Title: Telomestatin impairs glioma stem cell survival and growth through the disruption of telomeric G-quadruplex and inhibition of the proto-oncogene, c-Myb.

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Translational Relevance

In this manuscript, we addressed our hypothesis that a G-quadruplex ligand telomestatin eradicates glioblastoma stem cells (GSCs). For the majority of our assays, we used the patient-derived glioblastoma cells, grown as spheres that are enriched with GSCs. Telomestatin treatment of these cultures, as well as the glioblastoma spheres-derived mouse xenograft tumor models, resulted in growth suppression and induction of apoptosis of GSCs. Furthermore, we demonstrated that telomestatin has a novel target molecule, a proto-oncogene c-Myb. c-Myb was identified with the cDNA microarray experiments with telomestatin-treated cells, and pharmacodynamic analysis with the mouse model of glioblastoma confirmed the microarray results. Depletion of c-Myb by gene knockdown significantly reduced GSCs' growth both in vitro and in vivo. Lastly, immunohistochemistry of c-Myb with 90 clinical samples revealed a statistically-significant elevation of c-Myb expression in surgical specimens of glioblastoma, supporting the significance of this proto-oncogene in glioblastoma biology.
Abstract

**Background:** Glioma stem cells (GSCs) are a critical therapeutic target of glioblastoma multiforme (GBM).

**Methods:** The effects of a G-quadruplex ligand, telomestatin (TMS), were evaluated using patient-derived GSCs, non-stem tumor cells (non-GSCs), and normal fetal neural precursors *in vitro* and *in vivo*. The molecular targets of TMS were determined by immunofluorescence *in situ* hybridization (iFISH) and cDNA microarray. The data was then validated by *in vitro* and *in vivo* functional assays, as well as by immunohistochemistry against 90 clinical samples.

**Results:** TMS impaired the maintenance of GSC stem cell-state by inducing apoptosis *in vitro* and *in vivo*. The migration potential of GSCs was also impaired by TMS treatment. In contrast, both normal neural precursors and non-GSCs were relatively resistant to TMS. Treatment of GSC-derived mouse intracranial tumors reduced tumor sizes *in vivo* without a noticeable cell death in normal brains. iFISH revealed both telomeric and non-telomeric DNA damage by TMS in GSCs but not in non-GSCs. cDNA microarray identified a proto-oncogene, c-Myb, as a novel molecular target of TMS in GSCs and pharmacodynamic analysis in TMS-treated tumor-bearing mouse brains demonstrated a reduction...
of c-Myb in tumors in vivo. Knockdown of c-Myb phenocopied TMS-treated
GSCs both in vitro and in vivo, and restoring c-Myb by overexpression partially
rescued the phenotype. Lastly, c-Myb expression was markedly elevated in
surgical specimens of GBM compared to normal tissues.

Conclusions: These data indicate that TMS potently eradicates GSCs through
telomere disruption and c-Myb inhibition, and this study suggests a novel
GSC-directed therapeutic strategy for GBM.
Introduction

The development of effective therapies for glioblastoma multiforme (GBM) is a challenging endeavor due to the aggressive proliferation and the high migratory potential of this form of cancer. Recent studies have suggested the existence of a hierarchical organization of multiple heterogeneous cell populations in GBM having distinct tumor driving capacities (1). Among heterogeneous tumor cells, glioma stem cells (GSCs) are defined as a subpopulation that is capable of self-renewal and differentiation into multi-lineaged tumor cells with distinct tumorigenic potentials in vivo. The resistance of GSCs to current forms of therapy is likely related to the failure of current treatments (2). Identification of novel therapeutic strategies for GSCs in GBM remains a major hurdle to effectively attack this highly malignant tumor. GSCs exhibit phenotypic and genetic similarities to their somatic counterparts, neural stem cells (NSCs). Targeting shared pathways that regulate the survival of both GSCs and NSCs, therefore, may eradicate both types of stem cells. To accomplish the selective targeting of GSCs over NSCs, it is crucial to uncover the mechanisms that
specifically regulate the initiation of GBM and the maintenance of the stem cell-like phenotype of GSCs.

Human chromosomes are capped with telomeres, regions of repeating DNA that prevent the degradation of genes at the chromosomal ends (3). Due to incomplete replication of linear DNA ends, human somatic cells progressively shorten their telomeres during every round of cell division. Immortalized cancer cells have an increased ability to extend their telomeres, which is one of the reasons that cancer cells can bypass replication senescence and subsequent cell death (4, 5). If indefinite cell division is a property that is unique to tumor stem cells, one potential approach to target GSCs would be to disrupt telomere extension. In the process of telomere elongation, the enzyme telomerase plays a critical role. Despite the emerging hope for attacking telomerase in cancers based on this unique character, the major limitation of this approach in clinical settings is its delayed effectiveness.
Telomestatin (TMS) is a natural product isolated from *Streptomyces anulatus* 3533-SV4 (6) and stabilizes the G-quadruplex (7) that is postulated to be present in telomeric DNA (3) and in the promoter regions of several proto-oncogenes (8-11). Formed G-quadruplex structures function as transcriptional repressor elements (12). Treatment with TMS induces apoptosis of various cancer cells with relatively less of an effect on somatic cells (13,14). Although the effect of TMS on telomeric DNA has been well described, it is not clear if it is the only mechanism of higher sensitivity of cancer cells over somatic cells. In addition, the sensitivity of cancer stem cells to TMS has not been demonstrated yet.

Here we show that TMS triggers the preferential apoptosis of GSCs with less of an effect on normal precursors or non-GSCs in GBM. Immunofluorescence *in situ* hybridization (iFISH) detected the presence of damage in both telomeric and non-telomeric DNA regions in GSCs but not in non-GSCs. Analysis of a cDNA microarray identified a reduction in the proto-oncogene, c-Myb, following TMS treatment of GSCs. Decreased c-Myb expression was also observed in pharmacodynamic analyses of TMS-treated xenografted tumors. Moreover,
treatment of tumor-bearing mice demonstrated a statistically significant reduction in tumor sizes in vivo. Lastly, immunohistochemistry of clinical samples of GBM and normal tissues exhibited a statistically-significant elevation of c-Myb levels in tumors. Collectively, these data suggest that targeting GSCs with TMS has the potent effect on diminishing GBM growth in vitro and in vivo through disruption of telomeric DNA and inhibition of c-Myb.

Materials and Methods

Cell lines and Cell Culture

Characteristics of 8 GBM (GBM146, 157, 205, 206, 218, 1600, 2313, and 13) and 2 non-tumor human fetal brain specimens (f16w and 1105A) from aborted fetus was published previously (15-18). Methods for establishment of GBM sphere cultures and normal sphere cultures are published previously (16,18) and details are described in Supplemental information. All the experiments were performed with short-term cultures within 20 passages.

Chemicals

TMS was synthesized in the Biomedicinal Information Research Center, National
Institute of Advanced Industrial Science and Technology (Tokyo, Japan). The quality of TMS was assessed with HPLC (UPLC) system and the readout was based on the UV absorptions. The structure of TMS was also confirmed by $^1$H and $^{13}$C NMR spectra. Other anticancer drugs and chemicals were purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO).

**Flow cytometry analysis** Fluorescence-activated cell sorting (FACS), Sphere-forming assay, Immunostaining, Apoptosis assay, Cell growth assay, Xenograft, Western blot, tumor size measurement, Immunofluorescence *in situ* hybridization (iFISH) assay, transient transfection, lentivirus-mediated shRNA, and RT-PCR

Detailed methods are published previously (16,18-20) and described in supplemental information.

**Brain slice invasion assay**

Brain slice invasion assay was performed as described previously (21). Briefly, GBM spheres of 300 to 400 µm diameter treated with TMS or vehicle (DMSO) for 24 hours. Coronal brain slices obtained from neonatal mice were prepared for organotypic culture. Viable spheres that did not stain with Propidium Iodide were washed in slice medium, manually placed on the brain slices, and imaged by
fluorescence microscopy every 24 hours. Cells from spheres treated with TMS were also assessed for cell viability using an assay for reduction of soluble tetrazolium (Cell Titer kit, Promega).

**cDNA microarray**

RNA was extracted from GBM sphere samples treated with DMSO or 1 µM TMS for 24 hours with RNeasy Mini Kit following the manufacturer's protocol (QIAGEN). RNA samples were analyzed by the FGC using an Agilent 2100 Bioanalyzer Lab-On-A-Chip Agilent 6000 Series II chip to determine the integrity of the samples. Sample labeling and hybridization were performed according to the manufacturer's protocols. Scanning and Image Analysis: Microarray slides were hybridized overnight, washed, and then scanned with Agilent G2505C Microarray Scanner. The information about each probe on the array was extracted from the image data using Agilent Feature Extraction 10.5 (FE). The raw intensity values from these files are imported into the mathematical software package “R”, which is used for all data input, diagnostic plots, normalization and quality checking steps of the analysis process.

**Tissue microarray**

The slides of GBM tissue microarray were obtained from Department of
Pathology at the Ohio State University. After deparaffinization by Xylene, immunohistochemistry with the c-Myb antibody was carried out as described previously (22). Isogenic IgG control was used to confirm the specific signals for c-Myb immunoreactivity. The staining intensities were blindly categorized into three groups (negative, single positive, double positive) by two independent neuropathologists.

Statistics

Values are given as means ± SD, unless noted otherwise in the figure legend. The number of replicates is noted in the figure or legends. Absent error bars in the bar graphs signify SD values are smaller than the graphic symbols. Comparison of mean values between multiple groups was evaluated by an Tukey HSD Test for Post-ANOVA Pair-Wise Comparisons in a One-Way ANOVA unless noted otherwise in the figure legend. Comparison of mean values between two groups was evaluated by chi-square test or t-test. All statistical tests were two-sided. For all statistical methods a p-value less than 0.05 was considered significant.
Results

TMS is a relatively selective inhibitor of brain tumor cell lines and inhibits growth of patient-derived GBM spheres in vitro.

The effect of TMS in vitro was first tested on a panel of 39 human cancer cell lines (JFCR39) (23) (Figure 1A). Cells derived from tumors of the central nervous system (CNS) exhibited higher sensitivity compared with others. With these brain tumor cell lines, the concentration of TMS required for a 50% growth inhibition (GI50) ranged between 1 and 10 µM (Figure S1A). With patient-derived short-term GBM cell cultures (GBM1600 and GBM2313) propagated in serum-containing medium, the GI50 was approximately 5 µM (Figure S1B). This result suggests that TMS is a relatively potent and selective inhibitor of brain tumor-derived cells compared to other cancer-derived cells.

Next, the sensitivity of GSCs to TMS was examined. Sphere-forming potential is one unique characteristic of GSCs (24, 25). Using short-term cultures derived from 5 GBM patient specimens, we investigated the effect of varying doses of TMS on sphere formation (Figure S1C). Remarkably, in all samples treatment with 1 µM TMS completely abolished sphere formation. In 3 of these 5 samples,
treatment with 0.1 µM of TMS significantly reduced sphere numbers as well (Figure S1C) (p<0.05). These results suggest a prominent inhibitory effect of TMS on GSC phenotypic sphere formation capability in vitro.

**TMS preferentially inhibits survival of patient-derived GSCs compared to normal neural precursor cells.**

In several organs, a differential sensitivity to TMS treatment was previously observed between tumor cells and their somatic counterparts (13,14). We therefore sought to compare the effects of TMS treatment on the growth of patient-derived GSCs and normal neural precursors derived from human fetal brains. Normal neural precursors and GSCs were enriched in serum-free media with the supplement of basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) (Figure S2). When both of these cultures were stimulated with 10% fetal bovine serum (FBS) for differentiation, multiple types of lineage-committed cells were observed, as seen by a decline in Sox2 expression, and up-regulation of neuron-specific class III beta-tubulin (TuJ1) and glial fibrillary acid protein (GFAP) (Figure S2). Following examination of these precursor cells, we found that, compared to GSCs, neural precursor cells were
relatively resistant to TMS treatment (Figure 1B). The graph in Figure 1B demonstrates a significant difference of relative cell numbers between 3 GSC samples (GBM146, GBM157, and GBM206) and 2 normal neural precursor samples (f16w and 1105A) after treatment with 1 µM TMS for 96 hours. These data suggest that TMS treatment strongly impairs GSC growth, while normal neural precursors are less sensitive to TMS treatment. Likewise, we verified the effective dose of TMS required to affect the growth properties of human astrocyte primary cultures and immortalized human astrocytes overexpressing E6, E7, and hTERT (NHA). One phenotypic difference between these two cultures is that only NHA cells are tumorigenic in immunocompromised mouse brains (26). Following incubation of these two cultures with varying doses of TMS for 96 hours, we found that normal astrocytes are more resistant to treatment compared to NHA cultures (Figure S1D).

A cell surface protein, CD133, is not a universal marker for GSCs in GBM (27); however, we have found that in some of our clinical samples, positive CD133 expression correlates well with properties of GSCs, including tumorigenicity, in vitro clonogenic potential, multi-lineage differentiation capability, and high
expression of stem cell-related genes and proteins (18, 28). We therefore reasoned that if TMS preferentially eradicates GSCs, the proportion of CD133 (+) cells among total GBM cells should decrease with treatment. To explore this possibility, we treated tumor spheres derived from the 3 GBM cases with varying doses of TMS for 48 hours and performed fluorescence-activated cell sorting (FACS) to determine the proportion of CD133 (+) cells. As expected, TMS treatment reduced the proportion of CD133 (+) cells in a dose-dependent manner with the proportion of CD133 (+) cells being approximately half of the control samples at 1 µM concentration (91.5% vs. 62.2% in GBM146, 92.6% vs.43.5% in GBM157 and 60.7% vs. 24.3% in GBM206) (Figure 1C, upper panel and S3A). By separation of GBM157 cells based on cell surface CD133 expression, we confirmed that sphere forming potential is restricted to CD133 (+) cells, but not CD133 (-) cells (Figure S3B). When treated with TMS (1 µM), these sphere-forming cells were eliminated (Figure S3B). Immunocytochemistry with a CD133 antibody and Hoechst dye demonstrated that TMS treatment resulted in the condensed fragmentation of nuclei of CD133 (+) cells, suggesting the induction of apoptosis, but not differentiation of CD133 (+) cells into CD133 (-) cells by TMS (Figure 1C, lower panel). Taken together, these results
demonstrate that TMS treatment preferentially eradicates GSC through an apoptotic mechanism.

We then sought to determine the effect of TMS on GSC self-renewal. One means of estimating in vitro self-renewal is through the use of serial assays to test multiple rounds of sphere-forming capacity (29). We analyzed the capacity of these cells to form secondary spheres after TMS treatment (Figure 1D). Following 7 days of incubation with TMS, we carried out secondary sphere forming assays using drug-free culture conditions. A reduction in secondary sphere numbers was observed with even less concentrations of TMS (0.2-0.5 µM), indicating that treatment with lower doses of TMS abolishes self-renewal capacity of GSCs in vitro.

**Treating tumor-bearing mice with TMS reduces tumor growth in vivo.**

We next sought to test the effect of TMS treatment on tumor cell growth in vivo. First, we performed transplantation of 2 GBM sphere samples (GBM146 and 157) pre-treated with TMS (1 µM or 5 µM) into immunocompromised mouse
brains in accordance with Institutional Animal Care and Use Committee-approved protocols at OSU (Figure S4). These tumor-bearing mice were then sacrificed at 3 months post-transplantation and sizes of resultant tumors were determined by immunohistochemistry with human-specific Nestin and Vimentin antibodies. The average size of intracranial tumors was reduced with the lower dose (1 µM) (Figure S4, B and E) and almost completely abolished with the higher dose (5 µM) of TMS (Figure S4, C and F). Similar results were observed with the GBM146-derived model treated with 5 µM TMS (Figure S4, D and G). Collectively, pre-treatment of GSCs with TMS potently inhibits their in vivo tumor formation and growth.

Second, we evaluated the therapeutic efficacy of TMS by injection into the intracranial tumor cavities of immunocompromised mice. The mouse intracranial tumors were created by implantation of GBM157 spheres into the striatum (Figure 2A). We then injected TMS at 2 different time points; at day 0 (on the day of tumor implantation) and at day 14. The rationale for choosing these two time points is to investigate separately the effect of TMS on both tumor initiation and growth of established tumors. We then measured tumor sizes 3 months after...
transplantation. The effect of the treatment was evaluated by immunohistochemistry using a human-specific Nestin antibody and a Vimentin antibody (Figure 2B) (18). In both cases, local injection of TMS resulted in a 55-71% reduction in the overall tumor size, determined by Nestin immunoreactivity (Figure 2B, upper panels). Similarly, a 72-76% reduction was observed with Vimentin staining (Figure 2B, lower panels). In order to verify whether this procedure produces any cytotoxic effects on normal brain, we performed immunohistochemistry for activated caspase-3, a marker for apoptosis, in mouse brains bearing tumor cells 2 days after TMS injection. Activated caspase-3 immunoreactivity was strictly restricted within the TMS-injected tumor lesions and not in adjacent normal brain tissues including the subventricular zone of the lateral ventricles (Figure 2C, upper panels). These data were in concurrence with the results of Ki-67 staining which show a reduction in proliferating cells in TMS-treated tumors (Figure 2C, lower left panels). In control tumors treated with DMSO, few cells were positive for activated caspase-3, even in the area of tumor cell injection (Figure 2C, lower right panels). In summary, we confirmed that intratumoral TMS treatment significantly diminishes tumor size in vivo without causing the apparent death of
normal cells.

**TMS reduces migration of GSC in vitro and in vivo.**

One of the hallmarks of human GBM is its invasive and infiltrative nature. In agreement with recent studies that showed an enhanced ability of GSCs for migration and invasion (30), GSC-derived tumor models exhibit aggressive migration upon transplantation into mouse brains (Figure 3A). Of note, TMS-treated tumor cells showed significantly less migration into the contralateral hemisphere (Figure 3A, lower panels). This could be due to a reduced number of live tumor cells and/or to reduced invasive capacity. To further study this observation, we performed an assay for cell invasion on organotypic cultures of brain tissue which mimics brain cytoarchitecture and natural barriers to cell movement (Figure 3B) (31). First, we confirmed that undifferentiated GBM spheres (GSCs) possess greater migration potential into mouse brains than non-GSCs from the same samples (Figure 3C). We then treated GBM146 and GBM157 spheres with 3 different doses of TMS for 24 hours, seeded them onto mouse brain slices, and used fluorescence microscopy to evaluate invasion for up to 72 hours, as previously described (32). Both GBM sphere samples
dispersed aggressively through brain tissue in the absence of the drug (Figure 3D). In contrast, TMS significantly reduced cell migration in a concentration-dependent manner. Assessment of viability by in situ fluorescence and parallel viability assays indicated that this effect was specific for cell migration; since TMS did not affect viability of non-dissociated spheres until 48 hours (5 µM) to 96 hours (1 µM) after treatment (data not shown). Collectively, these data indicate that TMS abrogates both growth and migration of GSCs in vivo and in vitro.

TMS-treated GSCs, but not non-GSCs, undergo apoptosis through both telomeric and non-telomeric DNA damage.

Although previous studies have reported several different mechanisms of action for TMS, it appears to kill non-CNS tumor cells primarily through DNA damage-derived apoptosis (13,14, 33-35). When GBM sphere samples (GBM146, 157, and 206) were treated with TMS, we found a 17-35% increase in the number of cells with fragmented nuclei by Hoechst dye staining, a typical morphological feature of apoptotic cell death (Figure 4A). An abundance of apoptotic cells in TMS-treated GBM146 and 157 spheres was also confirmed by
staining with fluorescein-conjugated Annexin V antibody and labeling with Propidium Iodide (Figure S5A). Western blot analysis showed an induction of p53 in TMS-treated GBM spheres (Figure S5B).

Since our data in Figure 1 suggest the potency of TMS is predominantly in CNS tumor-derived cell lines and GBM spheres, we hypothesized that TMS has previously uncharacterized molecular targets in the non-telomeric DNA of GBM cells. To address this question, we performed the iFISH assay that enables the simultaneous localization of telomeres and 53BP1, a marker of the DNA damage response (36). As shown in Figure 4B, DMSO-treated control GBM146 GSCs exhibited uniform distribution of 53BP1 in the nucleoplasm, indicating that these cells did not initiate the DNA damage response under normal growth conditions. Conversely, TMS-treated GSCs rapidly developed punctate, nuclear 53BP1 foci in a dose-dependent manner (Figure 4B). Importantly, some of these foci colocalized with non-telomeric DNA (Figure 4C), therefore representing both telomeric and non-telomeric dysfunction-induced foci, a hallmark of deprotected DNA damage (37, 38). Meanwhile, consistent with their lower susceptibility to TMS-induced apoptosis, non-GSCs in GBM146 did not exhibit the 53BP1 foci
upon treatment with TMS or DMSO under the identical culture condition (Figure 3B and data not shown). Taken together, these results indicate that TMS induces telomeric and non-telomeric DNA damage preferentially in GSCs, and the loss of tumor stemness is likely associated with a failure in the DNA damage response in GSCs elicited by TMS.

**c-Myb is another target of TMS in GSCs.**

The presence of 53BP1 foci in DNA regions separate from telomeres led us to search for target genes of TMS. To this end, we performed cDNA microarray experiments using 4 GBM sphere samples with or without TMS treatment. Specifically, we focused on changes in G-quadruplex-interacting genes and telomere-related genes. Among the 15 genes identified, only c-Myb expression was significantly decreased in all 4 TMS-treated GBM sphere samples (Figure 5A). An analysis of genes that were previously reported as stemness-related genes or pro-neuronal, mesenchymal, or proliferative GBM (3834) did not identify any genes whose expression was significantly decreased by TMS treatment (Figure 5A). Downregulation of c-Myb in GBM spheres was further validated by RT-PCR and Western blot in all 4 samples (Figure 5B and Figure
S6). Conversely, c-Myb expression was not affected in TMS-treated non-GSCs or normal precursors, and as expected, the basal levels of c-Myb expression in these cell populations were substantially lower than those in GSCs (Figure 5B). Immunocytochemistry demonstrated the presence of c-Myb expressing cells in Nestin-positive GBM157 spheres (Figure 5C, left panels) and the decline in c-Myb expression in TMS-treated GBM spheres was also confirmed by immunocytochemistry (Figure 5C, right panels). In agreement with decreased expression *in vitro*, pharmacodynamic evaluation of mouse intracranial tumors at day 2 following TMS injection demonstrated an *in vivo* reduction of c-Myb immunoreactivity (Figure 5D). These data support the idea that c-Myb is another molecular target of TMS, and suggests c-Myb is involved in survival and/or growth of GSCs in GBM.

**c-Myb regulates GSC growth *in vitro* and *in vivo***.

We then sought to determine the functional role of c-Myb in GSCs both *in vitro* and *in vivo*. First, we evaluated the effect of c-Myb overexpression and knockdown with GBM157 spheres *in vitro*. c-Myb knockdown resulted in abrogated growth of GBM157 sphere cells, which phenocopied the results with
TMS treatment (Figure 6A, left panel and Figure S7). In turn, c-Myb overexpression in TMS-treated GBM157 spheres resulted in a full recovery of sphere cell growth at 0.5µM of TMS and a partial recovery at 1µM of TMS (Figure 6A, right panel and Figure S7). Next, we evaluated the effect of c-Myb knockdown on in vivo tumor growth. To this end, another short-term GBM sphere culture, GBM13, was infected with lentivirus harboring shRNA for either c-Myb or a non-targeting sequence (Figure S7) (26), and the survival periods of tumor burdened mice were determined (Figure 6B). Consequently, lentivirus-derived c-Myb knockdown prolonged the survival of mice with GSC-derived intracranial tumors in a statistically significant manner. Taken together, these data suggest that c-Myb plays an essential role in GSCs growth in vitro and in vivo, and is a target of TMS in GSCs.

c-Myb is highly expressed in some GBM tissues.

To evaluate the clinical relevance of the function of c-Myb in GBM, we investigated the expression of c-Myb in 66 surgical specimens of GBM and 24 normal brain tissues (Figure 6C). We performed immunohistochemical staining with these samples using an antibody for c-Myb (22). c-Myb immunoreactivity
was specifically observed in the nuclear compartments in both samples.

Representative pictures display the typical pattern of the staining (Figure 6C). As a result, c-Myb was abundantly expressed in nuclei of GBM cells in 62% of samples, whereas only 25% of normal brains showed positivity of c-Myb immunoreactivity (p=0.004) (Figure 6D).

Discussion

The cancer stem cell hypothesis postulates the existence of a subpopulation of poorly differentiated tumor cells with the capacity to self-renew and repopulate a diversity of lineage-committed tumor cells. By definition, in vivo tumor formation in immunocompromised mouse brains is the only direct evidence for the presence of GSCs within a pool of heterogeneous tumor cells. However, it is possible that this assay may select for tumor cells that have escaped from the immune system and/or human cells that have adapted to the microenvironment in mouse brains. These issues aside, the therapeutic significance of TMS treatment for human GBM is suggested by its effect on in vivo growth in mouse brains (Figure 2). In order to obtain clinically relevant data concerning the effect
of TMS treatment, we performed intratumoral injection of TMS. As a result, tumor size was significantly decreased. However, this treatment did not completely eliminate the tumors. It is possible that GSCs may contain a subpopulation of TMS-resistant cells. The relatively small number of samples tested in this study has allowed only limited interpretation concerning the genetic and phenotypic differences of GSCs as well as how universally effective TMS treatment would be on human GBM patients. To determine the therapeutic window of TMS, we need to address the question of whether or not the normal brain can tolerate doses of TMS required to kill tumor cells.

The cell sorting technique using cell surface markers is one potential means of isolating stem cells. This method has been widely adopted to determine the presence of GSCs in heterogeneous tumor cells (18, 24, 25). One of the limitations of this assay is the lack of universal and definitive markers for GSCs. CD133 is the most common antigen and AC133 is a monoclonal antibody against this cell surface protein (24). This antibody appears to correlate to some extent with the presence of GSCs in multiple different cell populations (24, 3940).
However, several studies have reported the presence of CD133-negative GSCs in GBM samples possibly due to patient-to-patient phenotypic and genetic differences (27). More recently, CD15 has gained attention as another potential marker for GSCs (4041). At this point, any of the currently available cell surface antibodies are only surrogate markers for GSCs and need to be tested on a case-by-case basis. In the samples of this study, CD133, but not CD15, correlated with enrichment of GSCs (data not shown).

TMS interacts with the G-quadruplex recognizing sequences in the genome which are present in the telomeric DNA (3), as well as the promoter regions of several proto-oncogenes (8-11). Previous studies have identified several potential target molecules including hTERT (13, 34), c-Myc (10) HIF1-alpha (11), RET (8) and PDGFR-beta (9), c-Myc (10), HIF1-alpha (11). The effective doses found in most of these studies were relatively high, from around the several micromolar range. In this study, patient-derived GBM sphere cells underwent apoptosis at approximately 0.5-1 µM, and no obvious change in expression of the above genes was detected. Instead, c-Myb, which also has the TMS-binding
sequences in its promoter region (4142), was the only gene showing a significant reduction in expression (Figure 5). Both reduction of c-Myb expression and foci-formation in telomeric DNA occurred in GBM spheres but not in non-GSCs following TMS treatment. Together, two distinct mechanisms of TMS action in GBM include, but may not be exclusive to, the direct disruption of telomere structure and inhibition of c-Myb.

In the CNS, c-Myb expression is restricted to the germinal zones of the adult brain and maintains progenitor cell proliferation and ependymal cell integrity (22). However, mice with the conditional brain-specific deletion of c-Myb exhibit only a mild defect in brain structure with hydrocephalus and ependymal cell abnormalities (22). To date, its function in gliomagenesis and maintenance of the immature state of GSCs has not been uncovered. We found that c-Myb expression is markedly elevated in clinical samples of GBM compared to normal brain tissues, and undifferentiated GBM spheres compared to normal spheres or non-GSCs. Our functional data using knockdown and overexpression (Figure 6) provide the first evidence that c-Myb plays a fundamental role in the growth of GSCs both in vitro and in vivo. Taken together, these data indicate that c-Myb is a potential new therapeutic target in the treatment of GBM.
TMS is a hydrophobic agent with a relatively large molecular weight (Molecular Weight of 583 kDa). The pharmacokinetics of this compound is not well defined and, more importantly, it is still unknown whether TMS penetrates the blood-brain barrier. In this study, we sought to use local (intratumoral) injection of TMS to deliver the drug directly to tumor cells. One of our aims is to identify safe and effective anti-GBM agents that can be applied to the removal cavity after tumor resection during surgery. Given that GBM is a tumor with an aggressive infiltrating nature, migrated tumor cells into the adjacent normal brain tissue are left behind during surgery. The presence of residual tumor cells makes relapse of the tumor inevitable within months or years of the surgery, despite adjuvant therapies. In order to achieve selective and effective eradication of the remaining tumor cells with minimal organ toxicity, we sought to identify anti-GSC agents that can be directly injected into the tumor cavity. Despite our positive data (Figure 2), several open questions still remain. For instance, we cannot completely exclude the possibility of normal cell death following local injection of TMS. Particularly, its effect on long-term self-renewal of normal NSCs in the subventricular zone and hippocampus in the adult brain needs to be fully addressed.
In this study, we demonstrated that TMS treatment preferentially targets GBM cells with stem cell-like characteristics. TMS decreased sphere-forming potential in vitro, reduced CD133 (+) cell population in vitro, and decreased tumor sizes in vivo. In addition, normal neural precursors treated with the same dose of TMS demonstrated less cytotoxicity. A search for target genes of TMS by cDNA microarray identified the proto-oncogene c-Myb and further functional analysis using GBM spheres demonstrated that c-Myb plays a critical role in the growth of GSCs both in vitro and in vivo. Lastly, a significant elevation of c-Myb expression in surgical specimens of GBM supports the clinical relevance of our findings. We believe that these data serve as a rationale for the design of a novel anti-GBM therapy. Future studies will shed light on the molecular functional role of c-Myb in GBM and the therapeutic window of TMS for GBM.

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37. Takai H, Smogorzewska A, de Lange T. DNA damage foci at dysfunctional

glioma predict prognosis, delineate a pattern of disease progression, and


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Competing Interest

All authors have seen and approved the manuscript, and there is no conflict of interest to declare.

Figure Legends

Figure 1. Growth inhibition activity of TMS against GSC.

(A) Sensitivity of 39 cancer cell lines to TMS for 48 hours. A panel of 39 human cancer cell lines (termed JFCR39) was previously described (20). Values below zero indicate resistance to TMS, and values above zero indicate sensitivity. Negative 1 indicates the treatment with highest dose of TMS did not reach below 50 % decrease in cell number. (B) Time course of relative cell numbers of 3 GSC
and 2 normal neural precursors at 1 µM of TMS. Asterisk (*) indicates statistical significance by two-sided t-test (p=0.00049). (C) Histograms indicating the proportions of CD133(+) cells in GBM146 spheres after treatment with varying doses of TMS for 48 hours (upper panel). Immunocytochemistry of GBM146 sphere cells stained with CD133 (red) after treatment with or without 1 µM of TMS for 48 hours (lower panel). Hoechst dye was used for nuclear staining. The arrow indicates a CD133 (+) cell with condensed fragmentation of nuclei. Original magnification; x40. (D) Experimental flow (left). Graphs (right) indicate the proportions of sphere-forming cells derived from TMS-pretreated 2 GBM samples (GBM146 and GBM157). Abbreviation; NS, neurospheres; D, DMSO. Asterisk (*) indicates statistical significance by two-sided t-test (p<0.05).

Figure 2. Reduction of GSC-derived tumor sizes with intratumoral TMS injection.

(A) Experimental flow. TMS was injected into the tumor cavity by 2 different regimens. One with 5 pMol of TMS at the same time of tumor cell xenograft (Day 0) (left). The other with 50 pMol of TMS at 14 days after tumor cell xenograft
(B) Representative pictures indicate human specific Nestin staining (upper panels) and Vimentin staining (lower panels) of immunocompromised mouse brains bearing GBM sphere-derived tumors with DMSO or TMS intratumoral injection at indicated time points. Original magnification; x2. Graphs indicate the average of overall tumor sizes determined by immunostaining with human-specific antibody in each group. The number of mice in each group is indicated in the graph. Asterisk (*) indicates statistical significance by two-sided t-test. Exact p-values are indicated in the figure. (C) Immunohistochemistry of mouse brains at 2 days following intratumoral injection of DMSO or 2.5 nMol of TMS with antibodies for human-specific Nestin, activated Caspase-3, and Ki-67. Pictures at the injection site are shown in upper left 2 panels and lower panels and pictures at the lateral ventricle are shown in upper right 3 panels. Original magnification; x20.

**Figure 3. Inhibitory effect of TMS on GSC migration.**

(A) Representative pictures of human-specific Nestin staining of immunocompromised mouse brains bearing human GBM157 sphere-derived
tumors (upper panel). Original magnification; x10. The asterisk in the photograph indicates the location of xenograft. The rectangle indicates the region of magnification in the lower panels. Human-specific Nestin staining indicates migrated human GBM cells to the contralateral side of mouse brains through the corpus callosum in the control (DMSO-treated) group (lower left panel) and TMS-treated group (lower right panel). Original magnification; x40. (B)

Experimental flow for the migration assay in organotypic cultures of mouse brain slices. (C) Graphs for the time course of migration of GBM spheres (GSCs) and serum-propagated cells (non-GSCs) derived from 2 GBM samples (GBM146 and 157). Quantitative analysis indicates cell dispersion (Area t_x / Area t_0). Asterisks (*** indicate statistical significance by two way ANOVA for repeated measures (p<0.001). (D) Representative pictures of GFP-expressing human GBM146 cells dispersing on brain slices after treatment with 1 µM of TMS or DMSO (left). Graphs indicate the relative dispersion of the cells normalized the initial size and fluorescence of the spheres (right). Asterisks indicate statistical significance by two way ANOVA for repeated measures (**p<0.05, ***p<0.001).
**Figure 4.** Formation of 53BP1 foci in telomeric and non-telomeric DNA in GSC, but not non-stem tumor cells.

(A) Representative pictures of Hoechst dye staining to detect chromatin condensations in late apoptotic cells (indicated by arrows) treated with indicated dose of TMS for 96 hours (left), and the graph indicating the proportion of apoptotic cells in 3 GBM spheres (right). Asterisk (*) indicates statistical significance (*p*<0.05). (B) iFISH analysis indicating telomeric and non-telomeric DNA damages in TMS-treated GBM spheres. GBM146 spheres (GSCs) or serum-propagated GBM146 cells (non-GSCs) were treated with 1 µM of TMS in serum-free medium for 96 hours and subjected to iFISH analysis (upper panels). Red, telomeric DNA; green, 53BP1; blue, DAPI staining for nuclear DNA. Quantitative graph of the 53BP1 focus-positive cells (lower panel). GBM146 cells were classified into the focus-positive or negative fractions according to the number of cells with punctate nuclear 53BP1 foci (n>2). Asterisk indicates statistical significance (*p*<0.001). (C) Magnified views of the representative telomere dysfunction-induced foci (arrow in left) and non-telomeric DNA damage foci (arrow in right) in GSCs derived from GBM146 treated with 1 µM of TMS for 96 hours.
Figure 5. c-Myb as a target of TMS in GSC.

(A) Identification of distinct gene expression profiles in TMS-treated GSCs. The fold changes in representative candidates for indicated genes (left panels). Scatter plot of normalized signal intensities of GSCs with TMS treatment (right panel). (B) Change of c-Myb expression level with TMS treatment in GBM146 spheres (GSCs), serum-propagated GBM146 cells (non-GSCs), and normal neural precursors (1105A) determined by RT-PCR and Western blot. Abbreviation; D, DMSO; T, TMS. (C) Immunocytochemistry of GBM157 spheres with c-Myb and human-specific Nestin antibodies. Hoechst is used for nuclear staining. Insets indicate staining results without the primary antibodies. Original magnification; x20. (D) Pharmacodynamic analysis of TMS or DMSO on c-Myb expression in GBM157 sphere-derived tumors in mouse brains. Two days post-injection of TMS (2.5 nMol), brains were stained with c-Myb antibody and counterstained with hematoxylin. Arrows indicate c-Myb (+) cells at the injection site. The proportions of c-Myb (+) cells in total cells per field are indicated at the bottom.
Figure 6. c-Myb is essential for GSC growth and its expression is elevated in surgical specimens of GBM.

(A) Right graph indicates the relative cell numbers of GBM157 spheres at day 7 post-transfection with indicated siRNA constructs. Left graph indicates the relative cell numbers of GBM157 spheres at day 7 post-transfection with indicated vectors with or without TMS treatment. (*; p<0.001) (B) Kaplan-Meier curves indicating proportion of live mice harboring intracranial tumors derived from GBM13 spheres infected with shRNA lentivirus for c-Myb (shc-Myb) or non-targeting sequence (shcontrol). (C) A single slide with 66 GBM specimens and 24 normal brain tissues stained with c-Myb antibody (left panel). Representative staining results with 2 GBM tissues and one normal brain is shown (brown in middle panels). IgG control shows the background staining intensity. (D) Graph indicates the proportion of c-Myb strongly positive (++), weakly positive (+), and negative (-) samples in normal brain tissues (n=24) and GBM tissues (n=66) (p=0.004).
## Table A

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### Diagram A

![Graph showing log fold change](image)

## Table B

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## Diagram B

![Western blot images](image)

## Table C

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## Diagram C

![Immunofluorescence images](image)

## Table D

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Telomestatin impairs glioma stem cell survival and growth through the disruption of telomeric G-quadruplex and inhibition of the proto-oncogene, c-Myb.

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