reported, recent evidence suggests a role for WT1 in cancer cell migration and invasion. Jomgeow et al. have demonstrated that WT1 increases the invasion of TYK ovarian cancer cells. The overexpression of WT1 causes an increase in cell invasion as measured by an in vitro invasion assay; this is associated with a change in structure possibly due to WT1-mediated changes in cytoskeletal components, a decrease in α-actinin and cofilin expression, and an increase in gelsolin expression. A separate study has demonstrated that WT1 regulates the E-cadherin promoter, a cell adhesion molecule involved in cancer cell invasion.

Studies to confirm the role of WT1 in GBM cell invasion and to determine the underlying molecular mechanism are ongoing in the laboratory.

Authors of several studies have examined the effect of WT1 overexpression on in vivo tumor growth in nude mice, but the results have been contradictory. The WT1(−KTS) isoform expression in transformed baby rat kidney cells increases the tumor growth rate, whereas the WT1(+KTS) isoforms suppress tumor growth. Transfection of all isoforms of WT1 in Wilms tumor cells increases tumor latency and decreases growth rate. The stable expression of WT1(−KTS) in prostate cancer cells decreases in vivo tumorigenicity, which is associated with increased apoptosis and decreased expression of the WT1 target Bcl-2. In contrast to the in vitro breast cancer cell data described above, the transfection of MDA-MB-231...
breast cancer cells with WT1 completely prevents tumor formation in nude mice, which is associated with decreased β-catenin expression and activity. Likewise, the subcutaneous or intraperitoneal injection of M1 murine leukemia cells overexpressing WT1(+KTS) results in decreased tumor formation in severe combined immunodeficient mice. To our knowledge, ours is the first study to be focused on the effect of WT1 silencing on in vivo tumor growth. The results of our study relative to others may be indicative of the well-described cell type–specific responses of WT1 or may reflect differences inherent in comparing the response of overexpression of an exogenous applied gene versus downregulation of an endogenously expressed gene. Although the results of our study are striking, the effect of WT1 silencing on the intracranial growth of glioma cells has not been addressed. We used the subcutaneous flank tumor as a preclinical model as we were primarily interested in evaluating the affect of WT1 silencing on tumor growth rate, and the flank tumor allows simple, frequent tumor measurements. The U251MG cells readily form tumors intracranially, and thus not only include the unique brain microenvironment, but also provide the opportunity to study intracranial delivery techniques. Currently, mouse xenograft models can accurately recapitulate the malignant glioma phenotype. An additional benefit is the marked invasive potential of some of the tumor cell lines. We have determined that similar to traditional GBM cell lines, several GBM xenografts cell lines express WT1 and have successfully used the same shRNA technique to silence the WT1 gene in GBM xenograft cell lines (data not shown). Experiments utilizing these models are currently ongoing in our laboratory.

Although stable expression of WT1-shRNA caused increased tumor latency and decreased tumor growth in vivo, the U251.Sh tumors reached 800 mm³ within 34 days of inoculation. The outgrowth of a tumor cell subpopulation could explain the accelerated growth rate after a latency period. In fact, when harvested, all tumors had regained WT1 expression, as demonstrated by Western blot. Protein contamination by nontumor cells in the tissue sample was not likely responsible for this observation as histological examination showed that anaplastic tumor cells comprised the majority of the specimen. This recovered ability to express WT1 suggests that the in vivo environment provided selective pressure for the outgrowth of clones, which had lost WT1-shRNA expression allowing increased proliferation. This finding clearly demonstrates the importance of WT1 in supporting GBM cell proliferation and provides further evidence in support of an oncogenic function of WT1 in GBM. Future studies focused on modalities capable of maintaining full WT1 silencing may be able to halt in vivo experimental GBM growth.

Conclusions

A GBM is a genetically heterogeneous tumor with many overlapping pathways apparently leading to a similar clinical outcome; therefore, molecular changes such as epidermal growth factor receptor overexpression or amplification and p53 mutation, although having an established functional importance in the biology of gliomas, do not correlate with prognosis. Nevertheless, our study demonstrates that anti-WT1 therapy may be a promising component of directed molecular GBM treatment. The WT1 gene is expressed by > 80% of GBM specimens and is not expressed by normal cells. In glioma progression, WT1 appears to be expressed early and thus may be a target for early treatment before the development of GBM.

Disclosure

The research presented in this paper was supported in part by a Medical Student Summer Fellowship from the American Brain Tumor Association and by the Hord Fund of the Medical College of Virginia Foundation. The Virginia Commonwealth University Microscopy Facility is supported in part with funding from National Institute of Neurological Disorders and Stroke Center Core Grant No. SP30NS047463.

Acknowledgments

The authors thank Amanda Richardson for invaluable technical assistance, and Harold F. Young, M.D., for critical support and advice.

References

11. Haber DA, Sohn RL, Buckler AJ, Pelletier J, Call KM, Hous-


---

Accepted November 4, 2008.
Please include this information when citing this paper: published online April 24, 2009; DOI: 10.3171/2008.11.JNS08368.

Address correspondence to: William C. Broaddus, M.D., Ph.D., Department of Neurosurgery, Virginia Commonwealth University, P.O. Box 980631, Richmond, Virginia 23298-0631. email: wcbroadd@vcu.edu.