Inhibitor of differentiation 4 drives brain tumor-initiating cell genesis through cyclin E and notch signaling

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Supplemental materials and methods

Serial passage of neural stem cell culture
For a serial passage culture to determine self-renewal capacity, spheres were collected and enzymatically dissociated using Trypsin-EDTA (10X, Sigma), and the dissociated single cells were then replated at a density of 200 cells/24-well plate. This step was repeated three times.

Plasmid, shRNA construction and retroviral infection
Cells were infected with retrovirus produced from the PT67 amphotropic packaging cell line (Clontech) transfected with retroviral vectors (pWZL-Id4-Blast, pWZL-NIC, and pBabe-cyclin E-puro). Cells were also infected with retrovirus with cyclin E-shRNA, Notch1-shRNA, Jagged1-shRNA, and Id4-shRNA, which were cloned into pSuperRetro-Puro, according to the instructions of the manufacturer (Oligoengine). The target sequences were mouse cyclin E-shRNA: aagacattctggatgagttac; mouse Notch1-shRNA: aagtcattctggatgagttac; and mouse Jagged1-shRNA: aagaccaacaacatcttctgc;
human Id4-shRNA-2: aagagagagagaggaagaaga.

**mRNA and protein analysis**
For semi-quantitative RT-PCR, a portion (1 ul) of the RT reaction was used to amplify Id4, EGFR, cyclin E, GFAP, Nestin, Cd133, Notch1, Jagged1, DLL1, PSN1, Hes1, Hes6, Hey1, Hey2, and GAPDH fragments using the corresponding gene-specific primer sets. Real-time RT-PCR was conducted using the iCycler IQ (Bio-Rad) and IQ Supermix with SYBR-Green (Bio-Rad). For Western blot analysis, protein in the extracts (30-100 ug) was incubated with anti-Id4 (H-70, Santa Cruz Biotechnology), anti-cyclin A (C-19, Santa Cruz Biotechnology), anti-cyclin D1 (H-295, Santa Cruz Biotechnology), anti-cyclin E (C-19, Santa Cruz Biotechnology), anti-pRB (Ser795, Cell Signaling), anti-CDK2 (M2, Santa Cruz Biotechnology), anti-CDK4 (C-22, Santa Cruz Biotechnology), anti-p27Kip1 (C-19, Santa Cruz Biotechnology), anti-Jagged1 (c-20, Santa Cruz Biotechnology), anti-Notch1 (S-20, Santa Cruz Biotechnology), anti-cleaved Notch1 (Val1774, Cell Signaling), and anti-α-tubulin (Sigma) antibodies.

**Subcutaneous and orthotopic implantations for tumorigenicity assay**
For the subcutaneous implantation assay, cells (1X10^6) were subcutaneously transplanted into nude mice (BALB/c nu/nu). Subcutaneous tumors were grossly visible at the injection sites after 10 days. For the orthotopic implantation assay, different numbers of cells (1X10^3, 1X10^4, and 5X10^4) were stereotactically injected into the left striata of nude mice (BALB/c nu/nu) (coordinates: anterior-posterior, +2; medial-lateral, +2; dorsal-ventral, –2 mm from the Bregma). All mouse experiments were approved by the animal care committee at the College of Life Science and Biotechnology, Korea University, and were performed in accordance with government and institutional guidelines and regulations.

**Luciferase reporter gene activity assay**
Notch transcriptional activity in the Ink4a/Arf wild-type astrocytes infected with pWGL-Id4-Blast, pWGL-NIC-Blast, and their control counterpart cells was determined by analyzing the relative luciferase activities of the pGL3-CSL plasmid using the Dual-Glo Luciferase Assay System (Promega). Transfection efficiency was normalized with the activity of Renilla luciferase, according to the instructions of the manufacturer (Promega).

**Immunofluorescence**
Cells fixed with paraformaldehyde were incubated with primary anti-cyclin E, anti-Nestin (MAB353, Chemicon), anti-GFAP (MP Biomedicals Immuno™), anti-Tuj1 (CBL412, Chemicon), anti-mouse Cd133 (prominin-1Ab, MACS), anti-human CD133 (AC133, MACS), anti-Sox2 (H-65, Santa Cruz Biotechnology), anti-Notch1, anti-CD31 (550274, BD), anti-NG2 (AB5320, Chemicon), and anti-Hes1 (AB5702, Chemicon) antibodies for 12 h at 4°C. Nuclei were then stained with DAPI (1 ug/ml) for 5 min. Whole sphere colonies were placed into a cryoprotective solution (20% sucrose in phosphate buffer) and sectioned at 10 um. Cryomicrodissected spheres were subject to an immunofluorescence assay using anti-Nestin and anti-Cd133 antibodies. Frozen brain tumor tissue slides (12-16 um) were incubated with the following antibodies: anti-Nestin, anti-Tuj1, anti-Cd133, anti-CD31, and anti-NG2 antibodies. Fluorescence images were obtained using a confocal laser scanning microscope (LSM5 Pascal, Carl Zeiss).

Tumor microarray and immunohistochemistry
The human GBM samples were collected from patients who had provided standard procedure consent. The tissue collection protocol was reviewed by the Institutional Review Board (IRB) for Human Research at the Samsung Seoul Hospital, SungKyunKwan University. Tissue microarrays containing 0.6-mm tissue cores of each sample (28 normal and tumor pairs and 40 tumor tissues) were constructed using an automated microarrayer (Beecher Instruments). Five-micrometer-thick sections of the TMAs were used for immunohistochemical studies. Immunohistochemistry experiments were performed using anti-Id4 antibody, according to the methods provided in the Vectastain ABC Kit (Vector).

Statistics
Data were statistically analyzed using a two-tail Student’s t-test. The level of statistical significance stated in the text was based on p values. p<0.05 was considered statistically significant.
Supplemental figure legends

Supplemental Fig. S1. Oncogenic transformation of Ink4a/Arf\(^{-/-}\) astrocytes by Id4 that is over-expressed in glioblastoma multiforme (GBM) tumors and GBM cell lines.

(A) Over-expression of Id4 mRNA in primary human GBM tumors was determined by semi-quantitative RT-PCR. Overexpression of EGFR mRNA was determined as a positive control for GBM tumors.

(B) Over-expression of Id4 mRNA and protein in normal human astrocytes (NHA) and human neural stem cell (HB1.F3), and GBM cell lines were determined by semi-quantitative RT-PCR and Western blot analysis, respectively. GAPDH and α-tubulin was used as a loading control.

(C) Tumor microarray using paired normal and tumor samples from 68 GBM patients (28 normal and tumor pairs and 40 tumors). Representative photos (10X and 40X) showing Id4 protein expression in normal brain and tumor regions from the same GBM patient. The expression level of Id4 protein showed relative heterogeneity within the same tumor sample (High and Low).

(D) In vitro growth rate of vector-transduced Ink4a/Arf\(^{-/-}\) astrocytes and Id4-transduced Ink4a/Arf\(^{-/-}\) astrocytes. Expression levels of Id4 in these cells were determined by Western blot analysis (inset).

(E) Tumor growth rate in the nude mice injected subcutaneously (n=6) with vector-transduced Ink4a/Arf\(^{-/-}\) astrocytes and Id4-transduced Ink4a/Arf\(^{-/-}\) astrocytes. Single asterisk indicates p<0.01.

Supplemental Fig S2. Distribution of Id4\(^{+}\) or CD31\(^{+}\) cells in the paired normal and tumor sections derived from human GBM specimen.

Immunofluorescence images showing Id4\(^{+}\) cells (green) or CD31\(^{+}\) endothelial progenitor cells (red) in a pair of normal and tumor sections of human GBM specimen.

Supplemental Fig. S3. Expression of Id4 during mouse brain development and development of glioma by injection of Id4-transduced Ink4a/Arf\(^{-/-}\) astrocytes.

(A) Expression of Id4 mRNA in different cerebral cortex developmental stages was determined by Northern blot analysis. Expression of GFAP (astrocyte marker) and Nestin (neural stem or progenitor cell marker) mRNAs was determined by semi-quantitative RT-PCR. E: embryonic day, P: postembryonic day. GAPDH as a loading control.
(B) Representative immunofluorescence images (40X) showing expression of S100β (astrocyte marker, Red) in vector-transduced Ink4a/Arf−/− astrocytes and Id4-transduced Ink4a/Arf−/− astrocytes. Nuclei are stained with DAPI (Blue).

(C) Relative cell populations that are CD133+/CD133−; Sox2+/Sox2−; Nestin+/Nestin− cells in the vector-transduced Ink4a/Arf−/− astrocytes and Id4-transduced Ink4a/Arf−/− astrocytes. Representative flow cytometry data showing expressions of neural stem cell markers (CD133, Sox2, and Nestin) in the vector-transduced Ink4a/Arf−/− astrocytes (Black) and Id4-transduced Ink4a/Arf−/− astrocytes (Purple).

Supplemental Fig. S4. Tumor formation in mouse brain by orthotopic injection of Ink4a/Arf−/− astrocytes-Id4-EGFP.

(A) Whole brains of nude mice injected with 50,000 Ink4a/Arf−/− astrocytes-Con-EGFP (no tumor) and Ink4a/Arf−/− astrocytes-Id4-EGFP were paraffin-sectioned and visualized by H/E staining. Arrows indicate injection sites.

(B) Representative immunofluorescence images (40X) showing expression of EGFP in the mouse brain injected with Ink4a/Arf−/− astrocytes-Con-EGFP and Ink4a/Arf−/− astrocytes-Id4-EGFP.

Supplemental Fig. S5. In vivo differentiation of Id4-transduced Ink4a/Arf−/− astrocytes to Tuj1+ and NG2+ cells in tumor tissues

(A) Immunofluorescence images (100X) showing Nestin+ cells (red) or Tuj1+ neuronal cells (green) or merge; Nestin+ cells (red) or NG2+ oligodendrocyte cells (green) or merge; Nestin+ cells (red) or CD31+ endothelial cells (green) or merge in brain tissues derived from orthotopically injected vector-transduced Ink4a/Arf−/− astrocytes (no tumor sections). Nuclei were stained with DAPI (blue).

(B) Immunofluorescence images showing Nestin+ (red), Cd133+ (red), or Tuj1+ cells (green) in tumor tissues derived from subcutaneous injection of Id4-transduced Ink4a/Arf−/− astrocytes.

Supplemental Fig. S6. Co-localization of Id4+ and cyclin E+ cells in the human GBM specimens.

(A) PCNA+ cells in tumors derived from Id4-transduced Ink4a/Arf−/− astrocytes, cyclin E-transduced Ink4a/Arf−/− astrocytes, and cyclin E shRNA-transduced Ink4a/Arf−/− astrocytes were determined by immunohistochemistry using anti-PCNA antibody.

(B) Immunofluorescence images showing Id4+ cells (green) or cyclin+ cells (red) in two human GBM specimens (#314 and #268 patients).
Supplemental Fig. S7. Expression of active Notch1 and Hes1 in control and Id4-transduced Ink4a/Arf<sup>-/-</sup> astrocytes, as well as co-localization of Id4 and Notch1 in the paired normal and tumor sections derived from human GBM specimen.

(A) Representative immunofluorescence images of active Notch1 and Hes1 proteins, which were upregulated in the Id4-transduced Ink4a/Arf<sup>-/-</sup> astrocytes.
(B) Immunofluorescence images showing Id4<sup>+</sup> cells (green) or Notch1<sup>+</sup> cells (red) in a pair of normal and tumor section (#159 patient) as well as one tumor section (#355 patient) of human GBM specimens.

Supplemental Fig. S8. Expression of active Notch1 in the various Ink4a/Arf<sup>-/-</sup> astrocytes.

Representative immunofluorescence images of active Notch1 (red) in the Ink4a/Arf<sup>-/-</sup> astrocytes-Puro, Ink4a/Arf<sup>-/-</sup> astrocytes-Id4+Con, Ink4a/Arf<sup>-/-</sup> astrocytes-Id4+Notch1-shRNA, and Ink4a/Arf<sup>-/-</sup> astrocytes-NIC.

Supplemental Fig. S9. Inactivation of Notch signaling by Jagged1-knockdown and DAPT treatment renders a loss of neural stem cell properties in Id4-transduced Ink4a/Arf<sup>-/-</sup> astrocytes.

(A) Loss of neural stem cell properties by depletion of Jagged1 expression in Id4-transduced Ink4a/Arf<sup>-/-</sup> astrocytes using a Jagged1-shRNA system. Number of neurospheres (>10 and >20 um) generated from Ink4a/Arf<sup>-/-</sup> astrocytes-Puro, Ink4a/Arf<sup>-/-</sup> astrocytes-Id4, and Ink4a/Arf<sup>-/-</sup> astrocytes-Id4+Jagged1-shRNA (4,000 cells/24-well plate) grown in NSC culture conditions for 10 days (right graph). Representative photos (40X) showing the morphologies of cells grown in neural stem cell cultures (left photos). Single asterisk indicates p<0.01.
(B) Expression levels of Jagged1, Notch1, Sox2, Nestin, and Cd133 mRNAs in Ink4a/Arf<sup>-/-</sup> astrocytes-Puro, Ink4a/Arf<sup>-/-</sup> astrocytes-Id4, and Ink4a/Arf<sup>-/-</sup> astrocytes-Id4+Jagged1-shRNA were determined by semi-quantitative RT-PCR.
(C) Decreased neurosphere formation in the Id4-transduced Ink4a/Arf<sup>-/-</sup> astrocytes due to treatment with 1µM DAPT, a γ-secretase inhibitor. Representative photos (40X) showing the morphologies of vector-transduced Ink4a/Arf<sup>-/-</sup> astrocytes and Id4-transduced Ink4a/Arf<sup>-/-</sup> astrocytes treated with DMSO and DAPT (right panel). Neurosphere numbers (>10 and >20 um) of Id4-transduced Ink4a/Arf<sup>-/-</sup> astrocytes (4,000 cells/24-well plate) treated with DMSO and DAPT (left graph). Single asterisk indicates p<0.01.
Supplemental Fig. S10. Notch signaling and cyclin E expression are regulated by Id4 in the human GBM cell lines.

(A) Depletion of Id4 using one Id4-specific shRNA [Id4-RNAi2; GenBank NM_001546 5’-aagagagagagaggaagaaa-3’ (1001-1021)] in human LN229 glioma cells led to downregulation of NIC, Jagged1, and cyclin E proteins as well as Jagged1 and Notch-downstream genes (Hes1, Hey1, and Hey2). Id4-RNAi1 [NM_001546 5’-aacaagaaagtcagcaaagtg-3’ (622-642)] was not shown to deplete endogenous Id4 in the LN229.

(B) Relative Notch transcriptional activity in the LN228-pSuperRetro-puro and LN229-pSuperRetro-Id4-RNAi-puro was determined by Notch/CSL luciferase-reporter assay using pGL3-control and pGL3-CSL-control vectors.

(C) In vitro growth rates of LN228-pSuperRetro-puro and LN229-pSuperRetro-Id4-RNAi-puro were determined by a standard cell counting (left graph) and low-density seeding assays (right photo).

(D) Over-expression of Id4 in human A172 glioma cells induced increases of Jagged1, NIC, and cyclin E proteins.

(E) Cell proliferation and neurosphere formation of A172 cells was increased by over-expression of Id4.

(F) Notch transcriptional activity in the A172 cells was elevated by over-expression of Id4.

(G) Expression levels of Id4, NIC, Jagged1, and cyclin E were increased in primary human glioma stem cells (NCI0822) compared to NHA (normal human astrocyte) and HB1.F3 (human neural stem cell line). Representative photos show cell morphology of NHA, HB1.F3, and NCI0822 grown in the NSC culture conditions (DMEM/F12+EGF+bFGF).

(H) Tet-On inducible expression of Id4 in the Ink4a/Arf−/− astrocytes led to induction of cyclin E, Jagged1 and NIC expressions.
A

Glioblastoma Multiforme

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B

Glioblastoma cell lines

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C

10 X

40 X

High

Low

GBM region

Normal brain region

D

Cell numbers (X10^3)

Days in culture

E

Tumor volume (cm^3)

Days post injection

Ink-/-ast-Id4

Ink-/-ast-Con

α-tubulin

*
Jeon-Supplemental Fig. S2

159-Normal

Id4

CD31

DAPI

Merge

159-Tumor

Id4

CD31

DAPI

Merge
Jeon-Supplemental Fig. S3

A  

Cerebral cortex

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Id4

GAPDH

GFAP

Nestin

GAPDH
Jeon-Supplemental Fig. S4

A

H/E staining

Ink-/− ast-EGFP

Ink-/− ast-Id4-EGFP

B

IF: EGFP/DAPI

Ink-/− ast-EGFP

Ink-/− ast-Id4-EGFP
**A**

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No tumor in orthotopic injection (Ink4a/Arf−/− astrocyte-Id4)

**B**

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Subcutaneous implantations (Ink4a/Arf−/− astrocyte-id4)
A  
IHC of tumor sections with α-PCNA

Ink/-/- ast-Id4  
Ink/-/- ast-cycE  
Ink/-/- ast-Id4-cycE-RNAi

B  
Id4  
cyclin E  
DAPI  
Merge

314-Tumor

268-Tumor
A

Notch1/DAPI

Hes1/DAPI

Ink-/ast Con

Ink-/ast Id4
IF: Notch/DAPI

Ink-/-ast puro

Ink-/-ast Id4+Con

Ink-/-ast Id4+Not-Ri

Ink-/-ast NIC
Jeon-Supplemental Fig. S9

A

Puro

Id4

Id4-Jgd1-Ri

B

Ink/- astrocyte

Id4+

Con Id4 Jgd1-Ri

Jagged1

Notch1

Sox2

Nestin

Cd133

GAPDH

C

Sphere number of Ink/-ast-Id4

Sphere number of Ink/-ast-Puro

Sphere number of Ink/-ast-Id4-Jgd1-Ri

DMSO DAPT

>20 um

>10 um

>20 um

>10 um

0

10

20

30 40

50

60 70

80

90

100

Neurosphere size

Sphere number

DMSO

DAPT

Con

Id4

*