

Inhibition of Notch Signaling in Glioblastoma Targets Cancer Stem Cells via an Endothelial Cell Intermediate

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ABSTRACT

Glioblastoma multiforme (GBM) is a highly heterogeneous malignant tumor. Recent data suggests the presence of a hierarchical organization within the GBM cell population that involves cancer cells with stem-like behavior, capable of repopulating the tumor and contributing to its resistance to therapy. Tumor stem cells are thought to reside within a vascular niche that provides structural and functional support. However, most GBM studies involve isolated tumor cells grown under various culture conditions. Here, we use a novel three-dimensional organotypic “explant” system of surgical GBM specimens that preserves cytoarchitecture and tumor stroma along with tumor cells. Notch inhibition in explants results in decreased proliferation and self-renewal of tumor cells but is also associated with a decrease in endothelial cells. When endothelial cells are selectively eliminated from the

explants via a toxin conjugate, we also observed a decrease in self-renewal of tumor stem cells. These findings support a critical role for tumor endothelial cells in GBM stem cell maintenance, mediated at least in part by Notch signaling. The explant system further highlighted differences in the response to radiation between explants and isolated tumor neurospheres. Combination treatment with Notch blockade and radiation resulted in a substantial decrease in proliferation and in self-renewal in tumor explants while radiation alone was less effective. This data suggests that the Notch pathway plays a critical role in linking angiogenesis and cancer stem cell self-renewal and is thus a potential therapeutic target. Three-dimensional explant systems provide a novel approach for the study of tumor and microenvironment interactions. STEM CELLS 2010;28:1019–1029

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The prognosis for the malignant brain tumor glioblastoma multiforme (GBM) has remained poor for decades, with a median survival of about 1 year [1]. In combination with surgery, the most effective treatment for GBM is radiation, but its efficacy is limited due to significant radiation resistance. A small subpopulation of “stem-like” cells is now thought to contribute to this radioresistant phenotype and lead to repopulation of the tumor after treatment [2–4]. Brain tumor stem cells share characteristics with normal neural stem cells, such as neurosphere formation, the capacity to differentiate into multiple lineages and possibly interaction with a complex stem cell niche that includes endothelial cells [5–7]. In fact normal neural stem cells have been proposed as the potential cells of origin of glioblastoma [8, 9].

Notch signaling is an evolutionarily well-conserved pathway that plays a key role in many aspects of development such as cell differentiation, proliferation, and apoptotic events, but the specific effects are highly context dependent [10, 11]. There are four human Notch receptors that consist of an extracellular peptide containing epidermal growth factor (EGF) receptor-like repeats and a transmembrane peptide. They display both overlapping and distinct tissue distributions as well as both redundant and distinct functions [12]. Notch 1 and Notch 2 are the most ubiquitously distributed whereas Notch 2 and Notch 4 are more specifically expressed in vascular smooth muscle and endothelial cells [13]. Ligand binding via the Jagged or Delta-like family of membrane proteins leads to cleavage of the receptor by members of the A Disintegrin And Metalloprotease (ADAM) and γ -secretase families of proteases. This results in the release of the Notch intracellular domain (NICD), which translocates to the nucleus where

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it interacts with members of the C-Promoter binding factor (CBF1), Suppressor of Hairless, Lag 1 (Longevity Assurance Gene) (CSL) family and recruits multiple coactivators and corepressors leading to activation of target genes as well as NICD degradation. Notch is thought to target the transcription of multiple genes, including members of the basic Hes (helix-loop-helix family) and Hey (hairy and enhancer of split related with XRPW modif) [14]. The Notch pathway plays a major role in maintenance of the stem cell state in the nervous system [15, 16] and in the regulation of angiogenesis in normal development as well as tumors including glioblastoma [17–19]. It also interacts closely with the vascular endothelial growth factor (VEGF) pathway in modulating tumor angiogenesis and is increasingly viewed as an important therapeutic target. However, the specific effect of Notch inhibition on tumor endothelial cells, and perhaps consequently cancer stem cells, is poorly defined.

Emerging data suggests that glioblastoma stem cells may reside in a protective vascular niche that could contribute to cell fate decisions and survival [20]. Interactions between cancer stem cells and the vascular niche are thought to confer a survival advantage following therapeutic insults as well as allowing maintenance of the stem cell population and thus the ability to repopulate the tumor. A greater understanding of the role of the stroma in modulating malignant progression in glioma and other tumors has led to attempts at re-calibration of therapeutic strategies to target both cell autonomous and environmental factors.

Laboratory studies of glioma biology have long relied on the use of dissociated glioma cell lines, which are evaluated in isolation from the tumor microenvironment. In view of the growing awareness of the role of the tumor stroma [21], specifically the vascular niche, to the integrity of the tumor stem cell pool, we optimized the organotypic or “explant” culture model in which the original cytoarchitecture, cell connectivity, and stroma—including blood vessels—are preserved [22, 23]. Using this system, we demonstrate that tumor endothelial cells are key components of Notch signaling in glioblastoma and likely mediate its effects on proliferation and self-renewal.

MATERIALS AND METHODS

Culture of Primary Tumors as Organotypic/Explant Cultures

GBM tumor specimens were collected from the surgical suite and placed on ice in sterile phosphate-buffered saline (PBS). The tumor tissue was treated with RBC lysis buffer (eBioscience, San Diego, CA, ebioscience.com) to remove red blood cells and washed twice with ice-cold PBS. Using sterile 1 ml 27.5G syringes (BD Biosciences, San Jose, CA, <http://www.bdbiosciences.com>), the tumor tissue was dissected under the microscope into ~ 1 mm³ pieces (explants). Five explants were placed on a sterilized culture plate insert (Millipore, Billerica, MA, <http://www.millipore.com>) that was pretreated with 1 μ g/ml fibronectin (BD Biosciences,) and placed in a 3-cm well. Culture media consisted of F12-DMEM (Gibco, Grand Island, NY, <http://www.invitrogen.com>) and N2 supplements (glucose, glutamine, sodium bicarbonate, 25 μ g/ml of insulin, 100 μ g/ml of human apotransferrin, 20 nM progesterone, 100 μ M putrescine, 30 nM sodium selenite [all components from Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>]) at a pH of 7.2 [24–26]. Sterile N2 media was added in the lower well and 10 μ l of media were added to the surface of each explant. The media was changed every other day. Bromodeoxyuridine (BrdU; Sigma-Aldrich, St

Louis, MO, <http://www.sigmaaldrich.com>, 10 μ M) was added to the media 8 hours before harvesting.

Tissue Processing

Tissue was fixed in 4% *p*-formaldehyde overnight at 4°C, then transferred to 30% sucrose at 4°C. Optimal cutting temperature compound (Sakura Finetek, Torrance, CA, <http://www.sakura-finetek.com>) was used for embedding and 7 or 200 μ m (for 3D reconstruction confocal microscopy) sections were cut on a cryostat (Leica, Deerfield, IL, <http://www.leica-microsystems.com>).

Fluorescence Immunohistochemistry

The sections were fixed with methanol for 5 minutes and permeabilized with acetone for 1 minute at -20°C . They were washed in PBS and blocked with 10% normal goat serum (Invitrogen) in PBS and 0.3% Triton X-100 for 1 hour. Primary antibodies were incubated overnight at 4°C and appropriate secondary antibodies (Alexa conjugates, Invitrogen) for 1 hour at 25°C. Slides were washed in PBS, counterstained with *t*-4',6-Diamidino-2-phenylindole (DAPI) (Invitrogen) and mounted in 70% glycerol or Prolong Gold anti-fade reagent (Invitrogen) for confocal and fluorescence microscopy. Antibodies included: Ki67 (1:100 Dako, Glostrup, Denmark, <http://www.dako.com>), CD105 (1:100, Dako), CD31 (1:100, BD Biosciences), NICD (NICD 1:000, Abcam, Cambridge, MA, Cambridge, U.K., <http://www.abcam.com>) and Hairy and Enhancer of Split 5 (HES5) (1:100, Chemicon, Billerica, MA, <http://www.chemicon.com>). BrdU and terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick end labeling expression was measured using the 5-bromo-2'-deoxy-uridine Labeling and Detection Kit I, and the POD (Horseradish peroxidase) In Situ Cell Death Detection Kit (both Roche Applied Science, Indianapolis, IN, <http://www.roche-applied-science.com>) according to manufacturer's guidelines.

Image Acquisition and Histological Analysis

Epifluorescence microscopy was performed using a BX51 microscope (Olympus, Center Valley, PA, <http://www.olympus-global.com>) equipped with a C4741-95 digital camera (Hamamatsu, Bridgewater, NJ, <http://www.hamamatsu.com/>). Images were processed using the SlideBook 4.2 image software (Olympus). Confocal microscopy was performed using a Leica TCS SP2 AOBs laser scanning DMRXA2 microscope. The maximum intensity and blend projections of the stacked images were computed using Bitplane Imaris 4.5 software (Zurich, Switzerland, www.bitplane.com). Thereafter, an isocontour model of blood vessels was generated to highlight the volume of the structures, then rotated, panned, and zoomed while recording the animation. The movie was later exported in .MOV format. Bright-field images were acquired using an Olympus IX71 microscope. Quantification of endothelial cells and ki67+ cells was performed on randomly selected high-power fields. On average, 4,000 cells per explant were counted.

Administration of Radiation and the γ -Secretase Inhibitor DAPT

The explants were allowed to attach to the insert overnight. A total of 25 μ g/ml of the γ -secretase inhibitor DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester, Sigma-Aldrich) were added and media changed every other day. Radiation was delivered via X-RAD 225C irradiator (Precision X-Ray, Inc., North Branford, CT, <http://www.pxinc.com/>). For the combination group, the explants were pretreated with 25 μ g/ml DAPT for 5 days then irradiated with 10 Gy. DAPT was maintained in the media for the next 5 days.

Each experiment represents a minimum of three (range: 3–5) different tumors, each of which is represented in three wells. In average each condition thus consisted of a total of 9–15 explants per tumor, with a range of 3–5 tumors per experiment. Explants are pooled in groups of 3–5 per well.

Neurosphere Assay and Flow Cytometry

The explants were dissociated into smaller pieces and placed in Hanks' balanced salt solution (HBSS, Invitrogen) containing 1 mg/ml DNase I (Roche Applied Science) for 15–30 minutes at 37°C. The tissues were then triturated every 5 minutes and ultimately passed through a 35- μ m cell strainer (BD Falcon, www.bd.com). The cells were washed using sterile 0.9 M sucrose in HBSS (pH 7.3) and resuspended in N2 media containing 20 ng/ml basic fibroblast growth factor-2 and EGF (both from R&D Systems, Inc., Minneapolis, <http://www.rndsystems.com>). The cells were plated in ultralow attachment plates (Costar, Lowell, MA, www.colepalmer.com) at high density (20,000 cells per milliliter) for 3 days. Primary neurosphere formation was obtained readily but was often associated with nonviable clumps. After clearing agglutinating debris, we re-plated the cells at clonal density (100 cells per milliliter) and formed secondary neurospheres over 10–14 days.

For flow cytometry, the explants were dissociated in Liberase (400 μ g/ml, Roche Applied Science). Cells were blocked with 1:10 FcR blocking reagent (Miltenyi, Auburn, CA, <http://www.miltenyibiotec.com>) for 15 minutes at 4°C and labeled with CD133 antibodies (AC141 and AC133 epitopes (1:1), Miltenyi) for 30 minutes at 4°C. Flow cytometry analysis was performed using FACSCalibur flow cytometer (BD, Franklin, NJ ([bd.com](http://www.bd.com))) and dead cells were excluded using 7-amino-actinomycin D (BD Pharmingen, San Diego, CA, http://wwwbdbiosciences.com/index_us.shtml).

Clonogenic Assays

We established three different cell lines from fresh glioblastoma specimens [27, 28]. Briefly, surgical specimens were dissociated and cultured in N2 media under neurosphere conditions and different treatment conditions (DAPT, radiation and/or combination). Formed neurospheres were passaged for two to four times, and then dissociated and replated at clonal density (100 cells per milliliter). To further confirm clonogenicity under attached culture conditions, cells replated on culture were coated with polyornithine, laminin, and fibronectin. Colony formation was visualized with gentian violet staining.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted using Trizol (Invitrogen). A total of 0.5 μ g of RNA was reverse-transcribed using SuperScript III First-Strand Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR) (Invitrogen). The resulting cDNA was subjected to real-time PCR amplification using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, <http://www.appliedbiosystems.com>). Primer sequences in supporting information Table 1.

Saporin-Conjugated Antibodies

The antibody CD105 (1:1,000) was incubated with goat anti-mouse IgG-saporin (ZAP, Advanced Targeting Systems, San Diego, CA, www.atsbio.com) in N2 media for 30 minutes at 37°C to allow binding and formation of a CD105 antibody-saporin complex [26], which was added to the explants or to hCMEC/D3 cell line (human cerebral microvessel endothelial cell line, courtesy of Babette Weksler) [29] used as control. Manufacturer directions were followed. As control, the CD105 antibody was incubated with a nontargeting goat IgG saporin that does not bind CD105. The antibody-saporin solution was injected into the explant under a dissecting microscope after gently incising the explant surface for better access.

VEGF Enzyme-Linked Immunosorbent Assay

VEGF into the culture media was measured as described previously [30], using a human VEGF enzyme-linked immunosorbent assay kit (Invitrogen).

Statistical Analysis

Statistical significance was determined using the student *t*-test (paired when indicated). *p*-values below .05 were regarded as significant. Errors are SEM. All experiments were performed in multiples ($n = 3-5$). Each *n* represents the number of independent tumors and a minimum of 10 explants per tumor.

RESULTS

The Tumor Microenvironment Is Preserved in the Explant Model of GBM

Our approach to the study of putative cancer stem cells in GBM was predicated on the maintenance of the tumor stem cell niche and tumor stroma including endothelial cells in vitro. To this end, we optimized a system for the culture of organotypic brain slices, first described by Stoppini for the study of the hippocampus [23]. This model was designed originally for the study of normal physiological properties in the central nervous system.

Tumor tissue was obtained directly from the surgical suite and was dissociated into small pieces or “explants,” which were maintained in a transwell system, allowing their growth and maintenance in culture at an air-liquid interface. Chemically defined media was used without additional growth factors or sera. The explants survived well and were kept in culture for up to 3 weeks with good viability. They flattened out and grew slowly over the course of a week (Fig. 1A). The original cytoarchitecture was preserved with strong similarities between the parent tumor and its corresponding explant (Fig. 1B). This is best demonstrated in the preservation of tumor stroma, including a fibrillary GFAP+ background and tumor endothelium and pericytes (supporting information Fig. 1). Three-dimensional architecture was also preserved as seen in the maintenance of a highly branched appearance of the capillary network within the explant (3D-reconstructions in Fig. 1C and supporting information Movie). Endothelial hyperplasia and vascular glomeruloid bodies, highly characteristic features of GBM, were also preserved in explants (Fig. 1B, inset). Tumor cells in the explant exhibit a high proliferation rate as demonstrated by BrdU incorporation and Ki-67 immunostaining (Fig. 1D). For a quantitative perspective, we analyzed the number of proliferating cells and of endothelial cells in sets of original tumors and their corresponding explants and found essentially identical values (Ki-67 of 18.53% and 16.67% and CD105 of 10.13% and 9.29%, respectively). The process of explant culture is also highly efficient with the likelihood of successful explant derivation per tumor dissected exceeding 90% once protocols for tissue handling and culture were optimized. Occasionally explants containing significant areas of necrosis and pseudopalisades do not survive well and have to be discarded. Thus explants of glioblastoma maintain a significant similarity to the original tumor specimens.

We also analyzed variability within explants over the course of in vitro culture. Time course fluorescence-activated cell sorting analysis of four GBM samples on day1, day5, and day10 revealed no time-dependent changes of CD133+ cells in the explants ($n = 3$ sets of explants for each time point, Fig. 1E). Time course analysis of in vitro uptake of BrdU over time also shows low variability that remains below statistical significance ($p < .16$) (supporting information Fig. 2A). Immunohistochemistry for GFAP (Fig. 1F), Nestin, and CD133 (supporting information Fig. 2B) also shows minimal variability of expression over the course of 10–14 days.

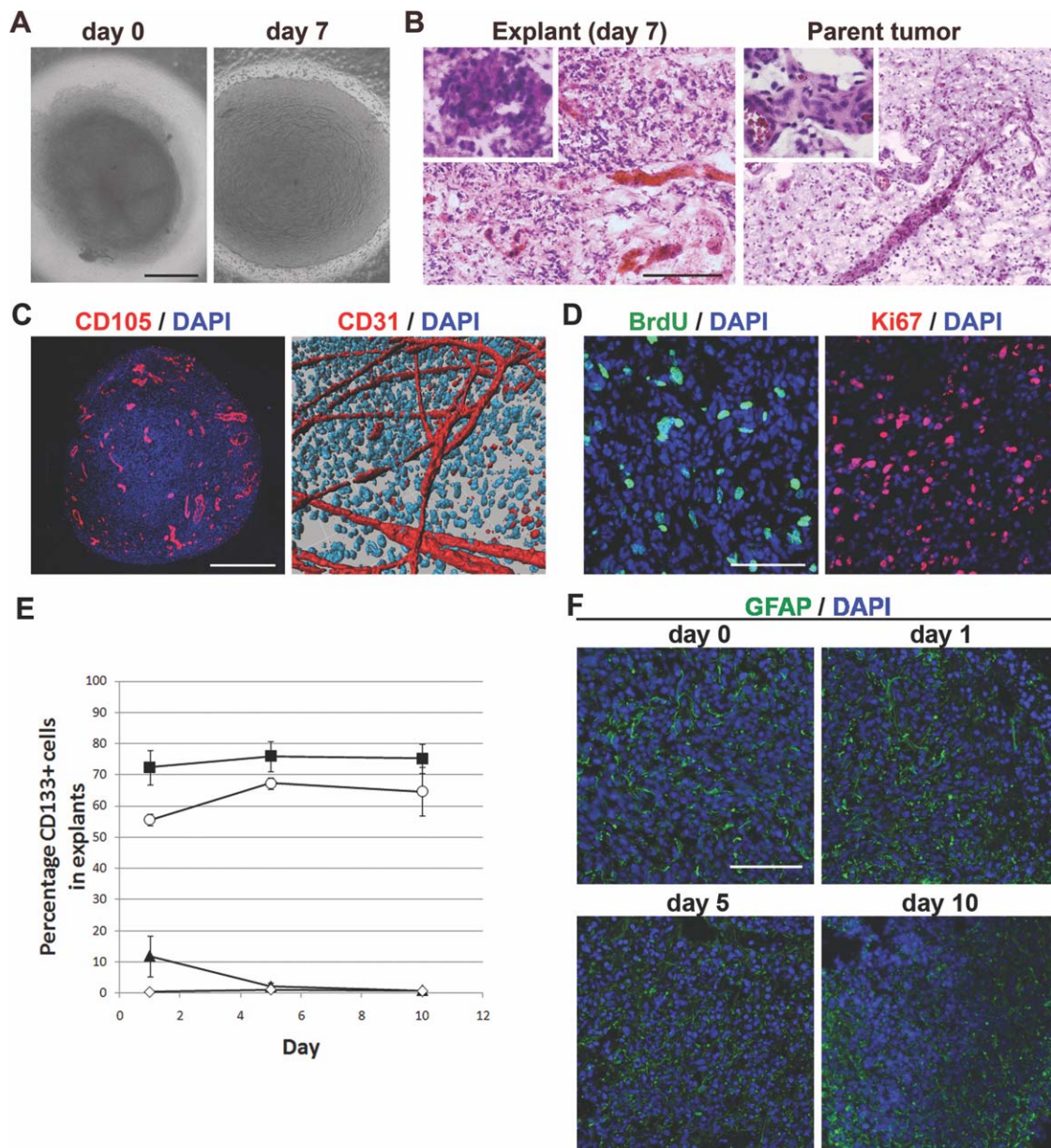


Figure 1. The tumor microenvironment is preserved in the explant model of glioblastoma multiforme (GBM). (A): Bright-field image of GBM explants attached to a fibronectin-coated membrane. The explants flattened out and grew slowly over 7 days. (B): Hematoxylin and eosin staining of an explant at 7 days in vitro showing good preservation of cytoarchitecture and blood vessels, in comparison with its parent tumor. Inset shows a higher magnification of a glomeruloid body in both parent tumor and explant. (C): Immunohistochemistry for the tumor endothelial marker CD105 demonstrates maintenance of high vascularity. The three-dimensional structure of tumor vessels is demonstrated using a reconstruction of confocal immunofluorescence images of an explant in the right panel. CD31 labeling in red highlights the blood vessels, nuclei in blue (grid: 50 μm). (D): Explants maintain a high level of proliferation, shown here at 5 days in vitro, by immunohistochemistry for BrdU and Ki67. (E): Flow cytometry analysis of CD133 in explants from four different tumors. The number of CD133+ cells within each tumor showed little variability over 10 days in vitro ($p > .05$ for all four tumors). Tumors often exhibited significant heterogeneity for CD133 expression. (F): Immunofluorescence images of GFAP show a well maintained cytoarchitecture and no significant change in GFAP expression in the explants over 10 days in vitro. Error bars are SEM [Scale bars: 500 μm in (A) and (C); 100 μm in (B) and (F); 50 μm in (D)]. Abbreviations: BrdU, bromodeoxyuridine; DAPI, 4',6-diamidino-2-phenylindole; GFAP, Glial Fibrillary Acidic Protein.

This data suggests stability of tumor cell composition and marker expression as well as proliferation in our in vitro system over time.

Variability among tumors is a predictable element in this system, in view of the recognized genetic, vascular, and tumor cell heterogeneity in glioblastoma. A common occurrence is the variability of CD133 expression among different tumors (Fig. 1E) despite consistently rigorous gating. Expo-

sure to radiation or chemotherapy treatments before surgery and specimen acquisition may result in significant selection and alterations in tumor response to in vitro. Stratification of tumors according to clinical and genetic variables is an area of intense research activity, which may lead to rapid testing and classification of GBM subtypes based on their transcriptome and/or proteome profiles [31]. In this manuscript, a total of 23 tumor specimens were used, originating from nine male

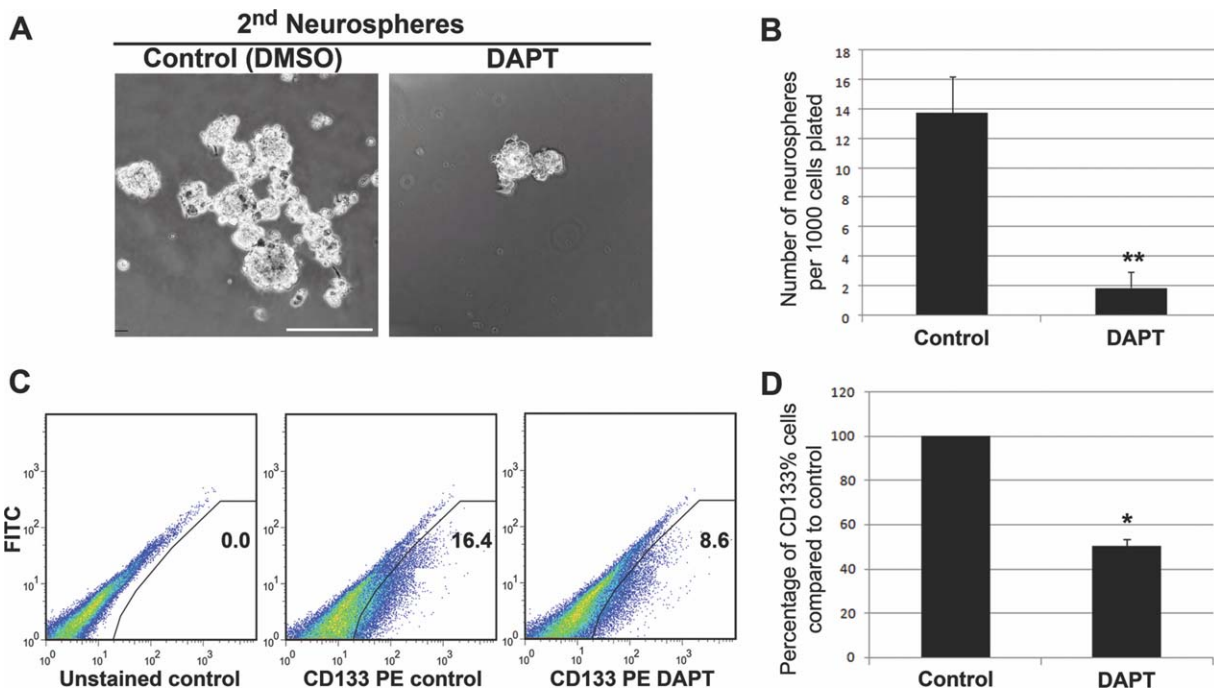


Figure 2. Notch inhibition decreases the number and function of stem cells in glioblastoma multiforme (GBM) explants. (A, B): After 5 days of DAPT treatment, the explants were dissociated into single cells and grown under neurosphere conditions. A significant (84%) decrease in secondary neurosphere formation was observed [in (B), $p = .0018$]. (C, D): Flow cytometry analysis for CD133 in DAPT treated explants. Despite large variability in % CD133+ cells among the primary GBM tumor samples, there was a consistent relative decrease in the number of CD133+ cells averaging 50% on treatment with the Notch inhibitor and in comparison with controls. Scale bar in (A): 200 μm . Error bars are SEM. Abbreviations: DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

and 14 female patients, of whom nine had recurrent tumors and 14 were newly diagnosed. All tumors were WHO grade IV glioblastomas with one tumor known to have progressed from a previous WHO grade III anaplastic astrocytoma. All but one of the recurrent tumors had received irradiation and temozolomide at the time of tissue collection. Limited clinical cytogenetics data were available for 19 of 23 specimens (supporting information Table 2).

Notch Inhibition Decreases Self-Renewal in GBM Explants

Recent data suggests a role for Notch pathway in maintenance of self-renewal by CD133+ cells in vitro. We analyzed the impact of Notch inhibition on GBM by exposing the tissue explants to the γ -secretase inhibitor DAPT for 5 days. To analyze the impact of Notch inactivation on self-renewal, we performed secondary neurosphere formation assays at clonogenic density. Five days after continuous DAPT treatment, the explants were dissociated and placed under neurosphere conditions. A significant decrease in secondary neurosphere formation by an average of 84% was observed after DAPT treatment, compared with controls ($n = 3$, $p = .0018$) (Fig. 2A, 2B). This was associated with a parallel decrease in the number of CD133+ cells as demonstrated by flow cytometry studies. Although the percentage of CD133+ cells varied among tumors, the relative decrease was very consistent (48–53%) (Fig. 2C, 2D). These findings suggest that inhibiting Notch results in a decrease in self-renewal potential of tumor cells as well as a decrease in the number of CD133+ cells or downregulation of CD133 expression. The overall effect is compatible with a decrease in stem cell-like potential within the tumor explants.

The Effects of Notch Inhibition Is Mediated by the Loss of Endothelial Cells

The Notch pathway plays a key role in development and in the regulation of angiogenesis. The NICD and the downstream target gene, *Hes5*, follow a vascular and perivascular expression pattern in tumor explants as well as parent tumor tissue (supporting information Fig. 3A). Treatment of explants with DAPT for 5 days resulted in a loss of the CD105 expressing cells averaging 50% decrease but reaching up to 75% in some tumors (range: 1.1- to 4.1-fold decrease, $p = .0001$) as seen by immunohistochemistry and RT-PCR (Fig. 3A, 3B). Other endothelial markers such as CD31, von Willebrand factor, and CD146 were also decreased (data not shown). This data suggest an antiangiogenic effect of Notch blockade with subsequent disruption of the vascular niche in the explants. The Notch downstream target genes *HES1* and *HES5* were significantly downregulated as expected (Fig. 3C). This effect was very consistent in all GBM's tested and reflects effective inhibition of Notch signaling. In view of the concomitant decrease in neurosphere formation as a consequence of Notch inhibition, we evaluated the secondary neurospheres for expression of endothelial or other stromal markers, specifically CD105 and CD73, and found them to be negative. Most neurosphere cells stained for nestin and CD133 (Fig. 3D).

Having shown above that Notch inhibition results in a decrease in neurosphere formation and CD133+ cells, we tested the hypothesis that it was in fact the effect on endothelial cells that mediates the decrease in brain tumor stem cell number and function.

We used a conjugated antibody system that targets CD105 (endoglin), a tumor endothelium marker that is widely expressed in glioblastoma [32, 33] and explants (Fig. 1C).

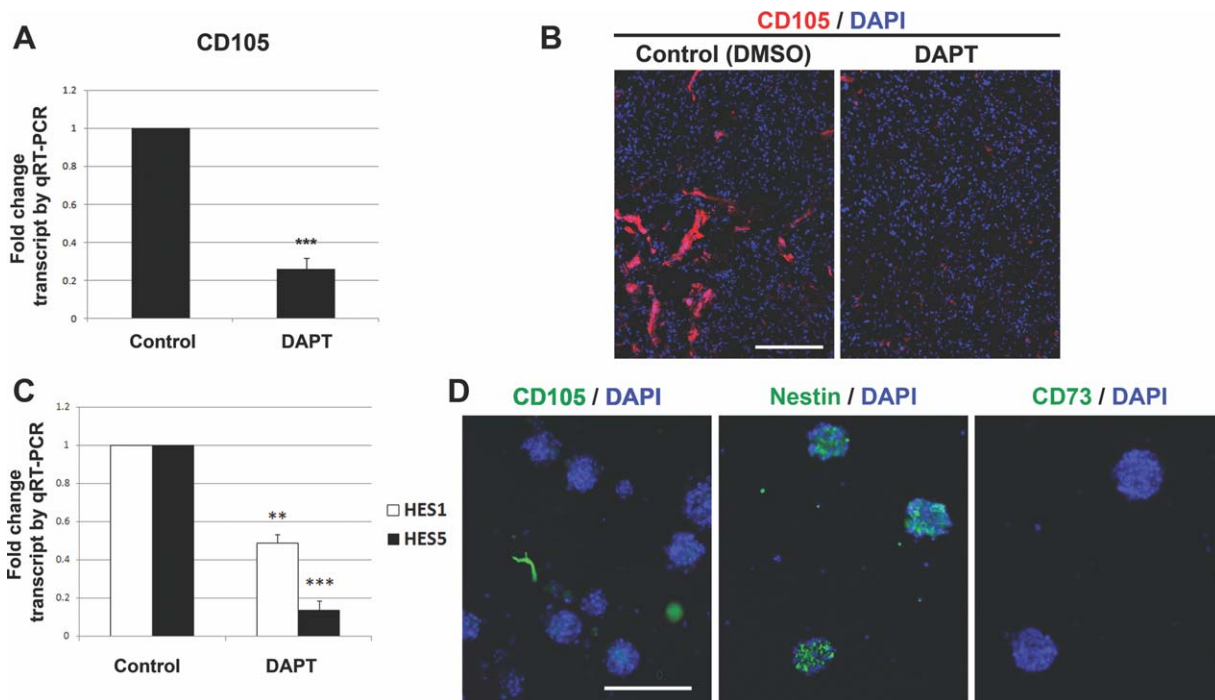


Figure 3. Notch inhibition exerts its effect in part by targeting endothelial cells. Following Notch inhibition with DAPT explants exhibit a decrease in expression of the endothelial marker CD105 as demonstrated by qRT-PCR [(A) 3.8-fold decrease, $p = .004$] and immunohistochemistry (B). (C): Downregulation of Notch target genes HES1 and HES5 ($p = .0071$ and $p = .00004$, respectively) on DAPT administration. (D): Immunohistochemistry for secondary neurospheres shows the absence of endothelial cells (CD105) and mesenchymal markers (CD73). Neurospheres are immunopositive for nestin and partially for CD133 (data not shown). Scale bars 100 μm in (A); 200 μm in (D). Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; DMSO, dimethylsulfoxide; HES1, Hairy and enhancer of split 1; HES5, Hairy and enhancer of split 5; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

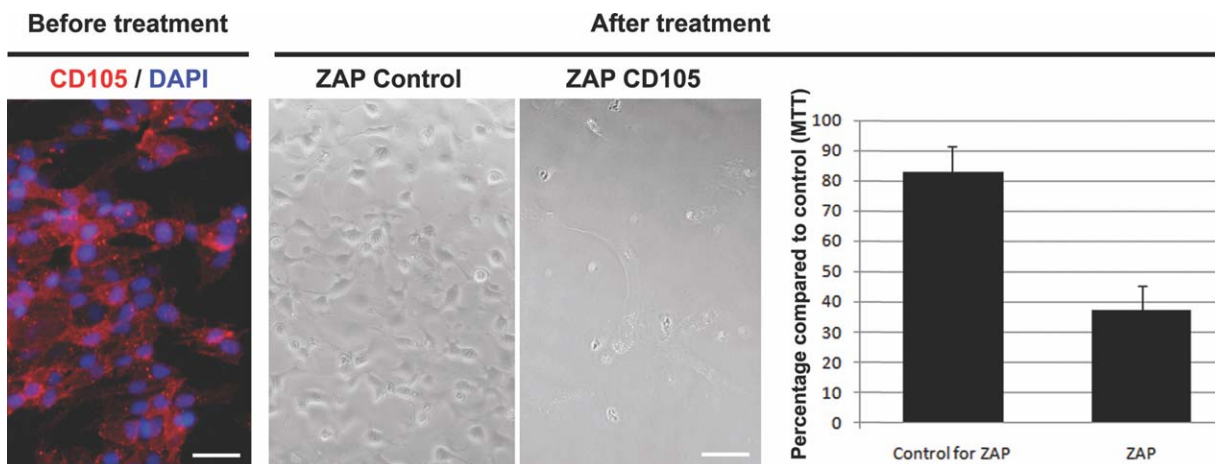


Figure 4. CD105-ZAP effectively kills human brain endothelial cells. The human brain endothelial cell line hCMEC expresses CD105 (red). Treatment with the anti-CD105 antibody conjugated to the saporin toxin but not the untargeted control resulted in cell death of the majority of human cerebral microvessel endothelial cell line (hCMEC) cells. The mitochondria activity based MTT assay confirmed this significant reduction in viability (44.9% reduction ($\pm 11.6\%$), $n = 3$, $p = .029$, error bars represent SEM). Scale bars 25 μm . Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide; ZAP, saporin immunoconjugate.

Anti-CD105 antibody is conjugated to a saporin toxin that is released in the cell on internalization of the antibody complex, resulting in cell death via ribosomal inhibition [34]. A brain endothelial cell line highly expressing CD105 (hCMEC, courtesy of Weksler) was used as a control and for dose optimization of the saporin-conjugated antibody. Significant cell death (37% compared with controls, $p = .028$) was obtained within 5 days of exposure to the anti-CD105 saporin complex

(Fig. 4). Subsequently, explants were treated with anti-CD105-saporin for 5 days. Control explants received the CD105 antibody preincubated with a nontargeting goat IgG saporin complex. A selective and significant decrease in endothelial cells was observed in the treated explants ($p = .0024$) as shown by immunohistochemistry and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Fig. 5A). Other endothelial cell markers such as CD31 were also

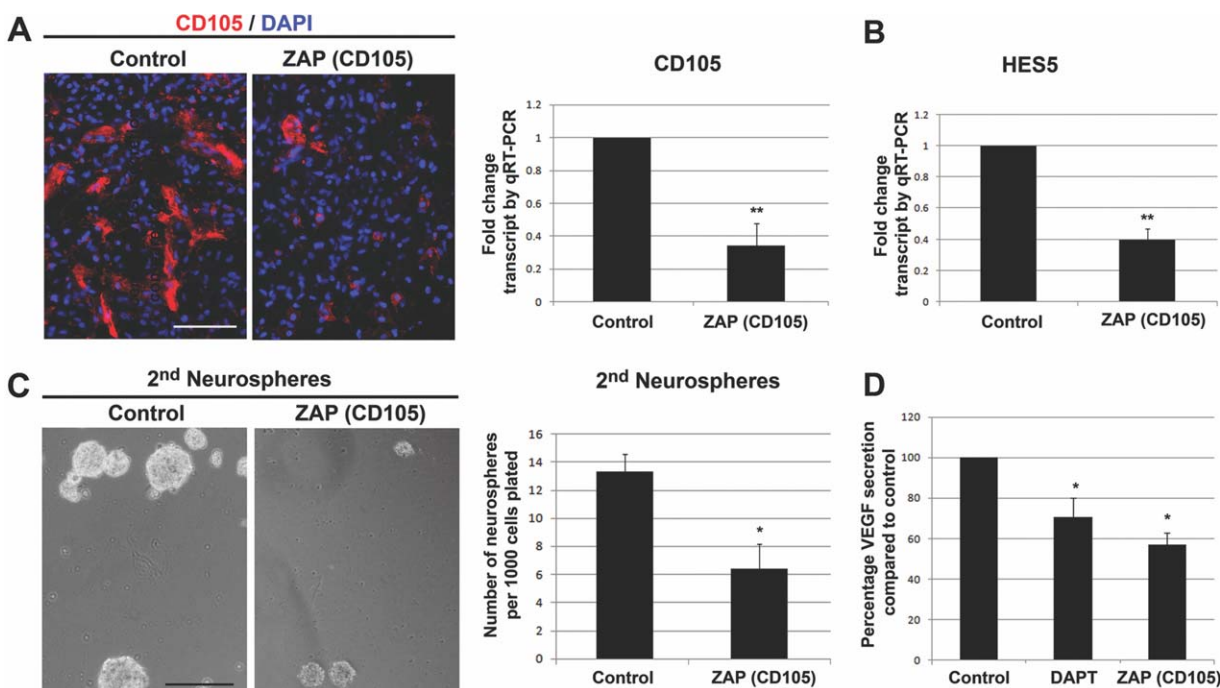


Figure 5. Effect of selective elimination of endothelial cells in glioblastoma multiforme (GBM) explants. **(A):** Treatment with the CD105-saporin complex reduces endothelial cells as shown by immunohistochemistry and mRNA levels for CD105 (2.9-fold decrease, $p = .001$). **(B):** A decrease in HES5 transcript was observed as well after 5 days treatment with the CD105-saporin complex (right panel, 2.5-fold decrease, $p = .002$) suggesting suppression of Notch activity as a consequence of the loss of endothelial cells. **(C):** Disruption of the CD105+ endothelial cell population results in a significant decrease in neurosphere-forming ability and a tendency toward formation of smaller spheres. **(D):** Vascular endothelial growth factor (VEGF) secretion in the media was measured by enzyme-linked immunosorbent assay. Pharmacological suppression of Notch activity resulted in a decrease of VEGF secretion in the media of a similar magnitude to that seen after the CD105-saporin mediated loss of endothelial cells (30% decrease with and 43% decrease, respectively, $p = .038$). Scale bars: 100 μm in **(A)**; 50 μm in **(B)**; 200 μm in **(C)**. Error bars represent SEM. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; HES5, hairy and enhancer of split 5; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; ZAP, saporin immunoconjugate.

decreased. This was associated with a significant downregulation of the Notch effector Hes5 by 60% compared with untreated controls (Fig. 5B). Interestingly, the loss of endothelial cells resulted in a decrease in neurosphere-forming ability to <50% of controls ($p = .04$) (Fig. 5C). CD133 expression was analyzed by qRT-PCR and was found to be essentially unchanged (supporting information Fig. 3B). Flow cytometry post-ZAP treatment was very suboptimal possibly due to crossreaction between the saporin-conjugated anti-CD105 and the anti-CD133 antibodies, both being IgG1 isotypes. This was further associated with a decrease in secretion of VEGF into the media, similar in magnitude to that seen under conditions of pharmacological inhibition of Notch (Fig. 5D). Thus the loss of endothelial cells mimicked a Notch inhibition like state and reduced self-renewal suggesting that the mechanism underlying the effects of Notch blockade involves disruption of the vascular niche. The reduction in VEGF may be the result of the loss of endothelial cells although the possibility of an inhibitory effect by Notch on the VEGF pathway cannot be ruled out.

Notch Inhibition Enhances the Effect of Radiation in GBM Explants but not GBM Cultures

The impact of radiation on GBM is often studied in cell lines in vitro, and their radiation resistance is well documented, but the response of tumor cells within their native microenvironment is not well studied. This is particularly relevant in view of the data supporting a role for endothelial cells in supporting self-renewing cells in some brain tumors [6]. Explants

from five different GBM specimens were pretreated with DAPT for 5 days followed by radiation with a single dose of 10 Gy. They were compared with explants from the same specimens receiving a single treatment (radiation or Notch) and untreated controls. The experiment was conducted over 10 days, to allow a 5-day pretreatment with the Notch inhibitor preceding radiation. Data analysis was performed 5 days postradiation. Single treatments, via DAPT or radiation, reduced proliferation in the explants by 50%. However, the combination of DAPT and radiation had a more profound effect, dramatically reducing the proliferation rate from 14.3% to 3.4% (Fig. 6A, 6B). Neurosphere formation was tested in all treatment and control groups (Fig. 6C, 6D). Notch inhibition resulted in a significant decrease in neurosphere-forming ability compared with controls ($p = .027$), whereas radiation alone did not have a statistically significant impact ($p = .450$). However, the combined treatment was most effective, reducing neurosphere formation from 28.57% in controls and 13.12% in DAPT alone to 8.12% ($p = .014$ and $.018$, respectively). The number of CD133+ cells decreased dramatically to 23.69% of control after DAPT and 33.42% after radiation ($p = .013$ and $.011$, respectively) (Fig. 6E). The combination treatment also resulted in a significant decrease compared with control (23.48% of the control value, $p = .041$). Although the combination group was more effective than radiation alone, its impact did not reach statistical significance ($p = .2$).

In comparison, we performed similar experiments on glioblastoma neurospheres dissociated from primary tumors and passaged for short periods (P2–P4). As noted previously, such neurospheres consist of nestin+ cells and are negative for

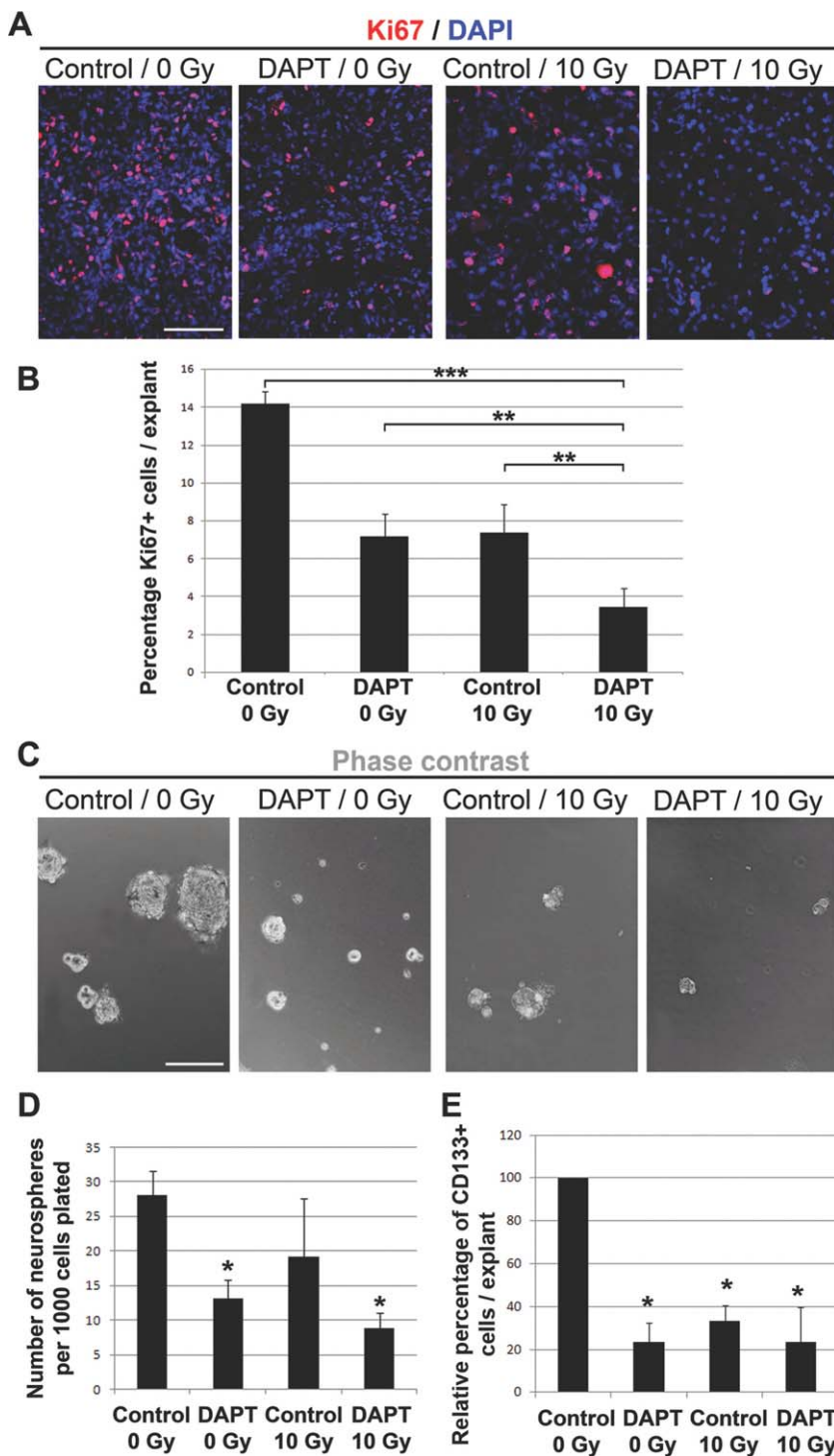


Figure 6. Notch inhibition enhances the effect of radiation in glioblastoma multiforme (GBM) explants. **(A):** Immunohistochemistry for Ki-67 (red) in explants exposed to DAPT, radiation or a combination treatment, demonstrating an antiproliferative response in all treatment conditions. **(B):** A quantitative assessment of the percentage of Ki-67+ demonstrates a decrease from $14.3 \pm 0.67\%$ to $7.2 \pm 1.19\%$ and $7.3 \pm 1.49\%$ following DAPT and radiation, respectively. The combination treatment reduced Ki-67- $3.4 \pm 0.98\%$. $p = .003$ (vs. DAPT), $p = .004$ (vs. radiation) and $p < 10^{-8}$ (vs. control). **(C, D):** Neurosphere-forming ability in control and treatment conditions demonstrates a lack of effect by radiation, while DAPT consistently reduced neurosphere formation. The combination treatment showed a more potent effect reducing neurosphere formation from 28.57% to 8.12% in controls. **(E):** Flow cytometry analysis of CD133 revealed a dramatic decrease in the combination treatments (23.5% , $p = .041$), but the effect was not significantly different compared with DAPT or radiation alone. Scale bars: $50 \mu\text{m}$ in **(A)**; $200 \mu\text{m}$ in **(C)**. Errors represent SEM. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester.

endothelial or mesenchymal stroma cells. Neurosphere cultures were treated with DAPT, XRT or a combination, for the same duration of time as the explants, passaged and replated at clonogenic density. The impact on cell growth was analyzed by dissociating the spheres into single cells and analyzing surviving cell numbers. Our data shows a significant decrease in cell number in response to DAPT, and a more substantial decrease in the radiation and combination groups (supporting information Fig. 4A). Neurosphere formation was also dramatically reduced after radiation and the combination

(from 18.8/1,000 cells to 1.9 and 1.5 neurosphere/1,000 cells plated in the radiation and combination groups, respectively, supporting information Fig. 4B). The number of CD133+ cells was highly variable among the tumors studied and trended down most significantly in response to the combination treatment (supporting information Fig. 4C). In comparison with the explant data, the impact of radiation alone on cell number and self-renewal in the dissociated cell group is much more significant and is not enhanced by Notch inhibition. The increased resistance to radiation seen within the

explants may be due to a variety of factors, including a protective role of the intact vascular niche on the tumor stem cells. It should be noted that unlike Notch inhibition, radiation treatment alone does not reduce the number of endothelial cells within explants (supporting information Fig. 3C,D). A more robust response to radiation is seen in the explants only in combination with loss of endothelial cells on Notch inhibition. In explants, there was a concomitant decrease in proliferation and the number of CD133+ cells in the group receiving radiation and yet an intact rate of neurosphere formation, suggesting that radiation alone does not impact putative cancer stem cell function. Alternatively, neurosphere-forming stem-like cells *in situ* may not always be CD133+, an observation with growing support in the literature.

Although the response to radiation is complex and warrants further investigation, this data supports a role for the microenvironment, specifically endothelial cells, in modulating the response to radiation and Notch inhibition. It further emphasizes the advantages of analyzing tumor response *in vitro* within the context of the tumor stroma.

DISCUSSION

Studies using glioblastoma cell lines have long suffered from poor reproducibility in view of recognized alterations in genomic profiles and *in vitro* behavior. Work on cell lines or neurospheres highlights specific subpopulations of cells but fails to analyze the interactions among cell species within a tumor. This is particularly relevant in the case of the Notch pathway where cell-to-cell interactions play a key role in ligand availability and receptor activation. The effects of Notch signaling are highly context dependent and have thus to be analyzed for individual cell types in specific microenvironments. In addition, there is emerging data that suggests that tumor stem cells reside in a perivascular niche structurally and functionally. Here, we show that the organotypic explant model is a useful tool for studying GBM biology. The preservation of blood vessels and cell connectivity in their original context offer a definite advantage over the more commonly used cell lines or tumor sphere systems. The dual effect of Notch inhibition on the tumor stem cells and on endothelial cells as illustrated here could not be demonstrated in cell lines or xenografts, which may recruit host-derived endothelial cells. Thus our organotypic model is exquisitely suitable to study the biology of glioblastoma and to model therapeutic response *in vitro*.

The description of stem-like cells in brain tumors and their potential contribution to therapeutic failure has led to significant expansion of research in this area [4]. The concept of cancer stem cells implies a hierarchical organization within the tumor, whereby tumor initiation and repopulation after cytotoxic therapy is ascribed to a small subpopulation of tumor cells. Although controversy remains about their lineage, phenotypic identification, and precise function [5], tumor stem cells are often defined *in vitro* as CD133-expressing cells with neurosphere-forming ability. However there is growing data to support that neurosphere formation, self-renewal and tumor formation is not exclusive to CD133 expressing cells. The expression of this marker whose physiological function remains unknown may also be dynamic and is likely dependent on culture conditions. It has also been suggested that the similarities between cancer stem cells and normal stem cells could be due to tumor initiation in the normal stem cell compartment, rather than dedifferentiation of more mature cells [9, 35–37]. These data stress the importance of identifying pathways that specifically target the stem cell-like cell populations.

The Notch pathway is a particularly attractive candidate due to its key role in promoting self-renewal in normal neural stem cells [15] as well as its involvement in angiogenesis. In fact Notch signaling has been invoked as a possible mediator of refractoriness to antiangiogenic agents that target the VEGF pathway [38]. The impact of Notch activity in glioblastoma is not completely defined. Some data suggest a role in proliferation [39, 40] and several Notch components are frequently upregulated in GBM transcriptomes [41]. More recent data suggest that Notch inhibition depletes CD133+ cells in glioblastoma and promotes increased responsiveness to radiation [42, 43]. Data in medulloblastoma cell lines [43] also suggested that Notch inhibition results in depletion of stem-like cells and a decrease in tumor forming capacity. In our experiments, Notch inhibition in GBM explants resulted in a decrease in the number of putative cancer stem cells and in self-renewal, compatible with literature reports. However, it also led to a decrease in endothelial cells suggesting a possible relationship between an intact endothelial compartment and the stem cell or self-renewing population. In fact the selective elimination of endothelial cells from the explants resulted in a state highly similar to pharmacological Notch inhibition, with downregulation of Hes5 and a decrease in neurosphere-forming ability and CD133 cells. These data suggest that the suppression of cancer stem cell function and number is at least in part mediated by endothelial cells and that the effect of Notch inhibition may be in part secondary to its antiangiogenic effect. They also highlight the advantages of analyzing tumor response to pathway manipulation within its original microenvironment. Current literature supports the coexistence of tumor stem cells within a vascular niche [6] that could provide a survival and functional advantage for tumor repopulation. Endothelial cells also play a critical role in the maintenance of self-renewal and the neurogenic potential of normal neural stem cells [16, 44]. Further studies are required to further characterize the role of Notch signaling in modulating the interaction of endothelium and tumor stem cells.

Additional experiments also highlighted differences in response to radiation between the explants and dissociated tumor cells. There was a more significant response to radiation by the neurosphere cultures in comparison with the explants. Radiation alone had a modest impact on self-renewal capacity in the explants and did not lead to a significant change in endothelial cells. The addition of Notch blockade potentiated the explants' response to radiation but had a minimal effect on the dissociated cells. This again highlights the role of the microenvironment, specifically endothelial cells in modulating the response to radiation. It could also reflect the existence of heterogeneous GBM cell subpopulations in the explants, such as neurosphere-forming cells that are either noncycling under homeostatic conditions or capable of bypassing radiation damage rapidly. This finding is compatible with clinical data whereby GBMs commonly exhibit a stable to a minor decrease in tumor volume followed by local recurrence after radiation. Resistant or previously non cycling stem-like cells may then re-enter the cell cycle and repopulate the tumor. An important caveat to this data interpretation is the possibility of selection of specific subpopulations under neurosphere culture conditions. In some of the experiments, there was a dissociated response between the number of CD133+ cells and neurosphere-forming ability. This may be in part due to the large variability of CD133 expression among tumors, but could also have implications as to the significance of CD133+ alone as a unique stem cell marker. Recent data suggest CD15 as a candidate marker for putative glioma stem cells [45], but that remains to be more extensively validated.

Notch inhibition can be achieved at various levels, including blocking ligand ubiquitination, altering receptor activation

or more directly interfering with downstream events. Monoclonal antibodies that target Notch ligands such as DLL4 or that inactivate Notch receptors have also been described. Gamma-secretase is a member of the I-CLIP protease family that has numerous transmembrane targets other than Notch receptors and what has been referred to as “promiscuous cleavage specificity” [46]. In addition to Notch and its ligands, it can also target Erb-B4, E- and N-cadherins, CD44, the low-density lipoprotein receptor and Nectin-1 [47]. Most of the available gamma-secretase inhibitors (GSI), including DAPT, have no preference for substrates, as is commonly observed for small molecule inhibitors/drugs. However, there are ongoing efforts to modify known inhibitors and allow greater affinity to specific target substrates. These efforts are particularly active in the Alzheimer field where there are reports of compounds (such as NSAIDs [48] or coumarindimer based molecules [49]) that can selectively target A β 42 (amyloid β precursor protein) thus alleviating the potential long-term toxicity of global γ -secretase inhibition. Nonetheless several nonspecific GSI molecules are currently in clinical trials for different cancers. Alternate and potentially more specific means of blocking the Notch pathway are under development, such as monoclonal antibodies that target the Notch receptors or ligands and targeted tumor delivery.

From a therapeutic perspective, by targeting the stem cell and the endothelial compartments, Notch inhibition addresses two prime targets: tumor repopulation and angiogenesis. Our data suggest that the effect of radiation is clearly enhanced when carried out in a background of Notch inhibition. In the absence of Notch signaling, tumor stem cells lose their self-renewal ability and likely transit into a progenitor-like state that is more vulnerable to radiation. Future studies will address the *in vivo* role of combined radiation and Notch inhibition.

CONCLUSION

We have presented a novel adaptation of the organotypic culture system to the study of glioblastomas. The three-dimen-

sional explant system preserves tumor cytoarchitecture and stroma including endothelial cells. This has allowed us to demonstrate that Notch inhibition also results in a decrease in the number of endothelial cells within the tumor, which likely disrupts the perivascular niche harboring tumor stem cells. In fact selective elimination of endothelial cells via an antibody-toxin conjugate resulted in a Notch-inhibition like state and a decrease in self-renewal, suggesting that endothelial cells play an important role in tumor stem cell maintenance that is at least partially mediated by Notch signaling. The explant system further highlighted differences in the response to radiation between explants and isolated tumor neurospheres. Combination treatment with Notch blockade and radiation resulted in a substantial decrease in proliferation and in self-renewal in tumor explants whereas radiation alone was less effective. This data suggests that the Notch pathway plays a critical role in linking angiogenesis and cancer stem cell self-renewal and is thus a potential therapeutic target. Three-dimensional explant systems provide a novel approach for the study of tumor and microenvironment interactions.

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DISCLOSURE OF POTENTIAL CONFLICT OF INTERESTS

The authors indicate no potential conflicts of interest.

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