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Archana Chidambaram

Helen L. Fillmore
Old Dominion University

Timothy E. Van Meter

Catherine I. Dumur

William C. Broaddus

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Novel report of expression and function of CD97 in malignant gliomas: correlation with Wilms tumor 1 expression and glioma cell invasiveness

Laboratory investigation

Archana Chidambaram, Ph.D.,[1] Helen L. Fillmore, Ph.D.,[2,3] Timothy E. Van Meter, Ph.D.,[1,2] Catherine I. Dumur, Ph.D.,[4] and William C. Broaddus, M.D., Ph.D.[1,2]

Departments of [1]Anatomy and Neurobiology, [4]Pathology, and [2]Neurosurgery/Harold F. Young Neurosurgical Center, Virginia Commonwealth University, Medical College of Virginia Campus, Richmond; and [3]Office of Research, Old Dominion University, Norfolk, Virginia

Object. The Wilms tumor 1 (WT1) protein—a developmentally regulated transcription factor—is aberrantly expressed in gliomas and promotes their malignant phenotype. However, little is known about the molecular allies that help it mediate its oncogenic functions in glioma cells.

Methods. The authors used short interfering RNA (siRNA) to suppress WT1 expression in glioblastoma (GBM) cells and evaluated the effect of this on GBM cell invasiveness. Gene expression analysis was then used to identify the candidate genes that were altered as a result of *WT1* silencing. One candidate target, *CD97*, was then selected for further investigation into its role by suppressing its expression using siRNA silencing, followed by proliferation and invasion assays.

Results. WT1 levels were reliably and reproducibly suppressed by siRNA application. This resulted in a significant decrease in cellular invasiveness. Microarray analyses identified the gene products that were consistently downregulated (27) and upregulated (11) with *WT1* silencing. Of these, *CD97* expression was consistently suppressed across the 3 different GBM cell lines studied and was found on further investigation to significantly impact GBM cell invasiveness.

Conclusions. Although *CD97* expression in gliomas has not been described previously, we conclude that the possible upregulation of *CD97* mediated by WT1 promotes cellular invasiveness—one of the most characteristic and challenging aspects of glial tumor cells. Further studies are needed to clarify the nature of this regulation and its impact, as CD97 could represent a novel target for antiglioma therapies.
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Key Words • **Wilms tumor 1** • **glioblastoma** • **invasiveness** • **target genes** • **oncology**

The WT1 protein was originally believed to function as a tumor suppressor. Early studies showed that the protein repressed the transcription of several oncogenic proteins, such as PDGF-A, transforming growth factor–β, EGF receptor, and insulin-like growth factor–I receptor, and increased the expression of genes that prevented malignant transformation, such as *E-cadherin*, and proapoptotic proteins (reviewed by Yang et al.[46]). However, subsequent studies showed that the ability of *WT1* to transcriptionally activate or repress a putative target depended largely on which cell type was being used, the isoforms studied, the number of WT1 binding sites present on the target promoter, and other factors (reviewed by Yang et al.[46]). Moreover, the escalating incidence of its discovery in malignant cells from several different tissue types (reviewed by Sugiyama[37]) implicated WT1 in oncogenesis. A key regulator of various developmental processes, WT1 expression was known to be switched off as tissues developed into the normal adult stage.[15,16,19,35] However, wildtype WT1 was found to be expressed in leukemias and other hematopoietic malignancies, lung and breast cancers, gastrointestinal cancers, sarcomas, tumors in the head and neck, reproductive organ neoplasms, and gliomas (reviewed by Sugiyama[37]). In most or all of these tumors, WT1 expression appeared to play a decisive role in promoting proliferation, invasion, and/or angiogenesis.[3,6,7,10,11,18,22,24–31,34,36,38,39,42]

We have previously demonstrated WT1 expression in approximately 80% of glioma cell lines and tumor speci-

Abbreviations used in this paper: ATP = adenosine triphosphate; EGF = epidermal growth factor; GBM = glioblastoma; PDGF = platelet-derived growth factor; qRT-PCR = quantitative reverse transcriptase polymerase chain reaction; SDS = sodium dodecyl sulfate; siRNA = short interfering RNA; VCU = Virginia Commonwealth University; WT1 = Wilms tumor 1.

mens.[5] The predominant WT1 isoform expressed in these cells contained a 17–amino acid peptide coded for by exon 5 and the tripeptide KTS between zinc fingers 3 and 4 (WT1 +/+). Our studies showed that glioma cells that had endogenous WT1 expression relied heavily on this protein for their growth and motility.[3,6] WT1 also conferred upon these cells resistance to radiation therapy and some chemotherapeutic agents.[3,4]

In the current study, we examined 2 additional GBM cell lines—U1242-MG and GBM-6—for the expression and function of WT1. Previous studies have shown that these 2 cell lines are highly invasive and mimic the pathology of GBM when implanted orthotopically in athymic mice.[12,49] Endogenous expression of WT1 was noted in both these cell lines. We also observed that WT1 profoundly influenced cellular invasiveness in these cell lines.

The question that arose from these observations then was: how was WT1 mediating these effects in glial neoplasms?

Given its structural identity—a zinc finger transcription factor—and its functional history, it seemed logical that WT1 might regulate the transcription (or posttranscriptional expression) of other genes that might then directly cause the above-mentioned effects. Therefore, to identify which genes might vary in glioma cells in their expression patterns as a result of WT1-mediated regulation, we used gene microarray analysis to characterize differential gene expression. This method permits rapid screening of thousands of genes to find the possible candidate genes that are differentially expressed as a result of any manipulations. Our analyses revealed the identity of some such presumed or established oncogenes whose levels paralleled that of *WT1* in glioma cells—*PDGF-D*, *TYMS*, *INPP5A*, *CD97,* and *FAM57A*. Genes whose expression levels were increased as a result of *WT1* suppression, on the other hand, included putative tumor suppressors, such as *LZTS1*, *TIMP3*, *MAFF,* and *WIPI1*. We then sought to ascertain if there was any correlation between the expression levels of WT1 and these candidate target genes in the U1242-MG and GBM-6 cells.

Our experiments showed that *CD97* was significantly downregulated in all 3 cell lines investigated—U251-MG, U1242-MG, and GBM-6. We selected *CD97* for further investigation, based on its consistent and direct correlation with *WT1* across the GBM cell lines examined and because of its putative roles in facilitating cell invasiveness and neoangiogenesis in gastrointestinal and thyroid tumors.[1,14,21,41,43] This is the first time that expression of this oncogene has been reported in neoplastic glial cells.

CD97 is a cell surface receptor that belongs to the adhesion G-protein–coupled receptor (GPCR) family. It is therefore characterized by heptahelical hydrophobic segments that form the 7 transmembrane domains, an extracellular N terminus containing EGF-like structural domains (comprising the α-subunit), and an intracellular C terminus.[47] Through alternative splicing of the mRNA transcript, 3 isoforms of the α-subunit of CD97 can be generated that differ in the number of EGF repeats they contain; these isoforms are hCD97 (EGF 1, 2, 5), hCD97 (EGF 1, 2, 3, 5), and hCD97 (EGF 1, 2, 3, 4, 5).[13] Also present in the amino-acid sequence of the extracellular segment, after the EGF-like repeats and before the first membrane-spanning sequence at position 318, is an RGD motif. This motif acts as a binding site for several classes of integrins, which are known to mediate attachment to the extracellular matrix and to other cells.[13] The ligands that have been found to bind CD97 include CD55/decay accelerating factor (DAF) (involved in protection from complement-mediated attack and lysis), chondroitin sulfate (a glycosaminoglycan that affects cell attachment), and $\alpha_5\beta_1$ integrin.[41] Interestingly, the intracellular signaling mechanism(s) by which CD97 and other adhesion-GPCR family members might act is (are) yet unclear.[20]

Expression of CD97 has been reported mainly in leukocytes, and in this group, it has been found predominantly in myeloid cells. CD97 expression has also been reported in smooth muscle cells and in epithelial tumors.[41] Its expression in thyroid carcinoma cells has been linked with promoting de-differentiation,[1] while in colorectal and gastric carcinomas and fibrosarcomas, its expression has been associated with cellular invasiveness.[20] Our results corroborated the findings from these earlier studies and we confirm that in glioma cells, also, CD97 appears to influence cellular invasiveness. Future studies will seek to confirm whether WT1 regulates expression of this protein at the transcriptional and/or post-transcriptional level(s).

Methods

Cell Culture

Cells from the human glioma cell line U251-MG were obtained from ATCC; U1242-MG cells were a kind gift from Dr. James Van Brocklyn (Ohio State University), and GBM-6 cells were generously provided by Dr. C. David James (University of California, San Francisco) via Dr. Paul Dent (VCU). All cells were cultured in DMEM containing 10% fetal bovine serum, glutamine, nonessential amino acids, and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO_2. Normal human astrocyte RNA (catalog #1805) was procured from ScienCell Research Laboratories.

Short Interfering RNA Transfections

Cells were plated in 6-well plates at a density of 1.5–2×10^5 cells per well. Twenty-four hours after plating, the cells were transfected as described previously.[4] Cells were divided into the following groups: those that had only Oligofectamine (Invitrogen) added to them (ctrl), those that were treated with nontargeting siRNA control (scr), and those that had been treated with siRNA targeting *WT1* or *CD97* (si). The final concentration of nontargeting siRNA control, anti-*WT1* siRNA, and anti-*CD97* siRNA was 100 nM, a concentration that was determined by performing optimization experiments. Twenty-four hours after transfection, 10% DMEM supplemented with 1% penicillin-streptomycin was added. Forty-eight hours after transfection, the cells were harvested with trypsin, and following resuspension of the cell pellet in 10% DMEM, the cells were replated for RNA extraction for qRT-PCR and microarray analyses, protein extraction for Western blotting, and cell proliferation and/or invasion assays.

RNA Extraction

RNA was extracted using Trizol (Invitrogen) according to the manufacturer's protocol. Following RNA extraction, the RNA content was quantified using a spectrophotometer, and approximately 12 μg of RNA per sample was subjected to DNase treatment using RQ1 DNase (Promega). Samples were then analyzed using qRT-PCR. Synthesis of probes and primers for anti-*WT1* siRNA was carried out at the VCU Nucleic Acid Research Facilities. The Taqman primer mixes for the target genes were purchased from Applied Biosystems. All qRT-PCR reactions were performed at the VCU Nucleic Acid Research Facilities.

Western Blotting

Protein was extracted from cell lines using SDS buffer (50 mM Tris-C1, 1% SDS, 10% glycerol) supplemented with protease inhibitors. The concentration of protein was determined by DC protein assay (Bio-Rad). For protein analysis of cells transfected with anti-*WT1* siRNA, 10 μg of total protein lysate was separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane according to the manufacturer's protocol (Invitrogen). The membrane was then blocked with 5% nonfat milk solution for 1 hour at room temperature. Mouse anti-WT1 monoclonal antibody (1:200 dilution, clone: 6F-H2, Dako) and rabbit anti-CD97 polyclonal antibody (1:200, Abcam) were diluted in blocking buffer, and the blots were incubated with the respective primary antibodies overnight at 4°C. After this, the membranes were washed 6 times in Tris-buffered saline containing 0.05% Tween-20 before and after a 1-hour incubation at room temperature with horseradish peroxidase–conjugated anti–mouse (1:2000) and anti–rabbit (1:3000) secondary antibodies directed against anti-WT1 and anti-CD97, respectively. Anticyclophilin A monoclonal antibody (1:30,000, Upstate Biotechnology) was used as a control for protein loading. Blots were developed using Pierce SuperSignal West Dura Substrate.

Cell Proliferation Assay

Cellular proliferation was measured at specified time points after transfection using the CellTiter-Glo luminescent cell viability assay (Promega). The cells that were plated in opaque 96-well plates 48 hours after transfection were lysed for 15 minutes in CellTiter-Glo ATP viability assay reagents as per the protocol. Relative luminescence was then detected on a LUMIstar luminescence plate reader (BMG Labtech).

Invasion Assay

Matrigel (BD Biosciences), previously aliquoted, was thawed at 4°C on ice overnight before use. Forty-eight hours posttransfection, cells were plated on Matrigel-coated 8.0-μm pore polycarbonate membrane inserts of 6.5-mm Transwell plates (Corning) at a density of 5000 cells/500 μl. Ninety-six hours following transfection, the medium was removed from the filters and the lower chambers, the upper part of each filter was scraped with cotton-tipped applicators to remove the cells that had not invaded, and each lower chamber was rinsed with phosphate-buffered saline. Then 200 μl of 1× trypsin-EDTA (prepared by diluting in phosphate-buffered saline a 10× solution of 0.5% trypsin-EDTA containing 5 mg/ml of trypsin with 4.81 mM Na-EDTA in 146.55 mM sodium chloride) was added to each chamber to harvest the cells, and after a 5-minute incubation at 37°C, 800 μl of 10% DMEM was used to neutralize the enzyme. The lower part of each filter was rinsed with medium to collect cells that had invaded through the Matrigel-coated insert but had not adhered to the base of the lower chamber. After centrifugation at 1000 rpm for 5 minutes to collect the cells, the supernatant was removed from each tube, and fresh medium was added to a volume of 500 μl. The entire volume was then plated (100 μl/well) into an opaque 96-well plate for assaying the ATP content as described above.

Gene Expression Profiling

The microarray reactions were performed using the Affymetrix GeneChip standard protocol as previously described.[9] Quality analyses of all RNA samples, as well as cDNA and cRNA synthesis products, were carried out before proceeding further by running 1 μl of every sample in RNA 6000 Nano or DNA 7500 LabChips on the 2100 Bioanalyzer (Agilent), following the manufacturer's protocol. Quality control criteria included cDNA and cRNA synthesis products within median lengths of 2.0 and 3.0 kb, respectively, and 3′/5′ ratios close to 1.00 for the housekeeping genes, *GAPDH* and *β-actin*.[8] The "significance-score" algorithm (S-score) developed by Dr. Li Zhang was used to produce a score for the comparisons of the expression summaries between cell groups.[48] The Minimum Information About a Microarray Experiment guidelines have been met, and the microarray raw data have been deposited with the National Center for Biotechnology Institute Gene Expression Omnibus—accession number GSE22578.

Statistical Analysis

All comparisons were between 2 cell groups: 1) cells treated with nontargeting siRNA or untreated cells; and 2) cells treated with siRNA directed against *WT1* or *CD97*. Each experiment was replicated at least 3 times, and results are presented as mean values with SDs. The Student t-test (2-tailed, paired) was used, and a calculated p value of ≤ 0.05 was considered statistically significant.

Results

Expression and Function of WT1 in U1242-MG and GBM-6 Cells

Using Western blotting, we detected WT1 expression in U1242-MG and GBM-6 cell lines (Fig. 1A). Cell extracts from the prostatic carcinoma cell line (PC3) were used as a positive control for WT1. Both GBM-6 and U1242-MG show the characteristic WT1 double band, suggesting the possibility of the + and – exon 5 variants that differ by 17 amino acids, at the predicted molecular weight level (52–54 kDa).

After using siRNA directed against *WT1*, we confirmed efficient silencing of this transcription factor by

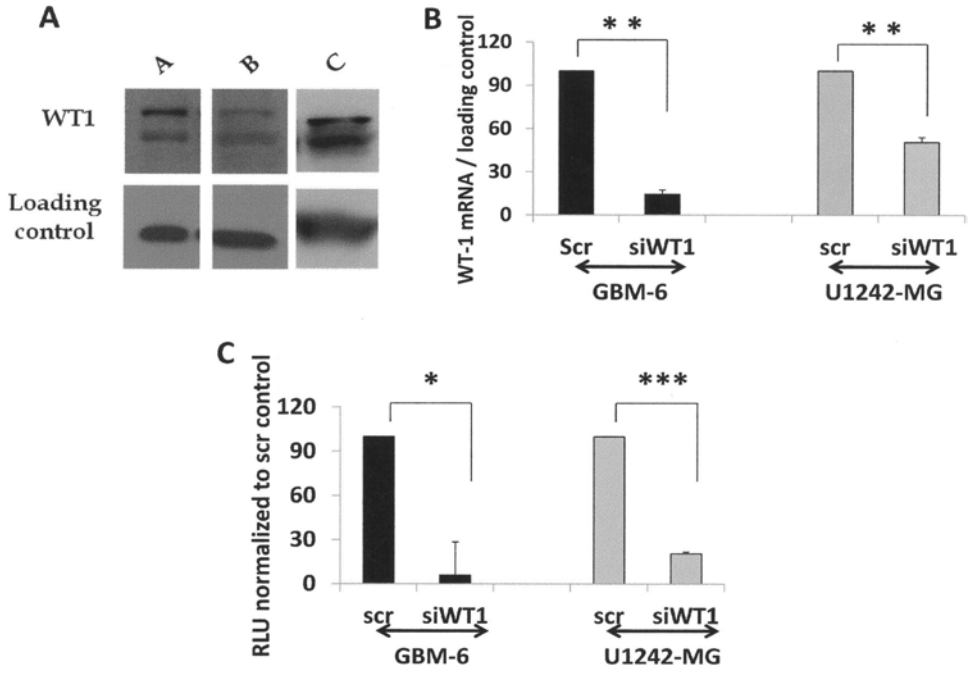

Fig. 1. Expression and function of WT1 in U1242-MG and GBM-6 cells. **A:** WT1 expression in GBM-6 and U1242-MG cells was detected using Western blotting. Twenty micrograms of protein was loaded in each lane. Cell lysates in Lanes A, B, and C are from PC3 (control), GBM-6, U1242-MG cells, respectively. **B:** *WT1* silencing was confirmed using qRT-PCR ($p \leq 0.001$). *WT1* mRNA levels in anti-*WT1* siRNA–treated cells are first normalized to the level of the loading control and expressed as a percentage of the control cells. **$p \leq 0.001$. **C:** *WT1* downregulation was associated with a decrease in GBM-6 and U1242-MG cellular invasiveness at Day 4 posttransfection (*$p < 0.05$, ***$p < 0.001$). Cell invasiveness in *WT1*-silenced cells was assessed by using the CellTiter-Glo assay (RLU is a surrogate for cell number) to first quantify the number of cells that had invaded through the Matrigel in the si groups versus the scr groups and then expressing this value as a percentage of that seen in control (scr) cells. All experiments were performed in triplicate with separate cultured samples of tumor cells treated in parallel with siRNA against *WT1* and corresponding controls.

qRT-PCR (Fig. 1B). On the 3rd day after transfection, we found that the level of *WT1* mRNA in GBM-6 cells treated with anti-*WT1* siRNA was 14.44% (± 2.89%) of that seen in their control counterparts that had been treated with nontargeting siRNA ($p \leq 0.001$). In U1242-MG cells, there was less knockdown (50.47% [± 3.49%] of control levels), which was nevertheless highly significant ($p \leq 0.001$).

We observed that these decreases in cellular content of WT1 remarkably depressed the invasive capacity of these cells (Fig. 1C). In GBM-6 cells, there was nearly a 95% decrease (± 22.65%) in the ability of the anti-*WT1* siRNA–treated cells to invade through the Matrigel-coated filter ($p \leq 0.05$) compared with the control cells, while in U1242-MG cells, silencing of *WT1* caused a decrease in cellular invasiveness to 20.62% (± 1.12%) of the control cells ($p \leq 0.001$). These differences in the ability to invade could potentially be accounted for by decreased proliferative rates in the siRNA-transfected cells versus control cells. Hence, we performed cell viability assays on these same groups of cells. Our results showed that the growth rates of control and siRNA-treated cells were not significantly different across 3 independent experiments (data not shown). Also, based on our previous experiments we found no statistically significant differences between untreated cells and cells treated with nontargeting siRNA (scr). Hence, we show only comparisons between groups treated with nontargeting and targeting siRNA.

Gene Expression Profiling: Putative Target Genes for WT1 in U251-MG Glioma Cells

To ascertain the identity of the potential target genes for WT1 in the particular context of glioma cells, we chose to perform gene expression profiling experiments. On 3 separate occasions, U251-MG cells were treated with Oligofectamine alone (ctrl), nontargeting RNA (scr), or siRNA against *WT1*. After confirming *WT1* knockdown with qRT-PCR in the siRNA-treated samples each time, microarray analyses were carried out using the Affymetrix GeneChip Standard protocol. Quality control checks were performed, and RNA samples that met the criteria described in detail in our previous studies[40] were used for microarray analyses. The S-score analyses of the data obtained in the 3 sets of experiments are summarized in Tables 1 and 2, revealing a total of 27 genes that were downregulated and 11 genes that were upregulated, respectively, in response to *WT1* silencing. These genes passed 2 separate levels of stringency for being declared significantly altered—the S-score, which establishes significance at the univariate level, and the Benjamini-Hochberg correction, which corrects for multiple comparisons.[38] The fold change of each gene in these tables is expressed as the mean value of the results for that gene from the 3 different microarray experiments. For clarity, standard deviations for these values are not shown, but they represented up to 30% of the mean values. Super-

TABLE 1: Gene expression profiling demonstrating the fold change of genes that are significantly downregulated in *WT1*-silenced U251-MG cells*

Gene Title	Gene Symbol	Fold Change	
		si vs ctrl	si vs scr
intestinal cell (MAK-like) kinase	**ICK**	**0.189**	**0.287**
inositol polyphosphate-5-phosphatase, 40 kDa	**INPP5A**	**0.25**	**0.1649**
chromosome 11 open reading frame 57	C11orf57	0.25	0.25
thymidylate synthetase	**TYMS**	**0.26**	**0.287**
platelet-derived growth factor-D	**PDGF-D**	**0.26**	**0.176**
LSM14A, SCD6 homolog A *(S. cerevisiae)*	LSM14A	0.287	0.26
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1, 7.5 kDa	NDUFA1	0.287	0.287
dihydrolipoamide branched chain transacylase E2	DBT	0.33	0.307
potassium channel tetramerisation domain containing 12	KCTD12	0.33	0.33
CD97 molecule	**CD97**	**0.33**	**0.307**
NECAP endocytosis associated 1	NECAP1	0.33	0.307
BTB (POZ) domain containing 1	BTBD1	0.33	0.307
endothelial PAS domain protein 1	**EPAS1**	**0.353**	**0.287**
hypothetical protein FLJ10357	FLJ10357	0.353	0.353
secretogranin II (chromogranin C)	SCG2	0.353	0.233
mitofusin 1	MFN2	0.353	0.33
carboxypeptidase, vitellogenic-like	CPVL	0.353	0.3789
LSM14A, SCD6 homolog A *(S. cerevisiae)*	LSM14A	0.353	0.33
chromosome 14 open reading frame 13	C14orf135	0.3789	0.3789
uroporphyrinogen III synthase (congenital erythropoietic porphyria)	UROS	0.3789	0.33
family with sequence similarity 57, member A	**FAM57A**	**0.3789**	**0.353**
serine carboxypeptidase 1	SCPEP1	0.3789	0.33
NSFL1 (p97) cofactor (p47)	NSFL1C	0.406	0.3789
hypothetical protein HSPC111	**HSPC111**	**0.406**	**0.353**
iron-sulfur cluster assembly 1 homolog *(S. cerevisiae)*	ISCA1	0.406	0.435
keratin 18	**KRT18**	**0.406**	**0.3789**
RNA binding motif, single stranded interacting protein 1	RBMS1	0.406	0.353

* A total of 28 genes were downregulated (including WT1, not shown in table). Bold type indicates genes that have putative or established roles in oncogenesis. A total of 3 independent microarray experiments were conducted.

TABLE 2: Gene expression profiling demonstrating the fold change of genes that are significantly upregulated in *WT1*-silenced U251-MG cells*

Gene Title	Gene Symbol	Fold Change	
		si vs ctrl	si vs scr
CD55 molecule, decay accelerating factor for complement (Cromer blood group)	CD55	2.46	2.828
spermidine/spermine N1-acetyltransferase 1	**SSAT1**	**2.639**	**2.639**
WD repeat domain, phosphoinositide interacting 1	**WIPI1**	**2.639**	**2.828**
calcitonin receptor–like	CALCRL	2.639	3.03
leucine zipper, putative tumor suppressor 1	**LZTS1**	**2.828**	**3.73**
TIMP metallopeptidase inhibitor 3 (Sorsby fundus dystrophy, pseudoinflammatory)	**TIMP3**	**2.828**	**3.03**
heme oxygenase (decycling) 1	HMOX1	2.828	2.828
SRY (sex determining region Y)-box 11	**SOX11**	**2.828**	**2.46**
v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	**MAFF**	**3.03**	**2.828**
RAR-related orphan receptor B	RORB	3.24	2.828
dihydropyrimidinase-like 3	**DPYSL3**	**3.48**	**3.73**

* A total of 11 genes were upregulated. Bold type indicates genes that have putative or established roles in suppressing cancers. A total of 3 independent microarray experiments were conducted.

vised cluster analyses from 3 independent experiments based on these 38 genes showed that the "ctrl" and "scr" cell groups cluster together and separately from the "si" group of cells that were treated with siRNA (Fig. 2). Thus, Fig. 2 represents an experiment performed in triplicate over the course of multiple weeks, with separate cultured samples of U251-MG cells treated in parallel for knockdown of WT1 expression with corresponding controls. To the left side of the heat map is a list of all the genes whose levels were found to be altered with this manipulation. *WT1* (highlighted in yellow) appears prominently downregulated as a result of our experimental treatment, confirming the successful downregulation using siRNA knockdown. Also, there is a high level of consistency in the patterns of up- or downregulation of the selected genes, among the corresponding replicates (ctrl, scr-, or si-treated) from the 3 experiments performed.

Among the genes that were downregulated with *WT1* silencing, implying a direct correlation, several (identified by searching the available literature) had either putative or established oncogenic roles in gliomas and/or other malignancies. These genes included *ICK*, *INPP5A*, *TYMS*, *PDGF-D*, *CD97*, *EPAS1*, *FAM57A*, *KRT18*, and *HSPC111* (Table 1). Conversely, the genes that inversely correlated with WT1 levels included some prominent tumor suppressors such as *SSAT1*, *LZTS1*, *WIPI1*, *TIMP3*, *SOX11*, *MAFF*, and *DPYSL3*.

Validation of Microarray Findings Using qRT-PCR

We used qRT-PCR to validate our microarray findings, because it was quicker and more sensitive and would provide information pertaining to the gene expression lev-

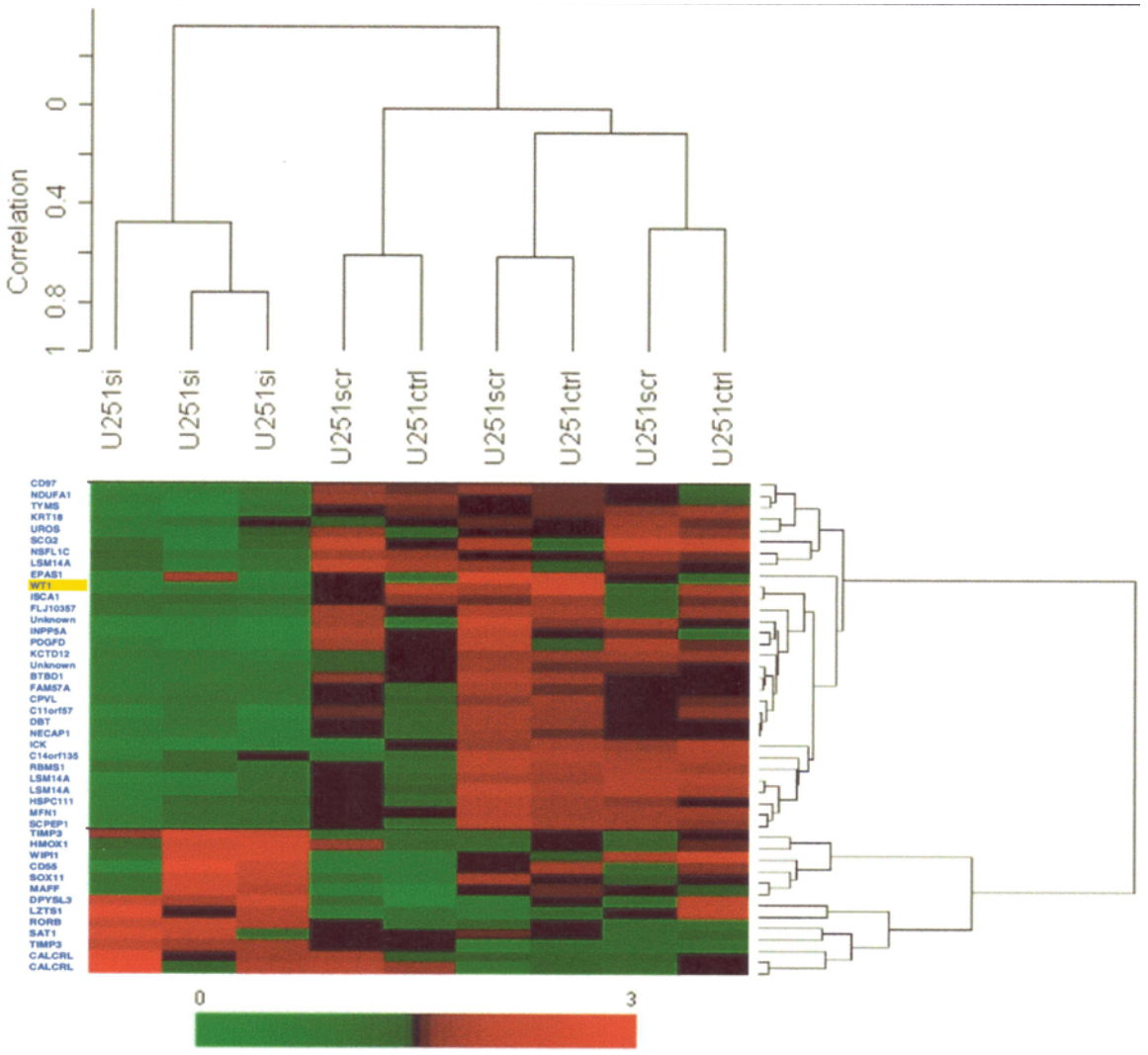

Fig. 2. Supervised cluster analysis. Two-dimensional hierarchical clustering of samples and genes using Pearson (centered) correlation and average linkage. Three independent transfection experiments were performed for each microarray analysis.

els, which was of particular interest to us since we were assessing the potential transcriptional targets of WT1. For our validation experiments, we selected from among all the targets identified by microarray only those that had been implicated as having a relevant role in oncogenesis or tumor suppression based on literature search. As with the microarray studies, siRNA-transfection, scr-transfection, and control (Oligofectamine alone) treated cells were prepared on 3 separate occasions, and RNA extracts were then subjected to qRT-PCR as described in *Methods* for the putative target genes. Furthermore, certain genes such as *ICK*, *KRT18*, and *TIMP3* are not represented in the graph owing to lack of reproducible results. The $2^{-\Delta\Delta CT}$ method was used to calculate fold changes in the mRNA expression levels of the candidate genes against β-actin mRNA. The values thus obtained for the si groups of cells were then compared with the corresponding values in the scr groups of cells. Figure 3 represents a comparison of the fold change of each candidate gene obtained from the qRT-PCR analyses (bar graph) against the corresponding value derived from the microarray analyses (line graph). We successfully substantiated the microarray results with qRT-PCR by showing that, with both sets of genes, there is a consistent trend toward down- or upregulation of their expression levels. In the case of the genes that were downregulated with *WT1* silencing, the magnitude of the fold-change values identified by means of qRT-PCR (range 0.25 ± 0.04 to 0.53 ± 0.22) was seen to be close to or only slightly different from that of the fold-change values obtained through the microarray experiments (0.16–0.35). With the inversely correlated genes, the fold-change values were much higher in the microarray results (2.6- to 3.7-fold differences) than in the qRT-PCR results (range 0.9 ± 0.21 to 1.9 ± 1.07).

Confirmation of Putative Target Genes in U1242-MG and GBM-6 Cell Lines

We subsequently investigated whether these genes that were dysregulated in U251-MG cells were similarly altered by *WT1* silencing in U1242-MG and GBM-6

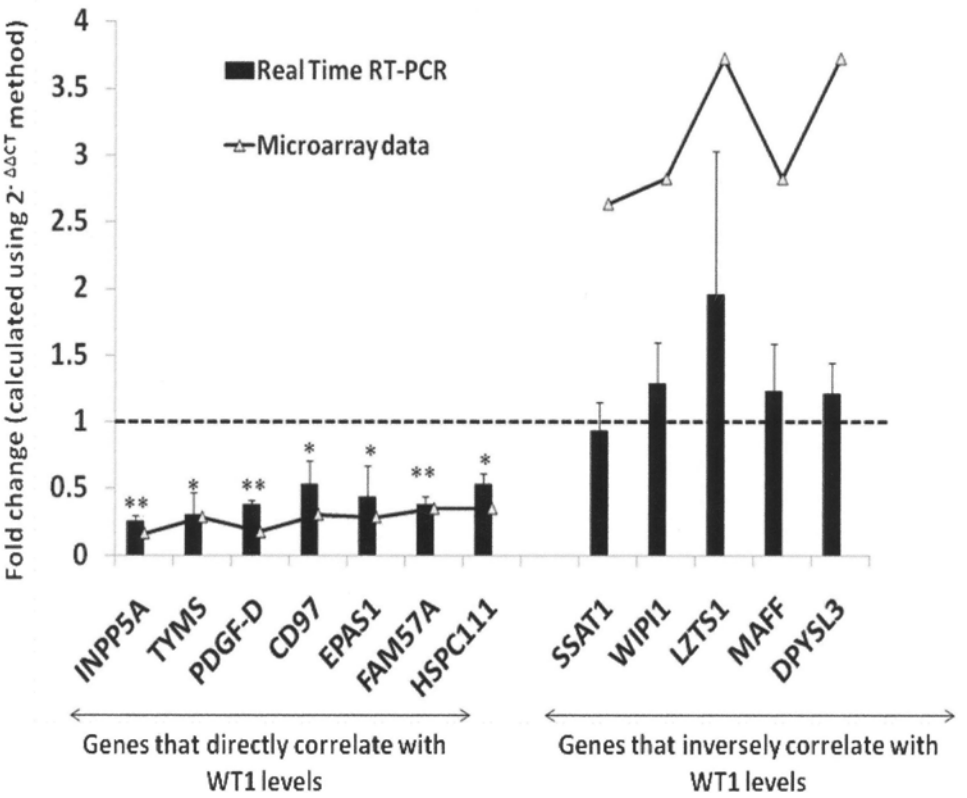

Fig. 3. Validation of microarray results. U251-MG cells were transfected with nontargeting siRNA (scr) or *WT1*-targeting siRNA (si). RNA samples extracted 48 hours following transfection were analyzed by qRT-PCR. **Bar Graph:** Fold-change values of siRNA-treated cells compared with scr controls (depicted by the *dashed line* at 1) calculated from qRT-PCR experiments. **Line Graph:** Fold-change values calculated using microarray analyses. Value of scr is set at 1 (depicted by the *dashed line*). Transfection was performed on 3 parallel sets of cell cultures to generate these results. *$p \leq 0.05$; **$p \leq 0.01$.

cells. Both of these cell lines were subjected to siRNA transfections targeting *WT1*, and nontargeting siRNA (scr) was used to generate appropriate controls. Again, for each cell line, results were generated from 3 independent experiments. Using qRT-PCR, we found that from among the genes whose expression levels paralleled those of *WT1*, *INPP5A*, *CD97*, and *TYMS* were downregulated in the si groups in both U1242-MG and GBM-6 cells compared with scr groups (Fig. 4). In GBM-6 cells, the extent of knockdown of these genes was as follows: *INPP5A*—51.77% ± 0.26% ($p \leq 0.05$); *CD97*—49.6% ± 0.14% ($p \leq 0.01$); and *TYMS*—52.4% ± 0.16%, ($p \leq 0.01$). Additionally, in GBM-6 cells, the expression levels of *PDGF-D* and *FAM57A* in the siRNA-treated groups of cells were significantly decreased to 16.2% (± 0.06%; $p \leq 0.01$) and 44.1% (± 0.23%; $p \leq 0.05$) of their scr counterparts, respectively. In U1242-MG cells, the extent of knockdown of these candidate target genes (as a percentage of scr control) was as follows: *INPP5A*—65.5% ± 0.11% ($p \leq 0.01$); *CD97*—30.6% ± 0.09% ($p \leq 0.05$); and *TYMS*—47.7% ± 0.04% ($p \leq 0.01$). Additionally, in U1242-MG cells the expression of *EPAS1*, a potent angiogenesis facilitator, was also decreased with *WT1* silencing. The siRNA-treated U1242-MG cells had 77.5% of *EPAS1* expression compared with their corresponding control values, although statistical significance could not be established. For the genes that microarray analyses demonstrated to be upregulated with *WT1* silencing (that is, inversely correlated with *WT1* expression), we found a trend toward an increase in *DPYSL* and *MAFF* (GBM-6) and *WIPI1* (U1242-MG), but the high variability across the 3 independent experiments precluded the establishment of statistical significance.

Analysis of Expression and Function of CD97 in GBM Cells

Based on our microarray findings and our confirmatory findings using qRT-PCR, we conducted promoter analyses to look for potential binding sites in the promoter regions of the genes that appeared to be differentially regulated by WT1 (data not shown). With the exception of *PDGF-D* (which had binding sites for Egr-1, but not specifically WT1), all the remaining candidate target genes were found to have at least 1 potential WT1 binding site in their promoter regions. This information, coupled with the consistent *CD97* downregulation in all 3 GBM cell lines that had been treated with anti-*WT1* siRNA, led us to further examine the role of *CD97* in these cells with respect to its expression levels and consequent effects on cellular invasiveness.

We first determined the expression of *CD97* at the protein level using Western blot analysis, which revealed that it was expressed in all 3 cell lines examined (Fig. 5A). We then compared the mRNA expression levels of

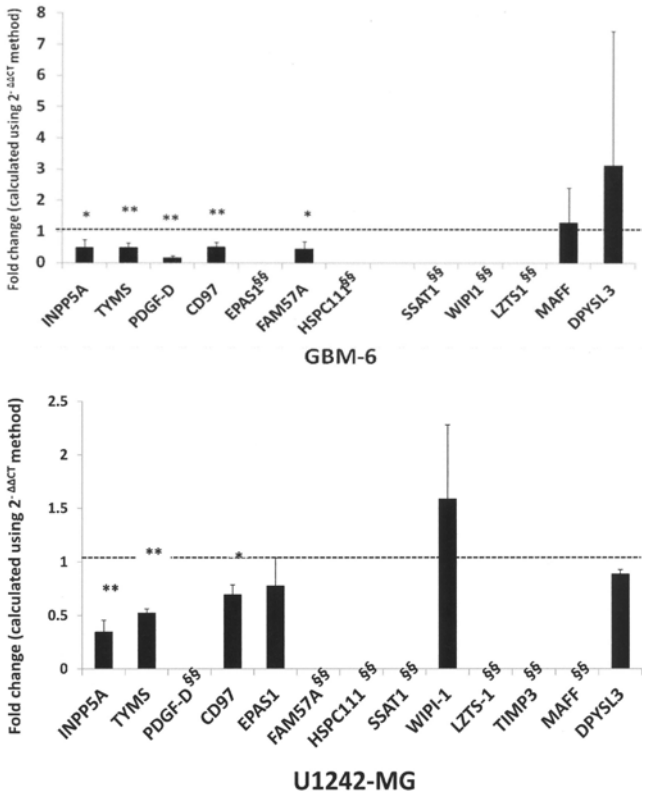

Fig. 4. Confirmation of altered regulation of target genes across different glioma cell lines transfected with anti-*WT1* siRNA. Experiments were performed in triplicate for both GBM-6 and U1242-MG cells. The x-axes represent the target genes and the y-axes the fold change in si cells (value of scr set at 1, *dashed line*). *$p \leq 0.05$; **$p < 0.01$. §§Genes that were not found to correlate/be expressed.

CD97 in normal human astrocytes to those in the GBM cell lines—U251-MG, U1242-MG, and GBM-6—and found 6- to 21-fold greater *CD97* mRNA levels in the 3 cell lines than in normal human astrocytes (Fig. 5B).

Subsequently, CD97 expression in all 3 glioma cell lines was downregulated by siRNA transfection methods, and the biological effects of this manipulation were examined. As shown in Fig. 5C, qRT-PCR analyses demonstrated a significant knockdown in all 3 cell lines even at Day 4 posttransfection ($p < 0.05$). In U251-MG cells, there was a nearly 50% decrease in *CD97* mRNA levels at this time point, whereas in GBM-6, there was a nearly 80% decrease. In U1242-MG cells, there was a less remarkable knockdown (20% decrease compared with the control cells), which was nevertheless statistically significant. When plated on Matrigel-coated filters of Transwell plates, we found that these decreases in *CD97* mRNA levels were associated with significantly lower cellular invasiveness capacities (Fig. 5D). In U251-MG cells treated with siRNA against *CD97*, the cellular invasiveness potential was decreased to roughly 53.85% of that of the control counterparts. In U1242-MG cells, even a modest decrease in *CD97* RNA levels was associated with a striking decrease in invasiveness (25.5% of control), whereas in GBM-6 cells, the 80% decrease in *CD97* mRNA levels caused a nearly 50% decrease in invasive capacity. Thus, it is clear that the expression of CD97 is functionally significant in glioma cells. We also examined the effects on cellular proliferation (if any) of knocking down CD97, even though such a role has not frequently been attributed to this molecule. Our findings showed that there were no significant differences between the proliferative rates of the control versus experimental groups across all the 3 cell lines (data not shown).

Discussion

We have identified, using gene expression profiling, genes whose expression levels correlate directly or inversely with WT1 levels in the specific context of malignant gliomas. Of the 27 genes whose expression patterns were positively correlated with that of *WT1* (that is, downregulated with suppression of *WT1* expression), 9 candidates were selected for having established or putative oncogenic functions, based on published literature. Conversely, of the 11 genes whose levels were inversely correlated (upregulated with *WT1* silencing), 7 have been implicated in the suppression of tumorigenesis. We have validated the microarray findings for these genes in U251-MG cells with qRT-PCR and have also confirmed these expression patterns in 2 other GBM cell lines. Our promoter studies revealed potential binding sites for WT1 in the promoter regions of all but one of the putative target genes. In the lone exception—*PDGF-D*—the promoter region did, however, show potential binding sites for the Egr-1 family of transcription factors. It has been established in previous studies[23,35,45] that WT1 shares the consensus binding sequences recognized and bound by the Egr-1 family of proteins.

CD97 was uniformly downregulated in all 3 cell lines with *WT1* silencing and displayed sites on its promoter that could be theoretically bound by WT1. Moreover, CD97 is well established as a facilitator of tumor cell invasiveness in other malignancies. Given these considerations, we further examined the role of this protein in gliomas. This novel undertaking revealed that in glioma cells, as in gastrointestinal malignancies and the fibrosarcoma cell line HT-1080,[20] the CD97 receptor fosters cellular invasiveness, and decreasing its endogenous expression caused a significant and striking decrease in the ability of the cells to invade through Matrigel.

The complexity of the WT1 protein has long posed a formidable challenge to those who have ventured to study its varied functions. Because of its vastly differing roles in different cell types, categorizing this protein as a tumor suppressor or an oncoprotein is difficult. In certain cells like the progenitor cells of the kidney, WT1 facilitates their exit from the cell cycle with subsequent differentiation.[15] In the neural and vascular progenitor cells, however, it has the exact opposite effect—facilitating proliferation and preventing differentiation.[15] Hence, any conclusions about its role(s) must be made strictly in the context of the cell type examined. We selected the microarray technique to study differences in gene expression arising out of manipulating the levels of WT1 in glioma cells that expressed it endogenously. Moreover, silencing the endogenous expression of the protein to look for the resultant differences was a more appropriate model to ac-

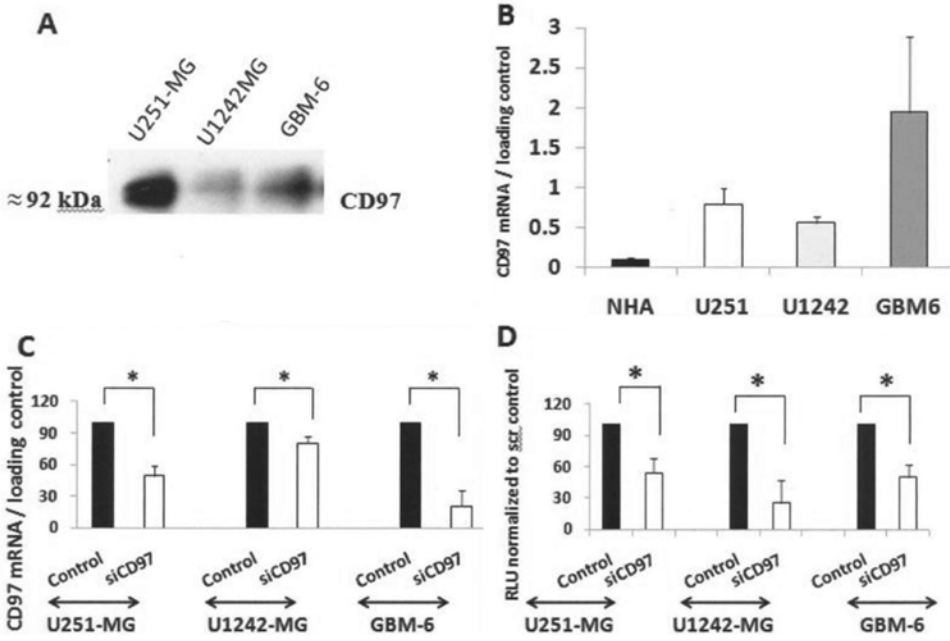

Fig. 5. Expression and function of CD97 in GBM cells. **A:** Western blot showing CD97 protein expression (≈ 92 kDa) in U251-MG, U1242-MG, and GBM-6 glioma cells. Ten micrograms of protein was loaded in each lane. Rabbit polyclonal Ab (Abcam) was used to detect CD97 (1:200). **B:** Quantitative RT-PCR demonstrated minimal expression of *CD97* in normal human astrocytes (NHA) and a 6- to 21-fold increase in expression in the GBM cell lines investigated. **C:** Using siRNA directed against *CD97*, we confirmed a significant knockdown in *CD97* RNA in the tumor cells on Day 4 posttransfection by qRT-PCR in U251-MG, U1242-MG, and GBM-6 cells. **D:** Treatment of U251-MG, U1242-MG, and GBM-6 cells with siRNA against *CD97* resulted in a significant decrease in their ability to invade through the Matrigel-coated filters of transwell plates compared with cells that were treated with nontargeting siRNA. Cell invasiveness in *CD97*-silenced cells was expressed by using the CellTiter-Glo assay to first quantify the number of cells that had invaded through the Matrigel in the si groups versus scr groups and then expressing this value as a percentage of that seen in control (scr) cells. Three independent cultures of U251-MG, GBM-6, and U1242-MG cells were transfected with corresponding controls. *$p < 0.05$.

curately assess the possible functions of WT1 rather than overexpressing WT1 in a WT1 null cell line, due to the myriad variables (WT1 isoforms and concentration) associated with the latter technique.

Identifying the target genes via which WT1 might influence its role in the neoplastic transformation of glial cells was important for many reasons. First, as a regulator of transcriptional and posttranscriptional processes, WT1 has the ability to coordinate many of the key aspects of glioma biology—proliferation, invasiveness, and angiogenesis—by regulating the expression of the molecules that mediate these processes. Moreover, given the rapid progress that is being made in the field of immunologically targeting WT1,[2,17,32,33] our findings help to fill in the missing links in understanding how it functions. Finally, knowing the identity of its molecular associates can help in devising therapeutic strategies that target one or more of these components. Since these components may belong to different intracellular signaling pathways, targeting them simultaneously could work synergistically to have even greater effects against malignant gliomas.

The identification of at least 9 candidate oncogenes that were suppressed and 7 putative tumor suppressors that were upregulated by *WT1* silencing confirmed our hypothesis that in gliomas, *WT1* has more of an oncogenic function. Our study has also revealed for the first time that CD97 is aberrantly overexpressed in GBM cells. This protein has well-documented roles in mediating tumor cell invasion and even angiogenesis[1,43] in other malignant cell types. In various gastrointestinal malignancies, an increased expression of CD97 has been demonstrated at the invading front of the tumor.[14,21] Likewise, we have shown here that in glial neoplastic cells, also, it has a significant role in conferring invasiveness upon the cells. From our findings, it appears that in U1242-MG cells, the extent to which WT1 decreases cellular invasiveness roughly equals that seen with silencing of *CD97* expression. In U251-MG and GBM-6 cells, on the other hand, *WT1* silencing causes a much more pronounced decrease in cellular invasiveness than that seen with *CD97* silencing alone. In both of these cell lines, expression of PDGF-D was also seen to be significantly decreased with lowered expression of WT1. It is well established that PDGF-D is a strong mitogenic and chemoattractant molecule that can initiate several malignant features, such as proliferation, invasion, and angiogenesis in brain tumors (reviewed by Wang et al.[44]). A more detailed examination of the relationship of this growth factor to WT1 undoubtedly merits further attention.

We restricted the scope of this study to examining the expression and function of CD97 in glioma cell lines to establish well-characterized in vitro model systems that will allow continuing investigation of the molecular mechanisms involved with WT1 overexpression and the

correlated CD97 expression. Further, we chose to compare CD97 expression in these cells to that in normal human astrocytes as there was very little data describing expression of this proinvasive receptor molecule in the normal brain tissue components. Clearly, evaluation of CD97 expression in vivo, both in animal models and in human tumor samples, will also be needed. Along these lines, we can confirm that preliminary data from studies conducted by our laboratory have revealed *CD97* mRNA expression by a high proportion of individual human glioma samples (data not shown). The detection of significantly higher levels of CD97 in the GBM cell lines and tumor specimens than is found in normal brain glia argues for a pathogenic role for this protein. Our results lay the groundwork for further analyses of the functional significance of CD97 using more sophisticated experimental techniques, such as brain slice invasion assays or in vivo studies.

We chose to downregulate CD97 expression as a more appropriate method in the initial evaluation of its inherent functional role. Future studies will seek to first identify the exact isoform(s) of CD97 (containing 3, 4, or 5 EGF repeats) that is (are) expressed in glioma cells. Upregulation of the identified isoform(s) in these cells can then be performed to confirm an increase in cellular invasiveness. Finally, although our promoter studies suggest putative binding sites for WT1 on the *CD97* promoter, definitive evidence is still required to demonstrate WT1-mediated transactivation of CD97 expression.

The ability to localize and therapeutically target the invading neoplastic cells in situ has for a long time been a challenge in the treatment of brain tumors. With the identification of CD97 expression and function in glioma cells, determining its ability to serve as a reliable marker of invasiveness using immunohistochemical staining of glioma tumor specimens opens up a potentially interesting line of investigation. Another option may be to devise a therapeutic strategy aimed at targeting the CD97 receptor, which could be relatively safe, since it is virtually absent in normal human astrocytes and the glia of normal brain (data not shown). Further investigations are also necessary to determine the effect of targeting the CD97 receptor on angiogenesis, as it is also known to affect this process.

Conclusions

Our studies emphasize the importance of WT1 in promoting GBM cellular invasiveness and reveal the identities of its putative target genes in glioma cells. We also show, for the first time in glioma cells, the expression and proinvasive function of CD97. Our study thus opens important new avenues in the field of glioma biology. More in-depth analyses of these molecules should carry us further in our attempts to find novel and more effective therapeutic targets for the treatment of GBM.

Disclosure

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Author contributions to the study and manuscript preparation include the following. All authors contributed equally to this work with respect to the following areas: conception and design, acquisition of data, analyses and interpretation of data, drafting the article, critical revision of the article, statistical analyses, and administrative/technical/material support.

References

1. Aust G, Eichler W, Laue S, Lehmann I, Heldin NE, Lotz O, et al: CD97: a dedifferentiation marker in human thyroid carcinomas. **Cancer Res 57:**1798–1806, 1997
2. Cheever MA, Allison JP, Ferris AS, Finn OJ, Hastings BM, Hecht TT, et al: The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. **Clin Cancer Res 15:**5323–5337, 2009
3. Chen MY, Clark AJ, Chan DC, Ware JL, Holt SE, Chidambaram A, et al: Wilms' tumor 1 silencing decreases the viability and chemoresistance of glioblastoma cells in vitro: a potential role for IGF-1R de-repression. **J Neurooncol 103:** 87–102, 2011
4. Clark AJ, Chan DC, Chen MY, Fillmore HL, Dos Santos WG, Van Meter TE, et al: Down-regulation of Wilms' tumor 1 expression in glioblastoma cells increases radiosensitivity independently of p53. **J Neurooncol 83:**163–172, 2007
5. Clark AJ, Dos Santos WG, McCready J, Chen MY, Van Meter TE, Ware JL, et al: Wilms tumor 1 expression in malignant gliomas and correlation of +KTS isoforms with p53 status. **J Neurosurg 107:**586–592, 2007
6. Clark AJ, Ware JL, Chen MY, Graf MR, Van Meter TE, Dos Santos WG, et al: Effect of WT1 gene silencing on the tumorigenicity of human glioblastoma multiforme cells. Laboratory investigation. **J Neurosurg 112:**18–25, 2010
7. Dudnakova T, Spraggon L, Slight J, Hastie N: Actin: a novel interaction partner of WT1 influencing its cell dynamic properties. **Oncogene 29:**1085–1092, 2010
8. Dumur CI, Nasim S, Best AM, Archer KJ, Ladd AC, Mas VR, et al: Evaluation of quality-control criteria for microarray gene expression analysis. **Clin Chem 50:**1994–2002, 2004
9. Dumur CI, Sana S, Ladd AC, Ferreira-Gonzalez A, Wilkinson DS, Powers CN, et al: Assessing the impact of tissue devitalization time on genome-wide gene expression analysis in ovarian tumor samples. **Diagn Mol Pathol 17:**200–206, 2008
10. Garrido-Ruiz MC, Rodriguez-Pinilla SM, Pérez-Gómez B, Rodriguez-Peralto JL: WT 1 expression in nevi and melanomas: a marker of melanocytic invasion into the dermis. **J Cutan Pathol 37:**542–548, 2010
11. Gerald WL, Haber DA: The EWS-WT1 gene fusion in desmoplastic small round cell tumor. **Semin Cancer Biol 15:**197–205, 2005
12. Giannini C, Sarkaria JN, Saito A, Uhm JH, Galanis E, Carlson BL, et al: Patient tumor EGFR and PDGFRA gene amplifications retained in an invasive intracranial xenograft model of glioblastoma multiforme. **Neuro Oncol 7:**164–176, 2005
13. Gray JX, Haino M, Roth MJ, Maguire JE, Jensen PN, Yarme A, et al: CD97 is a processed, seven-transmembrane, heterodimeric receptor associated with inflammation. **J Immunol 157:**5438–5447, 1996
14. Han SL, Xu C, Wu XL, Li JL, Liu Z, Zeng QQ: The impact of expressions of CD97 and its ligand CD55 at the invasion front on prognosis of rectal adenocarcinoma. **Int J Colorectal Dis 25:**695–702, 2010
15. Hohenstein P, Hastie ND: The many facets of the Wilms' tumour gene, WT1. **Hum Mol Genet 15 Spec No 2:**R196–R201, 2006
16. Huff V: Wilms' tumours: about tumour suppressor genes, an

oncogene and a chameleon gene. **Nat Rev Cancer 11:**111–121, 2011
17. Izumoto S, Tsuboi A, Oka Y, Suzuki T, Hashiba T, Kagawa N, et al: Phase II clinical trial of Wilms tumor 1 peptide vaccination for patients with recurrent glioblastoma multiforme. **J Neurosurg 108:**963–971, 2008
18. Jomgeow T, Oji Y, Tsuji N, Ikeda Y, Ito K, Tsuda A, et al: Wilms' tumor gene WT1 17AA(-)/KTS(-) isoform induces morphological changes and promotes cell migration and invasion in vitro. **Cancer Sci 97:**259–270, 2006
19. Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D, et al: WT-1 is required for early kidney development. **Cell 74:**679–691, 1993
20. Kwakkenbos MJ, Kop EN, Stacey M, Matmati M, Gordon S, Lin HH, et al: The EGF-TM7 family: a postgenomic view. **Immunogenetics 55:**655–666, 2004
21. Liu Y, Chen L, Peng S, Chen Z, Gimm O, Finke R, et al: The expression of CD97EGF and its ligand CD55 on marginal epithelium is related to higher stage and depth of tumor invasion of gastric carcinomas. **Oncol Rep 14:**1413–1420, 2005
22. Loeb DM, Evron E, Patel CB, Sharma PM, Niranjan B, Buluwela L, et al: Wilms' tumor suppressor gene (WT1) is expressed in primary breast tumors despite tumor-specific promoter methylation. **Cancer Res 61:**921–925, 2001
23. Mayo MW, Wang CY, Drouin SS, Madrid LV, Marshall AF, Reed JC, et al: WT1 modulates apoptosis by transcriptionally upregulating the bcl-2 proto-oncogene. **EMBO J 18:**3990–4003, 1999
24. Miyagi T, Ahuja H, Kubota T, Kubonishi I, Koeffler HP, Miyoshi I: Expression of the candidate Wilm's tumor gene, WT1, in human leukemia cells. **Leukemia 7:**970–977, 1993
25. Oji Y, Inohara H, Nakazawa M, Nakano Y, Akahani S, Nakatsuka S, et al: Overexpression of the Wilms' tumor gene WT1 in head and neck squamous cell carcinoma. **Cancer Sci 94:**523–529, 2003
26. Oji Y, Miyoshi S, Maeda H, Hayashi S, Tamaki H, Nakatsuka S, et al: Overexpression of the Wilms' tumor gene WT1 in de novo lung cancers. **Int J Cancer 100:**297–303, 2002
27. Oji Y, Miyoshi Y, Koga S, Nakano Y, Ando A, Nakatsuka S, et al: Overexpression of the Wilms' tumor gene WT1 in primary thyroid cancer. **Cancer Sci 94:**606–611, 2003
28. Oji Y, Nakamori S, Fujikawa M, Nakatsuka S, Yokota A, Tatsumi N, et al: Overexpression of the Wilms' tumor gene WT1 in pancreatic ductal adenocarcinoma. **Cancer Sci 95:**583–587, 2004
29. Oji Y, Ogawa H, Tamaki H, Oka Y, Tsuboi A, Kim EH, et al: Expression of the Wilms' tumor gene WT1 in solid tumors and its involvement in tumor cell growth. **Jpn J Cancer Res 90:**194–204, 1999
30. Oji Y, Yamamoto H, Nomura M, Nakano Y, Ikeba A, Nakatsuka S, et al: Overexpression of the Wilms' tumor gene WT1 in colorectal adenocarcinoma. **Cancer Sci 94:**712–717, 2003
31. Oji Y, Yano M, Nakano Y, Abeno S, Nakatsuka S, Ikeba A, et al: Overexpression of the Wilms' tumor gene WT1 in esophageal cancer. **Anticancer Res 24:**3103–3108, 2004
32. Oka Y, Sugiyama H: WT1 peptide vaccine, one of the most promising cancer vaccines: its present status and the future prospects. **Immunotherapy 2:**591–594, 2010
33. Oka Y, Tsuboi A, Fujiki F, Li Z, Nakajima H, Hosen N, et al: WT1 peptide vaccine as a paradigm for "cancer antigen-derived peptide"-based immunotherapy for malignancies: successful induction of anti-cancer effect by vaccination with a single kind of WT1 peptide. **Anticancer Agents Med Chem 9:**787–797, 2009
34. Perugorria MJ, Castillo J, Latasa MU, Goñi S, Segura V, Sangro B, et al: Wilms' tumor 1 gene expression in hepatocellular carcinoma promotes cell dedifferentiation and resistance to chemotherapy. **Cancer Res 69:**1358–1367, 2009
35. Scharnhorst V, van der Eb AJ, Jochemsen AG: WT1 proteins: functions in growth and differentiation. **Gene 273:**141–161, 2001
36. Scholz H, Wagner KD, Wagner N: Role of the Wilms' tumour transcription factor, Wt1, in blood vessel formation. **Pflugers Arch 458:**315–323, 2009
37. Sugiyama H: WT1 (Wilms' tumor gene 1): biology and cancer immunotherapy. **Jpn J Clin Oncol 40:**377–387, 2010
38. Tamaki H, Ogawa H, Ohyashiki K, Ohyashiki JH, Iwama H, Inoue K, et al: The Wilms' tumor gene WT1 is a good marker for diagnosis of disease progression of myelodysplastic syndromes. **Leukemia 13:**393–399, 1999
39. Ueda T, Oji Y, Naka N, Nakano Y, Takahashi E, Koga S, et al: Overexpression of the Wilms' tumor gene WT1 in human bone and soft-tissue sarcomas. **Cancer Sci 94:**271–276, 2003
40. Van Meter TE, Dumur CI, Hafez N, Garrett C, Fillmore HL, Broaddus WC: Microarray analysis of MRI-defined tissue samples in glioblastoma reveals differences in regional expression of therapeutic targets. **Diagn Mol Pathol 15:**195–205, 2006
41. Veninga H, Becker S, Hoek RM, Wobus M, Wandel E, van der Kaa J, et al: Analysis of CD97 expression and manipulation: antibody treatment but not gene targeting curtails granulocyte migration. **J Immunol 181:**6574–6583, 2008
42. Wagner N, Michiels JF, Schedl A, Wagner KD: The Wilms' tumour suppressor WT1 is involved in endothelial cell proliferation and migration: expression in tumour vessels in vivo. **Oncogene 27:**3662–3672, 2008
43. Wang T, Ward Y, Tian L, Lake R, Guedez L, Stetler-Stevenson WG, et al: CD97, an adhesion receptor on inflammatory cells, stimulates angiogenesis through binding integrin counterreceptors on endothelial cells. **Blood 105:**2836–2844, 2005
44. Wang Z, Ahmad A, Li Y, Kong D, Azmi AS, Banerjee S, et al: Emerging roles of PDGF-D signaling pathway in tumor development and progression. **Biochim Biophys Acta 1806:**122–130, 2010
45. Wang ZY, Qiu QQ, Enger KT, Deuel TF: A second transcriptionally active DNA-binding site for the Wilms tumor gene product, WT1. **Proc Natl Acad Sci U S A 90:**8896–8900, 1993
46. Yang L, Han Y, Suarez Saiz F, Minden MD: A tumor suppressor and oncogene: the WT1 story. **Leukemia 21:**868–876, 2007
47. Yona S, Lin HH, Siu WO, Gordon S, Stacey M: Adhesion-GPCRs: emerging roles for novel receptors. **Trends Biochem Sci 33:**491–500, 2008
48. Zhang L, Wang L, Ravindranathan A, Miles MF: A new algorithm for analysis of oligonucleotide arrays: application to expression profiling in mouse brain regions. **J Mol Biol 317:** 225–235, 2002
49. Zhao Y, Xiao A, diPierro CG, Carpenter JE, Abdel-Fattah R, Redpath GT, et al: An extensive invasive intracranial human glioblastoma xenograft model: role of high level matrix metalloproteinase 9. **Am J Pathol 176:**3032–3049, 2010

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Current affiliation for Dr. Van Meter: Pediatric Hematology-Oncology, Department of Pediatrics, VCU School of Medicine.

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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: wbroaddus@mcvh-vcu.edu.