Experimental Procedures:

Intracranial injection, histology and whole brain imaging. H&E sections were obtained from the coronal plane of the injection site. Qualitative analysis was performed for the following features: Necrosis (yes/no), tumor neovascularity (yes/no), mitoses (frequent/moderate/few), cellularity (high/medium), hemorrhage (yes/small/no), infiltration (high/medium/low), pleomorphism (high/medium/low), fascicles (yes/no), mutinodular (yes/no) and tumor area in section (large/medium/small). Tumor grade was then assigned according to established WHO criteria (Fuller 2008) based on the analysis of tumor necrosis, vascular changes and mitoses. For analysis of global patterns of growth image stacks obtained using confocal laser scanning microscopy were reconstructed in a single plane to generate a maximal intensity projection image which provides a representation of global tumor growth patterns.

Invasion assay. Cells were resuspended in proliferation media supplemented with 10ng/ml of b-FGF and EGF and loaded into top inserts (5 x 10^4 cells/500 µl). Proliferation media supplemented with 40 ng/ml of β-FGF and EGF was added to the lower chamber (750µl). Following incubation for 20 hr at 37 °C, cells that migrated to the underside of the membrane were fixed, stained, digitally imaged and counted. Differences in cell invasion were expressed as the percent of invading 18 mo Tr-NSPCs relative to invading 3 mo Tr-NSPCs.

γH2AX detection. For western blot analysis membranes were blocked in 5% nonfat milk followed by incubation with γH2AX primary antibody (rabbit polyclonal anti-Ser139 #2577, Cell Signaling Technology, Inc, Danvers, MA) and ß-actin antibody (mouse monoclonal # A5441 Sigma-Aldrich, St. Louis, MO). Corresponding secondary

antibodies conjugated to HRP (Pierce, Rockford, IL) were incubated for one hour at room temperature and signal was visualized using SuperSignal substrate (Pierce). For detection of γH2AX cell foci cells cultured in 8 well slide chambers coated with fibronectin for 48 hr were incubated with γH2AX primary antibody, washed and incubated with secondary antibody conjugated with AlexaFluor561 (Invitrogen) followed by counterstaining with DAPI followed by Fluorescent microscopy (see supplemental information).

Micronucleus and cytogenetic analysis. Cell cultures were treated with 4.5 μg/mL cyctochalasin B for 24 hours prior to harvest then collected by trypsinization, pelleted by centrifugation for 6 min at 100 x g, and incubated in 5 mL of 75 mM KCl for 15 min at 37 °C. One mL of ice-cold fixative, made fresh of 25:1 methanol:acetic acid, was added to the pellet and the cells pelleted as above. Cells were washed twice in 5 mL fixative, resuspended in a small volume of fixative and dropped onto glass slides. Cells were stained with the "Diff Stain" kit (IMEB inc.). Slides were viewed under light microscope, 1,000x total magnification, and 500 binucleate cells were scored for each sample. Micronucleus index was calculated as the percent of binucleate cells containing one or more micronuclei. The number of micronuclei per aberrant cell was calculated by dividing the total number of micronuclei by the number of cells containing micronuclei. Duplicate experiments were run for each sample, and means were compared using an unpaired t-test, calculated with Graphpad InStat (Version 3.06; Graphpad Software). For cytogenetic analysis of transformed NSPCs exponentially-growing cultures were incubated in 200nM colcemid for 2 hours before collection by trypsinization. Cells were fixed as described for the micronucleus assay with one modification; the fixative was 3:1 methanol:acetic acid. Slides were prepared, stained and scored as previously described

(Schwartz *et al.* 1999). Fifty metaphase cells were scored for each culture. Chromosome-type and chromatid-type aberration frequencies were measured as well as changes in chromosome number (aneuploidy, tetraploidy).

Cell cycle (FACS) analysis, cell growth and differentiation. For FACS analysis $5x10^5$ cells/well were seeded in a 6 well plate and harvested after 48h hours. Isolated nuclei stained with DAPI were analyzed using BD Influx cell sorter (BD Biosciences) followed by cell cycle analysis using Multicycle AV software (Phoenix Flow Systems, San Diego CA). Cell culture doubling times were determined by plating cells in 12 well plates in triplicate in proliferation media (see below) and counting viable cell numbers by trypan blue exclusion manually (hemocytometer) or automated (Vi-cell; Beckman Coulter. Inc.). To achieve similar times to log-phase growth, the 3mo TR-NSPCs were plated at 50,000 cells per well and harvested on days 2 and 4-7 after plating and 18mo Tr–NSPCs (which required higher cell densities to achieve exponential growth) were seeded at 90,000 cells per well and harvested on days 2-5 for cell counting. Cell numbers were plotted on a log scale. The doubling time was calculated using a linear regression on the log-scale plot with log2/slope defining the doubling time. Differentiation potency was determined by culturing transformed cells of each age under differentiating conditions based on previous methods. Transformed NSPCs were plated in 24-well plates on glass coverslips coated with 10% poly-L-lysine (Sigma) overnight in proliferation medium. The growth medium was then replaced by differentiation medium, and cultures were maintained for 10 days. Differentiation medium consisted of neurobasal medium with 2mM glutamine, supplemented with 2% B27 + 20 ng/ml NT3 for neurons, 1% N2 + 10% fetal bovine serum for astrocytes, or 100ng/mL IGF-I + 50ng/mL PDGF + 50ng/mL bFGF for oligodendrocytes. Cells were fixed in 4% paraformaldehyde for 5 minutes, rinsed with PBS, and blocked with 5% donkey serum. Neurons were

labeled with anti-MAP2 mouse monoclonal antibody (M1406, Sigma, 1:500), astrocytes with anti-GFAP rabbit polyclonal antibody (Z0334, Dako, 1:700), oligodendrocytes with anti-CNPase mouse monoclonal antibody (C5922, Sigma, 1:500). After incubation with appropriate FTIC secondary antibodies and DAPI, images were captured from random fields and analyzed by recording the total number of cells and cells positive for each lineage marker. Because of variable degrees of cell survival in oligodendroglial differentiation media the percentage of CNPase positive cells was not included in the analysis.

Cell viability, apoptosis and BrdU incorporation assays. Cells were plated at a density of 10,000 cells per well in 96 well plates and irradiated with 20 Gy (1 Gy/min) using a Cs^{137} source and 48hr later, cell viability was determined using the Alamar Blue (resazurin) reagent (O'Brien *et al.* 2000). Cells maintained in 65 mm plates (6 x 105 cells/plate) were analyzed for the induction of apoptosis using AnexinV-Cy5 16 hr after irradiation using Fluorescence Activated Cell sorting (BD Biosciences, San Jose, CA). The impact of age on the sensitivity of cell viability to the alkylating drug temozolomide (TMZ) was evaluated using Alamar Blue after exposure to 0, 20, 100, 400 and 1000 μM doses of TMZ for 24 hr in 96 well plates. To determine cell viability after more prolonged exposures to hypoxia, cells were exposed to 1% O2 or ambient oxygen for 7 days. To determine the effects of growth in vivo on glioma cell intrinsic hypoxic responses, cells from tumors derived from 3mo and 18moTr-NSPCs were isolated and cultured as described above. To determine proliferation responses after exposure to hypoxia, cells were incubated in 1% O_2 or ambient oxygen for 48 hr, and then treated with 2 μM of BrdU for 30 min in triplicate followed by fixation in 4% PFA. Fixed cells were incubated with anti-BrdU antibody (#32323, Santa Cruz, Santa Cruz, CA) followed by immunoperoxidase detection and counterstaining with hematoxylin. At least 600 cells

were counted and BrdU LIs were calculated as the number of BrdU positive cells over total cell number.

DNA repair enzyme activity assays. DNA repair activities were assayed in the 10,000 x g supernatants of whole cell homogenates prepared by lysis with non-ionic detergents in the presence of 600 mM NcCl (Blank *et al.* 2004). MGMT activity (fmol O^6-[^{3}H]methylguanine transferred/10^6 cells) was measured by standard biochemical assay that quantifies transfer of [^{3}H]methyl groups from O^6-[^{3}H]methylguanine in DNA to protein (Silber *et al.* 1999). Apurinic endonuclease (Ap endo) activity (fmol abasic sites incised/cell/min) was assayed by quantifying conversion of plasmid DNA from super-coiled to relaxed form caused by incision at an abasic site (Bobola *et al.* 2001).

Bioinformatic analysis of microarray data. Raw microarray data for 100 WHO grade III and IV astrocytomas were downloaded from the NCBI GEO repository GSE4271 (Phillips *et al.* 2006), processed and analyzed with Bioconductor (Gentleman *et al.* 2004) and normalized with the RMA method as implemented in the Bioconductor Affymetrix package. From the normalized data, genes with significant evidence for differential expression between young (ages < 40yrs) and old (ages > 55yrs) were identified using the Bioconductor limma package (Gentleman *et al.* 2004). The list of HIF-1 regulated genes (HRGs) was obtained from Benita *et al*. (Benita *et al.* 2009) and matched to the list of age dependent differentially expressed genes found in the analysis above. P-values were calculated with a modified t-test in conjunction with an empirical Bayes method to moderate the standard errors of the estimated log-fold changes. P-values were adjusted for multiplicity using Bioconductor's implementation of the Benjamini-Hochberg p-value adjustment method, which allows for selecting statistically

significant genes while controlling the estimated false discovery rate. Survival data available for each age group was used to analyze survival curves and differences in median survival.

Quantitative RT-PCR. Cells (5 x 10^5 cells/ 65mm plate) were incubated overnight in 95%air/5%CO_2, before being subjected to hypoxic conditions (*i.e.*, 1% O_2) for 48 hr prior to harvesting. RNA isolation, reverse-transcription, thermocycling and relative quantification were carried out as previously described (Mikheeva *et al.* 2010). A summary of primer pairs used for each gene is provided in supplemental data (Table 3S).

HIF-1 promoter reporter assay. Cells were plated on the fibronectin coated 6 well plates (4 x 10^5 cells/well). The next day cells were co-transfected with HIF-1 luciferase reporter (Bhattacharya *et al.* 1999) and galactosidase expression plasmid for normalization of transfection efficiency using Fugene 6 reagent (Roche, Indianapolis, IN). After 24 hours incubation cells were transferred to hypoxia chamber for 8 hours. Cells were lysed followed by analysis of luciferase and ß-galactosidase (Mikheev *et al.* 2000). Results are presented as a ratio of the normalized HIF-1 reporter activity under hypoxic conditions relative to normalized activity at normoxia.

Fluorescent microscopy. Fluorescent microscopic images were captured with 20x lens on an Axiovert 200 inverted microscope (Carl Zeiss Microimaging, Thornwood, NY) equipped with a cooled CCD camera (SensiCam, Cooke Corp, Auburn Hills, MI). All images were identically processed with Slidebook™ imaging software (Intelligent Imaging Innovations, Denver, CO). Cells with 2 or more positive foci located in nuclei were counted and presented as percent positive cells (labeling index, LI).

Supplemental Figures:

Figure 1S. Representative photomicrograph of 3mo and 18mo Tr NSPC derived gliomas.

Figure 2S. Micronuclei analysis in 3mo and 18mo normal NSPCs. The number of binucleate cells with micronuclei, the average number of micronuclei per cell are and the distribution of the number of micronuclei per cell in are shown. Cells of each age were from equivalent passage number.

Figure 3S. Cytogenetic analysis of 3mo and 18mo Tr-NSPCs. The mean chromosome numbers, chromatid breaks, excahnges and aneuploidy and standard errors for 100 cells of each age are shown. Significant differences were seen between the 2 ages for chromatid breaks ($p=0.018$).

Figure 4S. Differentiation potential of transformed NSPCs. Transformed 3mo and 18mo NSPCs grown in proliferation media were transferred to defined lineage specific differentiation media for 10 days and then fixed and stained for lineage specific markers MAP2 (neurons), GFAP (astrocytes) and CNPase (oligodendroglia). Representative images of 3mo Tr-NSPCs (top panel) and 18mo Tr-NSPCs in each culture condition and stained with the appropriate marker (MAP2 left, GFAP middle and CNPase right) are shown. The number of cells staining for each marker under differentiating conditions was then recorded. Cells immunoreactive for CNPase with oligodendroglial morphology were seen for each aged Tr-NSPC (see images on right from upper and lower panel). However, increased and variable cell death for both cell types in oligodendroglia differentiation media precluded informative quantification. However, quantitation was

possible for MAP2 and GFAP which showed no significant age-dependent differences in neuronal and astrocytic differentiation potential, respectively. Of note, no MAP2+ cells are seen in undifferentiated cells grown in proliferation media. A small number of bipolar, spindled GFAP+ cells (approximately 10%) are present in proliferation media but no significant differences in their number were detected (data not shown). The morphology of GFAP+ cells under non-differentiating conditions suggests these cells could represent GFAP+ neural progenitors while the GFAP+ cells generated after serum exposure (middle images) appear to be more typical of differentiated astrocytes.

Figure 5S. Differential expression of genes in young (<40yo) and aged (>55yo) human malignant glioma patients.

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