Expression of Notch-1 and Its Ligands, Delta-Like-1 and Jagged-1, Is Critical for Glioma Cell Survival and Proliferation

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Abstract

The Notch family of proteins plays an integral role in determining cell fates, such as proliferation, differentiation, and apoptosis. We show that Notch-1 and its ligands, Delta-like-1 and Jagged-1, are overexpressed in many glioma cell lines and primary human gliomas. Immunohistochemistry of a primary human glioma tissue array shows the presence in the nucleus of the Notch-1 intracellular domain, indicating Notch-1 activation *in situ*. Down-regulation of Notch-1, Delta-like-1, or Jagged-1 by RNA interference induces apoptosis and inhibits proliferation in multiple glioma cell lines. In addition, pretreatment of glioma cells with Notch-1 or Delta-like-1 small interfering RNA significantly prolongs survival in a murine orthotopic brain tumor model. These results show, for the first time, the dependence of cancer cells on a single Notch ligand; they also suggest a potential Notch juxtacrine/autocrine loop in gliomas. Notch-1 and its ligands may present novel therapeutic targets in the treatment of glioma. (Cancer Res 2005; 65(6): 2353-63)

Introduction

Gliomas are the most common primary brain tumors and confer a grave prognosis. Standard therapies, such as chemotherapy, surgery, and radiation, have had limited success in treating patients with high-grade gliomas. It is hoped that a greater understanding of the molecular pathways involved in glioma cell proliferation and survival will lead to more effective targeted therapies.

The Notch family of receptors consists of heterodimeric transmembrane proteins intimately involved in the determination of cell fate. Depending on the cell type, Notch signaling can positively or negatively influence proliferation, differentiation, and apoptosis (1, 2). To date, four Notch receptors have been identified (Notch 1-4) in humans, with five corresponding ligands including Delta-like-1, Delta-like-3, Delta-like-4, Jagged-1, and Jagged-2. The Notch-1 protein consists of an extracellular domain with 36 epidermal growth factor–like repeats, a single transmembrane domain, and an intracellular domain containing a RBP-JK-associated molecule (RAM) region, ankyrin domains, and a proline-glutamate-serine-threonine-rich (PEST) region. Activation of Notch-1 signaling is thought to occur via juxtacrine binding of an adjacent cell's Delta-like or Jagged at Notch epidermal growth factor regions 11 and 12. After this binding, two enzymatic cleavages occur to liberate the Notch intracellular domain (NICD) from the plasma membrane. Similar processing also releases the Delta-like and Jagged intracellular domains in the adjacent cell. The NICD translocates into the nucleus and binds to members of the CSL transcription factor family, thought to mediate most of the downstream effects of Notch signaling. Following NICD binding, the CSL family member CBF-1/RBP-JK, normally part of a co-repressor complex with histone deacetylase 1, becomes a transcriptional activator. Downstream targets of CBF-1 include a large family of β helix loop helix (*bHLH*) transcription factors known as the hairy/enhancer of split (*HES*) genes. Whereas the activated intracellular domains of Delta-like and Jagged are also thought to perform signaling functions, these functions are as yet uncharacterized.

The importance of Notch signaling in regulating differentiation and cell survival suggests its potential for aberrant regulation in cancer cells. Notch signaling has transforming potential, as was shown by transfection of a constitutively active NICD into E1A-expressing rat kidney cells (3). Similarly, a t(7:9) translocation resulting in a constitutively active Notch fusion protein transforms normal lymphoid progenitor cells into an immature T-cell lymphoblastic leukemia (4). Additionally, Notch and Notch ligands have been shown to be present or up-regulated in several human malignant diseases (5, 6). The Notch pathway also has major interactions with other critical cancer pathways, such as ras (7, 8). A recent report indicates that Notch-1 activity is necessary to maintain a cancerous phenotype in ras-transformed human cells (9). However, the role of Notch in cancers seems to be complex depending on factors, such as tissue type. In contrast to its cancer promoting role in lymphoid cells, Notch-1 signaling has been found to have a tumor-suppressive effect on murine skin tumors and in non–small cell lung cancer (10, 11). These findings indicate a variable role for Notch signaling in cancer.

A growing body of evidence suggests that Delta-like and Jagged are themselves important in development and carcinogenesis, possibly independent of their role as Notch ligands. A mouse homozygous knockout for Delta-like-1 is embryonic lethal and displays abnormal somite formation (12). A mouse knockout for Jagged-1 is also embryonic lethal but with a different phenotype marked by vascular defects (13). Alagille's syndrome in humans, marked by cholestasis/jaundice, characteristic facies, and arterial defects, has been traced to a defect in Jagged-1 (14, 15). Delta-like and Jagged have been found to be up-regulated in cervical cancers (16). A Delta-like family member, Delta-like-4, has been shown to cause T-cell leukemia/lymphoma when overexpressed in bone marrow cells (17). Interestingly, Jagged-1 transforms rat kidney epithelial cells in a manner requiring a PDZ ligand region in its

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intracellular domain, indicating the transformative potential of Jagged-1 signaling (18). More recently, the Jagged-1 intracellular domain has been shown to up-regulate activator protein 1 (AP-1) activity (19), a signaling pathway known to be important in many cancers.

To date, the Notch pathway has not been associated with gliomas. Preliminary work in our laboratory from phage display biopanning on human glioma cells resulted in the isolation of two peptides that share significant homology to regions of Jagged-1 and Delta-like-1, two Notch receptor ligands. These findings suggested the presence of Notch on human glioma cells, which was further supported by cDNA microarray data. This prompted us to confirm the expression of Notch-1 in glioma cell lines and to assess its biological relevance. In addition, we noted Notch activity in glioma cell cultures, suggesting the presence of Notch ligands in these cells and a possible juxtacrine/autocrine loop. In this work, we show the expression of Notch-1 and its ligands Delta-like-1 and Jagged-1 in both human glioma cell lines and primary human gliomas. Additionally, we show through knockdown of these targets that they play an important role in glioma cell proliferation and survival. These data suggest that Notch-1 and its ligands represent new and potentially promising targets in glioma therapy and show for the first time the importance of individual Notch ligands in cancer cell survival and proliferation.

Materials and Methods

Random Peptide Phage Display Biopanning. Biopanning experiments on the U87MG human glioma line were done with the Ph.D.-12 Phage Display Peptide Library kit from New England Biolabs (Beverly, MA). We used a modified version of the manufacturer's protocol. Confluent U87MG cells in a 10 cm dish were washed with PBS and fixed for 10 minutes with methanol at room temperature. Cells were blocked with PBS/2% nonfat dry milk for 1 hour at room temperature, then 2×10^{11} library phage in blocking buffer were applied to the cells for 1 hour at room temperature with gentle rocking. Nonadherent phage was aspirated and 10 washes done with PBS/0.05% Tween 20. Increasingly acidic solutions of citric acid 76 mmol/L (pH-adjusted with HCl to yield solutions from pH 4.4 down to pH 2.4 in increments of 0.2) were then applied for 10-minute intervals with gentle rocking. The phage eluted in the pH 2.4 solution were amplified according to the Ph.D.-12 kit protocol in ER2738 bacteria. Amplified phage were used in a second round of biopanning on U87MG as above. Phage clones from the resultant second round eluate were amplified and their DNA harvested by standard phenol extraction. For each, the DNA encoding the random peptide was sequenced (primer 5′-GCAAGCCTCAGCGACCGAATA-3′) to determine the corresponding peptide sequence.

Cell Culture. Glioma cell lines U87MG, U251MG, T98G, U373MG, U387, and A172 were all acquired from American Type Culture Collection. Two hundred ninety-three cells were acquired from American Type Culture Collection. U251MG human glioma cells were cultured in RPMI 1640 (Mediatech Inc., Herndon, VA) supplemented with 5% fetal bovine serum and 1% penicillin-streptomycin. T98G and U87MG human glioma cells were cultured in MEM (Mediatech) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. U373MG, U387, and A172 human glioma cells were cultured in DMEM (Mediatech) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. All cell lines were incubated at 37°C in an atmosphere of 5% CO_2.

Real-Time PCR. Gene expression levels in all tissue samples were measured by real-time PCR in a two-step procedure (reverse transcription being a separate step from the PCR) using ABI AmpliTaq Polymerase and fluorescent resonant energy transfer technology, on an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Taqman minor groove binder probes were used to decrease background from genomic DNA. Target gene expression levels in each tissue sample were subsequently normalized by the mRNA level of 18S rRNA or β-actin mRNA in the same mRNA sample. Relative gene expression ratios of each tissue sample were then compared with those of a common Universal Human Reference RNA (Stratagene, La Jolla, CA). All samples were measured in triplicate. Primers and probes were as follows:

- Notch-1 Forward primer 5′-GCAGTTGTGCTCCTGAAGAA-3′; Reverse primer 5′-CGGGCGGCCAGAAAC-3′ Probe FAM 5′-TCCTCCCTGTTGTTCTGC-3′ (reverse strand) NFQ (nonfluorescent quencher)
- Delta-like-1 Forward primer 5′-TGCAACCCTGGCTGGAAA-3′; reverse primer 5′-AATCCATGCTGCTCATCACATC-3′ Probe FAM 5′-ACTGCACAGAGC CGATCTGCCTGCCT-3′-NFQ
- Jagged-1 Forward primer 5′-GGAGGCGTGGGATTCCA-3′; reverse primer 5′-CCGAGTGAGAAGCCTTTCAATAAT-3′ Probe FAM 5′-GTAATGACACCGTTCAACCTGACAG-3′ -NFQ

Western Blotting. Whole cell lysates were prepared in 250 μL PBS containing 1% SDS, 2% NP40, 1 mmol/L sodium orthovanadate, and a protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Indianapolis, IN). Cell lysate samples were run on a precast 4% to 12% Bis-Tris gel (Invitrogen, Carlsbad, CA), transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia, Piscataway, NJ), and probed with Notch-1 antibody (1:100, Notch-1 Clone A6, Lab Vision NeoMarkers, Fremont, CA), Delta-like-1 antibody (1:200, sc-9102, Santa Cruz Biotechnology, Santa Cruz, CA), Jagged-1 antibody (1:200, sc-8303, Santa Cruz Biotechnology), or β-actin antibody (1:200, sc-1615, Santa Cruz Biotechnology). Horseradish peroxidase–conjugated secondary antibodies to rabbit immunoglobulin G and goat immunoglobulin G were used (1:5,000 and 1:10,000, respectively, Jackson Immunology Labs, Bar Harbor, ME). All antibodies were diluted in blocking buffer (5% w/v nonfat dry milk, 10 mol/L Tris-HCl, 100 mmol/L NaCl, 0.1% v/v Tween 20). All other buffers used during Western blotting were made following Invitrogen's NuPage protocol.

Reagents. Small interfering RNA (siRNA) duplexes were synthesized by Xeragon/Qiagen (Valencia, CA). Oligofectamine (Invitrogen) was used for transfection of siRNA into cells per manufacturer's instructions. Lipofectamine and Plus reagent (Invitrogen) were used for transfection of plasmids into cells per manufacturer's instructions. siRNA sequences were as follows:

- Notch-1: r(UGGCGGGAAGUGUGAAGCG)d(TT), r(CGCUUCACACUUCCCGCCA)d(TT)
- Delta-like-1: r(GCCGACAAGAAUGGCUUCAUU), r(UGAAGCCAUUCUUGUCGGCGC)
- JAG-1: r(CGCCAAAUCCUGUAAGAAU)d(TT), r(AUUCUUACAGGAUUUGGCG)d(TT)
- control (from Xeragon/Qiagen): r(UUCUCCGAACGUGUCACGU)d(TT), r(ACGUGACACGUUCGGAGAA)d(TT)

Cell Transfection. The effects of Notch-1 knockdown by siRNA were examined in U251MG, U87MG, T98G, U373MG, U387, and A172 cell lines. Cells were plated in six-well tissue culture plates at a density of 4.5×10^4 per well and transfected 24 hours later. Cells were transfected with Oligofectamine (Invitrogen) according to the manufacturer's protocol, with a concentration of 10 nmol/L siRNA. A second transfection was done 24 hours after the first. siRNA dosage was lowered to 10 nmol/L from the recommended concentration of 200 nmol/L because cell proliferation assays showed nonspecific toxicity from control siRNA at 200 nmol/L (data not shown).

Luciferase Assay. To measure downstream Notch activity, a U251MG cell line stably expressing a CBF1/luciferase reporter plasmid construct was generated (clone 17). The JH23A plasmid used in this stable cell line encodes luciferase under the control of four copies of a CBF1 promoter, and was a kind gift of S.D. Hayward (Department of Pharmacology and Molecular Sciences, Johns Hopkins School of Medicine, Baltimore, MD; ref. 20). To generate the stable cell line, the JH232A plasmid was co-transfected into U251MG MG cells with a plasmid encoding neomycin resistance and cells

then selected with the G418 antibiotic (Sigma-Aldrich Co., St. Louis, MO). The resultant stable clone was used in several assays with siRNA transfection. U251MG/CBF1-luciferase cells were plated at a density of 4.5 $\times 10^4$ per well in a six-well plate, doubly transfected as described above, and luciferase activity (luminescence) measured 2 to 3 days following the first transfection. Luminescence was assessed with the luciferase assay system (Promega Corp., Madison, WI) per manufacturer's protocol on an EG&G Berthold Lumat LB9507 (Oak Ridge, TN). To normalize luciferase activity, absolute luminescence for each sample was divided by the protein concentration for that sample. Protein concentration was determined by the DC Protein Assay kit II (Bio-Rad Life Science, Hercules, CA) per manufacturer's instructions.

Reverse Transcription-PCR. Cells were grown to confluence or near-confluence in six-well plates before lysis. Cell lysis and RNA purification were done with the RNEasy kit (Qiagen) per manufacturer's instructions. The reverse-transcriptase reaction was done with the Superscript II kit (Invitrogen) per manufacturer's instructions and PCR done using standard techniques. Primers for Notch-1 and β-actin were as follows:

- Notch-1 5′-GCCGCCTTTGTGCTTCTGTTC-3′; 5′-CCGGTGGT}-CTGTCTGGTCGTC-3′
- β-actin 5′-CGAGCGGGAAATCGTGCGTGACATTAAGGAGA-3′; 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′

Cell Proliferation Assay (alamarBlue). One-tenth volume of alamarBlue working solution (BioSource, Worcester, MA) was added to each well and the plate was incubated at 37°C until wells began to acquire a pink color. Fluorescence intensity was measured with excitation at 550 nm and emission at 590 nm using a Wallac Victor 1420 Multilabel Counter (Perkin-Elmer, Norwalk, CT). After measurement, the alamarBlue solution was aspirated and fresh media was added to each well. The alamarBlue assay is nontoxic and thus was done on the same cells on sequential days, not exceeding 3 days total.

Cell Proliferation Assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Cells were plated in a 96-well plate at 3,000 cells per well. To assay proliferation at the end of an experiment, one-tenth volume of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent (Cell Proliferation kit, Roche) was added to each well and the plate incubated at 37°C. After 4 hours, 100 μL of solubilization buffer (Cell Proliferation kit, Roche) was added to each well and the absorbance at 590 nm measured using a SpectraCount plate reader (Packard Bioscience, Co., Meriden, CT).

Flow Cytometry and Cell Cycle Analysis. U251MG cells were plated in 15 cm dishes at a density of 7.5×10^5 per dish and doubly transfected with Notch-1 siRNA as described above, using reagent concentrations proportional to the larger dish. Cells were harvested with minimal amounts of trypsin 48 or 72 hours following the first transfection and then washed twice in ice-cold PBS and counted. They were resuspended in 1 mL ice-cold PBS and 7 mL of ice-cold 80% ethanol added dropwise while gently vortexing. Cells were then fixed for 30 minutes on ice. After centrifuging, an equal number of cells per group were resuspended in 1 mL 2 N HCl/0.5% Triton X-100 while gently vortexing. Following incubation at room temperature for 30 minutes, cells were centrifuged and resuspended in 1 mL of 0.1 mol/L $Na_2B_4O_7 \cdot 10\ H_2O$ (pH 8.5). Cells were centrifuged and suspended in 250 μL 0.5% Tween 20/1% bovine serum albumin/PBS. Twenty microliters anti–bromodeoxyuridine (BrdUrd)-FITC were added per 1×10^6 cells and the cells incubated at room temperature for 30 minutes. They were then washed once in Tween/bovine serum albumin/PBS and resuspended in 1 mL of 5 μg/mL propidium iodide/PBS. FITC fluorescence was then read at excitation 495 nm, emission 519 nm, and propidium iodide fluorescence at 575 nm using a FACSVantage SE (Becton Dickinson, San Diego, CA).

Tissue Array. A tissue microarray containing cores from 80 gliomas was constructed at the Johns Hopkins Pathology Department Tissue Microarray Facility using standard techniques. Four cores, 0.6 mm diameter, were taken from each tumor. The array included 60 astrocytic tumors (20 each of WHO grades II, III, and IV) and 20 predominantly oligodendroglial tumors (WHO grades II and III). The tissue array was reviewed by two neuropathologists (C.G. Eberhart and I. Mikolaenko) to ensure that each core contained representative tumor. Immunohistochemical stains were done as previously described, using polyclonal antibodies raised against Notch-1, Delta-like-1, or Jagged-1 at a 1:200 dilution (Santa Cruz Biotechnology, C-20, H-265, and H-114, respectively). Nuclear staining (for Notch-1) or cytoplasmic staining (for Delta-like-1 and Jagged-1) was graded semiquantitatively by C.G. Eberhart and I. Mikolaenko.

Notch Intracellular Domain Retrovirus Rescue from Notch-1 siRNA. The pCLEN plasmid encoding the Notch-1 intracellular domain has been described previously and was a kind gift of G. Fishell (Department of Cell Biology, New York University School of Medicine, New York, NY; ref. 21). It was transfected with LipofectAMINE 2000 (Invitrogen) per manufacturer's instructions into a vesicular stomatitis virus G–expressing 293 producer cell line to generate NICD-encoding retrovirus. Retroviral supernatants were collected at days 2, 3, and 4 following transfection and pooled. Supernatants were frozen at −80°C until the day of usage. Control GFP-expressing retrovirus was derived similarly. Retroviral supernatant was added to glioma cells at an approximate multiplicity of infection of 10 on the day before siRNA transfection as described above.

Notch Inhibition with Notch Antisense and the Delta-Like-1/Fc Protein. Notch antisense and control plasmids under tetracycline control were a kind gift of L. Miele (Department of Biopharmaceutical Sciences and Cancer Center, University of Illinois at Chicago, Chicago, IL; ref. 9) They were transfected into U251MG cells with LipofectAMINE and Plus reagents (Invitrogen) per manufacturer's instructions. Antibiotic selection was used to derive a mixed population of stable transfectants. Proliferation of the Notch antisense–expressing and control construct–expressing cells was assessed with alamarBlue assay at 5 days after plating 45,000 cells/well in a six-well plate in the presence of tetracycline 2 μg/mL (Sigma). Two hundred ninety-three cells secreting a Delta-like-1/Fc fusion protein were a kind gift of G. Weinmaster (Department of Biological Chemistry and Molecular Biology Institute, UCLA School of Medicine, Los Angeles, CA). Concentrated Delta-like-1/Fc medium was prepared from these cells as previously described in parallel with medium from control 293 cells (22). Glioma cells were plated in 96-well plates at 3,000 cells/well and the concentrated media added the next day at a dilution of 1:50. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was done per manufacturer's instructions at 3 days after addition of concentrated media.

***In vivo* Murine Brain Tumor Model.** Before injection into mice, U251MG cells were transfected on 2 successive days as described above with Notch-1, Delta-like-1, Jagged-1, or control siRNA. They were harvested the next morning for injection. Before harvesting the cells, cells around the rim were aspirated and discarded because our experience suggests that these cells are not efficiently transfected by oligofectamine. Female nude mice (6-8 weeks) were anesthetized before being placed in a stereotactic apparatus (Stoelting Co., Wood Dale, IL) and the brain surface exposed. One hundred thousand transfected cells were stereotactically injected per mouse at a site corresponding to the caudate/putamen. Eight mice were used in each group. Mice were euthanized when they became moribund and the date recorded.

Statistical Analysis. Student's t test was done for most data with Microsoft Excel:mac 2001 (Microsoft Corp., Redmond, WA). For real-time PCR data, Student's t test (with Welch's correction for data sets with unequal variances) was done using Prism 4 (GraphPad Software, Inc., San Diego, CA). Log-rank analysis of Kaplan-Meier survival data was also done with Prism 4.

Results

Presence of Notch-1 in Human Glioma Cell Lines and Primary Human Gliomas. In an attempt to identify novel membrane targets on glioma cells, we did random peptide phage display biopanning on the U87MG glioma cell line. After two

rounds of biopanning, sequencing of the random peptide regions in phage clone genomes revealed several enriched sequences. Two of these peptides shared significant homology to regions of two Notch ligands, Jagged-1 and Delta-like-1, suggesting the presence of Notch on glioma cells (Fig. 1*A*). The homologous regions in Jagged-1 and Delta-like-1 were not in the Delta, Serrate, Lag-2 regions known to bind Notch, but these results suggest that other regions of these ligands may interact with Notch.

We sought to confirm the expression of Notch-1 in glioma cell lines. Notch-1 mRNA and protein were shown in all six glioma cell lines evaluated by real-time PCR and Western blot (Fig. 1*B* and *C*). In addition, we assessed Notch-1 mRNA expression in a series of primary human gliomas by quantitative real-time PCR. All 51 primary human gliomas evaluated showed Notch-1 mRNA expression, with means for each subtype significantly higher than non-neoplastic brain. Greater expression was noted in oligodendrogliomas and grade II-III astrocytomas than in glioblastomas (Fig. 1*D*). Notch-1 protein expression was then evaluated by immunohistochemistry in a tissue array containing a different set of 74 primary human gliomas (a total of 125 different gliomas were evaluated for either mRNA or protein expression). The antibody used specifically recognizes a region located within the NICD, thereby allowing one to visualize nuclear NICD, a surrogate marker for Notch-1 activation (23). Both cytoplasmic and nuclear Notch-1 staining was detected in almost all of the gliomas on the tissue array (Fig. 1*E*). Based on semiquantitative scoring of nuclear Notch-1 staining, the majority of grade II and III gliomas had moderate to high Notch-1 levels in the nucleus, whereas the signal in grade IV (glioblastoma) tumors was weak or undetectable. Cytoplasmic levels of Notch-1 generally seemed low and did not vary significantly between tumor grades. We also examined Notch-1 expression in non-neoplastic cerebral cortex, including surgically resected tissue adjacent to infiltrating gliomas and autopsy material (data not shown). In these non-neoplastic samples, Notch-1 immunoreactivity was relatively weak and almost entirely cytoplasmic, suggesting negligible Notch-1 activity in normal cortical tissue.

Presence of Notch Ligands in Glioma Cell Lines and Primary Human Gliomas. We next evaluated glioma cell lines and primary tumors for the presence of the most prevalent membrane-bound ligands for Notch, given the finding of Notch-1 activation in gliomas. Analysis of six human glioma cell lines showed Delta-like-1 and Jagged-1 mRNA expression by quantitative real-time PCR (Fig. 2*A*). The presence of Delta-like-1 protein and Jagged-1 protein in these six glioma lines was confirmed by Western blot (Fig. 2*B*). Similar to our findings in glioma cell lines, quantitative real-time PCR showed the expression of Delta-like-1 and Jagged-1 mRNA in all primary glioma samples evaluated (n = 58 and 54, respectively; Fig. 2*C*). Delta-like-1 mRNA expression was significantly higher in oligodendrogliomas than in normal brain ($P < 0.0001$), whereas grade II and III astrocytomas trended toward higher Jagged-1

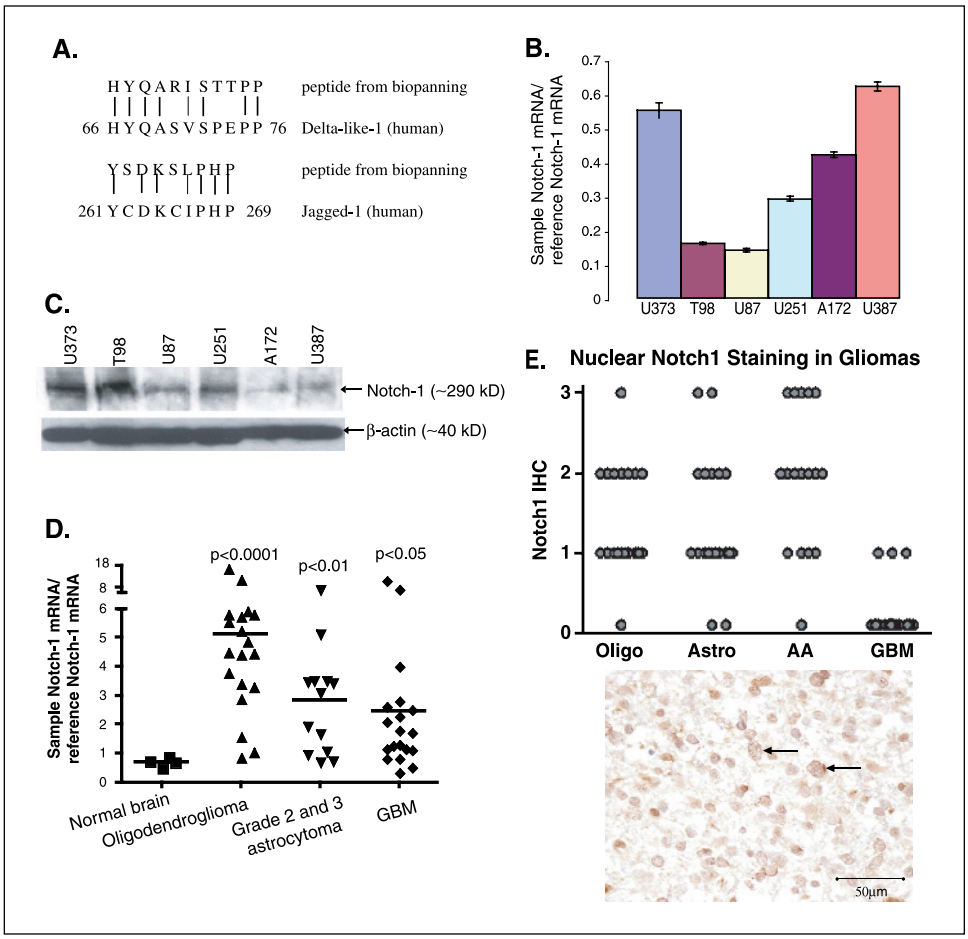

Figure 1. Presence of Notch-1 in human glioma cell lines and in primary human gliomas. *A*, phage display biopanning derived random peptides with homologies to Delta-like-1 and Jagged-1. The sequences of two peptides enriched after two rounds of biopanning against U87MG cells are shown compared with homologous regions in Delta-like-1 and Jagged-1. *Bold lines,* identical amino acids; *faint lines,* homologous amino acids. *B*, real-time PCR with Notch-1 probes of mRNA from six human glioma cell lines versus normal brain tissue. Results are normalized relative to the amount of β-actin mRNA and are plotted by the amount relative to reference sample. Samples were run in triplicate. *Columns,* mean. *C*, Western blot analysis of lysates from six human glioma cell lines showing Notch-1 bands present for each (*top*); corresponding β-actin bands (*bottom*). *D*, real-time PCR with Notch-1 probes of mRNA from 51 primary human gliomas versus normal brain tissue. Results are normalized relative to the amount of 18S mRNA and are plotted by the amount relative to reference sample. *Horizontal bar,* mean for each set. *P* value is for comparison to normal brain. *E*, plot of blinded scored values quantitating the degree of nuclear Notch-1 staining for tissue array samples of 74 gliomas different from those used for mRNA evaluation [glioblastoma (*GBM*), anaplastic astrocytoma (*AA*), low-grade astrocytoma (*astro*), and oligodendroglioma (*oligo*)]. Also shown is an example of Notch-1 nuclear staining in an anaplastic astrocytoma on the tissue array.

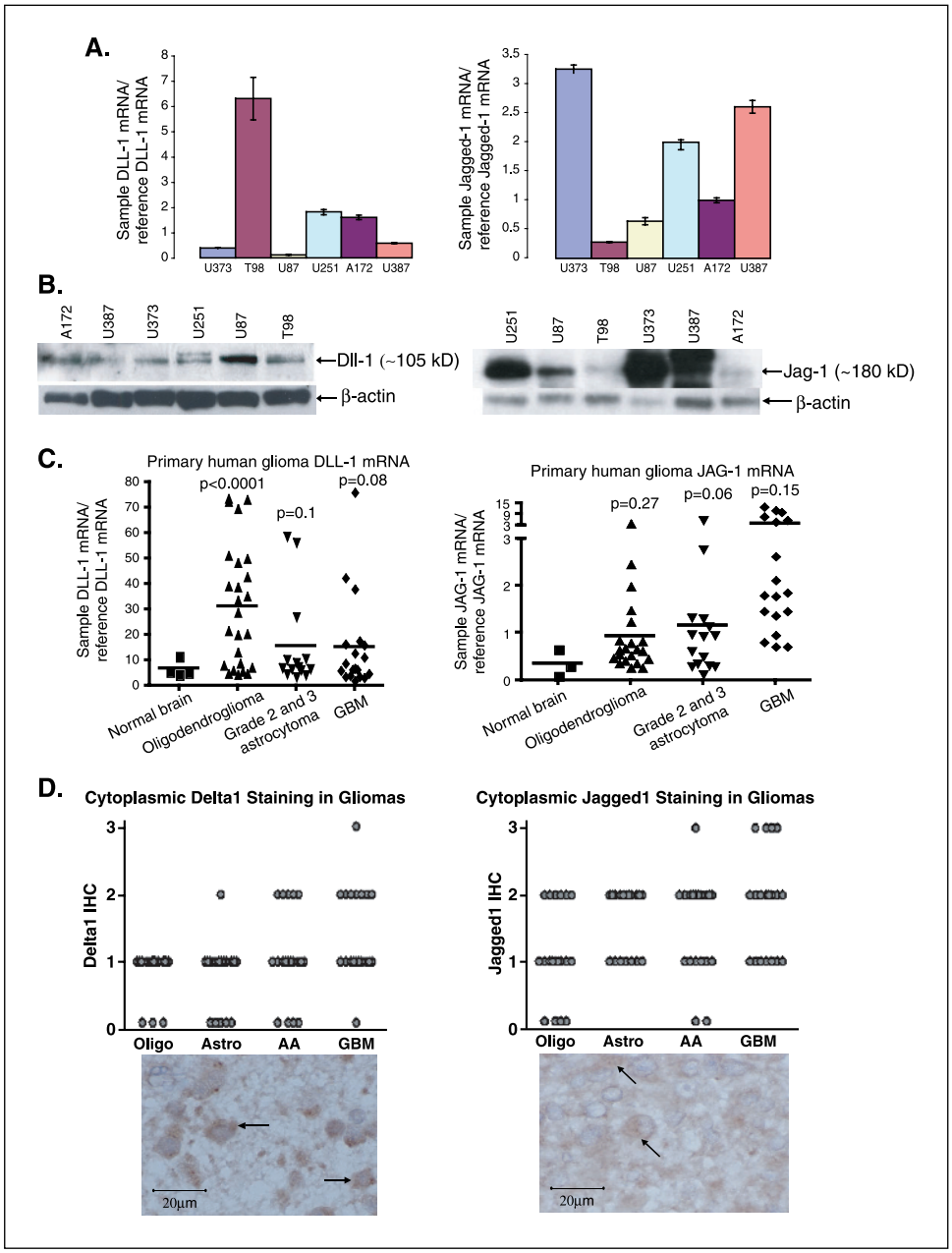

Figure 2. Presence of Notch-1 ligands in primary human gliomas. *A*, real-time PCR with Delta-like-1 and Jagged-1 probes of mRNA from six human glioma cell lines versus normal brain tissue. Results are normalized relative to the amount of β-actin mRNA and are plotted by the amount relative to reference sample. Samples were run in triplicate. *Columns,* mean. *B*, Western blot analysis of lysates from six human glioma cell lines showing Delta-like-1 and Jagged-1 bands present for each (*top*); corresponding β-actin bands (*bottom*). *C*, real-time PCR with Delta-like-1 and Jagged-1 probes of mRNA from primary human glioma samples versus normal brain samples. Results are normalized relative to amount of 18S rRNA and are plotted by amount relative to reference sample. *Horizontal bar,* mean for each set. *D*, immunohistochemical staining of human glioma tissue arrays. Plots of scored values quantitating degrees of cytoplasmic Delta-like-1 and Jagged-1 staining from tissue array samples of glioblastoma (*GBM*), anaplastic astrocytoma (*AA*), low-grade astrocytoma (*astro*), and oligodendroglioma (*oligo*). *Pictures,* representative Delta-like-1 and Jagged-1 staining of an anaplastic astrocytoma and a glioblastoma, respectively, at ×600 magnification.

expression than normal brain (P = 0.06). Although there were several glioblastomas with markedly increased levels of Jagged-1 expression, overall the mean level of expression in glioblastoma was not statistically different than that of normal brain due to highly variable expression between different tumors. Immunohistochemical analyses of a human glioma tissue array confirmed the presence of Delta-like-1 and Jagged-1 protein expression in 83% and 91% of the primary human gliomas, respectively (Fig. 2*D*). Some variation was observed between the relative quantities of mRNA indicated by quantitative PCR and the relative levels of their encoded proteins as determined by immunohistochemistry, possibly due to differences in mRNA processing, in enzymatic cleavage of the protein, or in the rate of receptor degradation due to ligand binding or other causes. Additionally, there could also be differences in translation efficiencies of these mRNAs between different gliomas given that glioblastomas have been shown to differentially recruit mRNAs to ribosomes versus polysomes secondary to Ras and Akt dysregulation (24).

Evaluating siRNA Knockdown of Notch-1, Delta-Like-1, and Jagged-1. To evaluate the functional importance of Notch-1 and its ligands in glioma cells, siRNAs targeting Notch-1, Delta-like-1, and Jagged-1 were used. The efficacy of each siRNA for knockdown of target mRNA and protein was confirmed through quantitative real-time PCR and Western blot analysis (Fig. 3*A* and *B*). For Delta-like-1 and Jagged-1, four siRNAs for each target were assessed and the most effective one used in subsequent experiments (data not shown). To determine the effects of these siRNAs on downstream Notch activity, we constructed a stable glioma cell line expressing luciferase regulated by a CBF1 promoter (U251MG/

CBF1-luciferase). A U251MG/CBF1-luciferase reporter cell line showed high basal levels of CBF1 activity, consistent with constitutive Notch activity in these glioma cells (Fig. 3*C*). After transfection with Notch-1 siRNA, CBF1 activity decreased over 90% ($P < 0.01$; Fig. 3*C*). This reporter cell line was also used with Delta-like-1 and Jagged-1 siRNAs to determine their relative contributions to downstream Notch activity. Transfection with Delta-like-1 siRNA yielded a roughly 50% decrease in CBF1 reporter activity ($P < 0.01$), whereas Jagged-1 siRNA transfection caused a more modest 25% drop ($P < 0.01$; Fig. 3*C*). This could reflect greater relative efficiency of the Delta-like-1 siRNA than the Jagged-1 siRNA but may also indicate a greater role for Delta-like-1 than Jagged-1 as a Notch ligand in this cell line. To confirm that Delta-like-1 and Jagged-1 siRNAs did not lower downstream Notch-1 activity by inhibiting Notch-1 transcription, semiquantitative reverse transcription-PCR was done with Notch primers on mRNA from glioma cells transfected with the most efficient Delta-like-1 or Jagged-1 siRNA or control siRNA (Fig. 3*D*). Notch-1 mRNA was found to be unaffected by Delta-like-1 or Jagged-1 knockdown.

Significance of Notch-1 in Glioma Cell Survival and Proliferation. We evaluated the effects of the Notch-1 siRNA on various human glioma cell lines. Two to three days following siRNA transfection, most glioma cells showed dramatic morphologic changes, characterized by cell rounding and extended processes (Fig. 4*A*). This was followed within 1 to 2 days by significant glioma cell cytotoxicity as evidenced by increased numbers of floating cells and by diminished numbers of viable cells in all six glioma cell lines evaluated (Fig. 4*B*). To explore the manner of cell death and decreased proliferation, cell cycle analysis was done by double-staining for BrdUrd DNA incorporation and propidium iodide uptake (Fig. 4*C*). A large decrease in dividing cells (gate R_4, 45-17.4%), as well as a significant increase in apoptotic cells (gates $R_1 + R_2$, 4.2-17.8%) was observed. Furthermore, a significant G_2-M block was observed in the surviving cells (gate R_5, 3.6-9.6%). Induction of

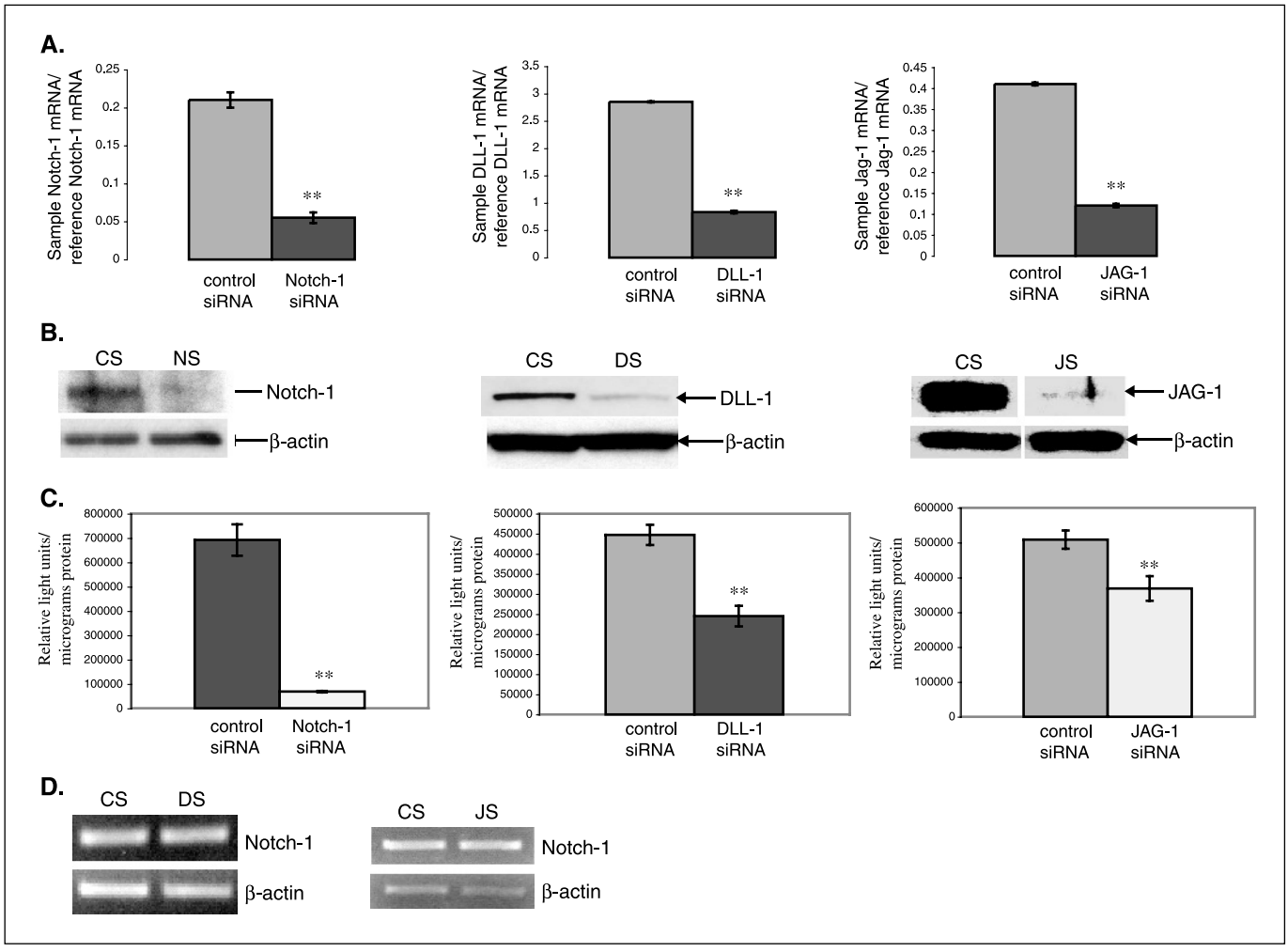

Figure 3. Efficacy of Notch-1, Delta-like-1, and Jagged-1 siRNAs. *A*, real-time PCR with indicated probes of mRNA from cells transfected with control siRNA or Notch-1, Delta-like-1, or Jagged-1 siRNA. mRNA was harvested at 72 hours after the first of two daily siRNA transfections. Transfected cells were U87MG for Notch-1 and Jagged-1 assays and T98G for Delta-like-1 assay. Results are normalized relative to the amount of β-actin mRNA and are plotted by the amount relative to reference sample. *B*, Western blot of protein lysates from U251MG cells at day 3 after the first of two transfections with Notch-1, Delta-like-1, Jagged-1, or control siRNA. Blots were stained with primary antibodies as indicated (*top*): *CS,* control siRNA transfected; *NS,* Notch-1 siRNA transfected; *DS,* Delta-like-1 siRNA transfected; *JS,* Jagged-1 siRNA transfected. Western blot with β-actin (*bottom*) shows equivalent loading. *C*, luciferase activity of U251MG/CBF1-luciferase cell line at day 3 after first of two transfections with Notch-1, Delta-like-1, or Jagged-1 siRNA compared with that with control siRNA. Luciferase activity of each lysate sample is normalized for protein concentration. $^{**}P < 0.01$ relative to control siRNA samples. *D*, semiquantitative reverse transcription-PCR with Notch-1 primers of mRNA from U251MG cells harvested 2 days after the first of two transfections with an efficient Delta-like-1 or Jagged-1 siRNA versus control siRNA. *Top,* PCR with Notch-1 primers; *bottom,* PCR with β-actin primers to indicate equivalent loading/amplification.

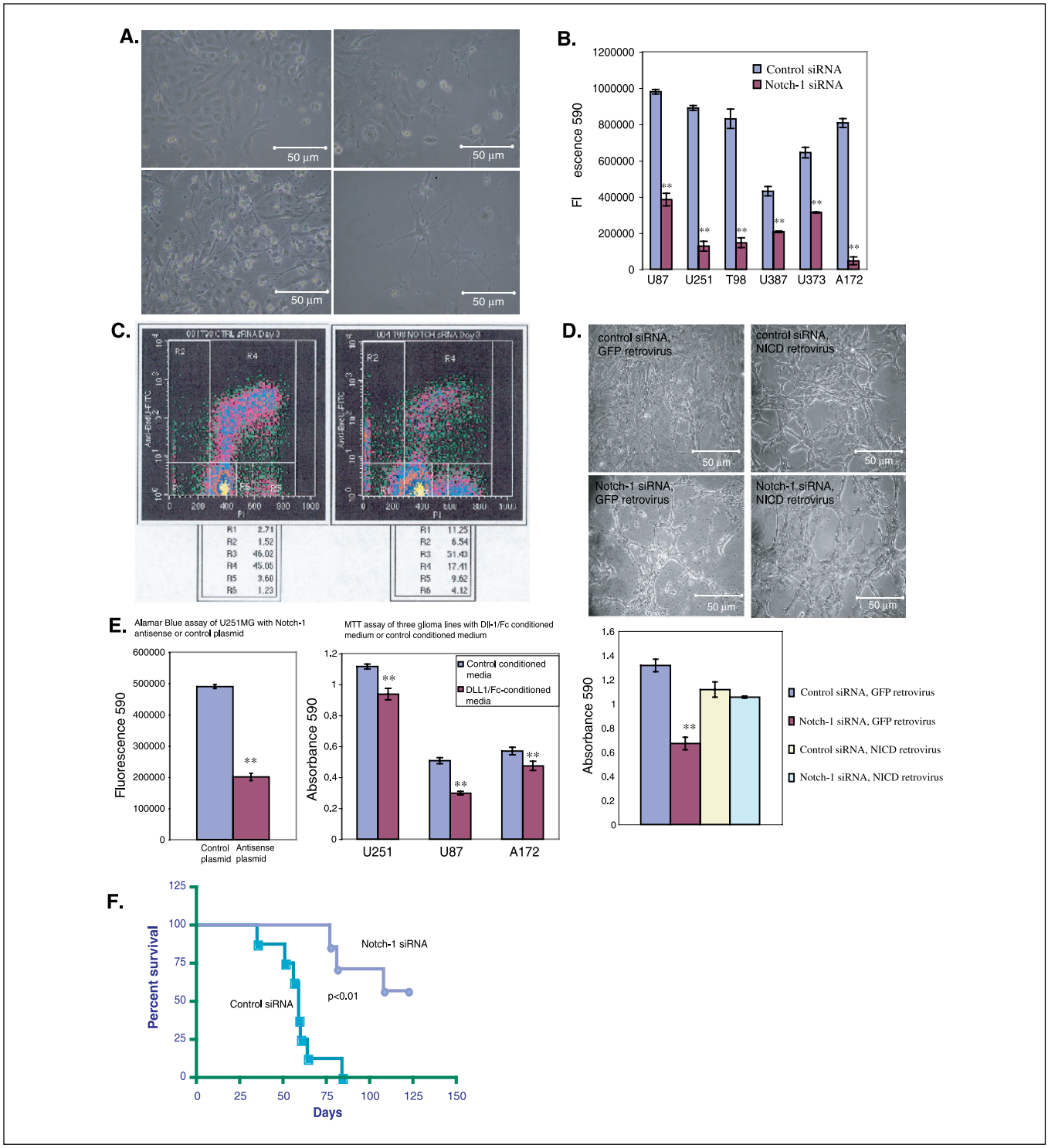

Figure 4. Effects of Notch-1 knockdown on glioma cell morphology and survival. *A*, light microscopic photographs of U251MG and T98G human glioma cells at 3 days after the first of two transfections with either Notch-1 siRNA or control siRNA. Note the long processes extending from the Notch-1 siRNA-transfected cells. *B*, alamarBlue assay of six human glioma cell lines after two daily transfections with Notch-1 siRNA or control siRNA. The assay was done at day 4, 5, or 6 due to different rates of proliferation of the cells. Wells were done in triplicate. *Columns,* mean. $^{**}P < 0.01$ relative to control siRNA samples. *C*, BrdUrd/propidium iodide cell cycle analysis of T98G cells at 72 hours after the first of two daily transfections with Notch-1 or control siRNA. *Y axis,* anti-BrdUrd staining, reflecting BrdUrd incorporation; *X axis,* propidium iodide uptake. Twenty thousand events are plotted. *D,* rescue of Notch-1 siRNA-transfected cells following transduction by a NICD-expressing retrovirus. Cells were transfected with either NICD retrovirus or control GFP retrovirus, then transfected with Notch-1 or control siRNA on 2 subsequent days. *Pictures,* morphology of cells by light microscopy 5 days after the first transfection; *chart,* data from a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay done the same day. $^{**}P < 0.01$ relative to control siRNA samples. *E*, alamarBlue proliferation assay of U251MG stably transfected with Tet-on or control plasmid in the presence of tetracycline. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay of three different glioma cell lines following a 3-day exposure to either control conditioned media or conditioned media with soluble Delta-like-1/Fc. $^{**}P < 0.01$ relative to control plasmid or control conditioned media samples. *F*, Kaplan-Meier survival curve of mice harboring intracranial U251MG gliomas transfected *ex vivo* with either Notch-1 siRNA or control siRNA ($P < 0.01$, log-rank analysis).

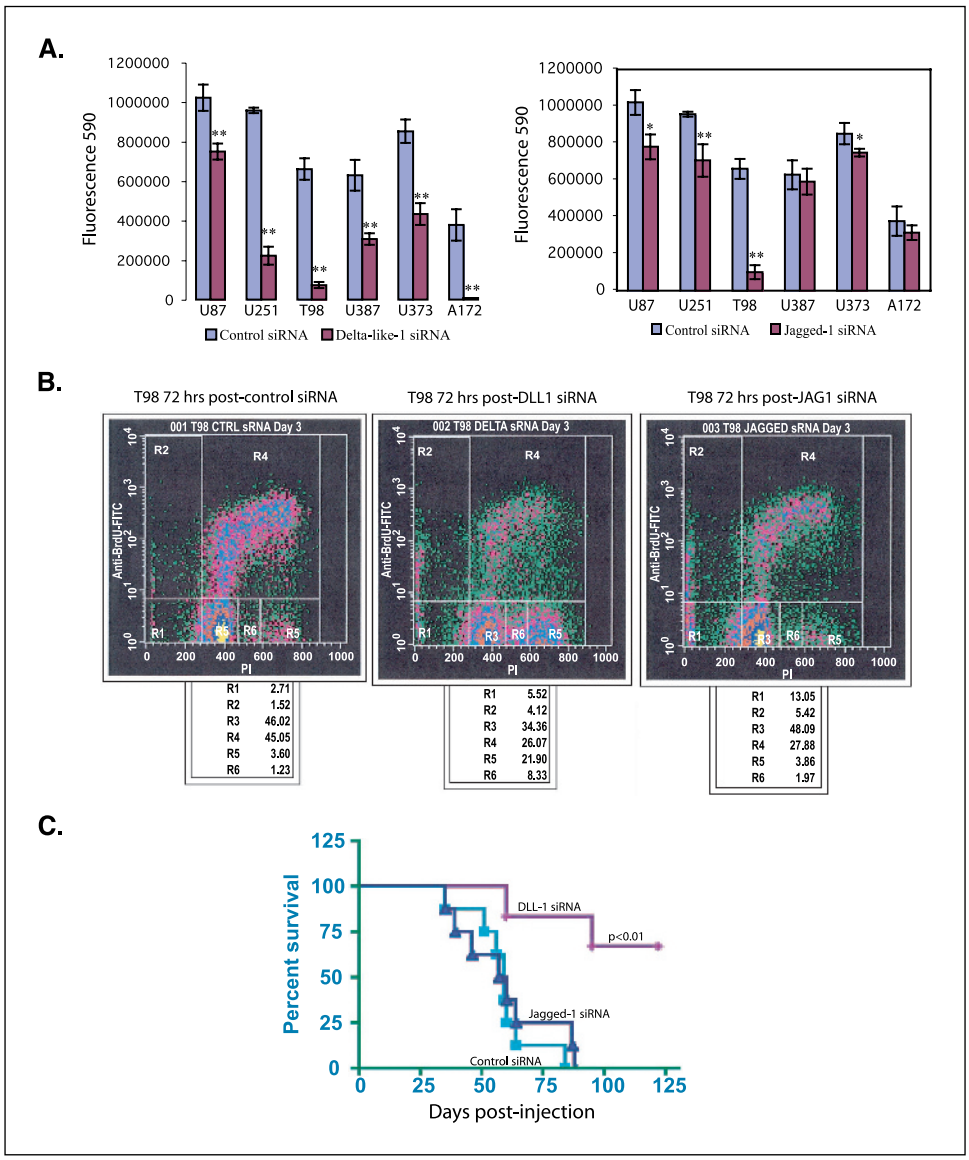

Figure 5. Effects of Delta-like-1 and Jagged-1 knockdown on glioma cell morphology and survival. *A*, alamarBlue assay of six human glioma cell lines after two daily transfections with Delta-like-1 or Jagged-1 compared with control siRNA. The assay was done at day 4, 5, or 6 due to different rates of proliferation of the cells. $^{*}P < 0.05$ relative to control siRNA samples; $^{**}P < 0.01$ relative to control siRNA samples. *B*, BrdUrd/propidium iodide cell cycle analysis of T98G cells at 72 hours after the first of two daily transfections with control, Delta-like-1, or Jagged-1 siRNA. *Y axis,* anti-BrdUrd staining, reflecting BrdUrd incorporation; *X axis,* propidium iodide uptake. Twenty thousand events are plotted. *C*, Kaplan-Meier survival curve of mice harboring intracranial U251MG gliomas transfected *ex vivo* with either Delta-like-1, Jagged-1, or control siRNA ($P < 0.01$, log-rank analysis).

apoptosis was further supported by Hoechst 33342/propidium iodide assay at 72 and 96 hours (Vybrant Apoptosis Assay kit #5, Molecular Probes, Inc., Eugene, OR; data not shown). These data suggest an important role for Notch-1 in glioma cell proliferation and survival.

The use of siRNA for mRNA knockdown has been accompanied by concerns for variable specificity and off-target effects (25). To show that the effects of the Notch-1 siRNA on glioma cell proliferation and survival were specifically due to Notch-1 inhibition, we did Notch-1 rescue experiments. Before transfection with Notch-1 siRNA, glioma cells were transduced by a retrovirus encoding the Notch-1 NICD, which does not include the sequence homologous to the Notch-1 siRNA (21). Cells transduced by the NICD retrovirus did not undergo the characteristic morphologic changes seen with the Notch-1 siRNA and were protected from cell death (Fig. 4*D*).

To further confirm the role of Notch-1 in glioma cell proliferation, we constructed a tetracycline-inducible Notch-1 antisense U251MG glioma cell line (9). Exposure of the stable cell lines to tetracycline resulted in a significant decrease in glioma cell proliferation compared with control tetracycline-treated glioma cells (Fig. 4*E*). Additionally, a fusion protein of the extracellular portion of human Delta-like-1 and a human Fc region was used to inhibit cellular Notch-1 ligand binding. Exposure of all three glioma cell lines to the fusion protein resulted in significant inhibition of glioma cell proliferation (Fig. 4*E*).

To determine whether the *in vitro* effects of Notch-1 knockout translated to delayed tumor growth *in vivo*, glioma cells were stereotactically implanted into the brains of nude mice after double transfection *in vitro* with either Notch-1 siRNA or control siRNA. The delayed effects after Notch-1 siRNA transfection allowed healthy-appearing cells to be harvested after two daily siRNA transfections before intracranial injection. As shown in Fig. 4*F*, animals implanted with siRNA-transfected U251MG cells survived significantly longer that animals implanted with control siRNA-transfected U251MG cells ($P < 0.01$, log-rank analysis).

Importance of the Notch-1 Ligands, Delta-Like-1 and Jagged-1, in Glioma Cell Survival and Proliferation. Given the expression of Delta-like-1 and Jagged-1 in primary human gliomas, we were interested in exploring their functional significance. Efficient Delta-like-1 and Jagged-1 siRNAs (see Fig. 3) were

transfected into six glioma lines and their effects assessed. Delta-like-1 knockdown produced dramatic effects, inducing a spindle-shaped morphology initially (not shown) with subsequent cell death. Significant decreases in viable cell number were evident in all six glioma cell lines as evaluated by alamarBlue assay (Fig. 5*A*). Jagged-1 knockdown slowed growth significantly in several of the glioma lines but had a potent cytotoxic effect only in the T98G cell line (Fig. 5*A*).

Effects of these Delta-like-1 and Jagged-1 siRNAs on cell cycle progression were also assessed. The T98G cell line was evaluated at 72 hours post–first siRNA transfection by double staining for BrdUrd incorporation and propidium iodide DNA staining. Both siRNAs caused large drops in percentage of dividing cells (those incorporating BrdUrd), from 45% with control siRNA to 26% with Delta-like-1 siRNA and 27% with Jagged-1 siRNA (Fig. 5*B*). Increases in the percentage of apoptotic, hypodiploid cells were also noted with both siRNAs (gates R_1 + R_2, 4.2% in control versus 9.6% in Delta-like-1 and 18.4% in Jagged-1; Fig. 5*B*). Additionally, a marked increase in percentage of cells arrested at the G_2-M checkpoint was observed with Delta-like-1 siRNA transfection (gate R5, 3.6% versus 21.9%; Fig. 5*B*). We also showed apoptosis by Hoechst 33342/propidium iodide assay at 72 and 96 hours, with the Delta-like-1 siRNA–inducing apoptosis in 43.9% of cells at 96 hours (Vybrant Apoptosis Assay kit no.5, Molecular Probes; data not shown). These data show a critical role for Delta-like-1 and Jagged-1 in glioma cell proliferation and survival.

The effects of Delta-like-1 and Jagged-1 knockdown on glioma cell growth *in vivo* were evaluated by stereotactically implanting Delta-like-1, Jagged-1, or control siRNA-transfected U251MG cells into the brains of nude mice. Mice harboring human glioma cells transfected with the Delta-like-1 siRNA had significant prolongation of survival compared with mice harboring glioma cells transfected with control siRNA ($P < 0.01$, log-rank analysis; Fig. 5*C*).

Discussion

Our data show, for the first time, the presence of Notch-1 in both glioma cell lines and primary human gliomas. Additionally, the nuclear localization of Notch-1 indicates Notch-1 activation in gliomas as its intracellular domain is only present in the nucleus following ligand-mediated receptor activation and enzymatic cleavage (26, 27). Along with Notch expression, we have also shown the expression of the Notch-1 ligands, Delta-like-1 and Jagged-1, in both glioma cell lines and primary human gliomas. To our knowledge, this is only the second example described in the literature of Notch ligand overexpression in a human malignant disease, with a previous report in cervical cancer (16).

Expression of Delta-like-1 in different glioma subtypes roughly parallels Notch-1 expression, although the up-regulation of Delta-like-1 mRNA seen with quantitative real-time PCR reaches statistical significance only in oligodendrogliomas. The similarity of expression between Notch-1 and Delta-like-1 patterns is consistent with a previous suggestion that Delta-like-1 expression increases with increased Notch-1 expression (9). In contrast, Jagged-1 expression seems to have an inverse relationship with Notch-1 expression in that Jagged-1 is up-regulated only in glioblastomas, which we found to express less Notch-1 than other gliomas. This divergent expression of Notch-1 and Jagged-1 is consistent with their roles in the developing nervous system, in which neural progenitor cells tend to express either Notch-1 or Notch ligand as they differentiate toward either a glial or neuronal fate, respectively (28). When compared with lower-grade gliomas, glioblastomas show an expression pattern more like that of neuronal precursors, with decreased Notch-1 expression and increased Notch ligand expression.

The reason for lower expression of Notch-1 by the most malignant gliomas, glioblastomas, relative to other primary human gliomas is unclear. However, it has recently been shown that invasive cervical carcinomas also down-regulate Notch-1 expression relative to lower-grade cervical tumors (29). Taken together, these two observations raise the possibility that down-regulation of Notch-1 is important for progression to a more aggressive phenotype in some cancers (19, 30). One potential explanation for this lies in the recent demonstration that Notch-1 inhibits AP-1 transcription factor activity (30). In contrast, Jagged-1 signaling has been shown to increase AP-1 activity (19). AP-1 is more highly expressed in glioblastomas and has been shown to drive transcription of genes encoding vascular endothelial growth factor, vascular endothelial growth factor-D, and matrix metalloproteinase-9, proteins important for angiogenesis and tumor cell invasion (31–33). AP-1 is also associated with ezrin activity (34), another protein important for tumor cell invasiveness and metastasis (31, 32, 35). Thus, Notch-1 down-regulation and Jagged-1 up-regulation may contribute to the highly angiogenic and invasive properties of glioblastoma through increased AP-1 activity. Nevertheless, despite their lower basal expression of Notch-1, glioblastomas may remain sensitive to further inhibition of Notch-1 activity, given that the cell lines we found sensitive to Notch knockdown were mostly derived from glioblastomas.

The presence of both Notch-1 and its ligands in gliomas suggests that there may be an autocrine or juxtacrine stimulatory mechanism operative in gliomagenesis. Our data indicate significant Notch-1 activity in cultures of glioma cell lines, supporting their ability to activate their own Notch-1. Whether this stimulation is autocrine or juxtacrine is unclear at this time, although the prevailing opinion is that physiologic Notch-1 signaling is juxtacrine only (5). In addition to the apparent ability of glioma cells to activate Notch-1, neighboring cells in the surrounding brain may serve as a source for activation of Notch-1 on glioma cells. Jagged-1 has previously been shown to be expressed by reactive astrocytes, and gliomas are typically surrounded by a profound reactive astrocytosis/gliosis (32). This could be a particularly important mechanism of Notch-1 activation for solitary infiltrating glioma cells *in situ*. Neural stem cells, which have been shown to migrate toward areas of brain involved by glioma and to express Jagged-1 and Delta-like-1, could also contribute to the juxtacrine stimulation of Notch-1 on glioma cells (36).

These data show not only the presence of Notch-1 in gliomas but also its potential role in glioma cell proliferation and survival. Inhibition of Notch-1 through siRNA, a Notch-1 antisense, or the Delta-like-1 fusion protein caused significant inhibition of glioma cell proliferation, although only the siRNA caused significant glioma cell apoptosis. We believe the cytotoxic effects of the Notch-1 siRNA compared with the other strategies for inhibiting Notch-1 reflects greater efficiency of siRNA for down-regulating Notch-1. This greater efficiency was confirmed in an experiment with our CBF1-luciferase reporter cell line (data not shown). The ability of the NICD-expressing retrovirus to rescue glioma cells from Notch-1 siRNA-induced apoptosis argues against the possibility that the siRNA-mediated cytotoxicity was due to an off-target effect of the

Notch-1 siRNA. Although prior studies have suggested a potential role for Notch-1 in cellular proliferation and inhibition of apoptosis, our data suggest a far greater dependence on Notch-1 in glioma cells than has previously been observed for other cell types (37).

Some of the glioma cell lines we utilized, such as U373MG and U387, are more resistant than others to the effects of Notch-1 knockout. Whether the greater resistance of these cell lines to Notch-1 inhibition reflects redundancy of Notch-1 function through other Notch family members remains to be determined. Nevertheless, even in these glioma lines that were more resistant to killing by Notch-1 knockdown, down-regulation of Notch-1 inhibited glioma cell proliferation and induced a distinctive morphologic phenotype marked by greatly increased formation of membrane processes reminiscent of neurites. This effect is consistent with previous reports showing that Notch signaling is involved with inhibition of neurite/dendrite formation (38, 39).

Our data indicate that Delta-like-1 and Jagged-1 are active ligands for Notch-1 in glioma cell lines, as shown by the decreases in CBF1 activity following their knockdown. Although different cell lines showed variable sensitivities to knockdown of the different Notch ligands, most cells seemed to be more sensitive to down-regulation of Delta-like-1. Delta-like-1 inhibition not only inhibited glioma cell proliferation but also induced glioma cell apoptosis, whereas Jagged-1 knockdown led to a general slowing of glioma growth but induced apoptosis in only one of the glioma cell lines tested. We believe that these siRNA-mediated effects were specifically due to knockdown of their intended targets and not secondary to off-target effects because the cytotoxic/cytostatic effects seen with several Delta-like-1 and Jagged-1 siRNAs correlated with their relative efficiency for knockdown of Delta-like-1 and Jagged-1 protein and mRNA, respectively (data not shown). Whether the more pronounced killing effect observed with Delta-like-1 knockdown versus Jagged-1 knockdown reflects quantitative differences in the efficiency of ligand inhibition, differences in their manner of Notch-1 activation, or other activities of Delta-like-1 and Jagged-1 remains to be determined. Regardless of the mechanism, these data represent the first demonstration that knockdown of a Notch ligand can inhibit cancer cell proliferation and induce apoptosis.

Whereas Delta-like-1 and Jagged-1 clearly play a central role in stimulation of Notch-1 in glioma cells, their significance in glioma cell biology may go beyond their effects on Notch-1 activation. It has recently been shown that Delta-like-1 and Jagged-1 are processed in a fashion similar to Notch-1, ultimately resulting in release of a nuclear-targeted intracellular domain (19, 40). Thus, Delta-like-1 and Jagged-1 seem to have their own signaling functions. The details of these signal transduction pathways have yet to be fully elucidated, although as previously mentioned the Jagged-1 intracellular domain serves to increase AP-1 transcription factor activity (19). The fact that Jagged-1 itself can have transforming activity requiring its intracellular PDZ-ligand domain suggests that it may have a signaling role in cancers (18). Thus, the response of glioma cells to Delta-like-1 and Jagged-1 inhibition may be secondary to the loss of Delta-like-1 and Jagged-1 signaling as well as a decrease in Notch-1 activation. Such a hypothesis is supported by our observation that the morphologic appearance of glioma cells significantly varies depending on whether Notch-1, Delta-like-1, or Jagged-1 has been inhibited.

These results suggest that Notch-1 and its ligands Delta-like-1 and Jagged-1 are potential therapeutic targets for the treatment of malignant gliomas. Genetic approaches utilizing vectors encoding antisense or siRNA hairpin structures to Notch-1 and/or its ligands, or alternatively vectors encoding Notch-1 ligand binding inhibitors, such as the Delta-like-1/Fc fusion protein, could be envisioned. Small molecule γ-secretase inhibitors might also be useful for therapeutic Notch-1 inhibition, given the dependence of Notch-1 on enzymatic processing for its activity (5, 9). Furthermore, therapeutically targeting Notch-1 in gliomas may have the added benefit of mediating an antiangiogenic effect, given the significant role of the Notch pathway in angiogenesis (41). Investigators evaluating the potential role of γ-secretase inhibition as a treatment for Alzheimer's disease due to the dependence of β-amyloid production on γ-secretase activity have voiced concern over the possibility for neurotoxicity following long-term Notch inhibition, given its important role in central nervous system development (42). Our data suggest, however, that a relatively short course of Notch inhibition may be sufficient to induce tumor cell apoptosis, in contrast to the long-term treatment that would likely be necessary for inhibiting β-amyloid production in a chronic disease (e.g., Alzheimer's). Shorter treatment courses would likely decrease the chance of any significant neurotoxicity, making γ-secretase a potentially promising target for the treatment of malignant gliomas.

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