Rapid reprogramming of primary human astrocytes into potent tumor initiating cells with defined genetic factors

Supplementary Data

Supplementary Fig. S1. Supplementary Fig. 1 related to Fig.1 Transformation scheme and expression of external genes in transformed cells.

Supplementary Fig. S2. Supplementary Fig. 2 related to Fig. 1. GEO analysis of gene expression patterns of transformed human astrocytes.

Supplementary Fig. S3. Supplementary Fig. 3 related to Fig. 2. Chromosome aberration analysis and hTERT gene expression in transformed astrocytes.

Supplementary Fig. S4. Supplementary Fig. S4 related to Fig. 4. In vivo growth of transformed astrocytes in nude mice.

Supplementary Table S1. Source of antibodies used in the current study.

Supplementary Methods. Additional details of experimental methods used in this study.

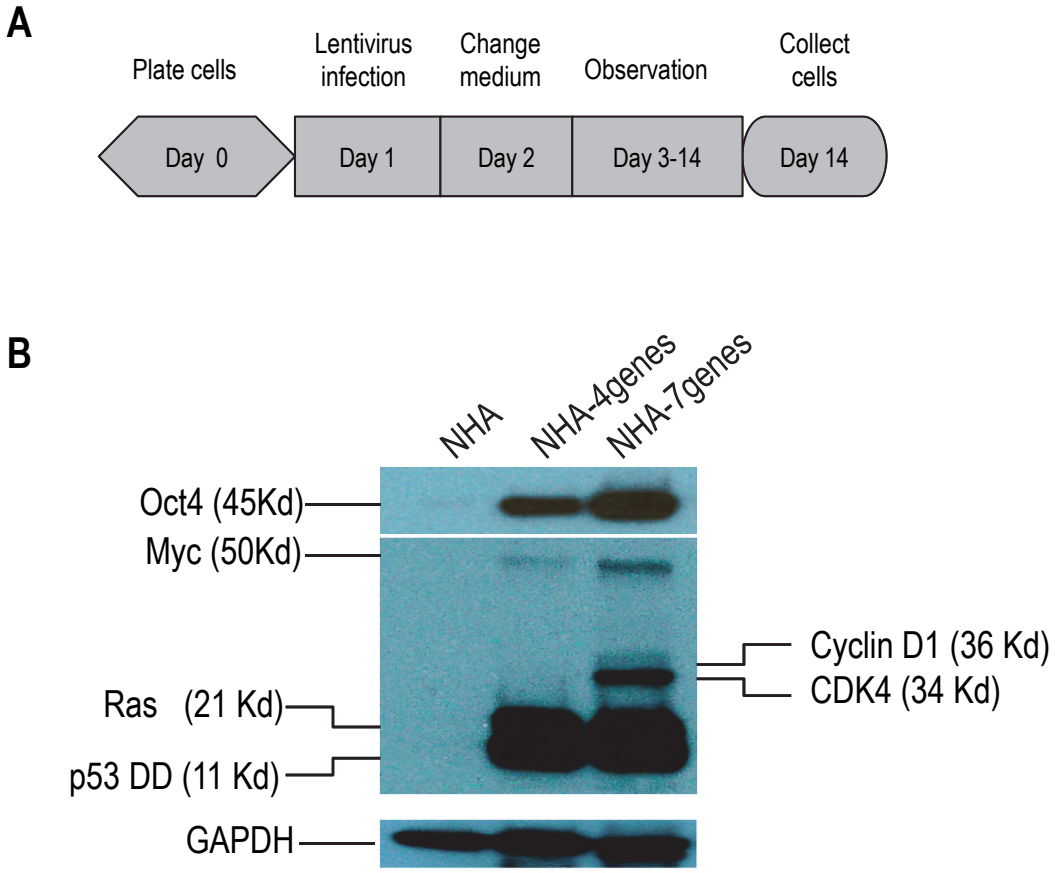

Fig. S1. Additional information on the transformation of primary human astrocytes.
A. Scheme for induction of transformation in primary human astrocytes.
B. Western blot analysis of expression of exogenous oncogenic factors in transformed astrocytes. NHA: normal human astrocytes. The oncogenic factors Myc, cyclin D1, CDK4, Ras, and p53DD were fused with an HA tag at its C-terminal end and were probed with an anti-HA tag antibody on the same blot (mid panel). On the other hand, the stem cell factor Oct4 and housekeeping protein GAPDH (loading control) were not tagged and probed separately with protein-specific antibodies (top and lower panels).

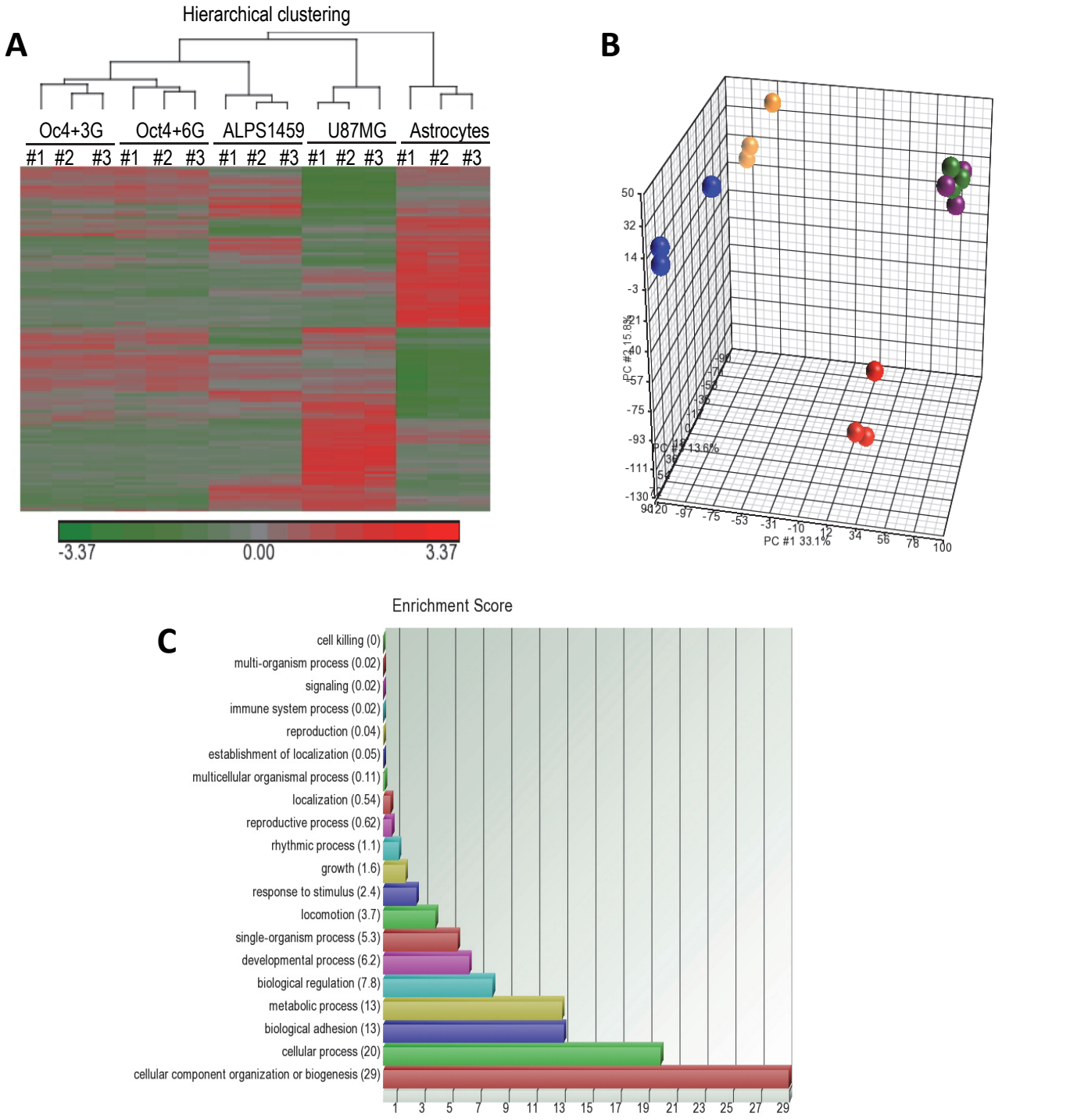

Fig. S2. Microarray analysis of reprogrammed/transformed astrocytes.
A. Cluster analysis of microarray gene expression data for Oct4+3G-, Oct4+6G-transformed astrocytes, ALPS1459 (a patient-derived human glioma stem cell line), U87MG, and control astrocytes. The three samples in each cell type are biological replicates.
B. Principal component analysis of the gene expression profiles of primary human astrocytes(•), 4G-transformed astrocytes (•), 7G-transformed astrocytes(•), ALPS1459 (a patient-derived glioma stem cell) (•), and U87MG(•). Three different cell populations from each cell type were analyzed for gene expression.
C. Biological pathways affected during the transformation process. The numbers in parenthesis represent the faction of genes among the ones that showed more than 2 fold difference in gene expression between OMRP-transduced and control astrocytes.

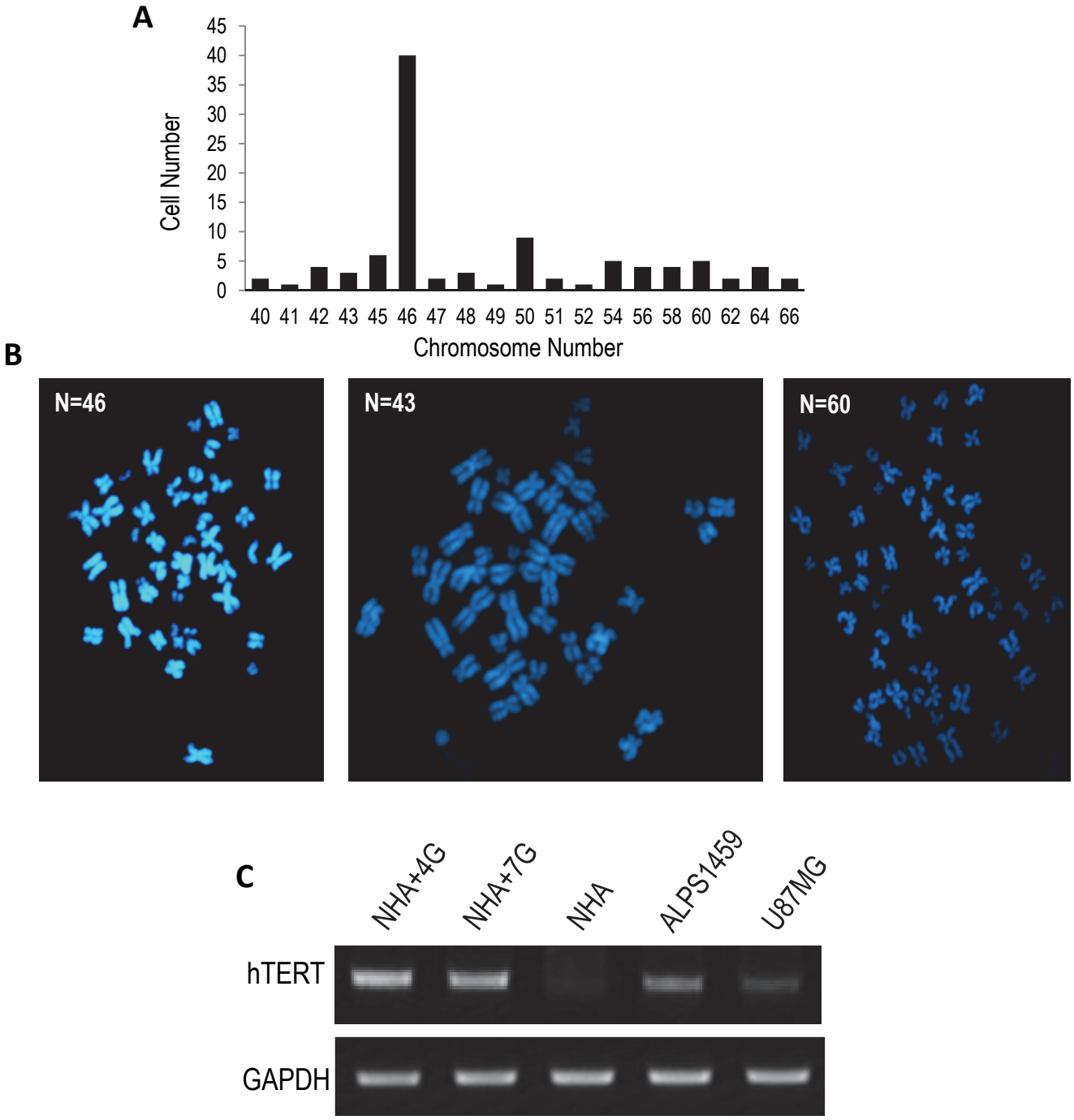

Fig. S3. Additional information on the characterization of transformed astrocytes.
A. Chromosome number analysis of chromosome spreads from 100 Oct4+6G-transformed astrocytes.
B. Examples of Oct4+6G-transformed cells with 46 (left), 43 (top right), and 60 (bottom right) chromosomes.
C. Semi-quantitative RT-PCR analysis of mRNA expression of human telomerase (hTERT) gene in control (NHA), OMRP transduced, and established glioma cells (ALPS1459 and U87MG) .

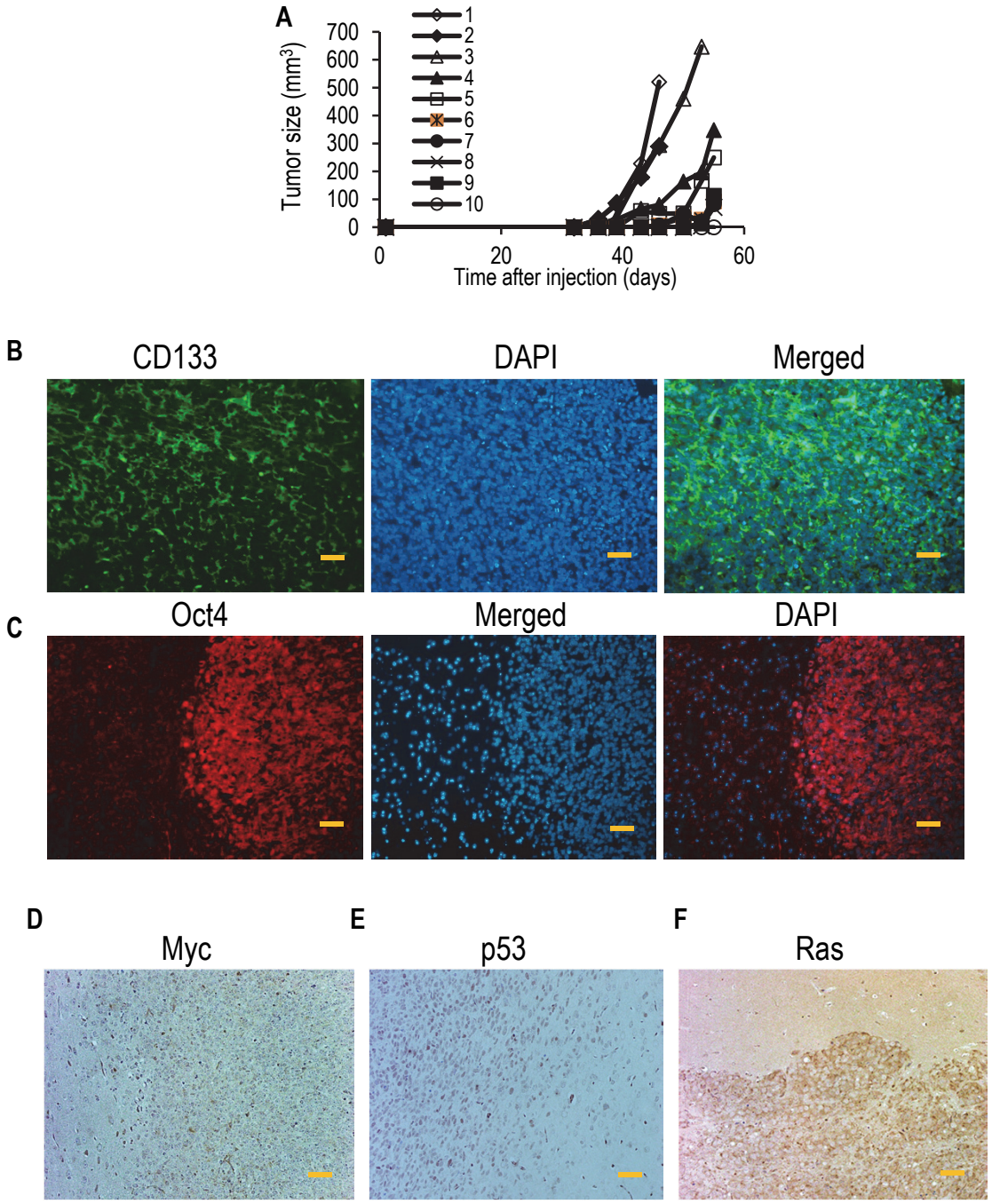

Fig. S4. Additional characterization of OMRP-transduced astrocytes *in vivo*.

A. Tumor formation in nude mice from 100 OMRP-transformed astrocytes injected subcutaneously in nude mice. Each of the mumbers represent a single mouse.

B. Immunofluorescence staining of CD133 in intracranial tumor tissues derived from OMRP-transduced astrocytes.

C. Immunofluorescence staining of Oct4 in intracranial tumor tissues derived from OMRP-transduced astrocytes.

D-F. Immunohistochemical staining of Myc, p53DD, and Ras in intracranial tumor tissues derived from OMRP-transduced astrocytes.

The scale bars in B-F represent 10 μm.

Supplementary Table S1. Antibodies used in this study.

Antibody name	Catalog	Company
Nestin (clone 10C2)	MAB5326	Millipore
IgM isotype	MABC008	Millipore
GFAP	AB5804	Millipore
Galc	MAB342	Millipore
Sox2	AB5603	Millipore
CD15	MAB4301	Millipore
Oct4	AB3209	Millipore
Oct 3/4 (N19)	sc-8628	Santa Cruz
CD133 (clone17A6.1)	MAB4399	EMD Millipore
TuJ1	MMS-435P	Covance
GFP	ab5450	Abcam
CD133	AB19898	Abcam
CD133/1 (AC133)-PE	130-080-801	miltenyibiotec
Mouse IgG1-PE	130-092-212	miltenyibiotec
CD90-PE	555596	BD Pharmingen
IgG1κ-PE	554680	BD Pharmingen
HA Epitope Tag	NB600-363	Novus
H-Ras Antibody (E-15)	sc-68743	Santa Cruz
P53(1C12)	2524	Cell Signaling
C-Myc	9402	Cell Signaling
Normal rabbit serum	011-000-001	Jackson Immunoresearch
biotin-SP-conjugated anti-mouse IgG	115-065-003	Jackson Immunoresearch
Peroxidase-conjugated affinipure goat anti-rabbit	111-035-003	Jackson Immunoresearch
Mouse IgG1	MAB002	R&D systems
Alexa Fluor® 555 anti-Mouse IgG	A-21427	Invitrogen
Alexa Fluor® 488 anti-Rabbit IgG	A-11008	Invitrogen

Supplementary Methods

Plasmids and lentivirus production

Most of oncogenes and stem cell genes used in this study were obtained from Addgene (Cambridge, MA). Among them, plasmids encoding human Oct-4, Sox2, Nanog, and Lin-28 genes were deposited by Dr. James A. Thomson of the University of Wisconsin (Madison, Wisconsin); plasmids encoding human Klf4 gene were deposited by Dr. Shinya Yamanaka of Kyoto University (Kyoto, Japan); plasmids encoding hTERT, MycT58A, KRasG12V, p53DD, Cyclin D1 and CDK4R24C were deposited by Dr. Christopher Counter of Duke University (Durham, NC). All of the above genes were transferred into the pLEX lentiviral vector (Open Biosystems, Huntsville, AL) by use of PCR-mediated subcloning.

To make lentivirus particles encoding the above genes, we used second generation lentiviral packaging plasmids psPAX2 and pMD2.G (both deposited at Addgene by Dr. Didier Trono of EPFL (Ecole Polytechnique Fédérale de Lausanne, Switzerland)). The pLEX plasmids encoding the individual genes were transduced into 293T cells together with packaging plasmids following a published, calcium phosphate-based Trono Lab protocol (http://tronolab. epfl.ch/ page58122.html). Supernatants were collected 48-72 hrs after transfection. The supernatants, which contain active recombinant lentiviral vectors, were then used in gene transduction experiments.

Microarray analysis

Total RNAs were extracted from cells by use of RNeasy kit (Qiagen, Alameda, CA). Microarray hybridization were conducted using Affymetrix® GeneChip® Microarrays (GeneChipRTM

Human Genome U133A 2.0, Affymetrics, Santa Clara, CA) by personnel at Microarray Core Facility of the Duke Institute of Genome Science and Policy. Microarray data were analyzed by use of the Parte**k** Genomics Suite 6.6 software **(**Partek Incorporated, St. Louis, MO) available through the Duke Microarray Facility.

Cell proliferation Assay

To obtain growth curves for the transformed cells, they were dissociated with 0.25% trypsin and the cells (1x 10^4/ml/well) were plated in 12-well tissue culture plates. Plated cells were harvested and counted using a Bio-Rad TC10 automated cell counter (Bio-Rad Laboratories**,** Hercules, CA) every day for 5 days.

Flow cytometry analysis of cell cycle distribution and cell surface marker expression

For cell cycle analysis, transformed cells were dissociated with 0.25% trypsin and EDTA (Invitrogen). A total of 1 x 10^6 of cells were harvested. The cells were washed twice with PBS and fixed in 70% ice-cold ethanol overnight. They were then spun down in centrifuge with ethanol decanted and resuspended in 0.5 ml PBS with the addition of 10 μl of propidium iodide (5 mg/ml) (Sigma, St. Louis, MO) and 10 μl of RNAase (10 mg/ml)(Sigma, St. Louis, MO). Cell cycle distribution of the cells was detected with FACScan Analyzer (BD Biosciences, San Jose, CA). For each sample a minimum of 1 x 10^4 cells were usually analyzed. Data were then analyzed with the Flowjo software package (Tree Star, Ashland, OR).

To detect expression of cell surface markers by flow cytometry, cells were stained for 10 minutes for PE-conjugated primary antibody or 20 minutes with unlabeled primary antibodies and then 20 minutes with fluorescent secondary antibodies, and analyzed by use of a flow cytometer.

Antibodies used for flow cytometry, immunofluorescence staining, immunohistochemical staining, and western blot analysis

Antibodies use in this study are listed in supplementary Table S1

TRAP assay for telomerase activities

The TRAPeze Telomerase detection kit (EMD Millipore, Cat#S7700, Darmstadt, Germany) was used to detect telomerase activities of control and transformed astrocytes. Manufacturer's protocol was followed to carry out the analysis.

Limited dilution assay.

Limiting dilution assay for tumor sphere formation assay was performed as described previously by Bellows and Aubin (reference 20) and Tropepe et al. (reference 21). Briefly, trypsinized individual cells were plated in 96-well tissue culture plates with 0.2 ml/well of neurosphere growth media. The final diluted average cell numbers ranged from 0.25 to 5 /well. Cultures were fed with 0.02 ml of media every other day until day 7. For each plate, the percentage of wells containing no tumor spheres was calculated (y-axis, log scale) and plotted against the average number of cells per well (x-axis). Regression lines were plotted and the equation derived. The average of number of cells (x-axis) that it takes so that 37% of wells in a plate have no tumor

spheres was calculated. Based on Poisson distribution, this number would represent the number of cells required to form one tumor sphere in every well on average.

Neurosphere differentiation assay

To evaluate of the ability of the transformed astrocyte-derived neurospheres to differentiate, free-floating neurospheres were centrifuged and dissociated with 0.05% trypsin into single cell suspensions. To differentiate into neurons, suspended single cells were plated on a polyornithine and laminin-coated culture dish (see next paragraph for coating procedures) in neuron differentiation medium (Neurobasal® medium, Invitrogen Cat#21103-049), supplemented with 2% B-27® Serum-Free Supplement(Invitrogen, cat# 17504) and 2mM GlutaMAX™-I (Invitrogen, cat# 35050). Medium was changed every 3-4 days. After 10 to 14 days, the emergence of neurons was then detected by immunofluorescence staining of TUJ1, a pan-neuron specific marker. To differentiate neurospheres into oligodendrocytes, sphere-derived single cells were plated on a polyornithine and laminin-coated culture dish in oligodendrocyte differentiate medium (supplementation of Neurobasal® medium with 2% B-27®, 2mM GlutaMAX™-I, and 30ng/ml T3 (Sigma, Cat# D6397)). After 10 to 14 days, the presence galactocerebroside (Galc) was detected by immunofluorescence staining. To differentiate neurospheres into astrocytes, sphere-derived single cells were plated on a Geltrex^{TM} coated culture dish in astrocyte differentiation medium (D-MEM (Invitrogen, Cat. no. 11995) with 1% N-2 (Invitrogen, cat# 17502) supplement, 2 mM GlutaMAX™-I, and 1% FBS). After 10 to 14 days, the cells were stained for the presence of GFAP through immunostaing.

Coating of cell culture plates with Geltrex™ Reduced Growth Factor Basement Membrane Matrix (Invitrogen, cat. no. 12760) was carried out following manufacturer's instruction. To coat the surface of the culture plates with Poly-L-Ornithine, the plates were incubated with 20 µg/ml poly-L-ornithine (Sigma, Cat# P3655) solution (2 mL for 35-mm dish) for 1 hour at 37°C. To coat the surface of the culture plates with laminin, the culture plates were incubated with 10 µg/mL Laminin (2 mL for 35-mm dish) for 2 hours at 37°C.

Immunofluorescence staining

To detect expression of intracellular proteins, cells grown on cover slip (MatTek Corporation, Ashland, MA) were fixed with 4% paraformaldehyde. They were then incubated in 5% goat serum with 0.2% Triton X-100 to block nonspecific binding and permeabilize membranes. They were subsequently stained overnight with primary antibodies and for 1 h with secondary antibodies. Coverslips were mounted on slides with mounting medium containing DAPI (Vector laboratories, Cat# H-1200, Burlingame, CA). Immunofluorescence was detected by use of a Zeiss Axiovision inverted microscope.

Immunohistochemical Study

Paraffin embedded, 5 µm formalin fixed tumor sections were mounted on microscope slides. Tissue sections were deparaffinized in xylene and rehydrated through graded alcohols. Antigen retrieval was performed by use of a microwave oven (heating in 10 mM sodium citrate (pH 6.0) for 10 mins). After blocking with 5% donkey serum, the sections were incubated with primary antibodies at 4ºC overnight and then with biotinylated secondary antibody 30 min, followed by incubation with streptavidin/peroxidase for 30 min at room temperature. Subsequently, the

sections were subjected to color reaction with 0.02% DAB (Cat#SK-4100, Vector Lab, Burlingame, CA) containing 0.005% H_2O_2 in PBS (pH 7.4), and counterstaining with hematoxylin lightly.

Temozolomide treatment

Cellular proliferation is evaluated by MTT assay using MTT cell proliferation assay kit (ATCC, Manassas, VA). Briefly, cells are plated in 96-well microtiter plates at a density of 2000 cells per well in a volume of 180 μL media. Temozolomide (Sigma) is prepared at 10 x the final assay concentration in media containing 1% DMSO. Twenty-four hours after cell plating, 20 μL of the 10x temozolomide at appropriate concentrations are added to plates in triplicate. The plates are assayed for cell numbers 5 days after the cells are plated. Percent inhibition is calculated by use of mock-treated cells as controls.