Published OnlineFirst July 25, 2011; DOI: 10.1158/0008-5472.CAN-10-4269

Endothelial Cells Create a Stem Cell Niche in Glioblastoma by Providing NOTCH Ligands That Nurture Self-Renewal of Cancer Stem-Like Cells

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Abstract

One important function of endothelial cells in glioblastoma multiforme (GBM) is to create a niche that helps promote self-renewal of cancer stem-like cells (CSLC). However, the underlying molecular mechanism for this endothelial function is not known. Since activation of NOTCH signaling has been found to be required for propagation of GBM CSLCs, we hypothesized that the GBM endothelium may provide the source of NOTCH ligands. Here, we report a corroboration of this concept with a demonstration that NOTCH ligands are expressed in endothelial cells adjacent to NESTIN and NOTCH receptor-positive cancer cells in primary GBMs. Coculturing human brain microvascular endothelial cells (hBMEC) or NOTCH ligand with GBM neurospheres promoted GBM cell growth and increased CSLC self-renewal. Notably, RNAi-mediated knockdown of NOTCH ligands in hBMECs abrogated their ability to induce CSLC self-renewal and GBM tumor growth, both *in vitro* and *in vivo*. Thus, our findings establish that NOTCH activation in GBM CSLCs is driven by juxtacrine signaling between tumor cells and their surrounding endothelial cells in the tumor microenvironment, suggesting that targeting both CSLCs and their niche may provide a novel strategy to deplete CSLCs and improve GBM treatment. *Cancer Res; 71(18); 6061–72. ©2011 AACR.*

Introduction

Glioblastoma multiforme (GBM) is the most lethal malignant brain tumor in adults without revolutionary improvement in treatment during the past 30 years (1, 2). Any treatment that can significantly prolong patients' overall survival for more than 3 months, which is the best achievement so far to treat GBM when using surgery, radiation therapy, and temozolomide, can be considered as a success (3). Emerging evidence shows that a small population of cancer stem-like cells (CSLC) within neoplasms is responsible for tumor propagation (4), including GBM (5, 6). Therapies targeting CSLCs bring hope for brain tumor patients (7–10). We have shown that activation of the NOTCH pathway in GBM CSLC is required for their growth *in vitro* and *in vivo* (10). However, the molecular mechanism by which NOTCH is activated in GBM CSLCs and if CSLCs, like their normal cognates, also need a niche to self-renew is unclear.

The stem cell niche is a microenvironment where stem cells reside. It is composed of stem cells, neighboring supportive cells, extracellular matrix, and other factors required for stem cell self-renewal (11). In the CNS, neural stem cells (NSC) are located at the subventricular zone (SVZ) of the lateral ventricle and subgranular zone of the dentate gyrus in the hippocampus (12–14). Some astrocytes and neuroblasts and endothelial cells in the SVZ are thought to contribute to these NSC niches by providing growth factors or membrane-bound ligands to NSCs for self-renewal (15). Although normal stem cells need to reside within a niche to self-renew (11), what a CSLC niche is composed of is largely unknown. As CSLCs share many properties with normal stem cells, CSLCs may also need a CSLC niche to self-renew. A recent report showed that endothelial cells function as a CSLC niche to promote CD133+ CSLC self-renewal in medulloblastoma and GBM (16). However, signaling pathways regulating the CSLC niche are still unclear.

The *Notch* locus was first described by Morgan in a strain of *Drosophila* with notched wing blades (17). Seventy years later, the gene was cloned as a cell surface receptor (18) playing a key role in the development of many different cell types and tissues, including neuron and glia (19–22). NOTCH signaling is initiated when transmembrane ligands on one cell bind NOTCH receptors on an adjacent cell and cause a gamma

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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secretase-mediated proteolytic release of the NOTCH intracellular domain (NICD) (23). NICD then translocates into the nucleus where it interacts with the transcriptional cofactor CBF1 and activates targets such as the *HES* and *HEY* genes to modulate cell fate (20, 21). In vertebrates, 4 NOTCH receptors (NOTCH 1-4), 5 ligands (JAGGED1,2, DLL1,3,4), and multiple effector molecules (*HES1-6*, *HEY1,2,L*) have been identified (24). During normal development, ligand expressing cells (signal-sending cell) generally have reduced NOTCH activity, whereas NOTCH receptor-expressing cells (signal-receiving cell) have elevated NOTCH activity (25). In general, signal-sending cells will undergo differentiation, whereas signal-receiving cells remain in an undifferentiated state (stem cell state). This phenomenon is called "lateral specification" (Supplementary Fig. S1; ref. 25). We and others have recently shown that GBM CSLCs have elevated NOTCH activity (10, 26, 27), and that NOTCH pathway blockade with a gamma-secretase inhibitor depletes GBM CSLCs, inhibits tumor growth, and prolongs survival of mice bearing intracranial xenografts (10). In this study, we investigated whether acquired NOTCH activity in GBM CSLCs comes from endothelial cells, which function as niche cells to promote GBM CSLC self-renewal by providing NOTCH ligands to NOTCH receptors expressed in GBM CSLCs.

Materials and Methods

GBM samples

Fresh primary GBM samples were obtained from the University of Michigan Hospitals with approval from the Internal Review Board.

Cell culture

Given the evidence that only some GBM cells fall in the CSLC hypothesis (28), we only choose GBM neurospheres that we have shown to fall in the CSLC hypothesis for this study (10, 29). GBM neurosphere cells (HSR-GBM1, HSR-GBM2, and HSR-GBM3) derived from 3 different GBM patients were cultured and maintained in NeuroCult proliferation medium (STEMCELL Technologies) supplemented with 10 ng/mL epidermal growth factor (EGF; PeproTech), 10 ng/mL FGFb (PeproTech), and 2 μg/mL heparin (Sigma; ref. 10). Human brain microvascular endothelial cells (hBMEC) and human umbilical vein endothelial cells (HUVEC) were purchased from Cell Systems Corporation (catalogue #ACBRI-376) and ATCC (catalogue #CRL-1730), respectively. hBMECs were maintained in CSC complete medium (Cell Systems) and HUVECs were cultured in F12K with 10% FBS, 0.1 mg/mL heparin, and 0.03 mg/mL endothelial cell growth supplement. For coculture system, neurospheres were seeded on top of the attached endothelial cells and maintained in the serum-free endothelial cell medium (Invitrogen) supplemented with 10 ng/mL EGF, 20 ng/mL FGFb, and 10 μg/mL heparin. For GBM cell differentiation, plates were coated with 15 ug/mL polyornithine for a minimum of 3 hours at 37°C. GBM cells were then cultured on the coated plates at the density of 1×10^5 cells/cm^2 in NeuroCult differentiation medium (STEMCELL Technology). The differentiated GBM cells were utilized 7 to 14 days later.

Lentivirus production and shRNA transduction

Lentiviruses were produced by transfecting BOSC 23 cells (ATCC, catalogue #CRL-11270) with 16 μg total of pSicoR (Addgene, catalogue #11579), psPAX2 (Addgene, catalogue #12260), and PMD.2G (Addgene, catalogue #12259) plasmids with a ratio of 5: 3.5: 1.75 in a 10-cm dish, using lipofectamine 2000 (Invitrogen; ref. 30). Tranfected cell media containing viruses were collected 48 hours later and filtered through a 0.45-μm polyvinylidene difluoride membrane. Virus-containing media were used immediately or stored at −80°C. Cells were transduced with lentiviruses by the centrifugation method (1,000 × *g*, 1.5 hours) with addition of 4 to 8 μg/mL polybrene. The medium was changed the next day, and gene expression was analyzed 1 week later (31). Transduction efficiency was monitored by green fluorescent protein (GFP) intensity with greater than 90% of cells being infected (30). shRNA sequences were designed by pSicoOligomaker 1.5 (courtesy of Dr. T. Jacks, MIT). The siRNA sequences used to knock down NOTCH ligands were 5′-GTGAGTGGTTGAATATGAT-3′ for JAG1 and 5′-GGAGAGAGGGGGCCAATGA-3′ for DLL4.

Orthotopic xenograft implantation

Mice were obtained and experiments were done in accordance with guidelines from the University Animal Care and Use Committee at the University of Michigan. Four to 5 week-old athymic nude-*Foxn1*^{nu} mice were purchased from Harland Laboratories for intracranial xenograft implantation (8, 10). A total of 50,000 single cells from GBM neurospheres with or without 50,000 endothelial cells were stereotactically injected into the brain (2 mm right, 1 mm back, 2 mm deep from the bregma; refs. 8, 10). Tumor growth was monitored by MRI (Supplementary Methods).

Statistical analysis

Statistical analyses were done using GraphPad Prism 4 (GraphPad Software). Data graphed with error bars represent mean and SE from experiments done in triplicate unless otherwise noted. A 2-sided Student's *t* test was used to determine the significance of any differences.

Results

NOTCH receptor-expressing cells are colocalized with NESTIN-positive tumor cells in primary GBM and have elevated level of NOTCH activity

Recently, we have shown that Notch pathway blockade depletes GBM CSLCs *in vitro* and prevents their propagation *in vivo* (10). To investigate whether NOTCH receptor-expressing cells in primary GBMs are CSLCs, we first examined NOTCH1 and NESTIN expression by immunofluorescence staining in primary GBM frozen sections. We found that NOTCH1-expressing cells colocalized with NESTIN-expressing cells in 3 different primary GBM samples (Fig. 1A). NOTCH2-expressing cells also colocalized with NESTIN-expressing cells in primary GBMs (Supplementary Fig. S2). Furthermore, NOTCH ligand JAG1- or DLL1-expressing cells are adjacent to the cells with NOTCH pathway activation as

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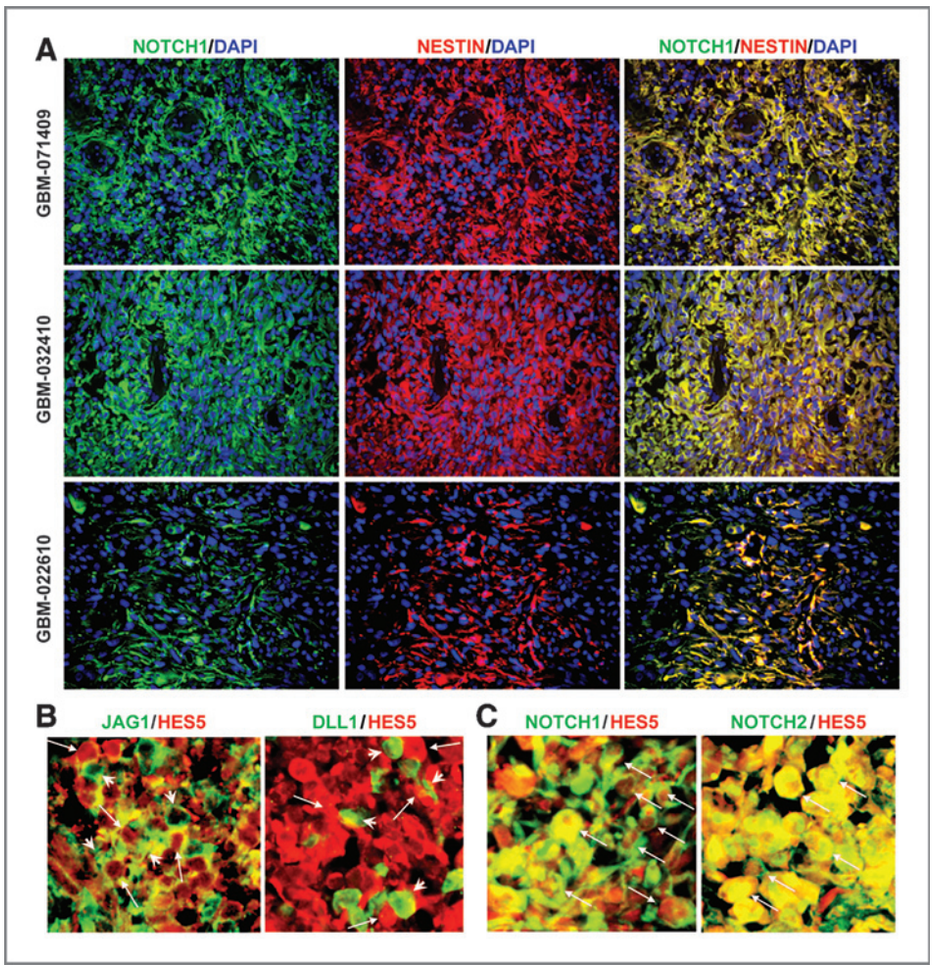

Figure 1. NOTCH receptor-expressing cells are colocalized with NESTIN-positive tumor cells in primary GBM and have elevated level of NOTCH activity adjacent to NOTCH ligand-expressing cells. A, expression of NOTCH1 and NESTIN was colocalized in the same cells in the primary GBM tumors (GBM-071409, GBM-032410, and GBM-022610). B, JAG1-expressing cells or DLL1-expressing cells (green, arrowhead) were adjacent to HES5-expressing cells (red, arrow) in primary GBM samples. C, NOTCH1- or NOTCH2-expressing cells were colocalized with HES5-expressing cells (arrow) in primary GBM samples.

indicated by HES5 expression (Fig. 1B). In addition, HES5-expressing cells are colocalized with NOTCH1- and NOTCH2-expressing cells in primary GBMs (Fig. 1C). These results suggest that the NESTIN-positive GBM CSLCs express NOTCH receptors and show elevated level of NOTCH activity. These data indicate that NOTCH ligand-expressing cells within the tumor may function as a CSLC niche by providing ligands to NOTCH receptors expressed in GBM CSLCs to activate the NOTCH pathway in CSLCs and to promote CSLC self-renewal, replicating the lateral specification phenomenon seen in normal tissue development (Supplementary Fig. S1).

NOTCH ligands are expressed in the endothelial cells surrounded by tumor cells in primary GBMs

Next, we sought to identify the nature of NOTCH ligand-expressing cells within GBM. First, we examined NOTCH ligands DLL1, DLL4, JAG1, and JAG2 expression in frozen sections of primary GBM samples using immunofluorescence. We found that DLL1 is expressed in most tumor cells, whereas DLL4 is exclusively expressed in endothelial cells (CD31+) within GBM (Fig. 2A). JAG1 or JAG2 is expressed in both endothelial cells and some tumor cells (Fig. 2A and data not shown). Interestingly, using Western blot we found that cells from GBM neurosphere line HSR-GBM1 also expressed DLL1 and JAG1, but not DLL4 (Fig. 2C). hBMECs expressed DLL4 and JAG1, but no detectable DLL1 (Fig. 2C). A second type of endothelial cell, HUVEC, also expressed JAG1 and DLL4, but not DLL1 (data not shown). The expression pattern of these NOTCH ligands detected by Western blot is consistent with our findings in primary GBMs by immunofluorescence (Fig. 2A). Furthermore, the NOTCH1 receptor and a CSLC marker, NESTIN, are expressed in the tumor cells adjacent to JAG1-expressing endothelial cells or tumor cells (Fig. 2B), recapitulating the "lateral inhibition" pattern of cell distribution commonly seen in NOTCH-regulated normal development (Supplementary Fig. S1; ref. 25). Taken together, these data suggest that endothelial cells within GBM may function as a CSLC niche by providing NOTCH ligands to NOTCH receptors expressed in CSLCs to activate the NOTCH pathway in CSLCs.

Better differentiated GBM cells express higher level of NOTCH ligands and reduced level of CD133 and are less tumorigenic in mouse

The fact that NOTCH ligand JAG1 is expressed in tumor cells adjacent to the NOTCH receptor- and NESTIN-expres-

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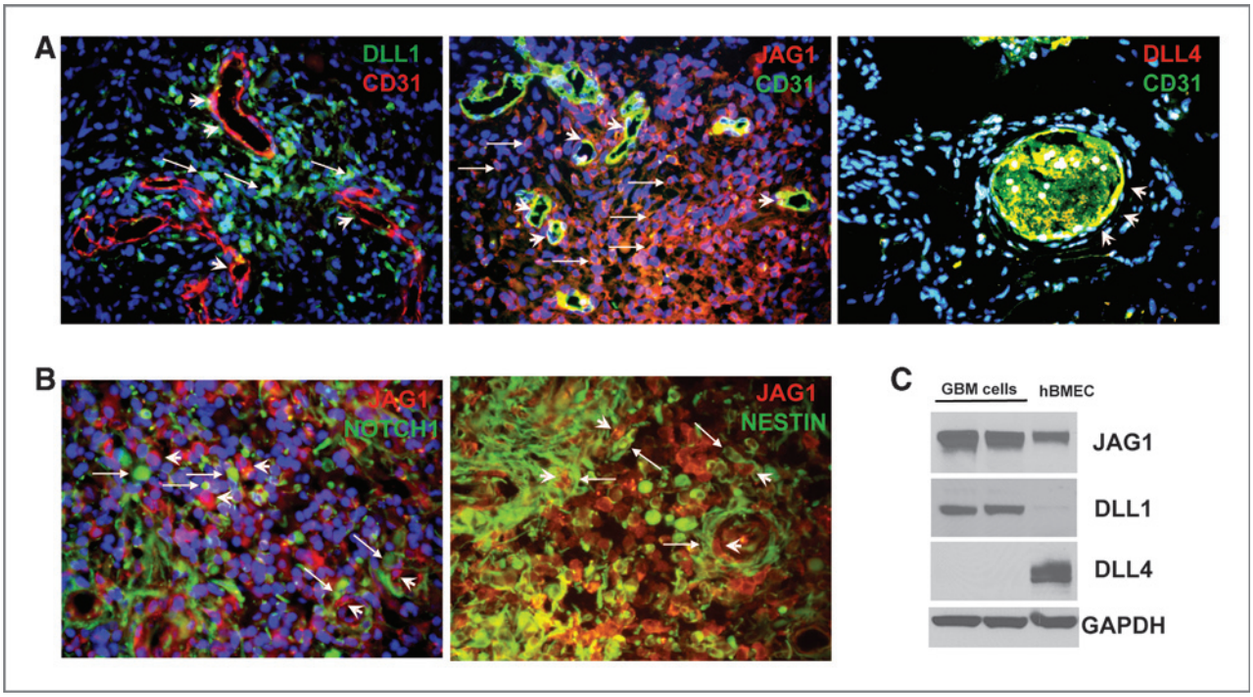

Figure 2. Expression pattern of NOTCH ligands in primary GBMs. A, NOTCH ligand DLL1 (green) was expressed in some GBM cells (arrow) and in some endothelial cells (CD31+) around the blood vessels (arrow head). JAG1 was expressed in both tumor cells (arrow) and blood vessels (arrow head). DLL4 was expressed in the blood vessels and colocalized with CD31 staining in endothelial cells (arrow head). B, NOTCH1 receptor and CSLC marker NESTIN were expressed in tumor cells (arrow) adjacent to JAG1-expressing endothelial cells or tumor cells (arrow head). C, Western blots showed expression of JAG1, DLL1, and DLL4 in GBM neurosphere cells (lane 1 and 2: same GBM cells) and hBMECs (lane 3), consistent with immunohistochemistry results. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

sing cells in primary GBMs (Fig. 2B) indicates some tumor cells may also function as a stem cell niche to provide NOTCH ligands to NOTCH receptors expressed in adjacent CSLCs. To test this, we first forced GBM neurosphere to differentiate, grow as mono-layer, and attach to the culture plate by switching to the differentiation medium. We found that the proliferation rate detected by Ki-67 staining was reduced and 3 lineage differentiation markers GFAP (glia), Tuj1 (neuron), and Gal-C (oligodendrocyte) were induced in the differentiated GBM cells (Fig. 3A). GFAP-positive cells were increased from 84.7 $\pm$ 4.8% to 95.9 $\pm$ 1.3%, Tuj-1-positive cells from 20.5 $\pm$ 6.1% to 88.6 $\pm$ 1.8%, and Gal-C-positive cells from 0 to 1.5 $\pm$ 0.9% ($P < 0.01$, t test). Expression of DLL1 was also induced in the differentiated cells (Fig. 3B). Furthermore, DLL1 is upregulated in all neurosphere lines HSR-GBM1-3 (Fig. 3B, Supplementary Fig. S3). Interestingly, HSR-GBM2 showed upregulation of all the ligands (Supplementary Fig. S3). In addition, expression of CSLC marker CD133 and NOTCH target HES1 were reduced in differentiated GBM cells (Fig. 3B), suggesting that GBM CSLCs lose NOTCH activity and "stemness" when they are differentiated. Consistent with these data, CD133-positive population was reduced in differentiated GBM cells (Fig. 3C). The CD133-negative GBM population also had elevated levels of NOTCH ligand DLL1 expression compared with the CD133-positive population (Fig. 3C). Finally, when we injected 50,000 neurosphere cells or 50,000 differentiated GBM cells into the forebrain of immunodeficient mice, 100% (5 of 5) of mice receiving GBM neurosphere cells formed large intracranial xenografts as detected by MRI (Fig. 3D), whereas only 40% (2 of 5) of mice injected with differentiated GBM cells formed tumors. Brain pathology confirmed the existence of intracranial xenografts and formation of larger tumor in the neurosphere-derived xenografts (Fig. 3D). GBM intracranial xenograft also shows a similar feature as human primary tumor (Supplementary Fig. S4). These data show that GBM neurospheres enrich tumor-initiating CSLCs and that they can be used as a CSLC-enrichment model whereas forced differentiated cells can be used as a CSLC-depletion model, consistent with previous reports (6, 8, 29). Taken together, these data suggest that GBM neurospheres enrich CSLC population and that differentiated GBM cells may also provide NOTCH ligands to NOTCH receptors expressed in adjacent GBM CSLCs.

NOTCH ligands promote GBM neurosphere growth *in vitro*

To examine whether NOTCH ligands expressed in endothelial cells and differentiated tumor cells may have functional effects on GBM cells, we treated GBM neurosphere with soluble JAG1 or DLL1 peptide and examined GBM neurosphere growth. We found that JAG1 peptide treatment for 5 days increased HSR-GBM1 and HRS-GBM2 neurosphere growth in a dose-dependent fashion (Fig. 4A). Furthermore, both JAG1 and DLL1 peptide induced HSR-GBM3 neurosphere

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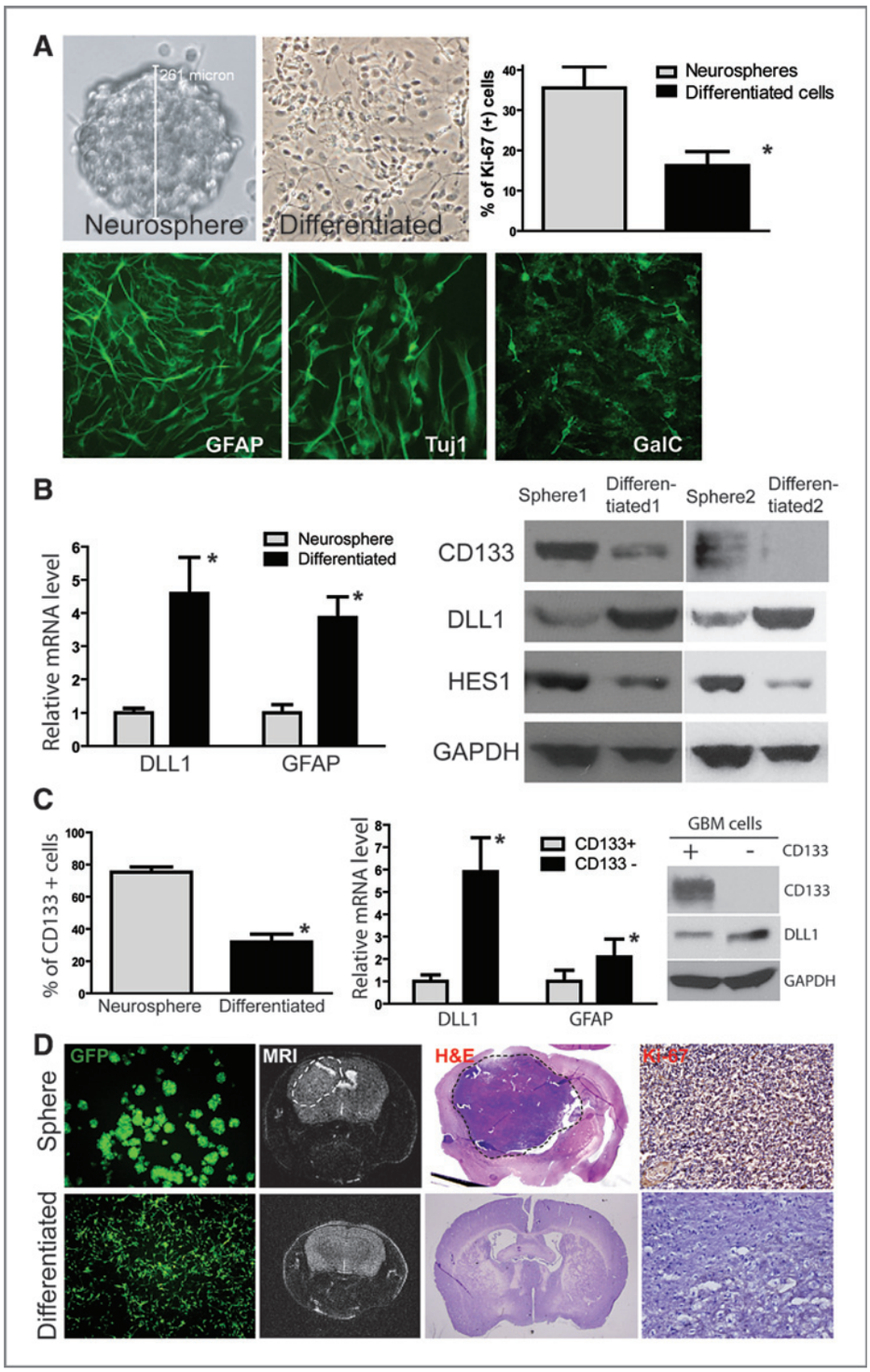

Figure 3. Differentiated GBM cells express NOTCH ligands and have reduced ability to form intracranial xenograft in mice. A, HSR-GBM1 neurosphere line was forced to differentiate and grow as a monolayer, which had reduced proliferation (*, $P < 0.05$, t test) and induced expression of GFAP (glial), Tuj1 (neuronal), and GalC (oligodendrocyte) markers. B, differentiated HSR-GBM1 cells expressed higher levels of DLL1 and GFAP at the mRNA level detected by quantitative RT-PCR (left). In addition, differentiated cells expressed a higher level of DLL1 and lower levels of NOTCH target HES1 and stem cell marker CD133 at the protein level as detected by Western blot in 2 different GBM neurosphere lines, HSR-GBM1 and HSR-GBM2. C, CD133-positive population was significantly reduced in differentiated tumor cells compared with GBM neurospheres (HSR-GBM1) detected by flow cytometry (left). In addition, CD133-negative population in HSR-GBM1 neurospheres expressed higher levels of DLL1 and GFAP at mRNA level compared with CD133-positive population (middle). Upregulation of DLL1 in CD133-negative population was also confirmed at the protein level by Western blot (right). D, when GBM neurospheres or differentiated cells from HSR-GBM1 labeled with GFP using lentivirus were injected into the mouse brain, neurosphere cells formed large xenografts as detected by MRI (dash circle), whereas differentiated cells had reduced ability to form xenografts. Pathology was confirmed by hematoxylin and eosin (H&E) staining and immunostaining of the proliferation marker Ki-67.

growth *in vitro* (Fig. 4A), suggesting that NOTCH ligand may induce NOTCH signaling in CLSCs through being immobilized by attachment to the extracellular matrix or the adjacent cells (32, 33). A weak growth response in HSR-GBM3 may be due to the different genetic/epigenetic backgrounds among different tumors.

In addition, expression of CSLC marker CD133 and CD15 were also induced in JAG1 or DLL1 peptide-treated GBM

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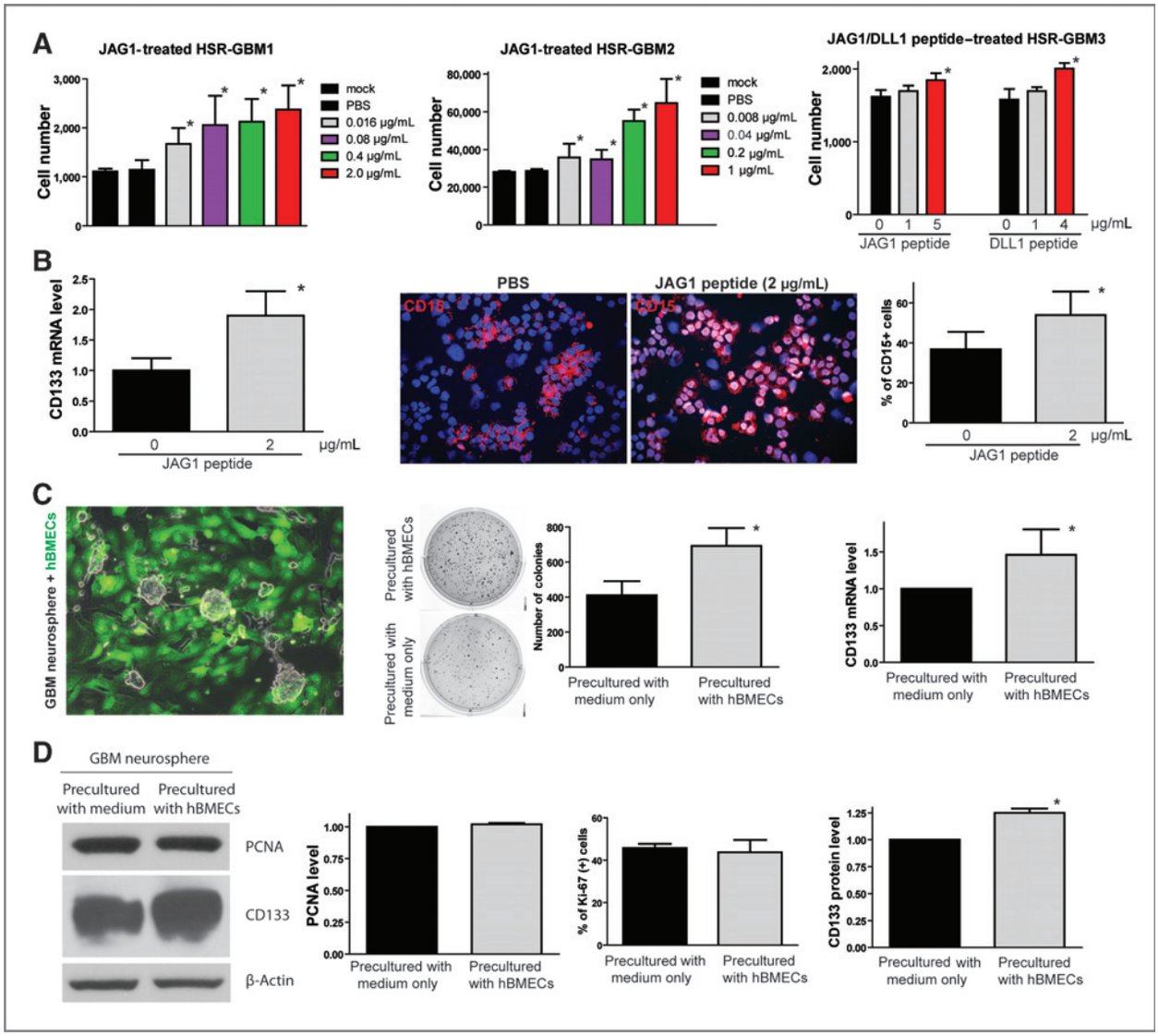

Figure 4. NOTCH ligand JAG1 or DLL1 promotes GBM neurosphere growth *in vitro*. A, soluble ligand JAG1 peptide treatment for 5 days induced growth of HSR-GBM1 and HSR-GBM2 in a dose-dependent fashion (left and middle). JAG1 or DLL1 peptide also induced growth of HSR-GBM3 (right). B, 2 μg/mL JAG1 peptide treatment increased CD133 mRNA expression in HSR-GBM1 (left). A total of 2 μg/mL JAG1 peptide treatment also increased the CD15-positive CSLC population in GBM neurospheres detected by immunofluorescence (right, $n = 6$ random fields, *, $P < 0.05$, t test). C, GBM neurospheres cocultured with GFP-labeled hBMECs were sorted by flow cytometry and examined clonogenesis by soft agar formation assay. GBM cells formed more colonies when precultured with hBMECs ($n = 6$ wells were counted, *, $P < 0.01$, t test). In addition, expression of CSLC marker CD133 was also induced at mRNA level in GBM neurospheres precultured with hBMECs compared with those precultured with medium only (right, $n = 6$ repeats of this experiment, *, $P < 0.01$, t test). D, there was no proliferation change in GBM neurospheres precultured with or without hBMECs, identified by PCNA protein expression using Western blot and percentage of Ki-67 positive population by immunofluorescence. However, CD133 expression was significantly induced in GBM neurospheres precultured with hBMECs (right). β-Actin was used as a loading control.

neurospheres (Fig. 4B), suggesting that activation of NOTCH signaling by JAG1 or DLL1 increases self-renewal of GBM CSLCs *in vitro*. Finally, we used clonogenesis as a functional CSLC marker to examine if hBMECs can induce GBM CSLC self-renewal. First, we labeled hBMECs with GFP by lentiviral vector and cocultured hBMECs with unlabeled GBM neurospheres (Fig. 4C) for 3 days. Then, we sorted GFP-negative GBM neurosphere cells and put them into soft agar to examine the changes of tumorigenicity. Medium alone cultured GBM neurospheres were used as a control. We found that hBMECs promoted clonogenesis of GBM neurospheres (Fig. 4C). CD133 expression was also induced in GBM neurospheres precultured with hBMECs, indicating CSLC population is increased (Fig. 4C). Interestingly, the proliferation rate of the entire GBM neurosphere culture (detected by proliferation marker PCNA expression using Western blot and percentage of Ki-67 positive population in the whole culture) did not change when precultured with hBMECs compared with

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precultured with medium only (Fig. 4D), whereas expression of CSLC marker CD133 was significantly increased (Fig. 4D). These data suggest that hBMECs only induce GBM CSLC self-renewal instead of proliferation of the whole culture (Supplementary Fig. S5). Taken together, these data show that NOTCH ligand JAG1 and DLL1 are sufficient to promote GBM CSLC self-renewal *in vitro*, suggesting that the endothelial or tumor cells that express higher levels of NOTCH ligands in GBM may provide these ligands to GBM CSLCs to activate the NOTCH pathway and promote self-renewal of GBM CSLCs.

Knockdown of NOTCH ligand expression in hBMECs by shRNA decreases CSLC population in cocultured GBM neurospheres *in vitro*

To further investigate whether NOTCH ligands expressed in endothelial cells are required for GBM CSLC growth, we also conducted loss-of-ligand function studies in endothelial cells cocultured with GBM neurospheres. As JAG1 and DLL4 are the predominantly expressed NOTCH ligands in hBMECs (Fig. 2), we first knocked down JAG1 expression in hBMECs using shRNA lentiviral vector pSicoR-shJAG1-GFP (Fig. 5A and B). Then, shJAG1 or control lentivirus infected hBMECs were

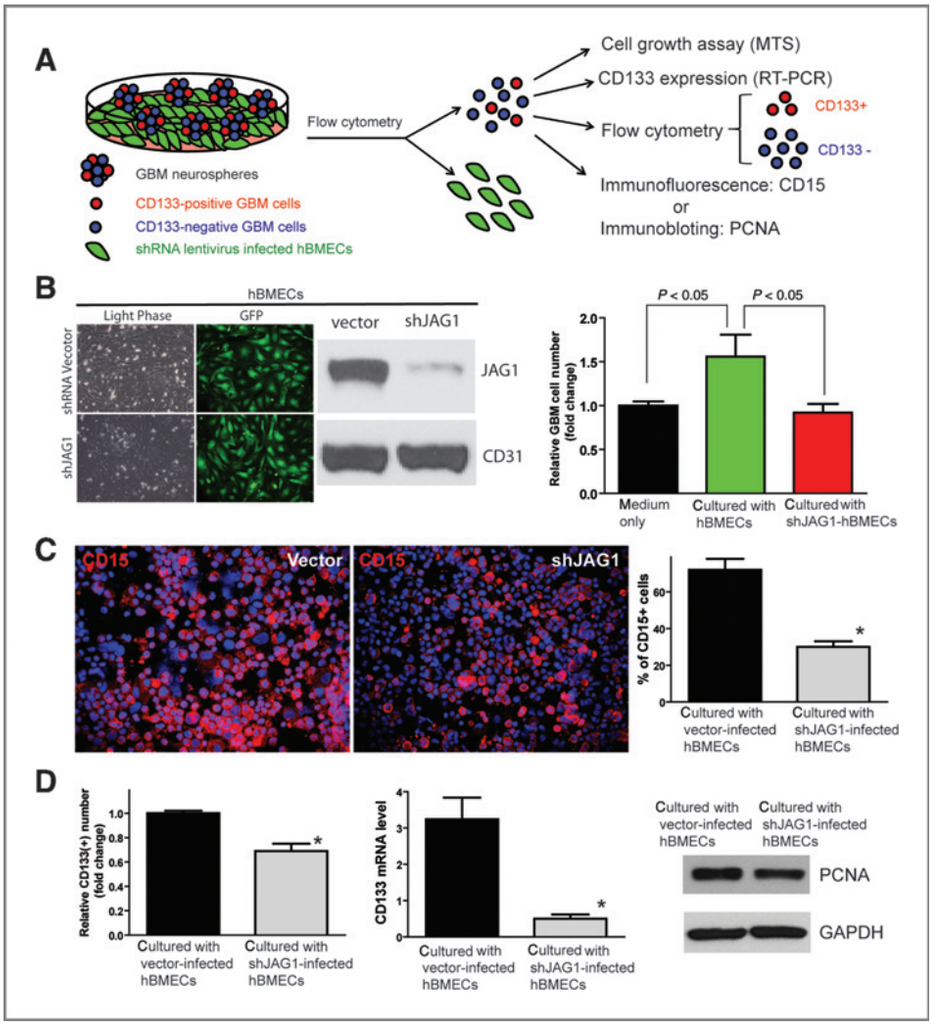

Figure 5. Knocking down JAG1 expression in hBMECs by shRNA decreases CSLC population in cocultured GBM neurospheres *in vitro*. A, schematic showing the experimental approach used to examine if NOTCH ligands expressed in endothelial cells are essential for cocultured GBM neurosphere growth. B, Western blot showed that JAG1 expression was significantly knocked down by shJAG1 lentiviruses in hBMECs to be cocultured with GBM neurospheres. CD31 was served as an internal control. HSR-GBM1 growth was increased when cocultured with hBMECs compared with neurospheres cultured by medium only, whereas knockdown of JAG1 expression in hBMECs abrogated hBMEC-induced HSR-GBM1 growth. C, CD15-positive CSLC population detected by immunofluorescence was also reduced in GBM neurospheres cocultured with shJAG1-infected hBMECs compared with GBM neurospheres cocultured with empty vector-infected hBMECs. D, knockdown of JAG1 expression in hBMECs decreased CD133-positive population in HSR-GBM1 neurospheres cocultured with hBMECs compared with HSR-GBM1 neurospheres cultured with empty vector-infected hBMECs (left). mRNA expression of CD133 was also lowered in HSR-GBM1 neurosphere cells cocultured with shJAG1-infected hBMECs compared with HSR-GBM1 cells cocultured with empty vector-infected hBMECs (middle). There was no proliferation change in HSR-GBM1 neurospheres cocultured with empty vector- or shJAG1-infected hBMECs, identified by PCNA protein expression using Western blot (right).

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cocultured with GBM neurospheres using serum-free endothelial cell culture medium for 3 days. GBM neurosphere cells (GFP-negative) were subsequently sorted by flow cytometry, and tumor growth and the CD133-positive population were assessed by MTS assay and flow cytometry, respectively (Fig. 5A). We found that GBM neurosphere growth was increased when cocultured with hBMECs compared with neurospheres cultured by medium only, whereas knockdown JAG1 expression by shRNA in endothelial cells abrogated hBMEC-induced GBM growth (Fig. 5B). CD15-positive population was also reduced in GBM neurospheres cocultured with shJAG1-infected hBMECs (Fig. 5C). Furthermore, knockdown of JAG1 expression in hBMECs decreased CD133-positive population and CD133 mRNA expression in GBM neurospheres cocultured with hBMECs (Fig. 5D). However, GBM neurosphere proliferation was not changed when cocultured with shJAG1-infected hBMECs (Fig. 5D). Taken together, these data showed that reduced CSLCs in GBM neurospheres when cocultured with shJAG1-infected hBMECs was due to reducing CSLC self-renewal, but not due to reduced proliferation of the whole culture (Supplementary Fig. S5), suggesting that NOTCH ligands expressed in hBMECs are essential for the maintenance of GBM CSLCs *in vitro*.

Knocking down JAG1 or DLL4 expression in hBMECs by shRNA inhibits growth of GBM intracranial xenografts derived from the mixture of GBM neurosphere cells and hBMECs

To examine whether expression of NOTCH ligands in endothelial cells is required for GBM CSLCs growth *in vivo*, shJAG1- or shDLL4-infected hBMECs were mixed with GBM neurosphere cells (ratio 1:10) and injected into the brain of immunodeficient mice (Fig. 6A). Tumor growth was monitored by MRI and tumor volume calculated (Fig. 6A). We found that GBM neurospheres cocultured with shJAG1- or shDLL4-infected hBMECs developed significantly smaller intracranial xenografts compared with those derived from a mixture of GBM neurospheres and empty vector-infected hBMECs (Fig. 6B and C). Furthermore, we examined the cell specificity of the NOTCH ligand effect by using another type of human endothelial cells, HUVECs, and found that knockdown of JAG1 expression in HUVECs also reduced growth of intrancranial xenografts (Fig. 6D). These data show that NOTCH ligands expressed in endothelial cells contribute to the growth of intracranial xenografts derived from the mixture of GBM neurosphere cells and endothelial cells, indicating that endothelial cells may function as a niche for GBM CSLCs by providing NOTCH ligands to NOTCH receptors expressed in GBM CSLCs.

CD133-positive population is reduced in GBM xenografts derived from mixture of GBM neurospheres and hBMECs with ligand knockdown

To examine the CSLC population changes in GBM xenografts derived from a mixture of GBM neurosphere and endothelial cells with or without ligand knockdown, first we sorted human GBM cells using mouse cell surface antigen (to remove mouse cells) and GFP (to remove human endothelial cells as they were infected with pSicoR-GFP lentiviral vector with or without shRNA against NOTCH ligand; Fig. 6A). Then we examined the CD133-positive GBM cell changes (Fig. 6A). We found that CD133-positive GBM CSLCs were significantly reduced in GBM xenografts mixed with shJAG1 or shDLL4-infected hBMECs (Fig. 7A). Similarly, CD133-positive population was also decreased in GBM xenografts mixed with shJAG1-infected HUVECs (Fig. 7A). Consistent with these data, CD15-positive GBM CSLC population was also reduced in GBM xenografts mixed with shJAG1-infected hBMECs (Fig. 7A). However, there was no significant change in tumor proliferation (Fig. 7B) and apoptosis (Fig. 7C) in the xenografts derived from the mixture of GBM neurospheres and hBMECs with JAG1 or DLL4 knockdown, suggesting that reduced GBM intracranial xenograft growth was due to loss of CSLCs (Supplementary Fig. S5), which need to receive NOTCH ligands from endothelial cells to activate NOTCH for their self-renewal. These data are consistent with previous findings that the endothelial niche mainly affects CSLCs instead of the proliferation and apoptosis of the whole tumor (16). Taken together, our results show that NOTCH ligands expressed in endothelial cells are critical for the propagation of GBM CSLCs.

Discussion

In this study, we explored the molecular mechanism underlying NOTCH activation in GBM CSLCs. We found that endothelial cells within the tumor function as a CSLC niche to promote CSLC self-renewal by providing NOTCH ligands to the NOTCH receptors expressed in GBM CSLCs (Supplementary Fig. S6).

To our knowledge, there is still no published documentation addressing whether cell contact-dependent signaling pathways contribute to CSLC self-renewal within their niche in GBM. Here, we first show that NOTCH ligands are expressed by endothelial cells and some tumor cells around the NESTIN- and NOTCH receptor-positive CSLCs in primary GBM samples. Then, we show that knockdown of NOTCH ligand JAG or DLL in endothelial cells decreases CD133-positive population in GBM neurospheres *in vitro* and inhibits cocultured GBM neurosphere propagation *in vivo*. Finally, we show that the reduced GBM xenograft growth is due to decreased GBM CSLC population. Taken together, our data provide experimental evidence that endothelial cells function as CSLC niche by providing NOTCH ligands to activate NOTCH signaling in GBM CSLCs (Supplementary Fig. S6). Therefore, several novel and innovative approaches can be developed based on interrupting the interaction between CSLCs and their niche(s). For example, a NOTCH ligand blocking antibody or peptide, in combination with chemo- and radiation-therapy and CSLC-targeting therapy, may result in improved GBM treatment.

In the CNS, that endothelial cells can function as a stem cell niche was initially found in normal NSCs, with endothelial cells promoting asymmetric self-renewal of NSCs from the SVZ (15). Later, it was confirmed that the vascular niche regulates self-renewal of NSCs *in vivo* (34) at both embryonic and adult stages (35). In addition, it has been shown that the

Published OnlineFirst July 25, 2011; DOI: 10.1158/0008-5472.CAN-10-4269

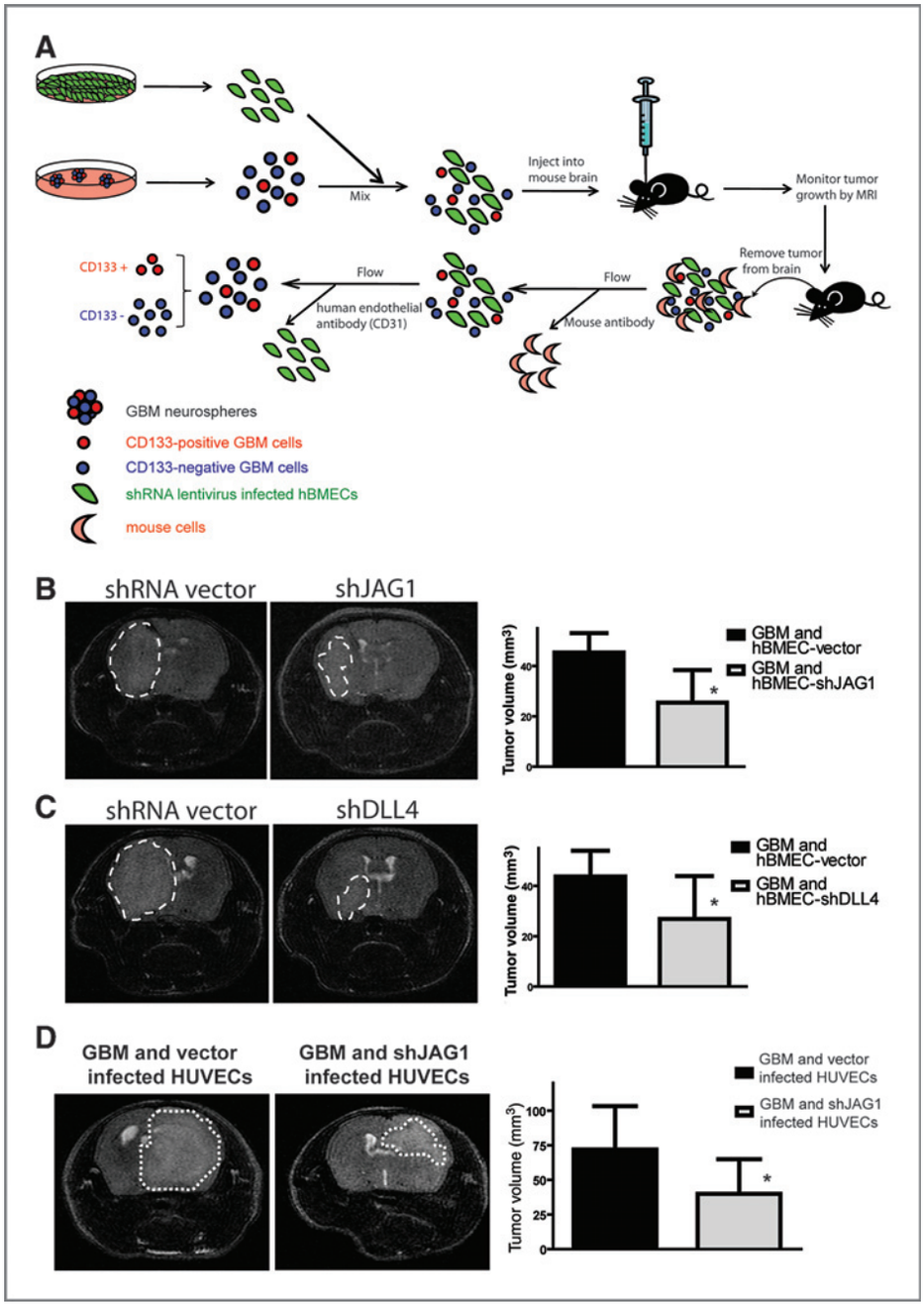

Figure 6. Knocking down JAG1 or DLL4 expression by shRNA in hBMECs inhibits growth of GBM intracranial xenografts derived from the mixture of GBM neurospheres and hBMECs. A, schematic showing the experimental approach used to examine if NOTCH ligands expressed in endothelial cells are essential for the growth of GBM intracranial xenografts derived from the mixture of GBM neurospheres and endothelial cells. B, MRI showed that the growth of GBM intracranial xenografts derived from a mixture of GBM neurospheres and shJAG1 infected hBMECs was much slower than those derived from a mixture with empty vector infected hBMECs ($n = 10$ per cohort, *, $P < 0.01$, t test). C, MRI scanning showed that the growth of GBM intracranial xenografts derived from a mixture of GBM neurospheres and shDLL4 infected hBMECs was much slower than those derived from a mixture with empty vector infected hBMECs ($n = 10$ per cohort, *, $P < 0.05$, t test). D, growth of GBM intracranial xenografts derived from a mixture of GBM neurospheres and a second type of endothelial cells (HUVECs) infected with shJAG1 was also significantly slower than those derived from a mixture with empty vector infected HUVECs ($n = 5$ per cohort, *, $P < 0.05$, t test).

NOTCH signaling pathway plays a critical role in regulating niche-dependent self-renewal of NSC in SVZ (36, 37). Furthermore, there is some experimental evidence showing that GBM may originate from SVZ NSCs (38). Consistent with the normal stem cell niche, it has also been shown that endothelial cells function as a cancer stem cell niche to promote CD133+ CSLC growth in GBM and medulloblastoma (16). Here, we show that the endothelial niche provides NOTCH ligands to GBM CSLCs to activate NOTCH signaling for their self-renewal. Together, these data indicate that GBM CSLCs share a common feature with their normal cognate in not only being endothelial niche dependent, but also NOTCH pathway dependent.

Although providing NOTCH ligands could be a mechanism by which endothelial niche maintains GBM CSLCs, the cause of expression of NOTCH ligands in the endothelial niche in GBM is still unclear. It has been shown that CD133-positive GBM CSLCs induce VEGF expression which may contribute to angiogenesis or endothelial niche formation in GBM (39). Previous studies have also shown that VEGF induces DLL4 expression in both physiologic and pathologic angiogenesis

Published OnlineFirst July 25, 2011; DOI: 10.1158/0008-5472.CAN-10-4269

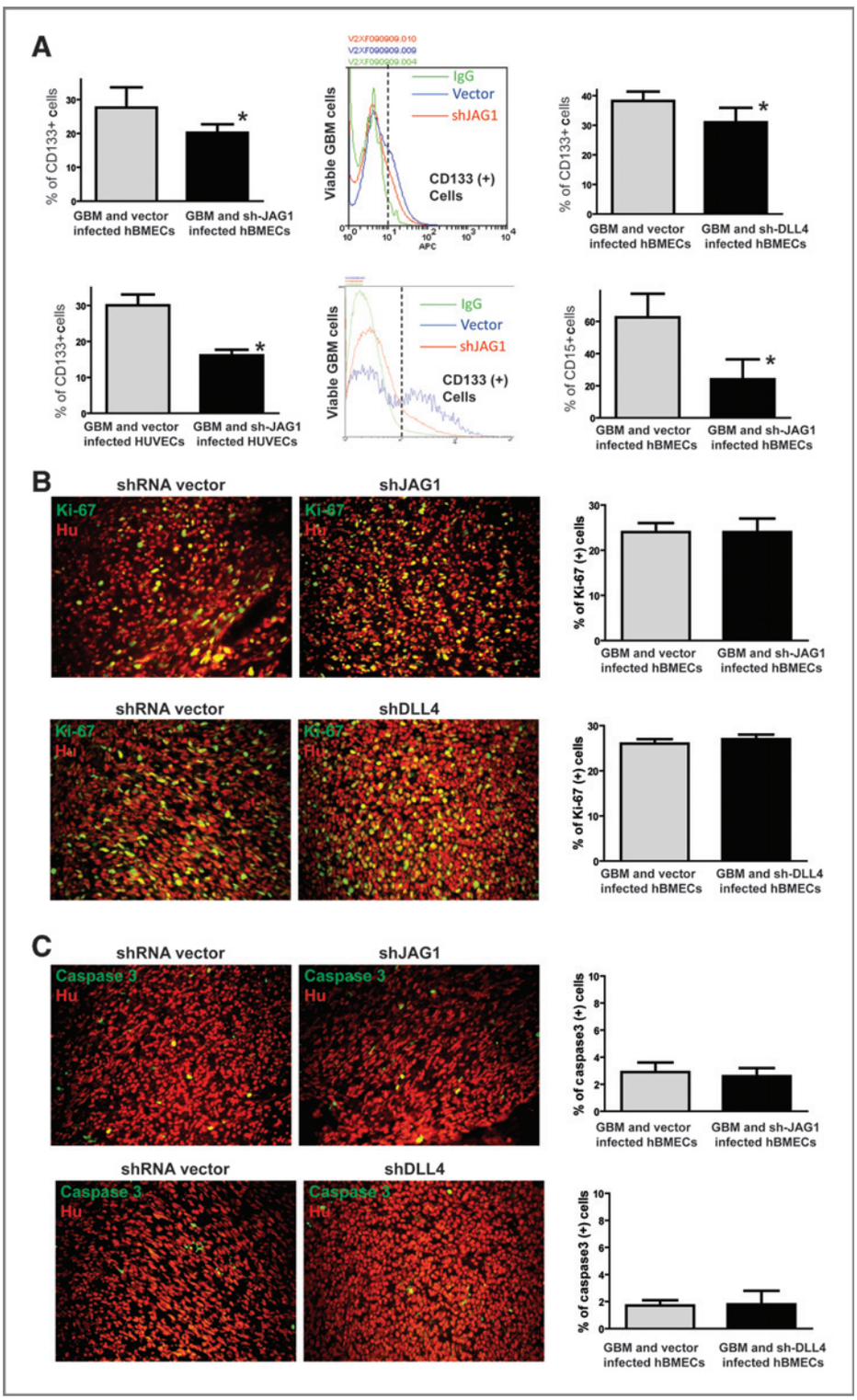

Figure 7. CSLC population is reduced in GBM xenografts derived from mixture of GBM neurospheres and hBMECs with ligand knockdown. A, intracranial xenografts derived from the mixture of GBM neurospheres and hBMECs were dissected and dissociated into single cell suspension to examine the CD133-positive population by flow cytometry. CD133-positive population was significantly decreased in the xenograft from GBM neurospheres and shJAG1 or shDLL4 infected hBMECs compared with the xenograft from GBM neurospheres and vector infected hBMECs (top). CD133-positive population was also significantly decreased in the xenograft from GBM neurospheres and shJAG1–infected HUVECs compared with the xenograft from GBM neurosphere and vector-infected HUVECs (bottom left and middle). In addition, CD15-positive population was also significantly decreased in the xenograft from GBM neurospheres and shJAG1 infected hBMECs compared with the xenograft from GBM neurospheres and vector infected hBMECs (bottom right). B, immunofluorescent staining of Ki-67 in the xenografts derived from the mixture of GBM neurospheres and hBMECs with JAG1 knockdown (top) or DLL4 knockdown (bottom) showed no proliferation changes. C, immunofluorescent staining of cleaved caspase 3 in the xenografts derived from the mixture of GBM neurospheres and hBMECs with JAG1 knockdown (top) or DLL4 knockdown (bottom) showed no apoptosis changes.

(40, 41). It is, therefore, possible that expression of NOTCH ligands in endothelial cells originates from GBM CSLCs through the VEGF pathway. In addition, endothelial cells may not be the only source of NOTCH ligands to GBM CSLCs. Our data show that some primary GBM cells also express DLL1 and differentiated tumor cells have elevated DLL1 expression compared with GBM neurospheres, indicating that differentiated tumor cells may also function as niche cells for

Published OnlineFirst July 25, 2011; DOI: 10.1158/0008-5472.CAN-10-4269

the maintenance of CSLCs. Further experimental evidence is needed to address this point.

The endothelial niche maintaining GBM CSLC self-renewal could be through multiple ways. In this study, we show that endothelial cells function as a stem cell niche for GBM CSLCs by directly providing NOTCH ligands. However, a recent study shows that nitric oxide (NO) released from tumor endothelium diffuses to neighboring glioma stem-like cells and activates the NOTCH pathway within these stem-like cells in a PDGF-induced mouse GBM model (42). It is, therefore, possible that activation of NOTCH signaling in GBM CSLCs maybe through both NOTCH ligand dependent and independent ways. In addition, 2 recent studies report that activation of HIF2α and PI3K pathway are required for CSLC growth within the endothelial niche in GBM and medulloblastoma (43, 44). These data not only further support the hypothesis that CSLCs share a common feature with their normal cognates in terms of dependence on an endothelial niche, but also suggest that the interaction between CSLCs and their endothelial niche could be through multiple mechanisms.

If GBM CSLCs reside within endothelial niche to self-renew, targeting both CSLC and its niche may be necessary to eliminate CSLCs. Indeed, inhibition of angiogenesis by VEGF blocking antibody, bevacizumab, has shown encouraging results in recurrent GBM patients (45). One possible reason is that bevacizumab depletes tumor blood vessels and self-renewing CSLCs in GBM (16). However, given the evidence of heterogeneity of GBM (46, 47), VEGF resistant GBM cells may develop. Therefore, targeting endothelial stem cell niche by NOTCH ligand blocking antibodies may be another alternative approach to treat GBM patients. Indeed, recent reports show that DLL4 blocking antibody reduces growth of tumor which are resistant to VEGF blocking antibody therapy (48–50). Thus, targeting the NOTCH ligand-dependent endothelial niche may be a novel way to treat GBM patients, particularly for those patients who are resistant to VEGF inhibitor therapy.

In summary, we have shown that endothelial cells function as a CSLC niche by providing NOTCH ligands to CSLCs for self-renewal. Targeting of CSLCs and stem cell niche via NOTCH inhibition and NOTCH ligand blockade may provide an innovative approach for GBM treatment, as niche cells and the NOTCH signaling pathway are essential for self-renewal of GBM CSLCs. Although the current study focuses on GBM, this approach may have relevance to multiple forms of cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was supported by Accelerate Brain Cancer Cure Project Award, American Brain Tumor Association Translational Grant, and Voices against Brain Cancer Research Grant to X. Fan.

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Received November 24, 2010; revised July 5, 2011; accepted July 14, 2011; published OnlineFirst July 25, 2011.

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Published OnlineFirst July 25, 2011; DOI: 10.1158/0008-5472.CAN-10-4269

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

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Endothelial Cells Create a Stem Cell Niche in Glioblastoma by Providing NOTCH Ligands That Nurture Self-Renewal of Cancer Stem-Like Cells

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Cancer Res 2011;71:6061-6072. Published OnlineFirst July 25, 2011.