

Supplemental Data

Restoring sGC expression and function blocks the aggressive course of glioma

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Supplemental Materials and Methods

Reagents

Polyclonal anti-sGC α 1 and monoclonal anti- α -tubulin antibodies were acquired from Sigma (St. Louis, MO), polyclonal anti-sGC β 1 antibodies were either generated by our lab or purchased from Calbiochem (Madison, WI); monoclonal anti-CD133 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and CD133 cell isolation kit was from Miltenyi Biotec (Bergisch Gladbach, Germany). Unless otherwise indicated, all routine chemicals and reagents were purchased from Sigma, and tissue culture media and reagents from Invitrogen (Carlsbad, CA).

Specimen collection, establishment of primary cultures

We obtained primary cell cultures from a set of pathologically confirmed human benign and malignant meningiomas and gliomas. Tissue was obtained from 10 patients who underwent therapeutic removal of primary intracranial tumors. Control tissue samples were obtained adjacent to each tumor from noneloquent white matter deemed normal based on radiographic and histological appearance. In addition, we measured human telomerase reverse transcriptase (hTERT) mRNA expression levels in both meningioma and glioma specimens using real-time PCR. hTERT levels were increased in malignant cells as expected (**fig. S3**). All samples were immediately used to establish primary cultures. To do so, tumor fragments were dispersed into individual cells by treatment with Dispase I for 15 to 30 min at 37°C. From each tumor, 10⁶ cells were then plated in a 100x20-mm tissue culture dish in low-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS)

plus 1% penicillin/streptomycin mixture. The cells were grown to confluence and then harvested, aliquoted, and stored in liquid nitrogen for future use.

Primary tumor sphere culture

Primary cultured human glioma cells were seeded in 24-well plates at 2×10^4 cells/well in DMEM/F12 medium containing 20 ng/mL EGF, 20 ng/mL bFGF, 10 ng/mL N2 supplement, plus 1% penicillin G/ streptomycin mixture (Gibco, USA). After primary spheres were formed and reached the size of 100-200 cells, they were harvested, dissociated into single cells and the CD133 positive cells were separated by magnetic cell sorting technique (MACS; Bergisch Gladbach, Germany). The CD133-positive cells were diluted to yield 1 to 2 cells/10 μ l and plated into 96-well plates; fresh medium was added up to 100 μ l. The formed spheres were used for experiments.

Quantitative real-time reverse transcription-PCR and reverse transcription PCR

Total RNA was isolated using UltraSpec reagent (Biotechx, Houston, TX) according to the manufacturer's instructions. Complementary DNA was synthesized by using a reverse transcription-PCR archive kit (Amersham Bioscience, Sweden) following the manufacturer's protocol. All quantitative real-time PCR tests were performed using the standard real-time PCR-protocol of the ABI Prism 7700, with a total reaction volume of 25 μ l as previously described (Bian et al., 2001). The temperature profile consisted of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C. The cycle threshold (Ct) for every sample is directly proportional to the amount of input template, so that with the use of a plasmid standard, the relative number of template molecules in the reaction was determined

by using a plasmid standard containing the desired amplicon. Q-PCR data were normalized to the level of acidic ribosomal phosphoprotein P0 (36B4; housekeeping gene) or β -actin.

Regular reverse transcription PCR was performed to identify the existence of PDE in U87 glioma cells, and identity of the bands was confirmed by sequencing. The primers used in reverse transcription PCR and the assay ID for real time PCR are listed in **supplemental Table 1** and **supplemental Table 2** respectively.

Western blot analysis

Cells were harvested and lysed by sonication in ice-cold PBS containing proteinase inhibitor cocktail. Equal amounts of protein (50 or 100 μ g/well) were loaded and separated by 7.5% SDS-PAGE. Separated proteins were transferred to a nitrocellulose membrane which was treated with 5% nonfat dry milk in TBS-T (20 mM Tris-HCl, 130 mM NaCl, pH 7.6 plus 0.1% Tween-20), then incubated at 4°C overnight with the specific antibodies. Secondary horseradish peroxidase-conjugated antibodies (Sigma) were used at 1:3000 dilution and protein bands were visualized by enhanced chemiluminescence (ECL Plus, Amersham Biosciences). A monoclonal antibody to α -tubulin was used to monitor equal loading of samples.

Generation of U87 stable transfectant lines

Coding sequences of sGC α 1 and sGC β 1^{Cys-105} were subcloned into the pcDNA3.1 and pMG transfer vectors (Invitrogen, Carlsbad, CA), respectively, and orientation was confirmed by restriction enzyme analysis and DNA sequencing. For generation of the sGC α 1 stable clone, pcDNA3.1-sGC α 1 plasmid was transfected into human U87 cancer cells using

Lipofectamine2000 according to manufacturer's protocol (Invitrogen; Carlsbad, CA). Control cells were transfected with the native pcDNA3.1 vector (mock transfectants). After 24 h, the medium was replaced with selective medium containing 800 µg/mL G418. After two weeks, neomycin-resistant clones were expanded, and successful transfection was confirmed by Western blot analysis using an anti-sGCα1 antibody. Four clones with the highest levels of sGCα1 were selected for further study. To generate sGCα1 / sGCβ1^{Cys-105} double transfection in U87 cells, the sGCα1 stable clones were transfected with a pMG-sGCβ1^{Cys-105} plasmid. The clones were selected on 800 µg/mL of G418 and 400 µg/mL of hygromycin B. Clones with successful transfection were confirmed by Western blot.

Immunohistochemistry

Paraffin embedded tissue sections were first deparaffinized and rehydrated. Then, primary anti-CD31 and anti-Ki67 (Cell Signaling, Danvers, MA) antibodies were applied to the sections. The biotinylated secondary antibody (Abcam, Cambridge, MA) was used and streptavidin-biotin-horseradish peroxidase kit (Vector Laboratories, Burlingame, CA) was further applied for signal detection. Diaminobenzidine (Vector Laboratories, Burlingame, CA) was used for staining and the sections were counterstained with hematoxylin. Endogenous peroxidase activity was blocked with 3% H₂O₂ (Fisher Scientific, Fair Lawn, NJ).

Cell viability assay

To quantify surviving/proliferating cells, 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added and incubated for 90 min. Medium and MTT were removed, dimethyl sulfoxide was added for 1 min to solubilize the dye, and

absorption was read at 570 nm in a spectrophotometer. In each experiment, cells were plated in quadruplicate and the average of the relative absorption (OD_{570}) was used as an estimate of the number of metabolically active cells. Percentage of surviving treated cells compared to control cells was calculated from the average OD_{570} values obtained in each experiment.

Colony formation assays

We adopted the traditional soft agar assay protocol for colony formation with some modifications for current tumorigenesis study. U87 glioma cells (3×10^3) were seeded in an agar-agarose semi-solid gel system (30mm cell culture dish) which was covered by culture medium containing FBS and the medium was changed every three days. After 21 days, the colonies were stained with 0.005% crystal violet solution for 1 h and washed with PBS three times and then photographed. The number and size of colonies were examined.

Establishment of xenograft tumors, efficacy evaluation, and characterization of intracranial xenografts

Animals—Protocol for animal use was approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center, and all experiments were done in accordance with National Institutes of Health guidelines.

Procedure— A total of 30 female mice, 8–10-weeks-old (*nu/nu* athymic; Charles River Laboratories) were used in the experiments. Human glioma cell lines with or without stable transfection and meningioma-derived stem cell like cells (at a concentration of 1×10^6 cells/ $5 \mu\text{L}$) were resuspended in PBS and injected into the right frontal lobe of nude mice using a guide-screw system implanted within the skull as described previously. Animals were

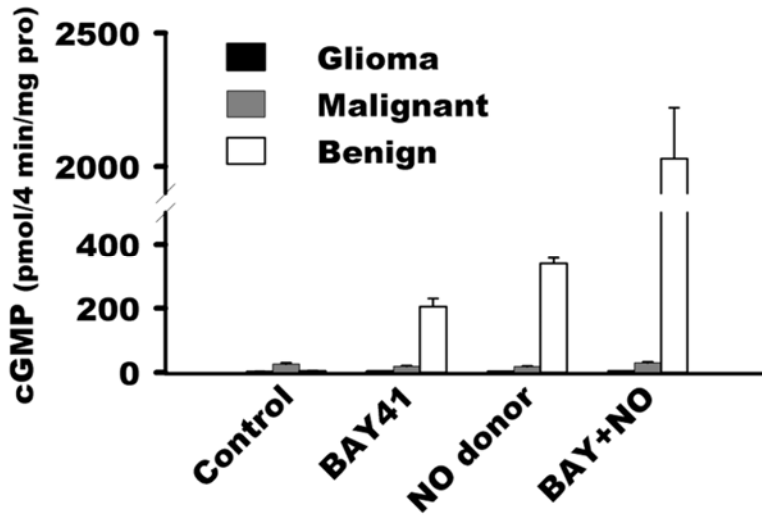
anesthetized with xylazine/ketamine during the procedure. When the animals became moribund due to tumor progression, they were euthanized and the brains were removed for histological and molecular analysis. The vasculature (CD31 staining) and cellular proliferation (Ki67 staining) in the tumors were evaluated with immunohistochemistry.

Table S1. Primers used in Reverse Transcription PCR.

Gene ID	Forward Primer	Reverse Primer	Product Length
PDE1A	agg tca ctt cca gca aat ta	cca cat agg aag aag ttt cg	371
PDE2A	gat cct gaa cat ccc tga cg	gca gaa gtg gga gac aga aaa g	583
PDE3A	caa cac tgt gtg tgt gtg tg	caa gtg gtg cat agc agt aa	337
PDE5A	gaa aag gac ttt gct gct ta	tga ttt tgt ttg cat cat gt	325
PDE6A	tgt tcc acc tga gtt acc tgc aca	tgg agg agg att cgg gat gac ttt	341
PDE8A	acc aat gta atg gat tct gc	tga gtt aca agc cct gag tt	377
PDE1B	gct ttg atg tct ttt cct tg	att ctg act tgg tct gga tg	351
PDE3B	gat gaa gaa gca aat ctt gg	ttc tcc acc tgc agt tta tt	389
PDE6B	agc tgg tca aat gcg gta tcc aga	agt agc tct tca gct tgc ctg tca	201
PDE1C	aga tat tag cca tcc agc aa	caa cgg aga tga cag aat	380
PDE6C	acc tgt aga tga aac tgg ctg ggg	gcc ttg gtg tgg ttc atg agc att	261
PDE9A	tgt cca cga caa cta cag aa	gtg gcc aag att aat gtg at	366
PDE10A	agc agg tac caa gat acg aa	tga ttc cag tcc agt acc tc	354
PDE11A	atg cag atc aac gac ttt tt	cgg ata gtt ttt cct cac tg	356
PDE8B	aga aca gga gga aag agt cc	cgt ttc ctg aca gtc ttc tc	327

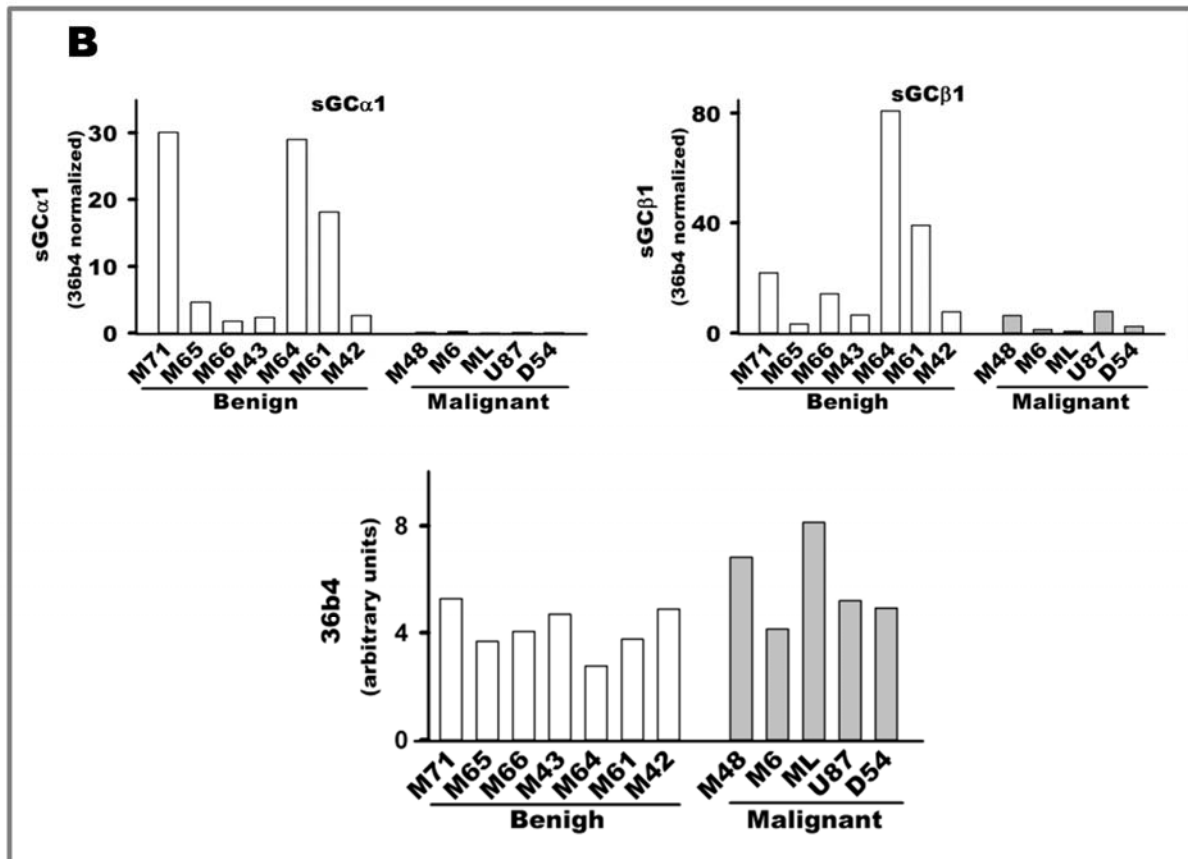
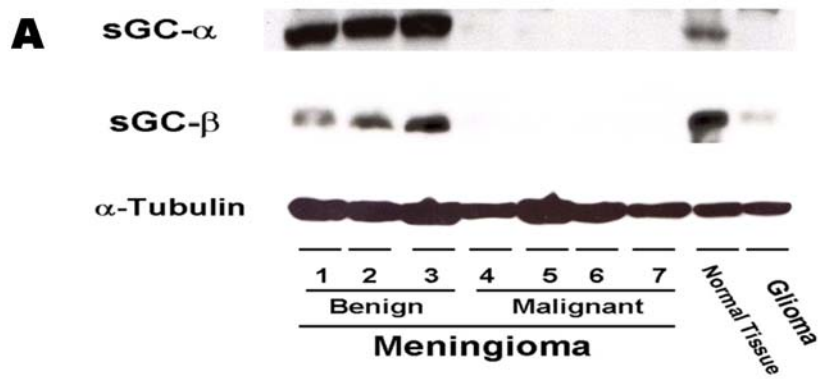
Table S2. Taqman Assays ID used in Quantitative real-time reverse transcription–PCR

Gene ID	Taqman Assay ID
β -actin	Hs03023880_g1
PDE5a	Hs00153649_m1
sGC- α 1,GUCY1A3	Hs00168325_m1
sGC- β 1,GUCY1B3	Hs00168336_m1
NPR1	Hs00418568_m1
NPR2	Hs00241516_m1
NPR3	Hs00168558_m1
hTERT	Hs99999022_m1
36B4	Hs99999902_m1
PRKG1	Hs00183512_m1
PRKG2	Hs00922440_m1



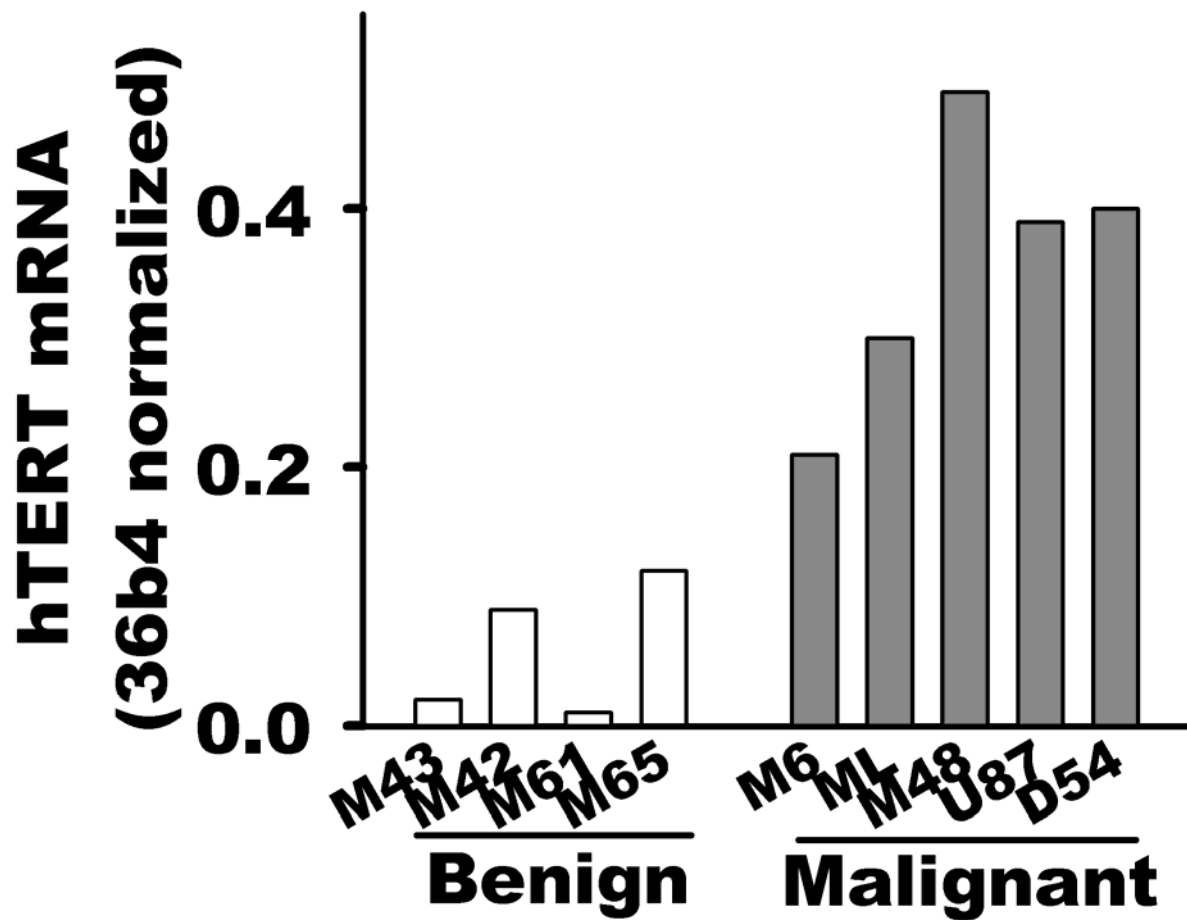
Supplemental Figure S1. Absence of NO-dependent signaling in malignant brain

tumors. Measurement of cGMP levels in benign and malignant meningioma as well as glioma cells treated with Bay41-2272 (1 μ M), spermine/NONOate (0.1 mM) or Bay41-2272 (1 μ M) plus spermine/NONOate (0.1 mM) revealed that cGMP levels in glioma and malignant meningioma cells were unresponsive to the stimulations. Data are mean \pm s.e.m. n = 6 per group, P < 0.01 (malignant vs benign in every experimental group).



Supplemental Figure S2. Altered expression of sGC in malignant brain tumors. Protein expression levels by Western blot (A) showed the 82 kDa sGC α 1 subunit was undetectable in primary cultures of malignant meningioma cells (benign meningioma cells were used as control) and in malignant glioma tissue (normal autologous brain tissue was used as control).

The sGC β 1 subunit (78 kDa) was also absent in human malignant meningioma and its level was very low in malignant glioma tissue, while the protein was abundant in normal brain tissue or benign tumor samples. RT-Q-PCR (B) demonstrated sGC α 1 mRNA was undetectable and sGC β 1 mRNA levels were very low in malignant tumors, while their levels were significantly higher in benign tumor samples.



Supplemental Figure S3. hTERT mRNA levels in benign vs malignant brain tumors. The quantitative real-time PCR analysis of hTERT mRNA levels in benign and malignant meningioma and glioma. The hTERT mRNA levels were quantified after normalizing to 36B4 (a housekeeping gene) mRNA. (M= meningioma; U87 and D54 are gliomas).