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Perivascular Nitric Oxide Activates Notch Signaling and Promotes Stem-like Character in PDGF-Induced Glioma Cells

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SUMMARY

eNOS expression is elevated in human glioblastomas and correlated with increased tumor growth and aggressive character. We investigated the potential role of nitric oxide (NO) activity in the perivascular niche (PVN) using a genetic engineered mouse model of PDGF-induced gliomas. eNOS expression is highly elevated in tumor vascular endothelium adjacent to perivascular glioma cells expressing Nestin, Notch, and the NO receptor, sGC. In addition, the NO/cGMP/PKG pathway drives Notch signaling in PDGF-induced gliomas in vitro, and induces the side population phenotype in primary glioma cell cultures. NO also increases neurosphere forming capacity of PDGF-driven glioma primary cultures, and enhances their tumorigenic capacity in vivo. Loss of NO activity in these tumors suppresses Notch signaling in vivo and prolongs survival of mice. This mechanism is conserved in human *PDGFR* amplified gliomas. The NO/cGMP/PKG pathway's promotion of stem cell-like character in the tumor PVN may identify therapeutic targets for this subset of gliomas.

INTRODUCTION

In the normal brain, capillaries located in the subventricular zone (SVZ) and hippocampus form the major structural entity of the neural stem cell niche (Riquelme et al., 2008), and the perivascular region is the location for neural stem cells (Palmer et al., 2000; Louissaint et al., 2002). This proximiy between neural stem cells and the vasculature is believed to facilitate intercellular communication between neural stem cells and endothelia that release soluble factors critical for promoting stem cell renewal (Shen et al., 2004; Ramírez-Castillejo et al., 2006).

Glioblastoma multiforme (GBM) is the most malignant and aggressive type of central nervous system tumor (Legler et al., 1999) and is classified into groups based on gene expression profiles where approximately 30% are designated "proneural" and show evidence of PDGF signaling (Phillips et al., 2006; Brennan et al., 2009). As gliomas progress to higher grade, they develop several histologic structures that define malignant behavior, including microvascular proliferation or hypercellular vasculature. Microvascular proliferating regions of these tumors are grossly disorganized angiogenic vessels that are a hallmark of malignant behavior and are surrounded by a perivascular niche that is a habitat for brain tumor stem-like cells (Calabrese et al., 2007). Soluble factors released from endothelial cells promote the self-renewal and proliferation of brain tumor stem-like cells (Calabrese et al., 2007; Folkins et al., 2007). In medulloblastomas, stem-like cells in the perivascular niche are resistant to radiation and are believed to give rise to tumor recurrence (Hambardzumyan et al., 2008b). The underlying mechanism(s) responsible for the formation of vascular stem cell niches that maintain tumor cells in a stem-like state are not understood; moreover, the mechanisms responsible for driving stem-like character in the perivascular niche and the endothelia-derived factors that support cancer stem-like cells of the perivascular niche in brain tumors have not been identified.

Nitric oxide synthases are a family of enzymes that produce nitric oxide (NO) from their substrate L-arginine. NO regulates many physiological processes through the NO/cGMP pathway, as well as through protein S-nitrosylation (Fukumura et al., 2006). During NO/cGMP signaling, NO produced from one cell diffuses to neighboring cells where it binds to its receptor soluble guanylate cyclase (sGC). sGC converts GTP to cGMP to activate several downstream effectors, including cGMP-dependent protein kinase (PKG). Many of the activities of NO signaling can be mimicked by cGMP analogs that activate PKG. The endothelial isoform of nitric oxide synthases, endothelial nitric oxide synthase (eNOS), is required for initiation and maintenance of human pancreatic tumor growth (Lim et al., 2008), and eNOS is elevated in various cancers (Fukumura et al., 2006), including human gliomas (Bakshi et al., 1998; Broholm et al., 2003), where its expression is correlated with glioma grade (Cobbs et al., 1995). Elevated levels of eNOS expression and activity in gliomas are often localized to the tumor vascular endothelium (Iwata et al., 1999). However, the specific role of eNOS in gliomagenesis has not been fully established.

We hypothesized that NO produced by eNOS in endothelial cells functions in a paracrine manner to activate signaling

pathways in glioma cells in the perivascular niche and thereby promotes or reinforces stem cell character. We used a genetically engineered mouse model of PDGF-induced gliomas to investigate the role of NO in gliomas. This model shows eNOS expression restricted to the tumor vascular endothelium and that a population of stem-like cells expressing Nestin and Notch1 are tightly apposed to the tumor endothelium. These Nestin-expressing stem-like cells also express sGC, the receptor for NO, and NO activates Notch in glioma stem-like cells through the NO/cGMP/PKG pathway. This NO-induced activation of Notch signaling in stem-like cells accelerates glioma initiation and tumor formation in mice. We further show that mice lacking eNOS have delayed gliomagenesis and subsequent enhanced survival correlating with decreased activation of the Notch pathway. We further demonstrate that this mechanism is conserved in human *PDGFR*-amplified gliomas. We also show that NO activates the Notch pathway to enhance the side population (SP) phenotype in cultured human glioma cells.

RESULTS

Nitric Oxide Stimulates *Nestin* and *Hes1* Promoter Activity in Cultured Human Glioma Cells

Factors derived from the tumor vascular endothelium reinforce the self-renewal of stem-like cells residing in the brain tumor perivascular niche (PVN) (Calabrese et al., 2007). Since elevated eNOS expression and activity is restricted to the tumor vascular endothelium, we investigated whether NO might be one factor promoting stem-like activity within the niche. In order to investigate the potential role of NO on stem-like character, we analyzed its effect on pathways known to regulate stem cell character, namely Notch, Shh, and Wnt (Taipale and Beachy 2001; Radtke and Raj 2003). Luciferase reporters coupled to the promoters of *Hes1*, *Gli1*, or *β-Catenin* were transiently transfected into U251 human glioma cells. As shown in (Figure 1A), S-nitrosoglutathione (GSNO), an NO donor, induced a more than two-fold increase in luciferase expression in the Hes1-luciferase-expressing cells relative to untreated controls (80.57 ± 0.55 versus 28.32 ± 0.13; $p < 0.0001$). This effect was specific to activation of the *Hes1* promoter, as there was no statistically significant difference in activation of *Gli1* or *β-Catenin* promoters (41.15 ± 0.81 versus 39.91 ± 0.37 and 5.44 ± 0.23 versus 4.23 ± 0.19, respectively) following GSNO treatment. Nestin, a well-characterized marker of stem/progenitor cells in brain tumors, is highly expressed in stem-like cells of the glioma PVN, and Notch signaling is known to activate the *Nestin* promoter in gliomas (Shih and Holland 2006). In addition, loss of eNOS was demonstrated to decrease *Nestin* expression in the brain in vivo (Chen et al., 2005). Therefore, we determined whether NO affects *Nestin* expression in human glioma cells. Transient transfection of the U251 cell line with a *Nestin*-luciferase reporter indicated that GSNO treatment led to an approximately 2-fold induction of the *Nestin* reporter relative to controls (77.95 ± 2.55 versus 38.84 ± 0.66; $p < 0.0001$) (Figure 1A). We confirmed activation of the Notch pathway in U251 cells by western blot for HES1 protein, following GSNO treatment (Figure S1A available online). In addition, we analyzed the mRNA transcripts encoding HES1, NESTIN, GLI1, and β-CATENIN in these cells, following treatment with GSNO. The transcriptional levels of *HES1* and *NESTIN* were significantly elevated relative to controls (10.8 ± 2.45 versus 1 ± 0.26 and 5.2 ± 1.36 versus 1 ± 0.29, respectively). Gene expression levels for *GLI1* and *β-CATENIN* were unchanged (1.4 ± 0.56 versus 1 ± 0.39 and 0.97 ± 0.22 versus 1 ± 0.28, respectively) (Figure S1D). These data indicate that NO can specifically activate the Notch pathway in human glioma cells.

eNOS and Active Notch1 Proteins Are Significantly Elevated and Are Expressed in Cells of the PVN in PDGF-Induced Mouse Gliomas

To further investigate the connection between NO and the Notch pathway in gliomas, we employed the RCAS/tv, a method for creating PDGF-induced gliomas in mice, because the well-characterized robust perivascular niche microenvironment and histological features of this model closely mimic those observed in human gliomas (Holland 2004). Western blot analysis demonstrated that both eNOS and cleaved Notch1 (Notch intracellular domain-NICD) were highly elevated in PDGF-induced mouse gliomas with respect to the contralateral side of the brain ($p < 0.0001$) (Figure 1B). Using immunofluorescence, we investigated their spatial relationship to one another within the glioma PVN. Immunostaining for total eNOS protein within the PDGF-induced gliomas indicated that eNOS colocalized with CD31-expressing endothelial cells (Figure 1C) surrounded by a population of Nestin-expressing cells that also coexpress Notch1 (Figures 1D and 1E). These Nestin-expressing perivascular cells also express soluble guanylyl cyclase (sGC, the major receptor for NO) (Madhusoodanan and Murad 2007), whose staining is limited almost exclusively to the perivascular niche (Figure 1F) and which therefore may represent a population of cells within the niche that can respond to NO signaling.

Nitric Oxide Activates Notch Signaling and the SP Phenotype in Primary Cultured Mouse Glioma Cells

The data above suggest a regional correlation between eNOS expression and Notch1 activation in vivo. In order to determine whether there is a direct link between NO signaling and Notch signaling within PDGF-induced mouse gliomas, we investigated whether NO could upregulate the Notch signaling pathway in culture. Western blot analysis of GSNO-treated PDGF-induced glioma primary cultures (PIGPCs) revealed a dose-dependent increase in Notch intracellular domain (NICD) indicating activation of the Notch pathway (Figure S1C). Cell viability was not adversely affected after 6 hr of treatment (data not shown). We then examined the effect of NO on the expression of Notch ligand proteins and the downstream protein targets of Notch, Hes1, and Hey1. GSNO treatment of PIGPC indicated specific activation of the Notch pathway, as evidenced by a substantial increase in components of the activated Notch pathway (Figure 2A). The expression of the Notch ligand proteins, Delta-like1 and 4 (DLL1 and DLL4), increased within 30 min, which coincided with elevated NICD. Expression of the transcription factor targets of Notch signaling, Hes1, and Hey1 was subsequently elevated at 1 hr.

Activation of the Notch pathway plays a critical role in promoting stem-like character in brain tumors (Fan et al., 2006). Therefore, we investigated whether NO was involved in mediating this effect in PDGF-induced gliomas using side

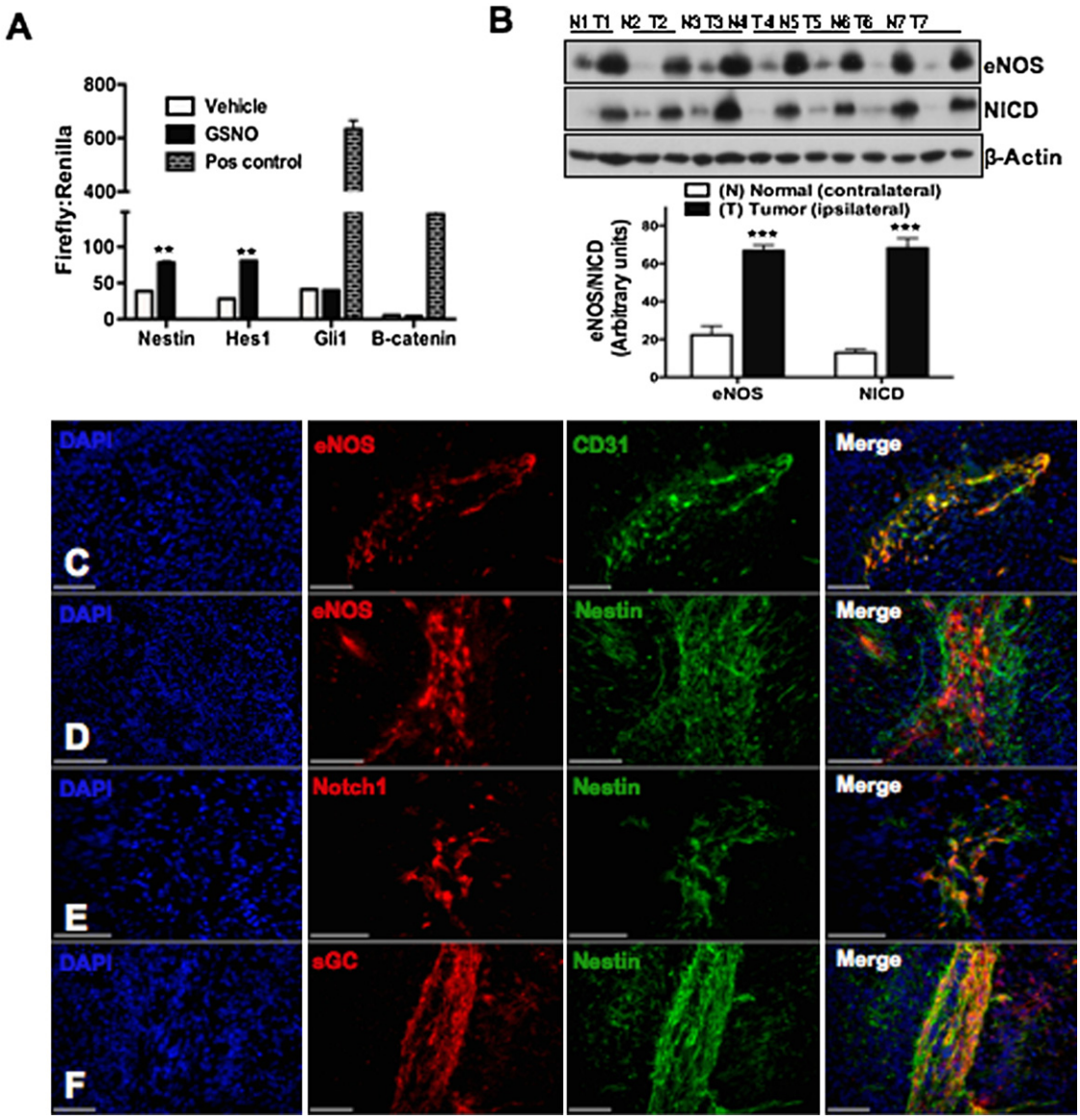

Figure 1. Nitric Oxide Stimulates Nestin and Hes1 Promoter Activity in Human Glioma Cells and Elevated eNOS and Notch1 Protein Expression Is Localized to Cells of the Glioma Perivascular Niche

(A) Luciferase assay of U251 glioma cells transfected with Hes1-, Nestin-, Gli1-, or β-Catenin-responsive luciferase reporters. Positive controls for Gli and Wnt reporters were cotransfections of CMV-gli- or CMV-beta-Catenin-expressing vectors, respectively (**$p < 0.005$ reports difference between Nestin and Hes1 promoter activity in control and GSNO-treated U251 cells). Error bars are the mean ± SEM.

(B) Western blot analysis of PDGF-induced mouse gliomas analyzed for total eNOS and Notch intracellular domain (NICD) proteins (n = 7). N and T represent the contralateral normal and tumor-containing hemispheres of the brain, respectively. Bottom: quantification of eNOS and NICD proteins. Data are represented as mean ± SEM (***$p < 0.005$ reports difference between the contralateral normal and tumor-containing hemispheres). Error bars are the mean ± SEM.

(C–F) Coimmmunofluorescence in mouse PDGF-induced gliomas of eNOS, Notch1, sGC (red), and CD31 and Nestin (green) expression. All nuclei are stained with DAPI. Scale bars, 75 μm.

population (SP) analysis. SP analysis is used for the identification of stem cells via fluorescence-activated cell sorting and is based on the capacity of stem cells to efflux Hoechst fluorescent dyes by the activity of ATP binding cassette transporters (ABC transporters) (Goodell et al., 1996). The SP cells in human glioma cell lines as well as other tumors are enriched in tumorigenic cells with stem cell properties, and the SP cells of PDGF-induced gliomas are more capable of growing as tumor neurospheres and are more tumorigenic than non-SP cells when transplanted in mice (Bleau et al., 2009). PDGF-induced glioma primary cultures (PIGPCs) were incubated with Hoechst 33342 in the presence or absence of GSNO and assayed for their SP. These PIGPCs contained an SP that ranged from ~3% to 20% at baseline depending on the tumor. Following 2–2.5 hr of NO treatment, a significant increase (2- to 5-fold) in the SP was observed relative to vehicle-treated cells derived from the same tumor

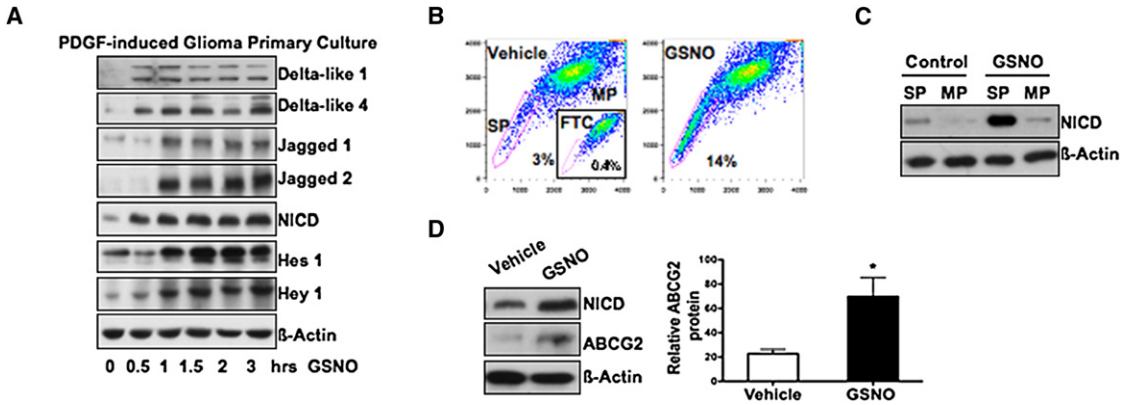

Figure 2. NO Activates Notch Signaling and the SP Phenotype in PDGF-Induced Glioma Primary Cultures

(A) PDGF-induced glioma primary culture (PIGPC) treated with GSNO (100 μM) for the indicated times (n = 6).

(B) SP analysis of GSNO-treated PIGPC. Inset shows cells treated with FTC (fumitremorgen C, an ABCG2 inhibitor). SP, side population; MP, main population. Data shows one representative graph of four independent experiments.

(C) GSNO-treated PIGPCs, sorted for their SP and MP, then analyzed by western blot for expression of NICD with respect to vehicle-treated controls.

(D) Left: western blot analysis of whole-cell lysates from PIGPC treated with GSNO or vehicle and probed for NICD, ABCG2, and β-actin proteins. Right: quantification of ABCG2 proteins using ImageJ software. Data are represented as mean ± SEM of three individual experiments (*$p < 0.05$ compared with control). Error bars are the mean ± SEM.

(15.50 ± 3.28 versus 4.25 ± 0.48; p = 0.015) (Figure 2B and Figure S2A). Both control and GSNO-treated cultures were judged to be approximately 90% viable, indicating that selection for viable cells was not occurring during the treatment (Figure S2B).

We hypothesized that NO might preferentially upregulate Notch signaling in a subpopulation of PIGPC cells. To address this possibility, primary cultures were pretreated with vehicle or GSNO, sorted for side and main populations, and analyzed by western blot for NICD. The relative increase of NICD seen above was greater in cells of the SP (Figure 2C), suggesting that NO activates the Notch pathway in a population of glioma cells, which may promote their SP phenotype or stem cell-like characteristics.

ABCG2 is expressed in glioma stem-like cells, and its expression was correlated with increasing glioma grade (Jin et al., 2009). Furthermore, *abcg2* gene expression is specifically upregulated in the cancer stem-like populations of mouse PDGF-induced gliomas (Bleau et al., 2009). We investigated whether NO might drive the expression of Abcg2 protein as an additional measure of NO activation of the Notch pathway. Therefore, we analyzed 4 PIGPCs treated with GSNO by western blot for the expression of Abcg2 relative to vehicle-treated controls. All four primary glioma cultures examined showed increased Abcg2 protein expression, following GSNO treatment versus controls (69.67 ± 15.48 versus 22.72 ± 3.21; p = 0.041) (Figure 2D).

Nitric Oxide Requires Notch Signaling to Enhance the SP Phenotype in PDGF-Induced Glioma Primary Cultures

To further investigate whether Notch signaling drives the SP phenotype in gliomas as it does in medulloblastomas (Fan et al., 2006), we treated these PIGPCs for 2 hours with the gamma secretase inhibitor (GSI) MRK-003 (Lewis et al., 2007). The baseline SP in these primary glioma cultures was reduced by GSI treatment, suggesting that Notch signaling is critical for the maintenance of the SP phenotype in PDGF-induced gliomas (Figure S3A). We investigated whether the increase in the SP phenotype induced by NO is dependent on Notch activation. PIGPCs were incubated for 2 hours with GSI in the presence or absence of GSNO, then analyzed for their SP. Treatment of these primary glioma cultures with GSI abolished the GSNO-induced increase of the SP (13.88 ± 1.78 versus 0.33 ± 0.13; p = 0.003) (Figure 3A and Figure S3B), suggesting that NO requires activation of the Notch pathway to drive the SP phenotype in PDGF-induced gliomas. Control, GSNO-, and GSI-treated cultures were approximately 90% viable by PI staining, which confirms that cell viability was not adversely affected by the treatments (Figure S3C). We confirmed the specificity of GSI-induced Notch inhibition with RNAi. *Notch1*-shRNA (*Notch1-SH*) knockdown of *Notch1* mRNA in PIGPCs resulted in a 50% reduction of Notch1 protein and significantly decreased the SP relative to a nonspecific scramble control (p = 0.0109) (Figures 3B and 3C). To further verify that Notch signaling mediates the enhanced SP phenotype observed above, PIGPCs were infected with a vector expressing constitutively active Notch (NICD) and then analyzed for their SP phenotype. NICD overexpression induced a more than 2-fold increase in the SP when compared with the empty vector control (14.3 ± 2.70 versus 6.59 ± 1.41; p = 0.0226) (Figure 3D). These data indicate that Notch signaling is necessary and sufficient for the NO-induced elevation in SP phenotype in these glioma cells.

Inhibition of Nitric Oxide Activity in PDGF-Induced Gliomas In Vivo Diminishes Notch Signaling and the SP Phenotype and Enhances Survival

We next investigated whether the relationship between NO and Notch signaling seen in culture was conserved in PDGF-induced mouse gliomas in vivo. Ten glioma-bearing mice were treated with the NOS inhibitor L-N^G-nitroarginine methyl ester (LNAME) for 24 hr. Tumor tissue and contralateral normal brain were analyzed by western blot for Notch cleavage (NICD) and Notch

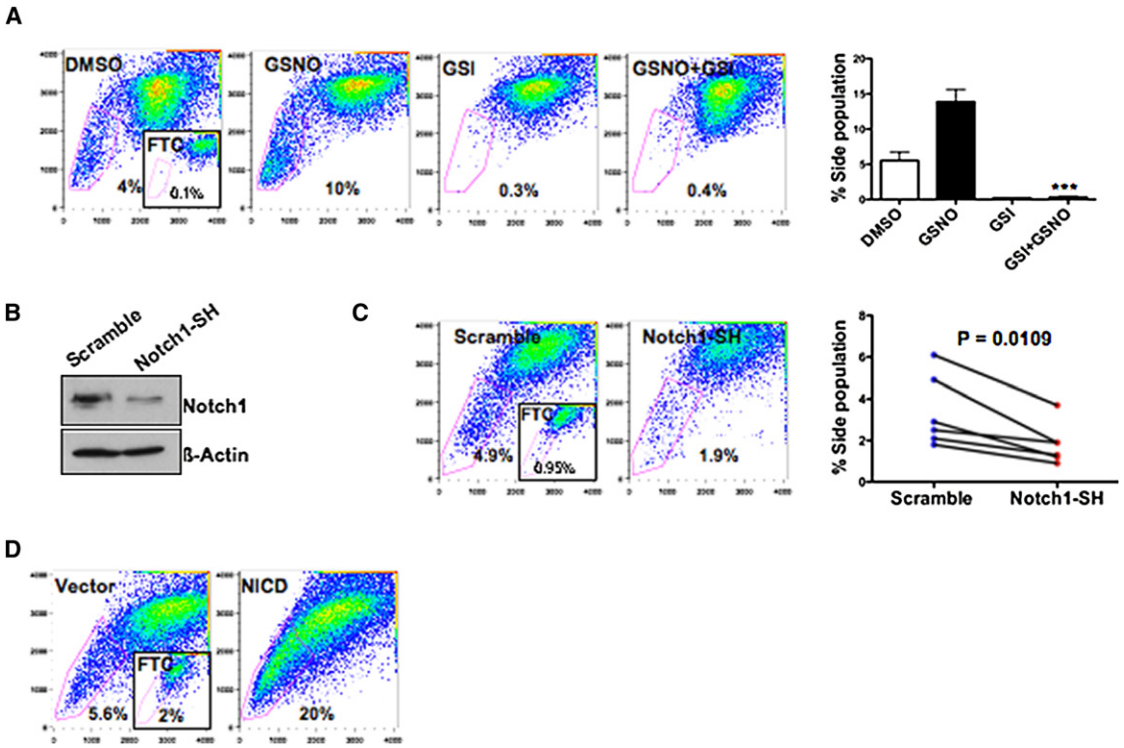

Figure 3. Notch Signaling Is Required for Nitric Oxide Enhancement of the SP Phenotype

(A) Left: SP analysis of GSNO (100 μM)- and GSI (3 μM)-treated PIGPCs. Inset shows cells treated with FTC control. Right: bar chart shows quantification of SP analyzed data on the left. Error bars are the mean ± SEM of three individual experiments (***p < 0.0005 compared with GSNO treatment).

(B) Western blot to confirm knockdown of Notch1 proteins.

(C) Left: SP analysis shows a decrease in the percent of SP cells by Notch1-sh-RNA (Notch1-SH) relative to nonspecific scramble control. Inset shows a negative control, FTC. Graph represents one of six independent PIGPCs samples. Right: quantification of Notch1-shRNA mediated knockdown of the SP in six independent PIGPCs.

(D) SP analysis of PIGPCs infected with empty vector or vector expressing constitutively active Notch (NICD). Inset shows cells treated with FTC.

ligands. Nine vehicle-treated PDGF glioma-bearing mice were used as controls. The amount of NICD in tumors was significantly elevated relative to the normal brain in all cases ($p < 0.0001$) (Figure 4A and Figure S4A). NICD was significantly diminished in LNAME-treated mice relative to untreated controls (79.35 ± 7.23 versus 130.4 ± 8.99; $p < 0.0004$), and the expression of the Notch ligand Jagged 2 was also significantly lower relative to the untreated controls (64.73 ± 12.57 versus 114.8 ± 3.51; $p < 0.05$) (Figure 4A). To determine if suppression of NO activity by LNAME would affect the SP in these PDGF-induced gliomas, we analyzed the SP of gliomas in six mice treated for 3 days with LNAME relative to six vehicle-treated tumor-bearing mice of the same age and background. We found that the SP for the treated group was significantly lower than the vehicle-treated controls (2.8 ± 0.22 versus 4.2 ± 0.43; $p < 0.0196$) (Figure 4B and Figure S5).

To assess whether complete loss of eNOS would affect the development of PDGF-induced gliomas in vivo, we crossed mice carrying a homozygous disruption of eNOS ($eNOS^{-/-}$) into Nestin tv-a (N-tva) mice and infected the progeny with RCAS-PDGF. We compared the survival of $eNOS^{-/-}$ mice with their respective wild-type littermates ($eNOS^{+/+}$). The survival of $eNOS^{-/-}$ mice ($n = 43$) was significantly longer than $eNOS^{+/+}$ mice ($n = 42$) ($p = 0.0042$) (Figure 4C). Since NO activates the Notch pathway in these PDGF gliomas, we tested whether the increased survival observed with the loss of eNOS correlated with a decrease in activation of the Notch pathway during tumor progression or at the time of death. Using western blot analysis, we compared the levels of protein expression of Notch pathway components between $eNOS^{+/+}$ mice (approximately 54 days, the median age of death in $eNOS^{+/+}$ mice) with respective $eNOS^{-/-}$ counterparts of the same age. $eNOS^{+/+}$ mice expressed significantly higher levels of NICD and the Notch ligand DLL1 relative to their $eNOS^{-/-}$ counterparts. However, the expression levels of NICD and Notch ligands Jagged 1 and DLL 1 and -2 in the tumors at death were similar in both populations (54 days for $eNOS^{+/+}$ and 84 days for $eNOS^{-/-}$) (Figure S4B), implying that the development of Notch signaling was delayed in the absence of eNOS, but eventually reached a level similar to $eNOS^{+/+}$ mice by the time the tumors were sufficiently aggressive to be lethal.

NO Activates Notch Signaling to Enhance the SP Phenotype through cGMP Activation of PKG

Downstream NO signaling can be cGMP dependent or cGMP independent. However, many physiological processes regulated by NO involve cGMP, which activates cGMP-dependent protein kinase (PKG) (Blaise et al., 2005). To investigate whether the

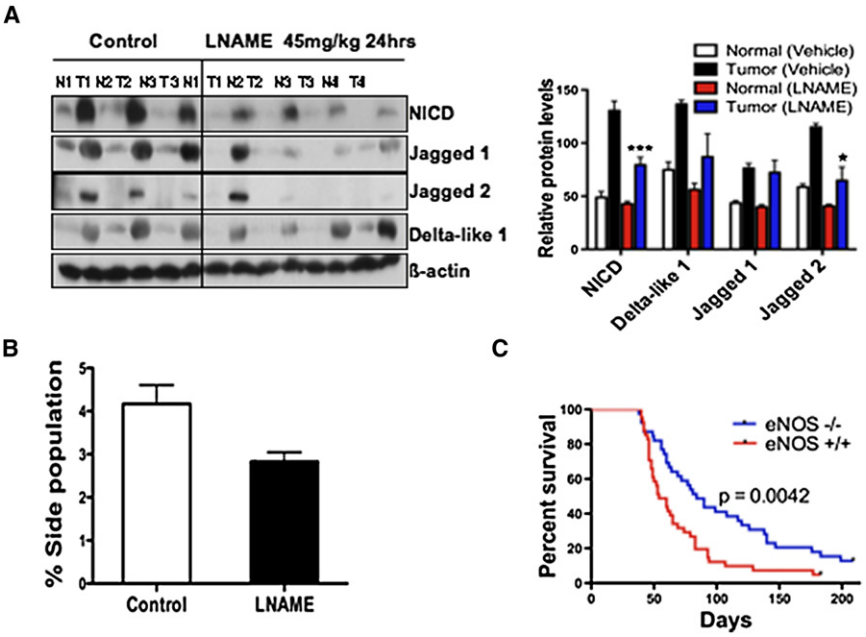

Figure 4. Suppression of NO Activity In Vivo Decreases Notch Signaling and the SP Phenotype and Enhances Survival In Vivo

(A) Western blot on whole-cell lysates of PDGF-induced gliomas derived from vehicle (water)- or LNAME (45 mg/kg; 24 hr)-treated mice using NICD, Jagged 1 and 2 and Delta-like 1 antibodies. N represents the contralateral nontumor hemisphere of mouse brain. T represents the tumor-bearing portion of mouse brain. Vehicle (n = 3); LNAME (n = 4). Bar chart represents quantification of proteins analyzed on the left using ImageJ software. (*$p < 0.05$ and ***$p < 0.0005$ reports the difference between NICD and Jagged 2 in vehicle and LNAME-treated PDGF-induced gliomas).

(B) Bar chart shows that suppression of NO activity in vivo decreases the SP phenotype in PDGF-induced gliomas (*$p < 0.05$ reports the difference between vehicle [n = 6]- and LNAME [n = 6]-treated groups).

(C) Kaplan-Meier survival curve shows loss of eNOS expression in PDGF-induced gliomas prolongs survival of mice ($eNOS^{+/+}$ n = 41; $eNOS^{-/-}$ n = 42).

increase in the SP phenotype induced by NO involved PKG, we determined if PKG activation could increase the SP phenotype in PIGPCs similar to NO. We utilized the nonhydrolyzable cGMP analog and potent activator of PKG, 8-Br-PET-cGMP (PET-cGMP), to address this question. PIGPCs were treated with vehicle or Pet-cGMP for 2 hr and then analyzed for their SP. When compared with vehicle-treated controls, PET-cGMP enhanced the percent of SP cells in PIGPCs to levels exceeding that achieved by GSNO alone (9.00 ± 5.03 versus 48.33 ± 7.67; 9.00 ± 5.03 versus 26.7 ± 7.84, respectively) (Figure 5A). This induction ranged from a 3- to 11-fold increase in the SP relative to vehicle treated controls (Figure S6). Furthermore, the increase in the SP observed with GSNO and Pet-cGMP combined was no more than that observed by either individual treatment (Figure 5A), suggesting that GSNO and Pet-cGMP act in the same pathway. To confirm activation of Notch signaling, PIGPCs were treated for 2 hr with Pet-cGMP, then analyzed by western blot for activation of the Notch pathway. Western blot analysis on these primary cultures revealed increased NICD and enhanced expression of the Notch downstream target Hes1 (Figure 5B). As an additional measure of the potential effect of PKG activity on Notch signaling, we used an alternative approach to enhance the activity of PKG in our glioma primary cultures. PIGPCs were treated with the phosphodiesterase 5 (PDE5) inhibitor, sildenafil, which enhances steady-state levels of cGMP by suppressing its degradation by PDE5 (Bender and Beavo, 2006). As shown in Figure 5C, PIGPCs treated with sildenafil for 1 hr show increased expression of NICD protein. The PKG target vasodilator-stimulated phosphoprotein (VASP) was phosphorylated at serine 239 (Butt, 2009), indicating that PKG was activated by Pet-cGMP and sildenafil (Figures 5B and 5C). Sildenafil is a direct inhibitor of ABC transporters (Oesterheld, 2009); therefore, SP analysis of primary glioma cultures treated with sildenafil could not be conducted. To confirm that PKG activity is required for NO-mediated enhancement of the SP phenotype, PIGPCs were treated with the PKG inhibitor KT5823 in the absence or presence of GSNO and analyzed for their SP characteristics. As shown in Figure 5D, in the presence of KT5823, induction of the SP by GSNO or Pet-cGMP was diminished relative to their respective individual treatments (6.11 ± 2.43 versus 17.30 ± 3.6 and 5.36 ± 0.87 versus 24.95 ± 0.55; p = 0.0027). VASP phosphorylation was used to confirm inhibition of PKG by KT5823 (Figure 5E). Similar results were obtained using a second inhibitor of PKG, Rp-8-pCPT-cGMP, which inhibits PKG by a different mechanism than KT5823 (data not shown). Collectively, these data indicate that NO enhances Notch signaling and the SP phenotype in PDGF-induced gliomas through activation of PKG. Endothelial cells are known to express ABC transporters and are a component of the SP in PDGF-induced gliomas (Bleau et al., 2009). Moreover, they can activate the cGMP/PKG signaling pathway in response to NO (Fukumura et al., 2006). We verified that the NO-induced increase in the SP phenotype was not due to the presence of contaminating endothelial cells within these PIGPC by analyzing six independent PIGPCs by western blot for the expression of the endothelial cell markers, eNOS and CD31 (Figure S1E).

Transient Activation of the NO/cGMP Pathway Enhances the Neural Stem Cell-Forming Capacity of PDGF Gliomas In Vitro

Thus far, we have shown that in a population of PIGPC cells, NO activates the SP phenotype and Notch signaling, both consistent with stem-like characteristics. These characteristics are observed after transient (2 hr) activation of the NO/cGMP pathway with GSNO or the cGMP analog, Pet-cGMP. Using a neurosphere formation assay, we then investigated whether this transient activation might have long-term effects on the overall capacity of these PIGPC to behave in a stem-like manner after withdrawal of treatment. When cultured with the growth factors EGF and bFGF, in the absence of serum, neural stem cells form neurospheres. Neurosphere-forming glioma cells

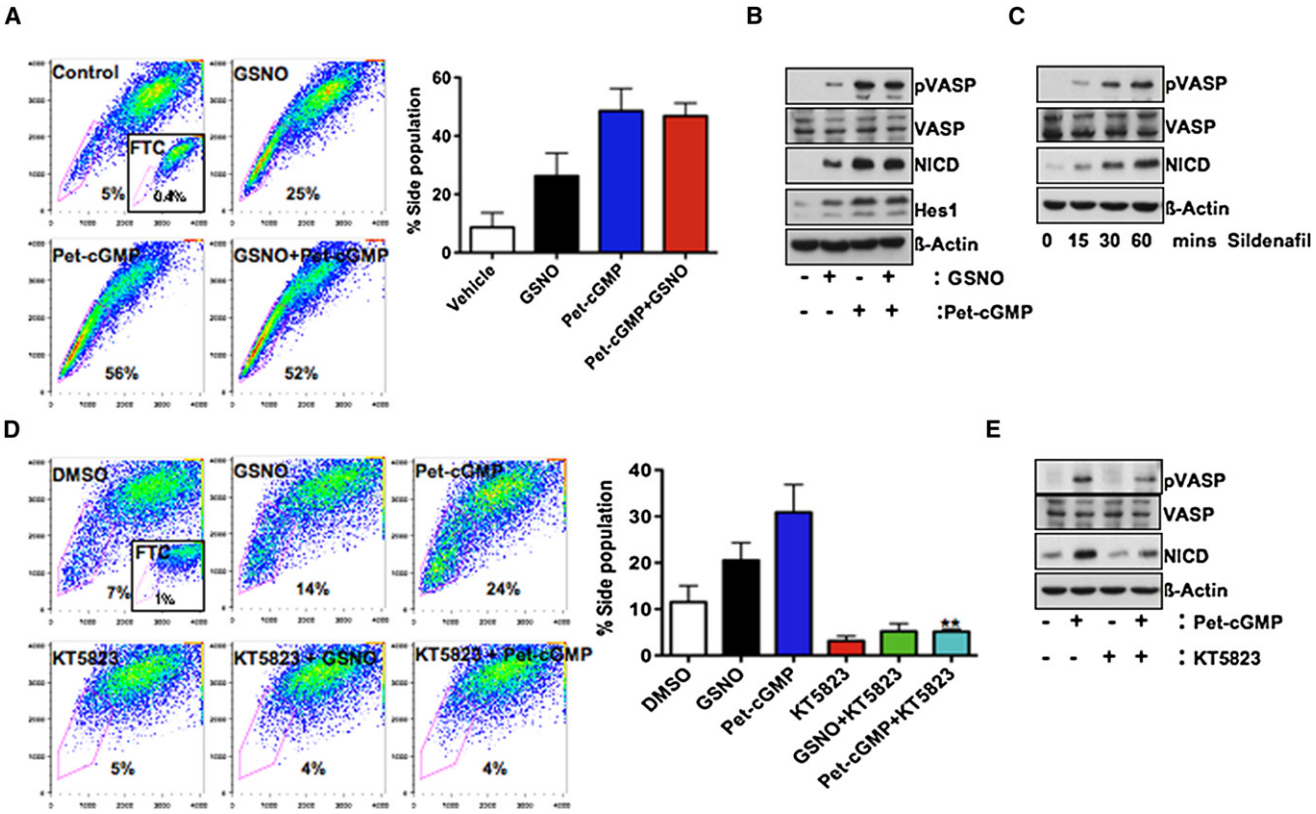

Figure 5. Nitric Oxide Induces the SP Phenotype through cGMP Activation of PKG

(A) Left: SP analysis of GSNO (100 μM)- and Pet-cGMP (200 μM)-treated PIGPCs. Inset shows cells treated with FTC. Right: quantification of SP analysis on the left. Error bars are the mean ± SEM of three individual experiments.

(B) Western blot analysis on whole-cell lysates of PIGPC analyzed in (A) and probed with pVASP, VASP (vasodilator-stimulated phosphoprotein), NICD, Hes1, and β-actin antibodies.

(C) Western blot analysis on whole-cell lysates of PIGPC treated with sildenafil (1μM) for the indicated times and probed with pVASP, VASP, NICD, and β-actin antibodies.

(D) Left: SP analysis on PIGPC treated with GSNO, Pet-cGMP, and the PKG inhibitor KT5823 (3 μM). Right: quantification of SP analysis on the left (**$p < 0.005$ reports the difference between Pet-cGMP alone and in the presence of KT5823). Error bars are the mean ± SEM.

(E) Western blot analysis on whole-cell lysates of PIGPC analyzed in (D) and probed with pVASP, VASP, NICD, and β-actin antibodies.

represent the more stem-like cell populations in brain tumors (Singh et al., 2003; Galli et al., 2004) and are enriched in the SP (Patrawala et al., 2005; Harris et al., 2008). In addition, SP cells generated from these PDGF-induced gliomas were previously shown to be enriched with the self-renewing stem-like cell populations, which propagate the neurosphere forming capacity of these gliomas (Bleau et al., 2009). Therefore, we pretreated PIGPCs individually with GSNO or Pet-cGMP for 2 hr, after which treatment was withdrawn. The cells were resuspended, plated at clonal density (1 cell/μl) in neurosphere medium, and then subjected to an in vitro tumor neurosphere-forming assay that was quantified by counting the number of neurospheres formed 2 weeks after treatment. Neurosphere formation in GSNO- or Pet-cGMP-treated glioma primary cells was approximately twice as fast and more than double the number observed in vehicle-treated plates (48.83 ± 8.167 versus 20.33 ± 2.171; $p < 0.05$ and 76.17 ± 5.845 versus 20.33 ± 2.171; $p < 0.005$) (Figure 6A). In addition, tumor neurospheres generated from each group could be serially passaged to generate secondary and tertiary neurospheres, demonstrating their capacity for self-renewal. These neurospheres also stained with antibodies against the stem cell markers Nestin, Nanog, Musashi, and Oct-4 (Figure 6B). These results indicate that short-term activation of the NO/cGMP pathway in a population of PIGPCs can induce a fundamental change in the behavior of these tumor cells to a more stem cell-like phenotype.

Transient Activation of the NO/cGMP Signaling Pathway in PIGPCs Enhance Their Capacity to Establish Tumors upon Transplantation

The SP cells of PDGF-induced gliomas are enriched with the self-renewing stem cell-like populations and are more tumorigenic than non-SP cells when injected into neonatal mice (Bleau et al., 2009). The data observed from the neurosphere formation assay above suggest that transient activation of the NO/cGMP pathway in vitro in a population of PIGPCs is sufficient to effect long-term changes toward a more stem cell-like phenotype within these gliomas. Therefore, we then determined if the pro-stem-cell-like characteristics achieved in these glioma cells by transient activation of the NO/cGMP pathway affected

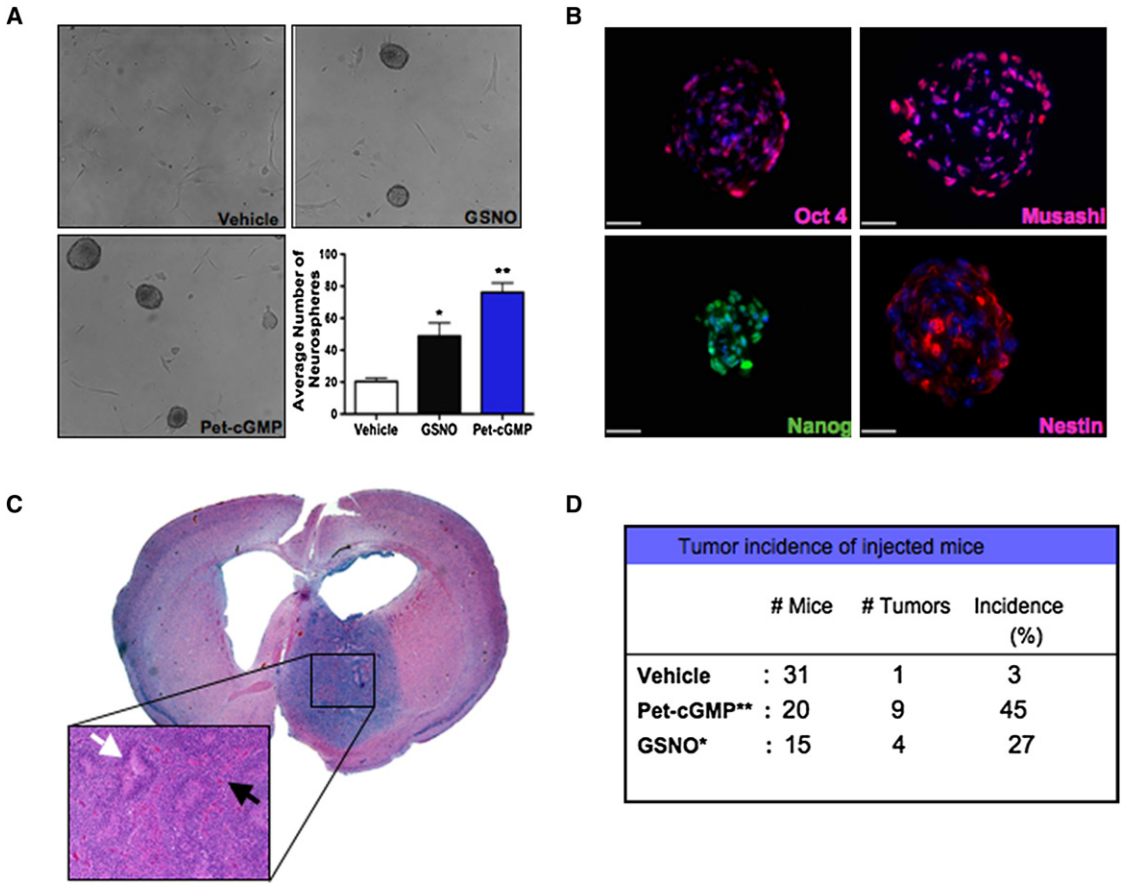

Figure 6. Enhanced NO/cGMP Signaling Promotes the Neural Stem Cell-Forming Capacity of PDGF-Induced Gliomas and Enhances Tumorigenicity

(A) PIGPCs pretreated with vehicle, GSNO, or Pet-cGMP for 2.5 hr then placed in NSC (neural stem cell) medium. Micrographs show NSC formation after 2 weeks. Lower right: bar chart represents quantification of number of neurospheres shown above as an average of three experiments performed in duplicate (*$p < 0.05$ and **$p < 0.01$ reports the difference between vehicle-treated controls and GSNO or pet-cGMP treatments, respectively). Error bars are the mean ± SEM.

(B) Oct-4, musashi, Nanog, and Nestin immunofluorescence on NSCs generated in (A). Scale bars, 40 μm.

(C) Representative whole-mount H&E of tumors generated from Pet-cGMP-treated PIGPC. Arrows indicate high-grade features such as pseudopalisades (white arrow) and microvascular proliferation (black arrow).

(D) A comparison of tumor incidence between vehicle (water)-, Pet-cGMP-, and GSNO-treated primary glioma cells (Fisher's exact test; *$p = 0.033$ and **$p = 0.0004$ reports the difference between vehicle-treated controls and GSNO and pet-cGMP treatments, respectively).

tumorigenesis in vivo. PIGPC cells were treated for 2 hr with Pet-cGMP or GSNO as above and immediately injected into the cortex of neonatal mice (8×10^4 cells per pup, n = 20 Pet-cGMP population; n = 15 GSNO population). As a control, the same number of untreated PIGPC cells were injected into the cortex of a second population of neonatal mice (n = 31). Mice were closely monitored and sacrificed if they developed symptoms of hydrocephalus, lethargy, or cachexia. Tumor formation was confirmed by histology, which showed tumors to be diffuse and some to exhibit histological features of high-grade gliomas such as the presence of microvascular proliferation and pseudopalisading necrosis (Figure 6C). As shown in Figure 6D, mice injected with PIGPCs pretreated with the cGMP analog Pet-cGMP developed tumors with both higher incidence and shorter latency than their control counterparts. Injection of Pet-cGMP-treated primary glioma cells resulted in tumors at 45% incidence (9 of 20), compared with a 3% incidence (1 of 31) obtained from injections of untreated primary glioma cells ($p = 0.0004$). In addition, GSNO-treated PIGPCs pretreated for 2 hr and injected into a similar number of mice of the same background resulted in a tumor incidence of 27% (4 of 15) compared with the lower incidence seen in untreated controls ($p = 0.033$). Moreover, the tumor incidence from GSNO-treated PIGPCs was approximately 20% less than the 45% seen with PET-cGMP and consistent with the relative effects of these two treatments on the SP phenotype described above.

Activation of the NO/cGMP Signaling Pathway Also Enhances Notch Signaling and the SP Phenotype in Human Glioma Cell Cultures and Diminishes Survival

Thus far, using the RCAS/tv, a mouse model, we demonstrated that activation of NO/cGMP signaling in PDGF-induced gliomas promotes stem cell-like characteristics. In light of these data from this mouse model, along with previous findings showing

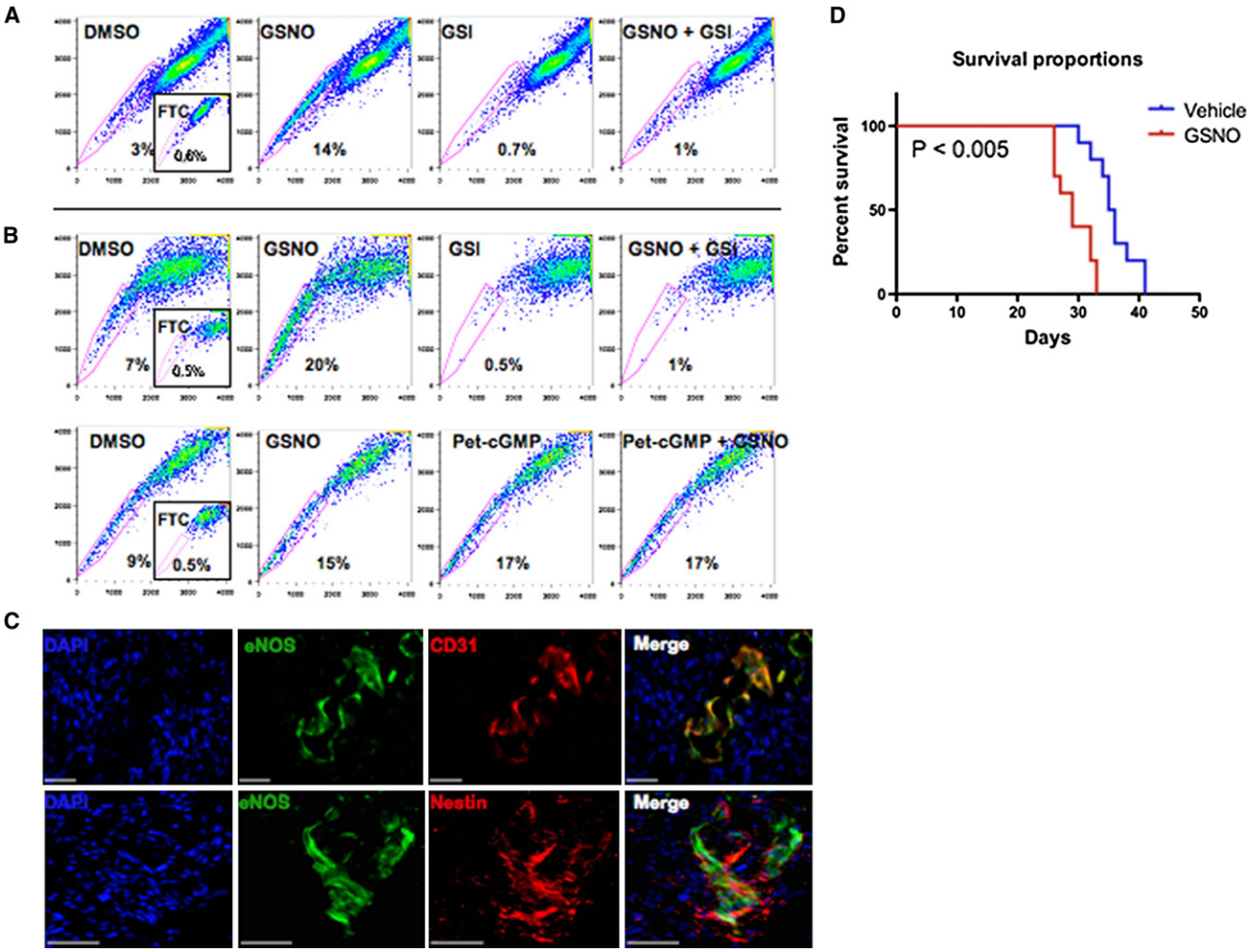

Figure 7. NO Enhances the SP Phenotype in Human Glioma Cells and Decreases Survival

(A) SP analysis of GSNO- and GSI-treated T98G human glioma cell lines. Inset shows cells treated with FTC.

(B) Above: SP analysis of GSNO (35 μM)- and GSI (3 μM)-treated human tumor neurospheres. Below: SP analysis of GSNO- and Pet-cGMP (80 μM)-treated human tumor neurospheres. Inset shows cells treated with FTC. Plots represent one of three experiments.

(C) Coimmmunofluorescence on PDGFR-amplified human gliomas to detect eNOS (green), CD31, and Nestin (red) protein expression. All nuclei are stained with DAPI. All scale bars, 75 μm.

(D) Kaplan-Meier survival curve shows GSNO-activated human glioma primary neurosphere cultures shortens the survival of mice. Median survival of vehicle- and GSNO-treated human primary glioma cultures was 36 and 29 days, respectively (n = 10).

elevated expression of eNOS in human gliomas, (Cobbs et al., 1995; Iwata et al., 1999), we wondered if NO might play a role in promoting stem cell-like activity within the PVN in the context of human gliomas. The T98G human glioma cell line was previously shown to contain a SP (Chua et al., 2008). We, therefore, treated these cells with GSNO and analyzed them for their SP phenotype relative to controls. GSNO induced an approximate 5-fold increase in the SP when compared with vehicle treated controls (13.87 ± 0.22 versus 3.43 ± 0.15; $p < 0.0001$) (Figure 7A). The induction of the SP by GSNO was blocked in the presence of the GSI (13.87 ± 0.22 versus 1.16 ± 0.04; $p < 0.0001$), suggesting a role for Notch in mediating this effect. Confirmation of Notch pathway activation was performed by western blot analysis (Figure S1B). As the PDGFR amplified subtype of human gliomas is accurately modeled in the PDGF-induced mouse model, we treated human primary cultured neurospheres from a PDGF receptor (PDGFR)-amplified human glioblastoma sample with GSNO. Following NO treatment, there was an approximate 3-fold induction of the SP when compared with vehicle treated controls (18.87 ± 0.79 versus 6.87 ± 0.37; $p < 0.005$). This GSNO-induced increase in the SP was blocked in the presence of the GSI (18.87 ± 0.79 versus 2.87 ± 1.14; $p < 0.005$) (Figure 7B), indicating that NO induction of the SP phenotype through activation of Notch is conserved in a subset of human gliomas. When combined, the percent increase in the SP by GSNO and Pet-cGMP was no more than with either individual treatment, as was seen in our PDGF-induced glioma mouse model. In addition, we analyzed five PDGFR amplified human glioma tissue samples by immunohistochemistry for the expression of ENOS, SGC, and NESTIN.

In all five PDGFR-amplified human glioma samples, ENOS showed vivid expression in the tumor vasculature, and in three of five samples, NESTIN expression was enhanced in cells surrounding the tumor vascular endothelium (Figure 7C), with the remaining two samples showing diffuse NESTIN staining throughout the tumor. The SGC staining paralleled the NESTIN expression in these tumors (data not shown). Identical immunohistochemical analysis of six human EGFR amplified human gliomas did not reveal similar ENOS and NESTIN expression limited to the PVN. (Figure S7).

To assess the tumorigenic potential of NO-activated human glioma primary neurospheres, immunocompromised mice were injected with 2.5×10^5 GSNO-treated primary glioma cells and compared to their age-matched controls injected with an equal number of the same vehicle-treated human primary glioma cells (10 mice per group). All mice developed tumors; however, the survival difference between the two groups was statistically significant ($p < 0.005$) with a shorter survival for mice receiving the NO-activated cells. The median survival of vehicle- and GSNO-treated human primary glioma cultures was 36 and 29 days, respectively (Figure 7D). These data suggest that within the PDGFR gene amplified subset of human gliomas, elevated nitric oxide activity within the tumor vasculature may play a role in promoting stem cell-like characteristics within some of these tumors.

DISCUSSION

Stem cells within the brain are localized to specific microenvironments that regulate their maintenance (Doetsch 2003). A perivascular location for stem cells has been described for normal (Shen et al., 2004; Ramírez-Castillejo et al., 2006) and brain tumor stem-like cells (Calabrese et al., 2007; Hambardzumyan et al., 2008a). In the context of brain tumors, regions of microvascular proliferation represent these vascular stem-like cell niches. As progression to more aggressive gliomas is associated with a prominent increase in the density of these vascular structures (known as microvascular proliferation), their presence in gliomas is a criteria for malignant histology by the WHO grading system (Kleihues et al., 1995). This association with increasing grade has led to proposals that the perivascular niche might directly contribute to progression of brain tumors. One proposal for the perivascular location of stem-like cells has been that factors released from endothelial cells intricately associated with the niche and activate one or more signaling pathways in adjacent cells that reinforces their stem-cell like character (Calabrese et al., 2007). Our finding suggests that one of these mechanisms is the activation of Notch signaling by NO produced from the endothelial cells. We propose that NO released from the tumor endothelium diffuses to neighboring glioma stem-like cells, tightly associated with the tumor vasculature, and activates the Notch pathway within these stem-like cells (Figure S8).

The tumor perivascular niche is a complex microenvironment with multiple cell types and signaling factors involved in the crosstalk between endothelial cells and stem-like cells residing within the niche. The contribution of aberrant NO signaling within the niche is not the only component involved in the crosstalk between the tumor endothelium and perivascular stem-like cells. In vivo, glioma stem-like cells are likely influenced by the convergence of signals from neighboring cells within the perivascular niche. In addition to glioma stem-like Nestin-expressing cells, there are other cell types known to reside within the brain tumor perivascular niche, such as reactive astrocytes that mediate Shh signaling (Becher et al., 2008) known to promote stem cell renewal in gliomas (Clement et al., 2007) and pericytes (Hambardzumyan et al., 2008a) that likely contribute either through direct cellular interactions or in a paracrine fashion to regulate the activity of glioma stem-like cells.

It is worth noting that transient activation of the NO/cGMP pathway by GSNO or Pet-cGMP activates Notch signaling and increases the percentage of SP cells in PIGPC within 2–2.5 hr. These cells then show long-lasting fundamental changes in their behavior, as demonstrated by their increased capacity for neural stem cell formation 2 weeks after treatment withdrawal and increased capacity for tumor formation more than 4 weeks after tumor cell transplantation. This effect might involve the conversion of a subpopulation of glioma cells within our primary cultures, with an inherent capacity to be primed by NO/cGMP signaling to a more stem cell-like state. The data strengthens the argument that stem cell character can be acquired by specific signaling from the tumor microenvironment. As the RCAS/tv-a PDGF mouse model mimics the PDGFR subtype of human gliomas, this observation may be limited to this subgroup of human gliomas. We have identified PKG as a critical mediator of NO-induced Notch pathway activation in PDGF-induced mouse gliomas; however, the mechanism through which PKG activates the Notch pathway remains to be determined.

This additional role for NO in promoting gliomagenesis through induction of stem-like activity complements its established role in tumor angiogenesis and underscores the emerging role of the glioma perivascular niche as a direct participant in the disease process. This work also highlights several potential therapeutic targets, such as inhibitors of endothelial nitric oxide synthase (eNOS), PKG, and Notch. Although these effects were ascribed to inhibition of eNOS-induced angiogenesis, it is possible that these therapies additionally modified stem-like characteristics within these tumors. In addition, the use of anti-angiogenic agents that disrupt these aberrant glioma vascular stem cell-like niches could potentially sensitize tumors to conventional treatments by depriving the stem-like cells of niche signals, such as NO, required for their maintenance. Such synergy was previously demonstrated with bevacizumab, which after treatment of glioma-bearing mice, resulted in depletion of tumor blood vessels and a significant reduction of tumor stem cells (Calabrese et al., 2007). Since antiangiogenics, such as endostatin and thrombospondin, exert their effects through inhibition of eNOS (Fukumura et al., 2006), the effect observed with bevacizumab, could also involve modifications to stem-like character within these tumors. Targeting of the Notch pathway was demonstrated to be an effective anti-tumor strategy in brain tumor preclinical studies (Fan et al., 2006). Pharmacologic suppression of the Notch pathway in medulloblastoma cells significantly decreased their cancer stem-cell populations, as indicated by SP analysis, CD133, and Nestin expression, and further diminished their capacity to form tumors following transplantation in mice. These cells were also more sensitized to apoptosis than more differentiated cells (Fan et al., 2006). This finding supports the notion that targeting the Notch pathway

in gliomas could potentially sensitize the stem cell-like fractions to apoptosis. The prediction from the preponderance of the literature is that these approaches might be more effective when combined with radiation and chemotherapy where perivascular resistant cells contribute to tumor recurrence after conventional therapy.

EXPERIMENTAL PROCEDURES

Hoechst 33342 Staining and Flow Cytometry

PDGF-induced glioma primary cultures (PIGPC) were suspended at 1 × 10^6 cells/ml in 15 ml falcon tubes in DMEM+10FBS medium, then preincubated at 37°C for 35 min in the presence of vehicle (water), GSNO (100 μM) (Sigma Aldrich, USA), Pet-Br-cGMP (200 μM) (Axxora Platform, San Diego, CA), Fumitremorgin C (FTC), and ABCG2 inhibitor (5 μM) (Axxora Platform, San Diego, CA) or the gamma secretase inhibitor (GSI)-MK-003 (3μM), a kind donation from Merck, USA. Cells were then incubated with Hoechst 33342 (5 μg/ml) for 90 min at 37°C with periodic shaking. Following Hoechst staining, cells were incubated on ice for 10 min then washed 2× in ice-cold PBS. Hoechst dye was excited at 407 nm using a trigon violet laser, and dual wavelength detection was performed using 450/40 (Hoechst 33342-Blue) and 695/40 (Hoechst 33342-Red) filters. Dead cell exclusion in control, GSNO-, pet-cGMP-, and GSI-treated glioma primary cultures occurred by forward and side scatter gating and the exclusion of PI-positive populations. Data were analyzed using FlowJo (Ashland, OR).

Generation of PDGF-Induced Primary Glioma Cultures

Neonatal Nestin tv-a (N-tva) INK4A/$Arf^{-/-}$mice described previously (Uhrbom et al., 2005) were injected intracranially with DF1 chicken fibroblasts, producing RCAS-PDGF retroviral particles to generate gliomas as described previously (Shih et al., 2004). PDGF-induced gliomas were dissected and enzymatically digested for 15 min in 1× Earles balanced salt solution containing 12% papain (Worthington, Lakewood, NJ) and 10 μg/ml DNase at 37°C. The digestion was stopped with 1 mg/ml ovomucoid (Worthington, Lakewood, NJ). Cells were washed and resuspended 3× in basal medium. Single-cell suspensions were plated in DMEM+10% FBS, and cells were grown as a monolayer. The medium was replaced 24–48 hrs later. Primary culture experiments were performed using PDGF-induced tumors from different mice; however, control and treated groups compared in all experiments were generated from the same tumor. Primary cultures used in all experiments were performed using 10 cm cultures dishes at 50%–60% confluence. In addition, all PIGPC were used between passage numbers p0–p4.

Intracranial Injection of Mouse Glioma Cells

To assess the tumorigenic capacity of PIGPC following GSNO treatment, approximately 8 × 10^4 vehicle- or GSNO-treated (2 hr) PIGPC were injected into the cortex of newborn pups. Tumor development occurred between 8–14 weeks.

Injection of human cells was done by stereotactic injection. NOD-SCID mice between 5–10 weeks old were anaesthetized by i.p. injection of ketamine (0.1 mg/g) and xylazine (0.02 mg/g). 1μl of 2.5 × 10^5 cells were injected into the cortex. Stereotactic coordinates were taken relative to bregma, right hemisphere: AP, 0 mm from bregma; Lat, 3.0 mm (left or right); depth, 1.0 mm from dural surface. Locations were determined using mouse atlas (Franklin and Paxinos, 2007). Tumor cells were injected using a Hamilton syringe. Mice were closely monitored for tumor development, as assessed by the presence of hydrocephalus, lethargy, or cachexia.

Statistical Analysis

Comparisons between two groups were made using two-sided t tests. Chi square test was use to compare groups in Kaplan Meier graphs. Two-way ANOVA was used to analyze data from luciferase assays. Fisher's Exact Test was used to compare tumorigenicity between vehicle- and pet-cGMP-treated primary glioma cells. Data represent the mean of three experiments unless otherwise noted. p values of <0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

The Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.stem.2010.01.001.

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