# SUPPLEMENTAL EXPERIMENTAL PROCEDURES

## Isolation of Glioma Stem Cells, Nonstem Glioma Cells, and Normal Neural Progenitors

Matched cultures enriched or depleted for GSCs were isolated from primary human brain tumor patient specimens or human glioblastoma xenografts as previously described (Bao et al., 2006a; Bao et al., 2006b) from patients providing informed consent under a protocol approved by the Duke University Institutional Review Board. Briefly, tumors were disaggregated by Papain Dissociation System (Worthington Biochemical) and filtered by 70μm cell strainer to remove tissue pieces according to the manufacturer's instructions (http://www.worthington-biochem.com/PDS/default.html). Cells were then cultured in stem cell culture medium supplemented as detailed below for at least four hr to recover surface antigens. Cells were then labelled with an allophycocyanin (APC)- or phycoerythrin (PE)-conjugated CD133 antibody (Miltenyi Biotec), and sorted by fluorescence-activated cell sorting (FACS). Alternatively, cells were separated by magnetic sorting column using microbead-conjugated CD133 antibodies (Miltenyi Biotech). CD133 positive cells were designated as GSCs whereas CD133 negative cells utilized as nonstem glioma cells. The cancer stem cell nature of the CD133 positive cells was confirmed by serial neurosphere assays and tumor formation assays (data not shown). Of note, we confirmed that these tumors could be serially transplanted with similar efficiency but some change in tumor latency (Table S2, S3). In contrast, cultures depleted of cancer stem cells did not self renew and did not initiate tumors. Normal human neural progenitors were obtained from Lonza (see detailed information at: https://bcprd.lonza.com/group/en/products_services/products/catalog_new.ParSys.0007.File0.tmp?path=eshop/IMS_DOCS/DD/DD0FDB384FB3BBF1B5C900110A5E23B5.pdf).

**Real Time Polymerase Chain Reaction**

mRNA was extracted by RNeasy Mini Kit (Qiagen, Inc), followed by cDNA synthesis using SuperScript® III First-Strand Synthesis Kit (Invitrogen, Inc). To investigate single gene expression pattern, individual gene primers and Master Mixes were purchased from SuperArray Bioscience Corporation. For real time PCR array, 384 wells Human Hypoxia Signaling Pathway PCR Array Kits were purchased from SuperArray Bioscience Corporation (APHS-032E).

**Western Analysis**

Equal amounts of cell lysate were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore) and detected using an enhanced chemiluminescence system (Pierce Biotechnology). Rabbit Polyclonal Antibodies for human HIF1α (NB100-449), HIF2α (NB100-122) were purchased from Novus Biologicals, Inc. Anti-α-tubulin was purchased from Sigma-Aldrich.

**VEGF Enzyme-Linked Immunosorbant Assay (ELISA)**

Glioblastoma stem and nonstem cells were infected by lentivirus to knockdown HIF1α, HIF2α, or nontargeting control sequences for 48 hr before medium was removed. Cells were cultured in parallel with fresh neural basal medium or same medium with 200 μM DFX for 24 hr and conditioned media were then collected. VEGF ELISA was performed with the Human VEGF ELISA Kit (R&D Systems) according to manufacture's instruction.

**CellTiter Assay**

5000 lentiviral infected glioblastoma stem or nonstem cells isolated from the glioblastoma samples were put into each well of 96-well plates and cultured in normoxia or hypoxia . Cell titers were determined

after the indicated number of days using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega).

**Caspase-3/7 Activity Assay**

5000 lentiviral infected GSCs isolated from T3565, T3691, or T3956 specimens passaged short term in immunocompromised mice or directly from a T080714 specimen were placed in each well of 96-well plates. 48 hr after infection, Caspase-3/7 activity was analyzed by Caspase-Glo®-3/7 Assay Systems (Promega).

**VEGF Transcriptional Reporter Assays**

100,000 lentiviral infected glioblastoma stem or nonstem cells were transfected in parallel with 2 μg of empty pGL2basic luciferase reporter vector as control or pGL2 basic containing the full length (2.7 kb) VEGF promoter upstream of luciferase (purchased from ATCC). Transfection efficiency was controlled by simultaneously transfecting 0.1 μg of a renilla luciferase reporter (phRL-null vector; Promega). After transfection, parallel cultures were subjected to either normoxic or hypoxic conditions (200 μM DFX) for 24 hr. Luciferase activity was assessed using the Dual-Luciferase Reporter Assay System (Promega) with a Fluostar Optima luminometer (BMG Labtech). To confirm HIF knockdown, remaining cell lysate were analyzed for HIF1α and HIF2α expression by Western.

**Endothelial Cell Coculture Studies**

For transwell coculture studies, 10,000 HMVEC endothelial cells were planted into each well of 12-wells coculture plates (BD Biosciences). Transwell inserts containing 0.4 μm diameter membrane pores that allow the passage of diffusible molecules, but not cells, were then placed into each well and

50,000 lentiviral infected GSCs were put into inserts. After 72 hr, inserts were removed. HMVEC proliferation were measured through [$^3$H]-thymidine incorporation. In another independent set of experiments, HMVEC cells number were counted after cells were fixed by 4% PFA and then stained by toludine blue.

**Immunohistochemistry**

Paraffin-embedded primary human glioblastoma sections or intracranial glioblastoma xenograft underwent IHC staining for CD133 (ab19898/Abcam), Olig2 (kind gift from Dr. John Alberta), HIF2α (MAB3472/Chemicon), HIF1α (NB100-479/Novus), or CD31 (ab28364/Abcam) following protocol as previously described (Sathornsumetee et al., 2008). Citrate buffer (PH 6.1) was used for antigen retrieval in most cases except for HIF2α staining which used Tris-EDTA (PH9.0). To minimize background, CD133 and HIF2α staining were performed by alkaline phosphatase (AP) conjugated secondary antibody, while Olig2, HIF1α and CD31 staining were performed using horseradish peroxidase (HRP) conjugated secondary antibody.

**Immunofluorescence**

Double immunofluorescence staining of CD133 (Ab19898/Abcam) and HIF2α (MAB3472/Chemicon) were performed on primary human glioblastoma frozen sections. Briefly, slides were fixed by 4% paraformaldehyde for 15 minutes at room temperature and blocked by 10% goat serum. Slides were stained by CD133 and HIF2α antibodies for 16 hr at 4 degree followed by Rhodamine Red-X-AffiniPure Donkey Anti- Rabbit secondary antibody and Fluorescein (FITC) AffiniPure Donkey Anti Mouse secondary antibody (Jackson-Immunoresearch). At last, mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories) was used and pictures were taken by Leica SP5 Confocal microscope (Duke Light Microscopy Core Facility).

## SUPPLEMENTAL TABLES

| Specimen | Age | Gender | Tumor Stage | Histopathology | FISH | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Chromosome 7 | EGFR | Chromosome 10 | PTEN |
| T3359 | 31 | M | Newly Diagnosed | Glioblastoma | Polysomy 62% | Polysomy 70% | Polysomy 80% | Polysomy 57% |
| T3691 | 59 | F | Newly Diagnosed | Glioblastoma | Polysomy 77% | Polysomy 83% | Loss 54% | Loss 49% |
| T3565 | 32 | M | Newly Diagnosed | Anaplastic Astrocytoma | Polysomy 52% | Polysomy 59% | Polysomy 73% | Polysomy 76% |
| T3832 | 75 | F | Newly Diagnosed | Glioblastoma | Polysomy 82% | Polysomy 97% | Loss 70% | Loss 73% |
| T3946 | 65 | F | Newly Diagnosed | Glioblastoma | Polysomy 64% | Polysomy 61% | Loss 61% | Loss 64% |
| TB080066 | 11mo | F | Newly Diagnosed | Medulloblastoma | | | | |
| TB080076 | 68 | F | Newly Diagnosed | Glioblastoma | Polysomy 54% | Polysomy 62% | Polysomy 49% | Polysomy 43% |
| TB080090 | 60 | F | Recurrent | Glioblastoma | | Amplification 86% | Loss 65% | Loss 72% |

**Supplemental Table 1. Characteristics of Brain Tumor Patient Specimens.** Patient and pathological information associated with brain tumor samples used for the isolation of cancer stem cells and nonstem cells is provided. The age and gender of patients as well as the tumor stage and histopathology are included. FISH (Fluorescence in situ hybridization) data determining Chromosome 7, Chromosome 10, EGFR, and PTEN status is included where performed.

| T3359 Stem | | | | |
|---|---|---|---|---|
| 1st Round | Cell number | 5000 | 1000 | 500 |
| | Incidence | 3/3 | 3/3 | 3/3 |
| | Median Survival | 48 | 52 | 57 |
| 2nd Round | Cell number | 5000 | 1000 | 500 |
| | Incidence | 3/3 | 3/3 | 3/3 |
| | Median Survival | 36 | 44 | 48 |

**Supplemental Table 2. Demonstration of Serial *In Vivo* Transplantation Efficiency of Glioma Stem Cells From Xenografts.** An *in vivo* limiting dilution assay was performed with GSCs isolated from T3359 glioblastoma specimen

| | Human Specimen | Cell Number | Median Survival | Incidence |
|---|---|---|---|---|
| 1st Round | T08-0090 | 2000 Stem | 35 | 3/3 |
| | T08-0076 | 2000 Stem | 35 | 3/3 |
| 2nd Round | T08-0090 | 3000 Stem | 25.5 | 2/2 |
| | T08-0076 | 3000 Stem | 40 | 2/2 |

**Supplemental Table 3. Demonstration of Serial *In Vivo* Transplantation Efficiency of Primary Glioma Stem Cells.** 2000 GSCs isolated directly from T08-0090 or T08-0076 glioblastoma patient specimen were implanted into the right frontal lobes of immunocompromised mice (n=3 per arm). 3000 GSCs isolated from the xenografted patient specimen in the first round were then injected into animals and monitored as above (n=2) for the serial transplantation.

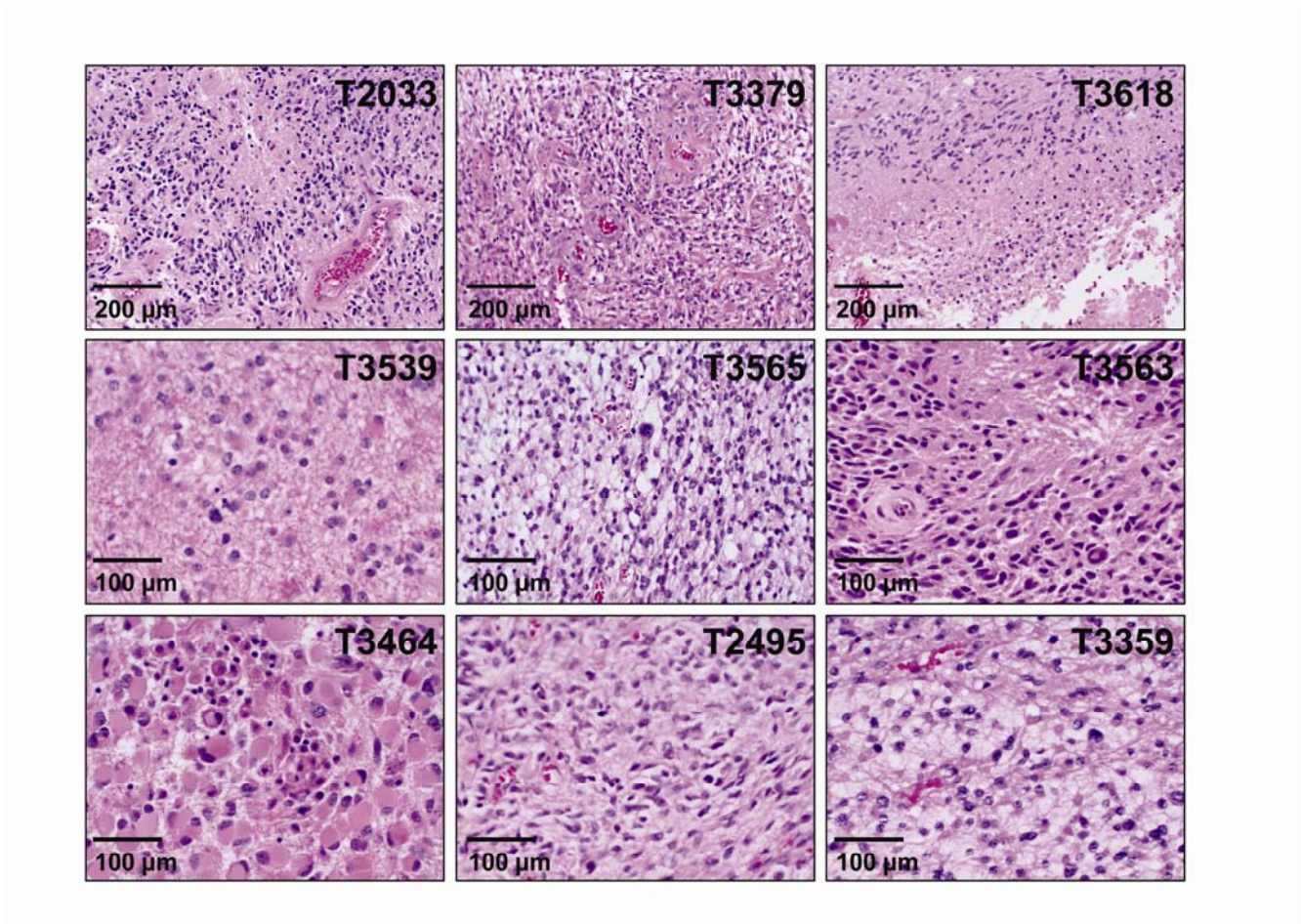
| | D456MG Stem | | D456MG Nonstem | | T3359 Stem | | T3359 Nonstem | |
|---|---|---|---|---|---|---|---|---|
| | Normoxia | Hypoxia | Normoxia | Hypoxia | Normoxia | Hypoxia | Normoxia | Hypoxia |
| ADM | 0.186 | 1.911 | 1.000 | 9.105 | 0.540 | 4.666 | 1.000 | 2.209 |
| AGPAT2 | 1.485 | 0.609 | 1.000 | 5.144 | 1.000 | 0.781 | 1.000 | 2.990 |
| AGTPBP1 | 1.950 | 1.050 | 1.000 | 1.068 | 3.366 | 4.157 | 1.000 | 4.033 |
| ANGPTL4 | 8.577 | 1.698 | 1.000 | 27.760 | 0.794 | 0.216 | 1.000 | 2.711 |
| ARD1A | 0.995 | 0.549 | 1.000 | 0.490 | 0.630 | 0.803 | 1.000 | 0.658 |
| ARNT2 | 1.756 | 0.852 | 1.000 | 0.643 | 1.301 | 0.864 | 1.000 | 0.938 |
| BAX | 0.952 | 0.794 | 1.000 | 0.745 | 7.787 | 11.062 | 1.000 | 2.758 |
| BHLHB2 | 0.424 | 2.717 | 1.000 | 5.272 | 0.756 | 2.665 | 1.000 | 2.548 |
| BIRC5 | 0.859 | 0.290 | 1.000 | 0.324 | 0.500 | 0.056 | 1.000 | 0.113 |
| CASP1 | 0.019 | 0.060 | 1.000 | 1.096 | 0.027 | 0.063 | 1.000 | 1.210 |
| CAT | 0.934 | 0.746 | 1.000 | 0.842 | 1.225 | 0.491 | 1.000 | 1.861 |
| CDC42 | 2.028 | 0.910 | 1.000 | 1.143 | 0.962 | 0.791 | 1.000 | 0.777 |
| CHGA | 6.750 | 0.968 | 1.000 | 0.417 | 2.474 | 0.474 | 1.000 | 2.504 |
| COL1A1 | 0.439 | 0.133 | 1.000 | 1.169 | 0.126 | 0.046 | 1.000 | 1.112 |
| CREBBP | 4.768 | 3.846 | 1.000 | 4.274 | 1.323 | 0.946 | 1.000 | 0.399 |
| CSTB | 1.102 | 0.663 | 1.000 | 0.790 | 1.260 | 1.230 | 1.000 | 1.407 |
| CYGB | 1.739 | 1.225 | 1.000 | 3.059 | 0.532 | 0.820 | 1.000 | 1.193 |
| DAPK3 | 1.011 | 0.653 | 1.000 | 0.903 | 0.602 | 0.806 | 1.000 | 0.665 |
| DCTN2 | 0.959 | 0.730 | 1.000 | 0.728 | 1.228 | 1.455 | 1.000 | 2.632 |
| DR1 | 0.982 | 0.820 | 1.000 | 0.972 | 1.580 | 1.144 | 1.000 | 0.981 |
| ECE1 | 1.791 | 2.153 | 1.000 | 1.069 | 0.665 | 0.658 | 1.000 | 1.800 |
| EEF1A1 | 1.150 | 1.075 | 1.000 | 1.000 | 0.795 | 1.395 | 1.000 | 0.601 |
| ENO1 | 1.009 | 1.328 | 1.000 | 1.229 | 0.818 | 0.715 | 1.000 | 1.099 |
| EP300 | 1.587 | 1.238 | 1.000 | 1.291 | 0.664 | 0.660 | 1.000 | 0.778 |
| EPO | 3.588 | 2.845 | 1.000 | 0.840 | 7.431 | 9.544 | 1.000 | 1.269 |
| GNA11 | 1.399 | 1.471 | 1.000 | 1.347 | 1.181 | 1.495 | 1.000 | 0.848 |
| GPI | 0.624 | 0.985 | 1.000 | 2.617 | 1.175 | 1.197 | 1.000 | 1.201 |
| GPX1 | 0.646 | 0.572 | 1.000 | 0.581 | 0.655 | 0.883 | 1.000 | 0.896 |
| HBB | 0.507 | 1.602 | 1.000 | 0.197 | 0.980 | 2.333 | 1.000 | 6.007 |
| HIF1A | 1.098 | 0.979 | 1.000 | 1.012 | 1.041 | 1.171 | 1.000 | 1.098 |
| HIF1AN | 1.261 | 1.042 | 1.000 | 0.854 | 0.977 | 1.218 | 1.000 | 0.905 |
| HIF2A | 8.354 | 30.336 | 1.000 | 3.839 | 4.000 | 11.877 | 1.000 | 1.523 |
| HIF3A | 2.331 | 2.080 | 1.000 | 1.821 | 0.659 | 0.602 | 1.000 | 1.173 |
| HK2 | 0.698 | 1.095 | 1.000 | 2.168 | 0.397 | 0.514 | 1.000 | 0.603 |
| HMOX1 | 1.283 | 1.863 | 1.000 | 3.583 | 0.816 | 1.329 | 1.000 | 2.957 |
| HTATIP | 1.025 | 0.539 | 1.000 | 0.441 | 0.306 | 0.100 | 1.000 | 0.278 |
| HYOU1 | 1.363 | 0.820 | 1.000 | 1.139 | 2.000 | 0.724 | 1.000 | 0.655 |
| IGF2 | 248.678 | 177.178 | 1.000 | 5.246 | 8.576 | 10.201 | 1.000 | 1.425 |
| IL1A | 3.364 | 2.363 | 1.000 | 3.914 | 3.077 | 2.819 | 1.000 | 3.678 |
| IL6 | 1.001 | 9.092 | 1.000 | 4.800 | 14.986 | 72.869 | 1.000 | 5.061 |
| IL6ST | 1.134 | 1.166 | 1.000 | 0.980 | 0.829 | 0.464 | 1.000 | 1.630 |
| IQGAP1 | 0.969 | 0.775 | 1.000 | 0.921 | 0.973 | 0.900 | 1.000 | 0.630 |
| KHSRP | 1.030 | 0.651 | 1.000 | 0.496 | 1.566 | 1.836 | 1.000 | 0.483 |
| KIT | 1.691 | 0.930 | 1.000 | 1.384 | 0.101 | 0.026 | 1.000 | 0.471 |
| LCT | 4.269 | 3.042 | 1.000 | 1.536 | 0.319 | 0.102 | 1.000 | 0.925 |
| LEP | 2.256 | 1.447 | 1.000 | 2.528 | 0.251 | 0.239 | 1.000 | 0.564 |
| MAN2B1 | 0.691 | 0.905 | 1.000 | 1.045 | 1.267 | 1.224 | 1.000 | 0.893 |
| MOCS3 | 0.224 | 0.354 | 1.000 | 0.353 | 1.004 | 2.543 | 1.000 | 0.957 |
| MT3 | 8.829 | 0.586 | 1.000 | 7.939 | 8.247 | 1.113 | 1.000 | 1.444 |
| MYBL2 | 1.244 | 1.351 | 1.000 | 1.125 | 1.758 | 1.347 | 1.000 | 0.553 |
| NOS2A | 0.204 | 0.126 | 1.000 | 0.992 | 0.342 | 0.294 | 1.000 | 0.175 |
| NOTCH1 | 3.117 | 0.902 | 1.000 | 0.463 | 4.011 | 1.557 | 1.000 | 0.505 |
| NPY | 0.369 | 0.331 | 1.000 | 1.438 | 2.639 | 4.182 | 1.000 | 1.972 |
| NUDT2 | 1.024 | 0.969 | 1.000 | 0.940 | 0.847 | 0.900 | 1.000 | 0.925 |
| PDIA2 | 1.161 | 1.895 | 1.000 | 1.163 | 0.666 | 0.342 | 1.000 | 1.368 |
| PEA15 | 0.871 | 0.859 | 1.000 | 0.874 | 0.485 | 0.204 | 1.000 | 1.655 |
| PIP3-E | 0.497 | 0.388 | 1.000 | 0.412 | 0.955 | 1.033 | 1.000 | 1.133 |
| PLAU | 0.153 | 0.212 | 1.000 | 1.035 | 0.659 | 0.368 | 1.000 | 0.286 |
| PLOD3 | 0.550 | 0.461 | 1.000 | 0.833 | 0.805 | 0.569 | 1.000 | 0.855 |
| PPARA | 1.110 | 0.739 | 1.000 | 0.999 | 0.795 | 0.525 | 1.000 | 0.793 |
| PPP2CB | 1.116 | 1.591 | 1.000 | 1.239 | 1.334 | 1.544 | 1.000 | 0.971 |
| PRKAA1 | 0.816 | 0.435 | 1.000 | 0.568 | 1.076 | 0.564 | 1.000 | 1.058 |
| PSMB3 | 0.771 | 0.439 | 1.000 | 0.736 | 0.662 | 0.663 | 1.000 | 0.596 |
| PTX3 | 13.766 | 13.406 | 1.000 | 1.852 | 2.056 | 8.207 | 1.000 | 4.137 |
| RARA | 0.700 | 1.285 | 1.000 | 2.112 | 0.635 | 1.027 | 1.000 | 1.684 |
| RPL28 | 0.953 | 0.865 | 1.000 | 0.851 | 1.071 | 1.037 | 1.000 | 1.244 |
| RPL32 | 1.199 | 1.117 | 1.000 | 0.908 | 1.195 | 1.087 | 1.000 | 1.071 |
| RPS2 | 1.082 | 0.962 | 1.000 | 0.923 | 1.092 | 1.094 | 1.000 | 1.301 |
| RPS7 | 1.066 | 0.920 | 1.000 | 0.856 | 1.017 | 1.016 | 1.000 | 0.976 |
| SAE1 | 1.103 | 0.703 | 1.000 | 0.520 | 3.234 | 2.055 | 1.000 | 0.588 |
| SLC2A1 | 1.507 | 3.847 | 1.000 | 3.734 | 1.579 | 9.518 | 1.000 | 3.383 |
| SLC2A4 | 1.476 | 1.114 | 1.000 | 0.652 | 1.006 | 0.519 | 1.000 | 1.506 |
| SNRP70 | 0.503 | 0.519 | 1.000 | 0.456 | 1.320 | 0.950 | 1.000 | 0.922 |
| SPTBN1 | 1.577 | 1.172 | 1.000 | 1.009 | 1.303 | 1.298 | 1.000 | 0.563 |
| SSSCA1 | 0.665 | 0.521 | 1.000 | 0.550 | 1.227 | 0.822 | 1.000 | 0.582 |
| SUMO2 | 1.254 | 0.927 | 1.000 | 0.799 | 1.334 | 1.257 | 1.000 | 0.850 |
| TH | 3.372 | 0.554 | 1.000 | 0.779 | 0.243 | 0.971 | 1.000 | 2.300 |
| TST | 1.417 | 0.857 | 1.000 | 0.627 | 5.069 | 2.552 | 1.000 | 0.807 |
| TUBA1 | 1.281 | 1.120 | 1.000 | 2.639 | 1.251 | 0.970 | 1.000 | 0.905 |
| UCP2 | 0.919 | 0.527 | 1.000 | 0.650 | 0.791 | 0.696 | 1.000 | 1.213 |
| VEGFA | 1.414 | 5.437 | 1.000 | 2.698 | 1.157 | 3.007 | 1.000 | 1.416 |

**Supplemental Table 4. Differential Expression of Hypoxia Responsive Genes in Glioma Stem Cells.** Glioblastoma stem and nonstem cells isolated from an established xenograft (D456MG) or patient specimen (T3359) were cultured under normoxia (20% oxygen) or hypoxia (1% oxygen) for 24 hr and RNA harvested with RNeasy Mini Kit (Qiagen). cDNAs were synthesized using SuperScript® III First-Strand Synthesis Kit (Invitrogen, Inc). Real time PCR was performed using Human Hypoxia Signaling Pathway PCR Array Kits (SuperArray Bioscience Corporation). Data were normalized to GAPDH and Beta-actin expression and then to the expression in nonstem cells under normoxia.

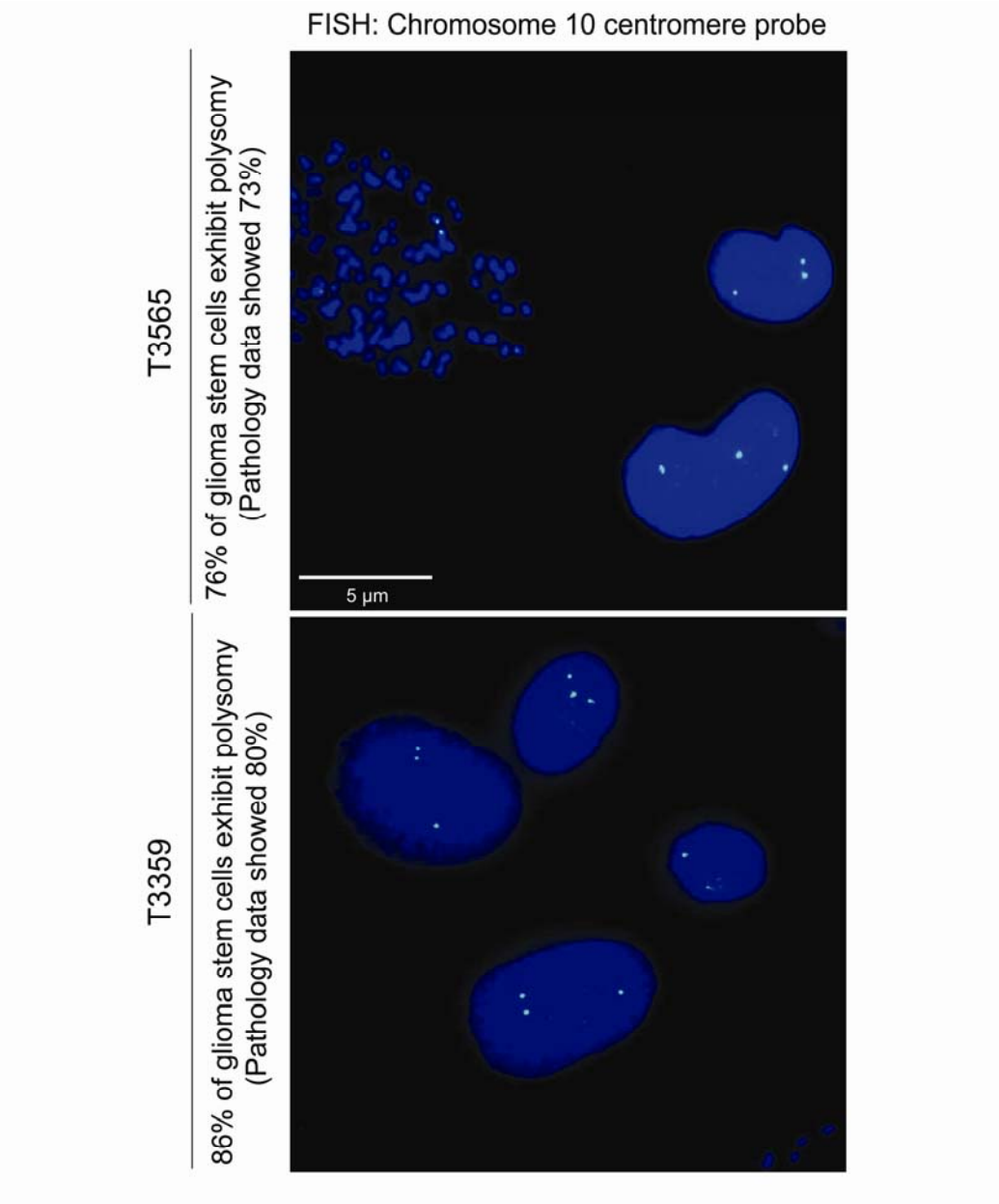
| Tumor | HIF2a Staining Score | | | | | |
|---|---|---|---|---|---|---|
| | PARENCHYMAL | NECROTIC 1 | NECROTIC 2 | NECROTIC 3 | NECROTIC 4 | NECROTIC 5 |
| T1783 | 1 | 5 | 5 | | | |
| T2155 | 1 | 8 | 5 | 14 | 1 | 3 |
| T1855 | 3 | 3 | | | | |
| T3522 | 1 | 1 | | | | |
| T3072 | 4 | 7 | 3 | 20 | 15 | 17 |
| T2654 | 0.1 | 7 | 10 | | | |
| T3243 | 0.1 | 12 | 20 | 5 | | |
| T3552 | 1 | 20 | 3 | | | |
| T3543 | 1 | 5 | 17 | | | |
| T890 | 2 | 1 | 1 | 5 | 10 | 15 |
| T864 | 0.3 | 4 | 2 | 2 | 1 | 1 |
| T1358 | 2 | 15 | 15 | 10 | 12 | |
| T1017 | 0.1 | 12 | 2 | 3 | 0 | |
| T1406 | 3 | 10 | 9 | 20 | 2 | 6 |
| T3837 | 0.1 | 1 | 0 | 0 | 1 | |
| T922 | 1 | 1 | 1 | 3 | | |
| T1189 | 0.1 | | | | | |
| T1074 | 0.1 | 10 | 0 | 0 | | |
| T809 | 7 | 20 | 15 | 5 | | |

**Supplemental Table 5. HIF2α Staining in Primary Glioblastoma Specimens.** Immunohistostaining of HIF2α was performed on 19 primary glioblastoma specimens. Pathologic review of the resulting slides determined a large variation of HIF2α expression level among samples and additional intratumoral heterogeneity. HIF2α staining was predominately located around regions of necrosis, but there greater variation in the percentaged stained in areas of necrosis than in the solid, non-necrotic regions (parenchymal). To identify the range of variation around necrotic foci, the mean percentage of HIF2α labeled cells for five different necrotic regions per tumor were evaluated when observed in tumor sections. Tumor cells were counted in the regions about the necrosis, as macrophages entrapped in the necrosis appeared to bind the stain. Data is presented as a single mean percentage of HIF2α labeled cells for parenchymal regions.

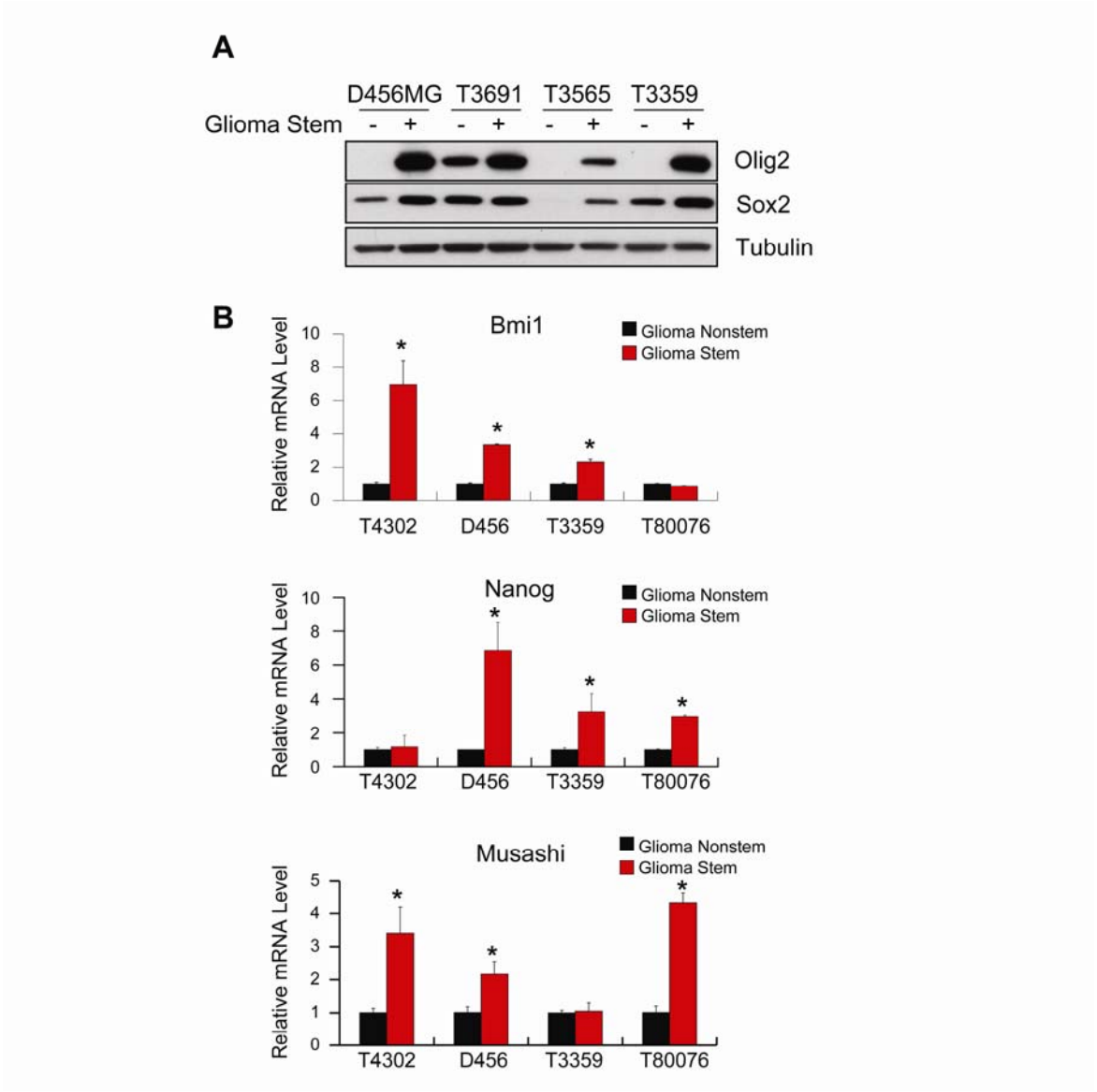
## SUPPLEMENTAL FIGURES



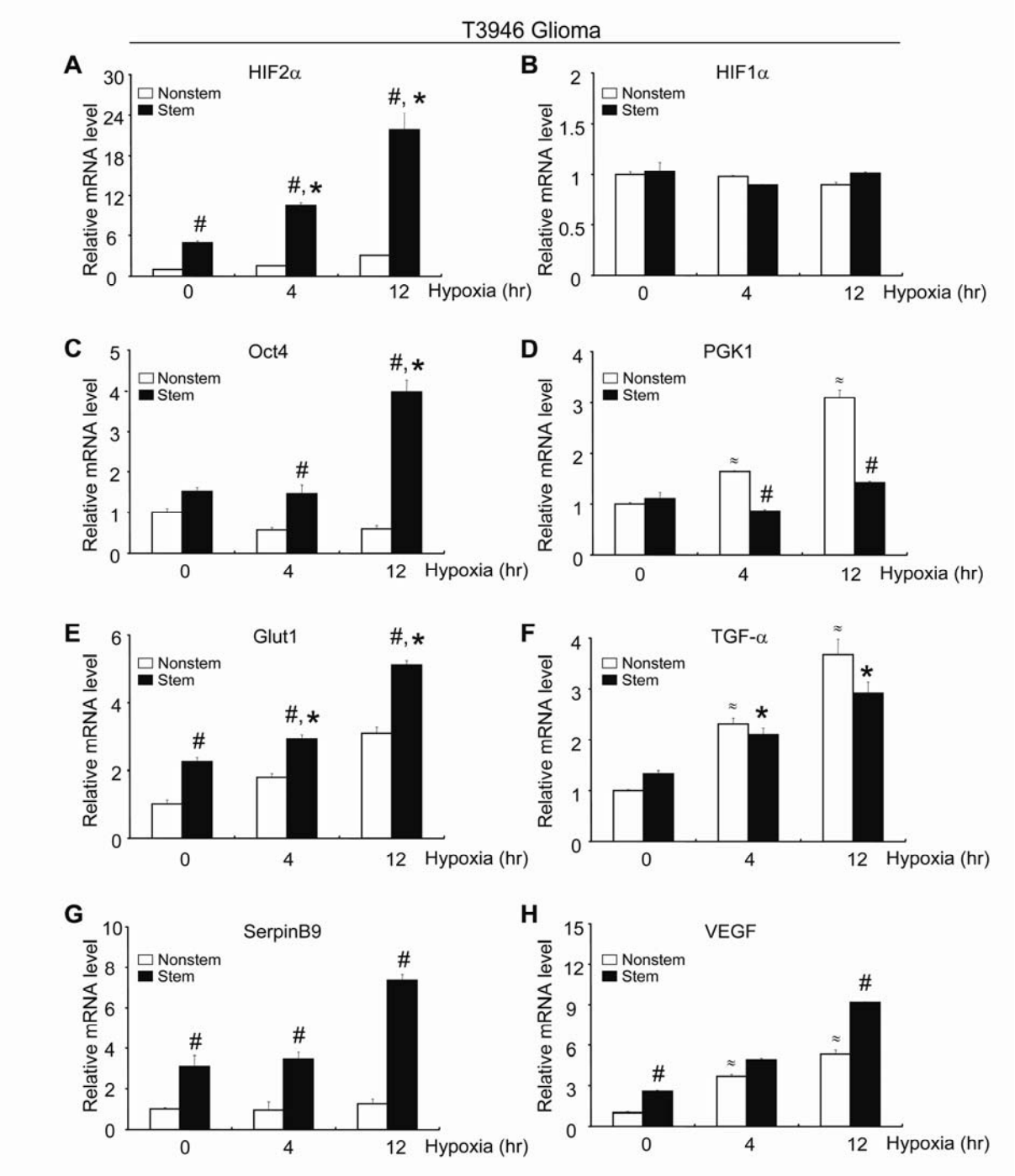

**Supplemental Figure 1. Histopathology of Glioma Patient Specimens.** Representative images of hematoxylin and eosin staining performed primary glioma samples (including T3565 and T3359 which were used for the isolation of cancer stem cells in this study) are shown. Glioblastoma samples demonstrated typical pathological features such as hyperchromatism, pleomorphism, extensive necrosis, and proliferating blood vessels.



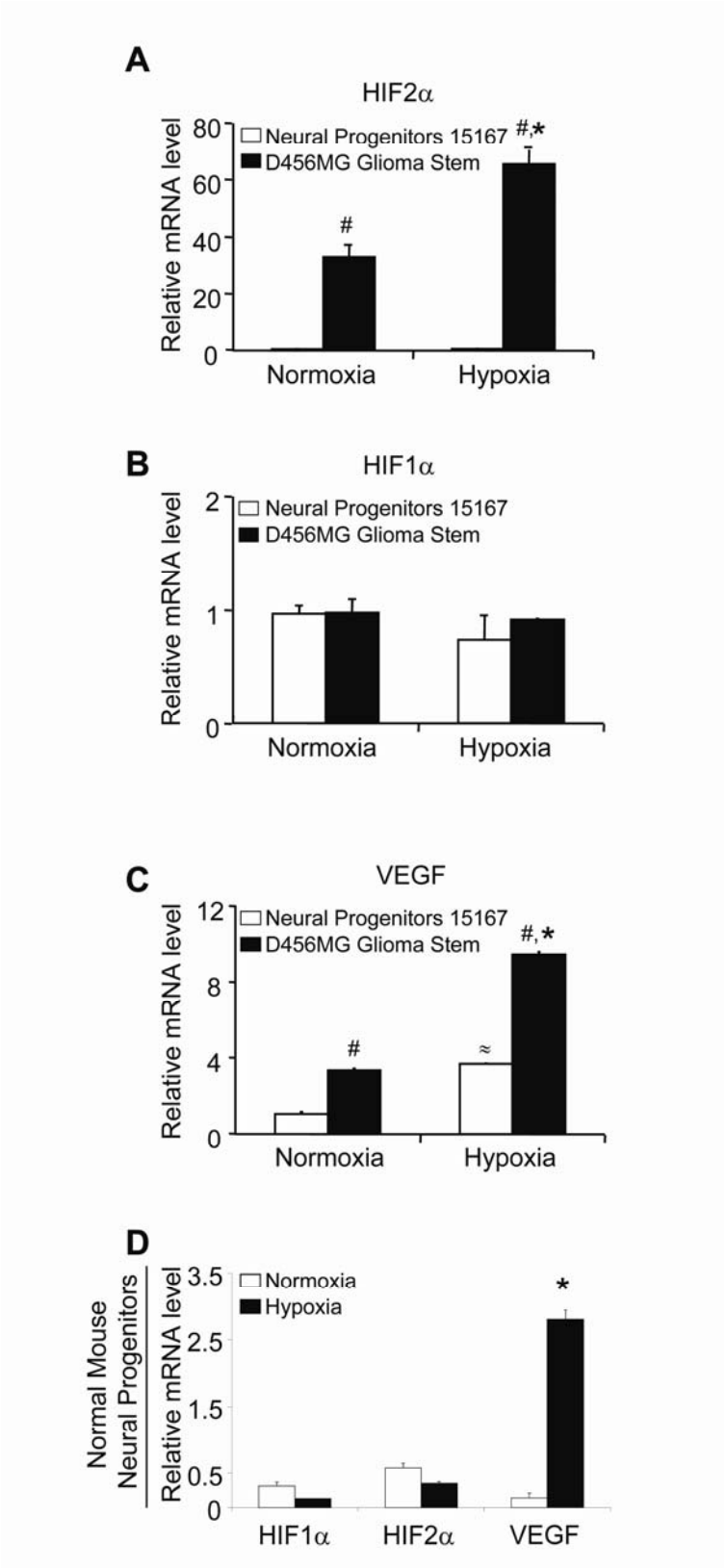

**Supplemental Figure 2: Glioma Stem Cells Had Chromosomal Alterations Identical to the Original Patient Specimen.** FISH (Fluorescence in situ hybridization) experiments were performed on isolated T3565 and T3359 GSCs using a Chromosome 10 centromere probe. Polysomy of chromosome 10 occurs in both samples (76% in T3565 and 86% in T3359) at levels consistent with the original patient specimen.



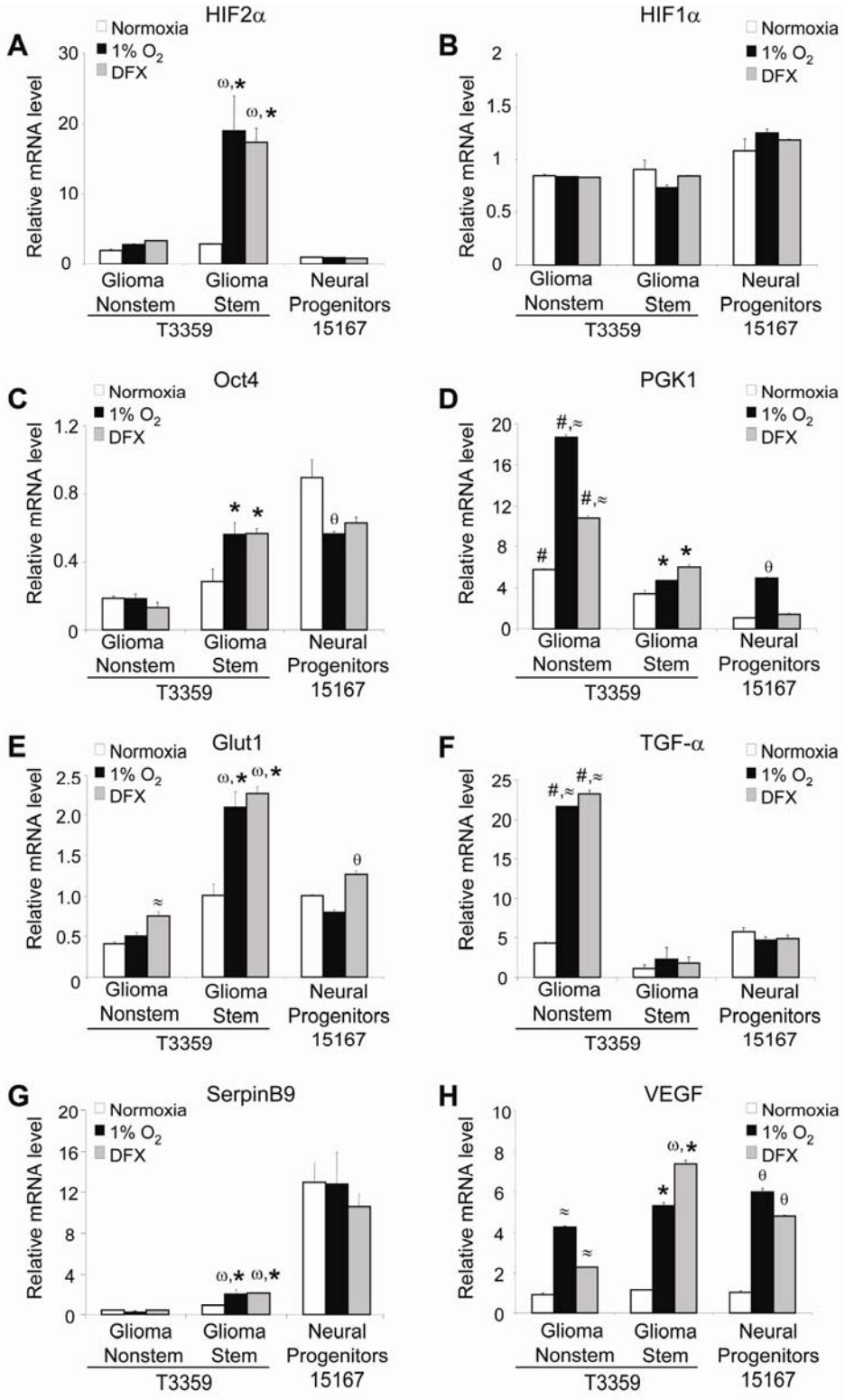

**Supplemental Figure 3. Glioma Stem Cells Preferentially Expressed Stem Cell Markers.** GSCs and nonstem glioma cells were isolated from the glioblastoma xenograft D456MG or the glioma patient specimens T3691, T3565, or T3359 short-term passaged in immunocompromised mice. (A) GSCs express higher levels of the stem cell markers Olig2 and Sox2 in comparison to matched nonstem glioma cells as analyzed by Western. (B) GSCs express elevated levels of the stem cell markers Bmi1, Nanog, and Musashi as determined by Real Time PCR in comparison to matched nonstem glioma cells.


T3946 Glioma
A
HIF2α
Nonstem
Stem
Relative mRNA level
Hypoxia (hr)
B
HIF1α
C
Oct4
D
PGK1
E
Glut1
F
TGF-α
G
SerpinB9
H
VEGF

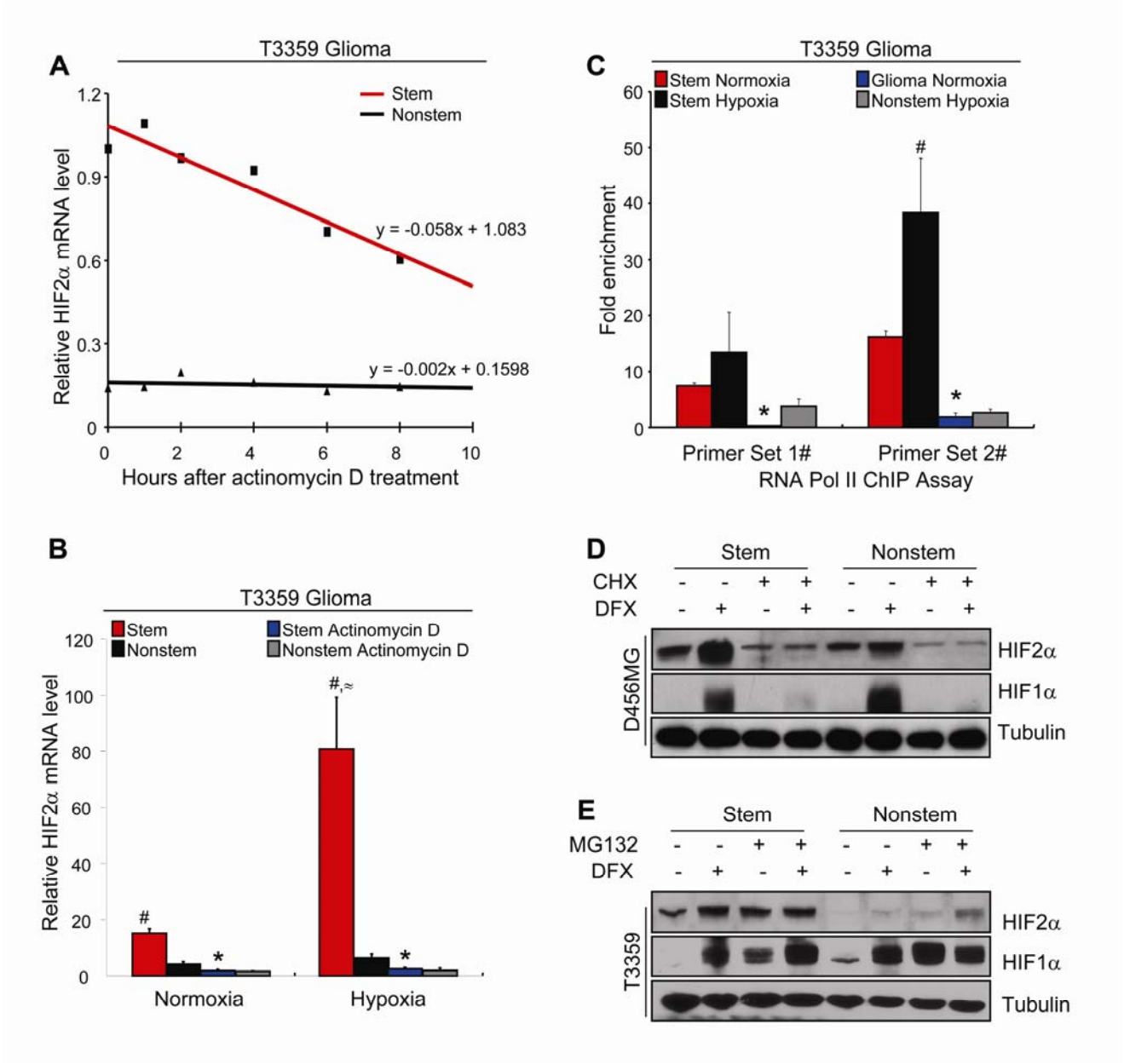
**Supplemental Figure 4: Glioblastoma Stem and Nonstem Cells Differentially Expressed Hypoxia Response Genes.** Glioblastoma stem and nonstem cells isolated from glioblastoma T3946 were treated with 200 μM desferrioxamine (DFX) to mimic hypoxia for the time indicated and total RNA harvested. Synthesized cDNAs were used for real time PCR analysis with primers specific for HIF2α (A), HIF1α (B), Oct4 (C), PGK1 (D), Glut1 (E), TGF-α (F), SerpinB9 (G), and VEGF (H). Data were normalized to GAPDH, Ubiquitin C, and SDHA. PGK1 is hypothesized to be specifically regulated by HIF1α whereas Oct4 and SerpinB9 are thought to be specifically regulated by HIF2α in response to hypoxia, suggesting HIF1α dependent genes are relatively upregulated in nonstem glioblastoma cells whereas HIF2α and HIF2α dependent genes are relatively upregulated in GSCs. #, $P < 0.01$ with ANOVA comparison of stem cells to nonstem cells with identical treatments. *, $P < 0.01$ with ANOVA comparison of stem cells under hypoxia vs. normoxia. ≈, $P < 0.01$ with ANOVA comparison of nonstem cells under hypoxia vs. normoxia.


A
HIF2α
Neural Progenitors 15167
D456MG Glioma Stem
Relative mRNA level
80
60
40
20
0
#
#,*
Normoxia
Hypoxia
B
HIF1α
Neural Progenitors 15167
D456MG Glioma Stem
Relative mRNA level
2
1
0
Normoxia
Hypoxia
C
VEGF
Neural Progenitors 15167
D456MG Glioma Stem
Relative mRNA level
12
8
4
0
#
≈
#,*
Normoxia
Hypoxia
D
Normal Mouse
Neural Progenitors
Relative mRNA level
Normoxia
Hypoxia
3.5
2.5
1.5
0.5
0
*
HIF1α
HIF2α
VEGF

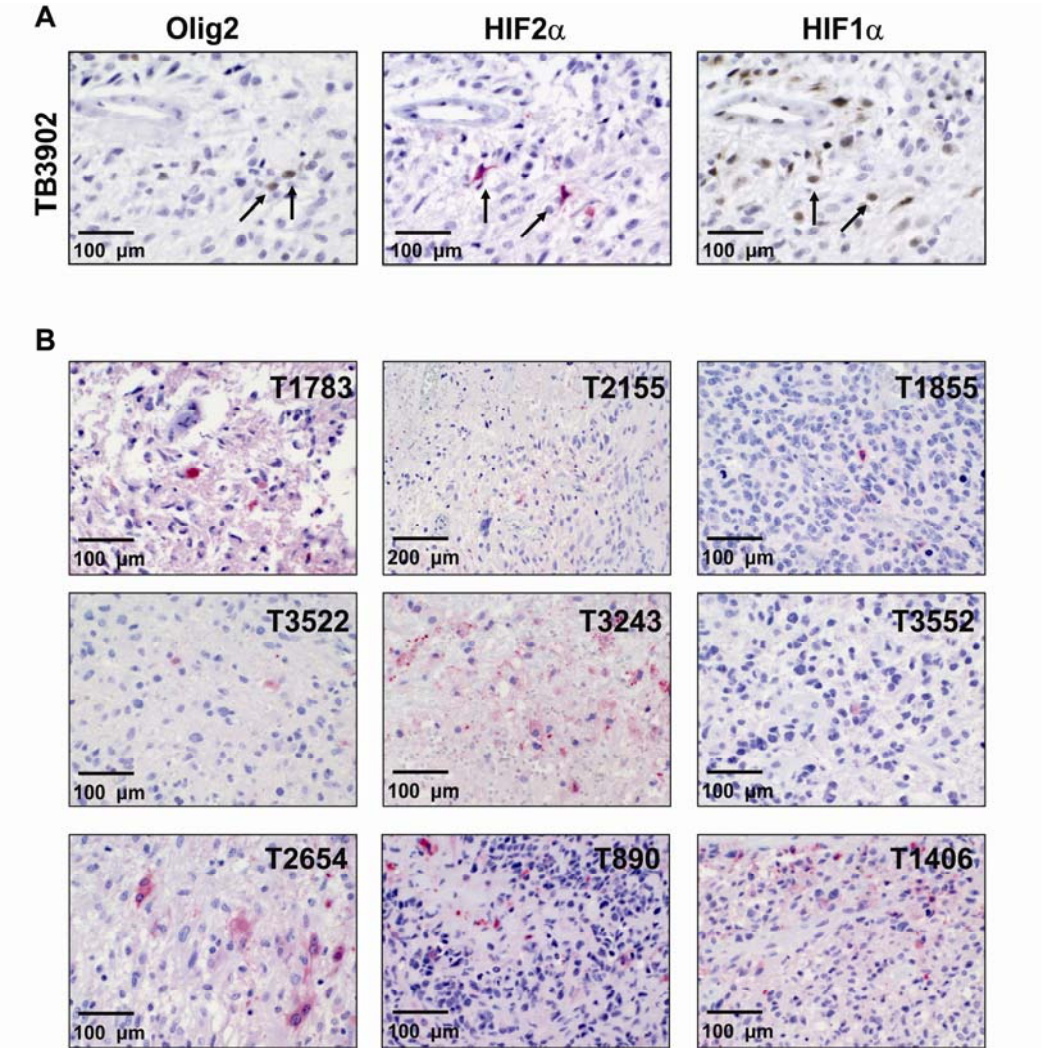
**Supplemental Figure 5. Elevated HIF2α Expression in Glioma Stem Cells in Comparison to Normal Neural Progenitors.** (A-C) GSCs were isolated from a D456MG xenograft. Normal fetal neural progenitors were purchased from Lonza. CDS133+ cells were isolated via FACS analysis with αCD133-APC from Miltenyi Biotech. All cells were treated with 200 μM DFX to mimic hypoxia for 8 hr. Real Time PCR analysis was performed with primers specific for HIF2α (A), HIF1α (B), and VEGF (C). #, $P < 0.001$ by ANOVA comparison of GSCs to neural progenitors with identical oxygen treatment. *, $P < 0.001$ with ANOVA comparison of GSCs under hypoxia vs. normoxia. ≈, $P < 0.001$ with ANOVA comparison of neural progenitors under hypoxia vs. normoxia. (D) Normal adult mouse neural progenitors were isolated from the brains of 2 month old C56B6 animals and cultured under 1% oxygen overnight. Real Time PCR analysis was performed with primers specific for mouse HIF2α, HIF1α, and VEGF. *, $P < 0.001$ with t-test comparison of adult mouse neural progenitors under hypoxia vs. normoxia.


A
HIF2α
Normoxia
1% O2
DFX
Relative mRNA level
30
20
10
0
ω,*
ω,*
Glioma Nonstem
Glioma Stem
Neural Progenitors
T3359
15167
B
HIF1α
Normoxia
1% O2
DFX
Relative mRNA level
2
1.5
1
0.5
0
Glioma Nonstem
Glioma Stem
Neural Progenitors
T3359
15167
C
Oct4
Normoxia
1% O2
DFX
Relative mRNA level
1.2
0.8
0.4
0
*
*
θ
Glioma Nonstem
Glioma Stem
Neural Progenitors
T3359
15167
D
PGK1
Normoxia
1% O2
DFX
Relative mRNA level
20
16
12
8
4
0
#,≈
#,≈
#
*
*
θ
Glioma Nonstem
Glioma Stem
Neural Progenitors
T3359
15167
E
Glut1
Normoxia
1% O2
DFX
Relative mRNA level
2.5
2.0
1.5
1.0
0.5
0
ω,*
ω,*
≈
θ
Glioma Nonstem
Glioma Stem
Neural Progenitors
T3359
15167
F
TGF-α
Normoxia
1% O2
DFX
Relative mRNA level
25
20
15
10
5
0
#,≈
#,≈
Glioma Nonstem
Glioma Stem
Neural Progenitors
T3359
15167
G
SerpinB9
Normoxia
1% O2
DFX
Relative mRNA level
20
16
12
8
4
0
ω,*
ω,*
Glioma Nonstem
Glioma Stem
Neural Progenitors
T3359
15167
H
VEGF
Normoxia
1% O2
DFX
Relative mRNA level
10
8
6
4
2
0
≈
≈
ω,*
*
θ
θ
Glioma Nonstem
Glioma Stem
Neural Progenitors
T3359
15167

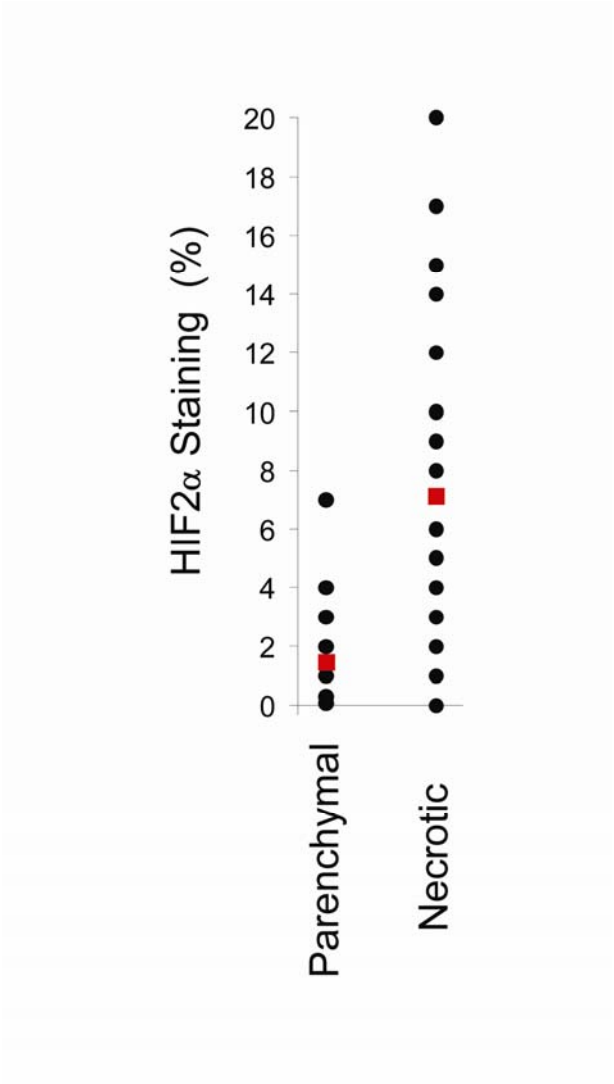
**Supplemental Figure 6. Differential Expression of *HIF2A* and Hypoxia Response Genes in Glioma Stem Cells Is Similar when Hypoxia Is Induced by a Chemical Mimetic or Low Oxygen Tension.** Glioblastoma stem and nonstem cells isolated from T3359 and normal neural progenitors were treated with 200 μM desferroxamine (DFX) for 8 hr to mimic hypoxia or incubated in 1% $O_2$ in a hypoxia chamber overnight and total RNA were harvested. Synthesized cDNAs were used for real time PCR analysis with primers specific for HIF2α (A), HIF1α (B), Oct4 (C), PGK1 (D), Glut1 (E), TGF-α (F), SerpinB9 (G), and VEGF (H). Data were normalized to β-actin. ω, $P < 0.01$ with ANOVA comparison of GSCs to nonstem cells and normal neural progenitors with identical treatments. *, $P < 0.01$ with ANOVA comparison of GSCs under hypoxia vs. normoxia. θ, $P < 0.05$ with ANOVA comparison of normal neural progenitors under hypoxia vs. normoxia. #, $P < 0.01$ with ANOVA comparison of glioblastoma nonstem cells to GSCs and normal neural progenitors with identical oxygen conditions. ≈, $P < 0.05$ with ANOVA comparison of glioblastoma nonstem cells under hypoxia vs. normoxia.


A
T3359 Glioma
Stem
Nonstem
Relative HIF2α mRNA level
y = -0.058x + 1.083
y = -0.002x + 0.1598
Hours after actinomycin D treatment
B
T3359 Glioma
Stem
Nonstem
Stem Actinomycin D
Nonstem Actinomycin D
Relative HIF2α mRNA level
Normoxia
Hypoxia
C
T3359 Glioma
Stem Normoxia
Stem Hypoxia
Glioma Normoxia
Nonstem Hypoxia
Fold enrichment
Primer Set 1#
Primer Set 2#
RNA Pol II ChIP Assay
D
Stem
Nonstem
CHX
DFX
D456MG
HIF2α
HIF1α
Tubulin
E
Stem
Nonstem
MG132
DFX
T3359
HIF2α
HIF1α
Tubulin

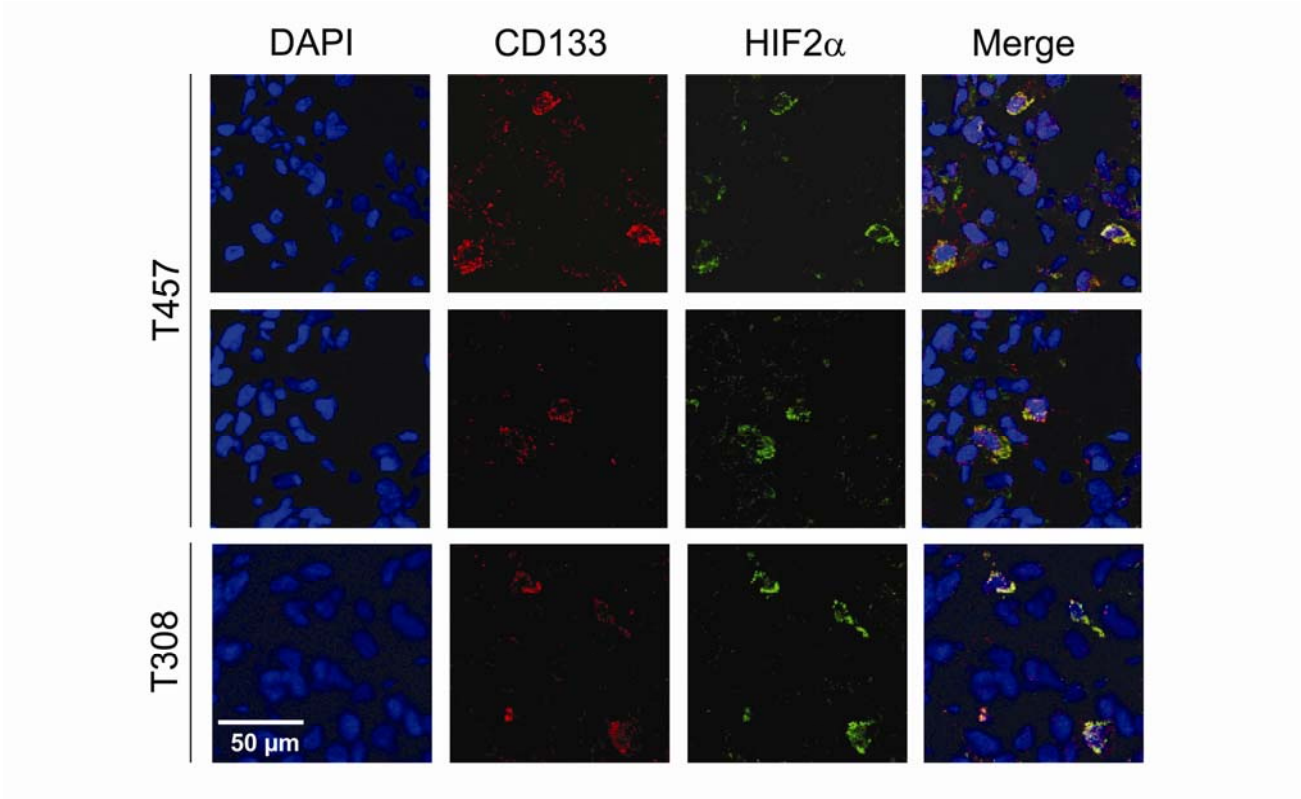
**Supplemental Figure 7. *HIF2A* Upregulation Induced by Hypoxia in Glioma Stem Cells Is Due to Enhanced Transcription.** (A) HIF2α mRNA degradation is greater in GSCs than matched nonstem cells. Therefore, stabilization of HIF2α mRNA cannot explain the elevation of HIF2α mRNA after hypoxia in stem cells. (B) *De novo* transcription is necessary for stimulation of HIF2α mRNA expression under hypoxia. Treatment with the transcription inhibitor Actinomycin D prevents the induction of HIF2α mRNA expression in GSCs treated with hypoxia. #, $P < 0.01$ with ANOVA comparison of stem cells to nonstem cells with identical oxygen conditions. *, $P < 0.01$ with ANOVA of GSCs under identical oxygen conditions treated with actinomycin D. ≈, $P < 0.01$ with ANOVA comparison of GSCs under hypoxia and normoxia. (C) Chromosome Immunoprecipitation (ChIP) assay using RNA polymerase II antibody indicates the HIF2α promoter is preferentially activated in GSCs. (D) New protein synthesis is necessary for the induction of HIF2α protein expression under hypoxia. GSCs isolated from a D456MG xenograft were treated with 200 μM desferrioxamine (DFX) for 8 hr to mimic hypoxia. 1 hr prior to DFX treatment, 2 μg/ml of the translation inhibitor cycloheximide (CHX) was added. (E) Inhibition of proteosome mediated degradation stabilizes HIF protein. Glioblastoma stem and nonstem cells isolated from T3359 were treated by 200 μM DFX for 8 hr and 10 μM of the protease inhibitor carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132) was added 4 hr before harvesting.



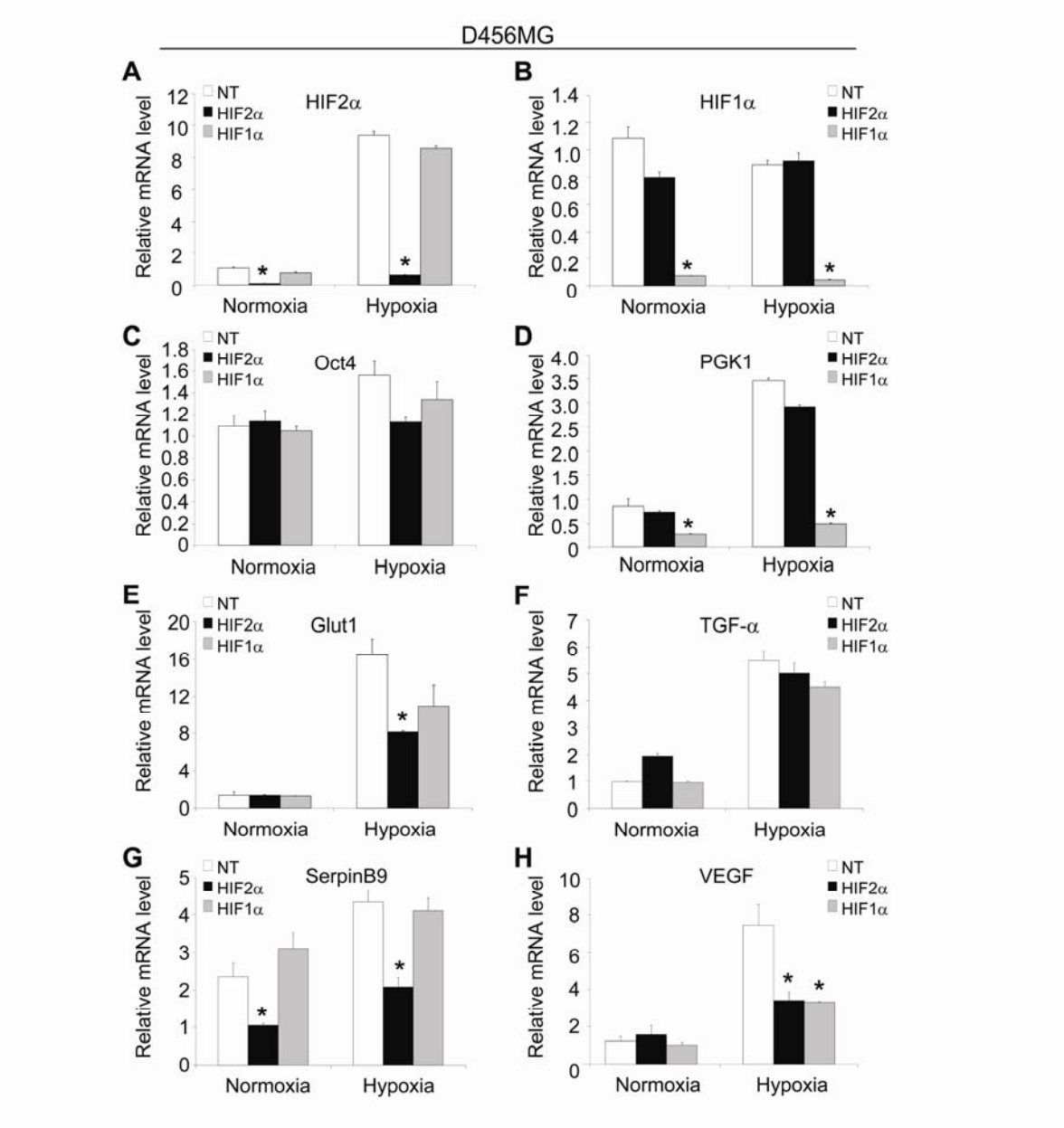

**Supplemental Figure 8. HIF2α Is Co-Expressed with Glioma Stem Cell Marker Olig2 in Glioblastoma Samples** (A) HIF2α but not HIF1α is expressed in pattern similar to the GSC marker Olig2. Olig2 and HIF2α immunostaining were more restricted and more similar than HIF1α immunostaining in adjacent sections from human brain tumor patient specimens. (B) Representative images of immunohistostaining for HIF2α in multiple glioblastoma samples. Glioblastoma samples express HIF2α at variable levels with a higher percentage of stained cells around regions of necrosis as pictured in samples 2155, 3243, and 890.



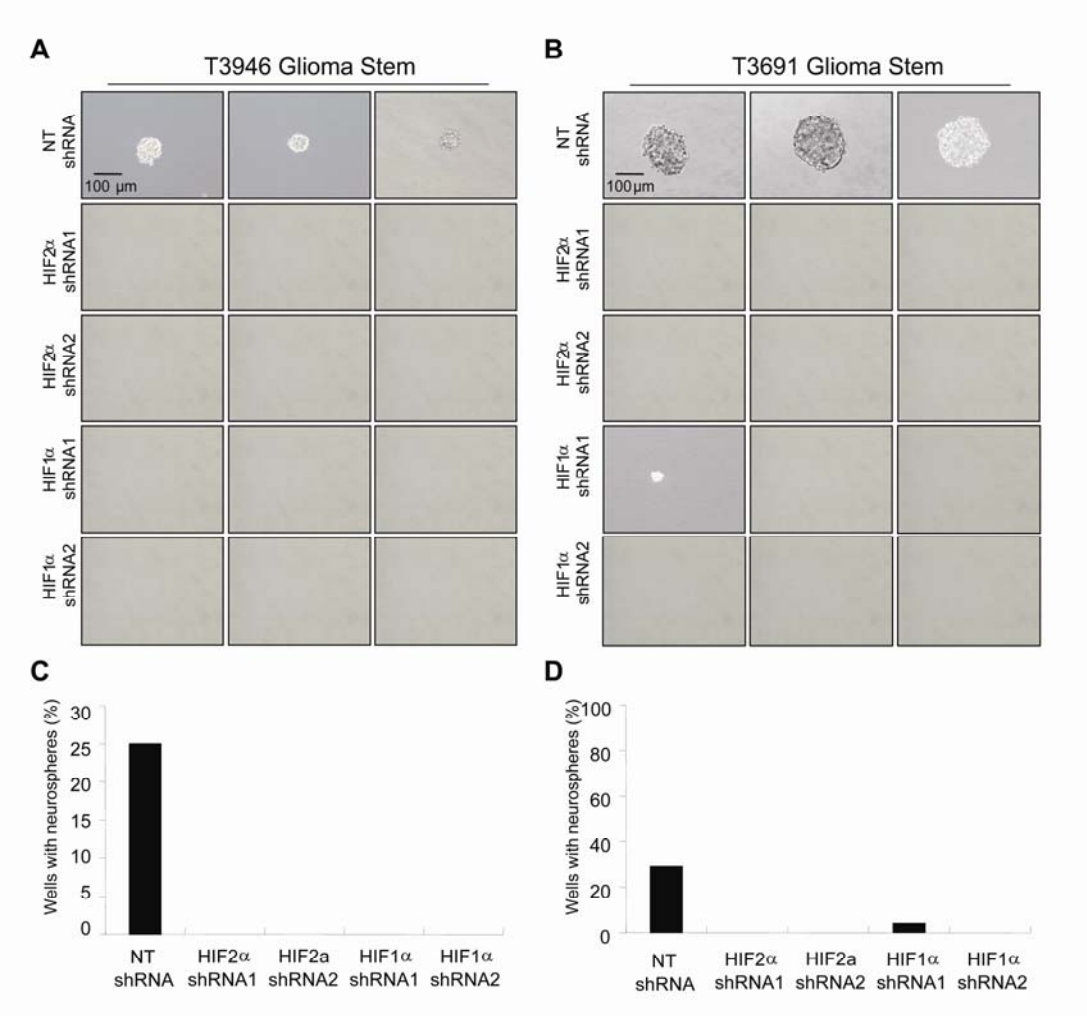

**Supplemental Figure 9. HIF2α Staining in Primary Glioblastoma Specimens.** Immunohistostaining of HIF2α was performed on 19 primary glioblastoma specimens. The values in the Supplemental Table 5 are presented in graphical format to illustrate the variation of the individual data points (black) and the average (red) percentage of HIF2α positive cells in each region.



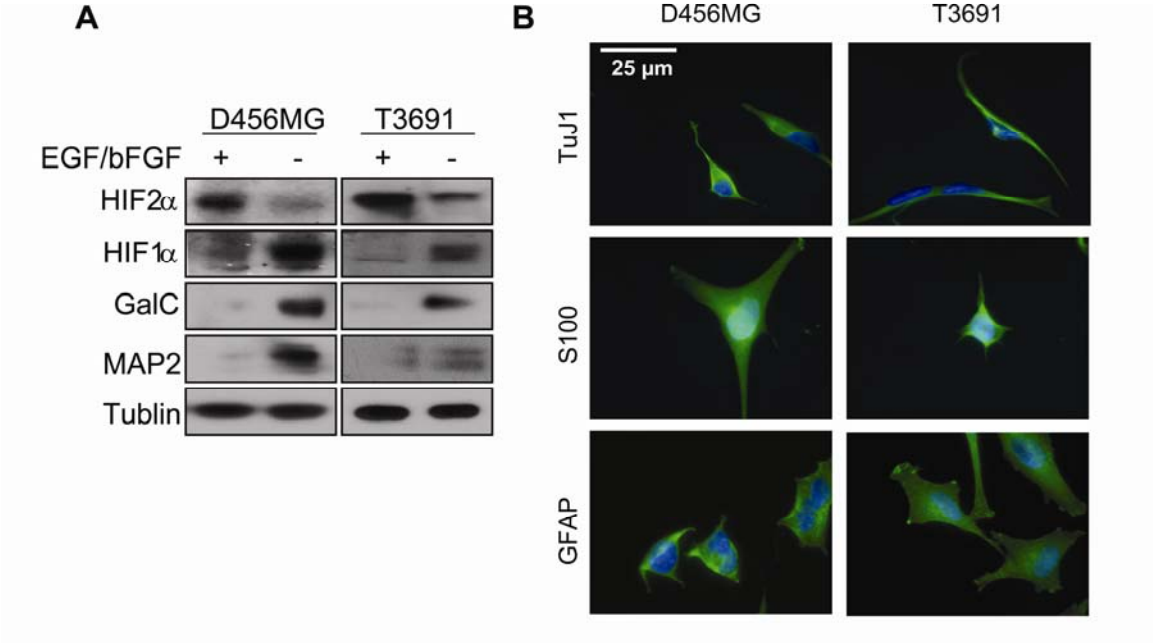

**Supplemental Figure 10. HIF2a and CD133 Co-Localized in Cells in Sections of Human Brain Tumor Patient Specimens.** Immunofluorescence demonstrates that cells staining positive for CD133 expression also express HIF2α. Primary human specimen slides were fixed by 4% paraformaldehyde for 15 minutes at room temperature and blocked by 10% goat serum. Slides were stained by CD133 and HIF2α antibodies for 16 hr at 4 degree followed by Rhodamine Red-X-AffiniPure Donkey Anti- Rabbit secondary antibody and Fluorescein (FITC) AffiniPure Donkey Anti Mouse secondary antibody (Jackson-Immunoresearch). At last, mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories) was used and pictures were taken by Leica SP5 Confocal microscope (Duke Light Microscopy Core Facility).



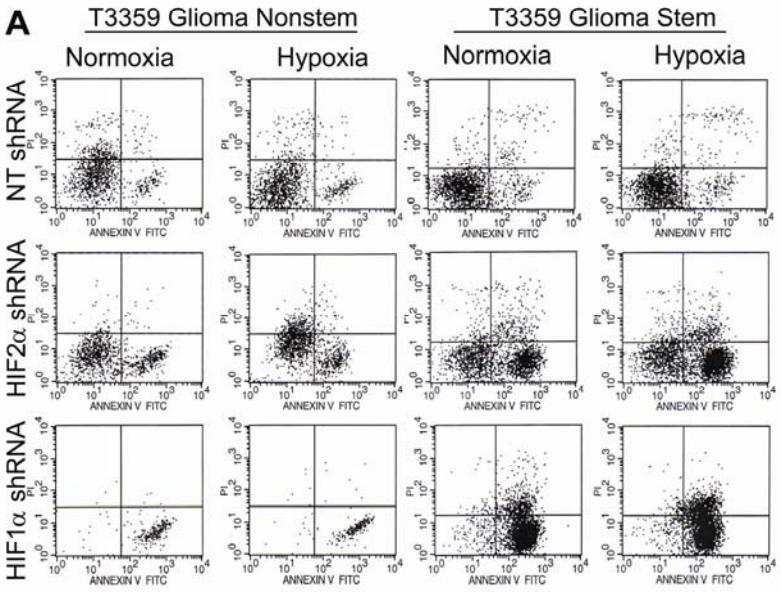

**Supplemental Figure 11. HIFs were Differentially Required for Hypoxia Response Genes in Glioma Stem Cells.** GSCs infected with the indicated shRNAs were treated with 200 μM desferrioxamine (DFX) for 8 hr to mimic hypoxia and total RNA harvested. Synthesized cDNAs were used for real time PCR analysis with primers specific for HIF2α (A), HIF1α (B), Oct4 (C), PGK1 (D), Glut1 (E), TGF-α (F), SerpinB9 (G), and VEGF (H). Data were normalized to β-actin. *, $P < 0.01$ with ANOVA comparison to nontargeting control (NT) under identical oxygen conditions.

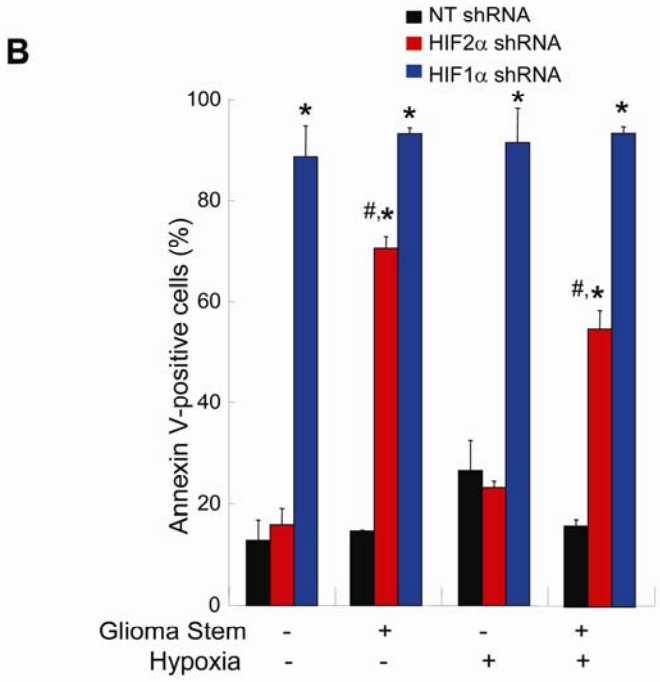



**Supplemental Figure 12. HIFα Knockdown Altered Glioma Stem Cell Neurosphere Formation.** GSCs isolated from T3946 or T3691 specimens passaged short-term in immunocompromised mice were infected with lentivirus expressing the indicated shRNAs. 48 hr later, puromycin was added to select infected cells. 48 hr after selection, live cells were plated at 1 cell/well in 24 wells plates in the presence of puromycin. 2 weeks later, neurospheres were counted and representative pictures were taken. (A) Representative images of spheres are shown at the indicated magnifications. While the nontargeting group contains normal neurospheres, none of the HIFα knockdown groups exhibit any neurosphere growth. (B) The percentage of neurosphere containing wells in each group.

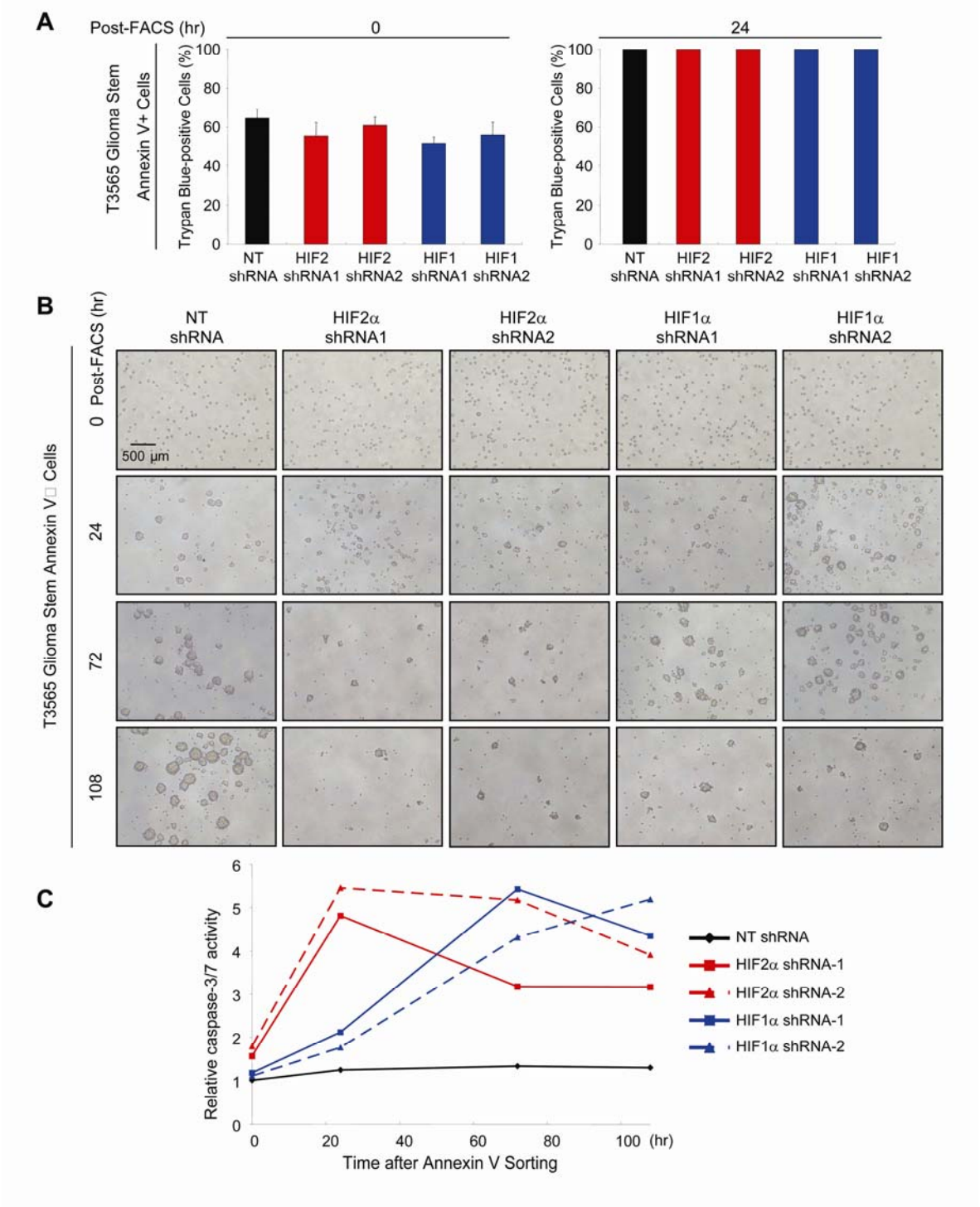



**Supplemental Figure 13. Differentiation of GSCs Was Associated with Decreased HIF2α and Increased HIF1α Expression.** (A)GSCs isolated from D456MG xenografts or T3691 patient specimen passaged short term in immunocompromised mice were induced to differentiate through withdrawl of EGF and bFGF. 10 days later, cells were treated with DFX for 12 hr and harvested for western blot analysis. Increased expression of the oligodendrocyte differentiation marker GalC and the neuronal differentiation marker MAP2 indicated that cells were differentiated. Differentiated cells have decreased HIF2α levels in comparison to maintained GSCs. In contrast, HIF1α is strongly increased in differentiated cells in comparison to GSCs. (B) Upon bFGF withdrawl, GSCs demonstrate morphology of glia and neuron cells as well as the expression of differentiation markers including Tuj1 (neurons), S100 (astrocytes), and GFAP (astrocytes).

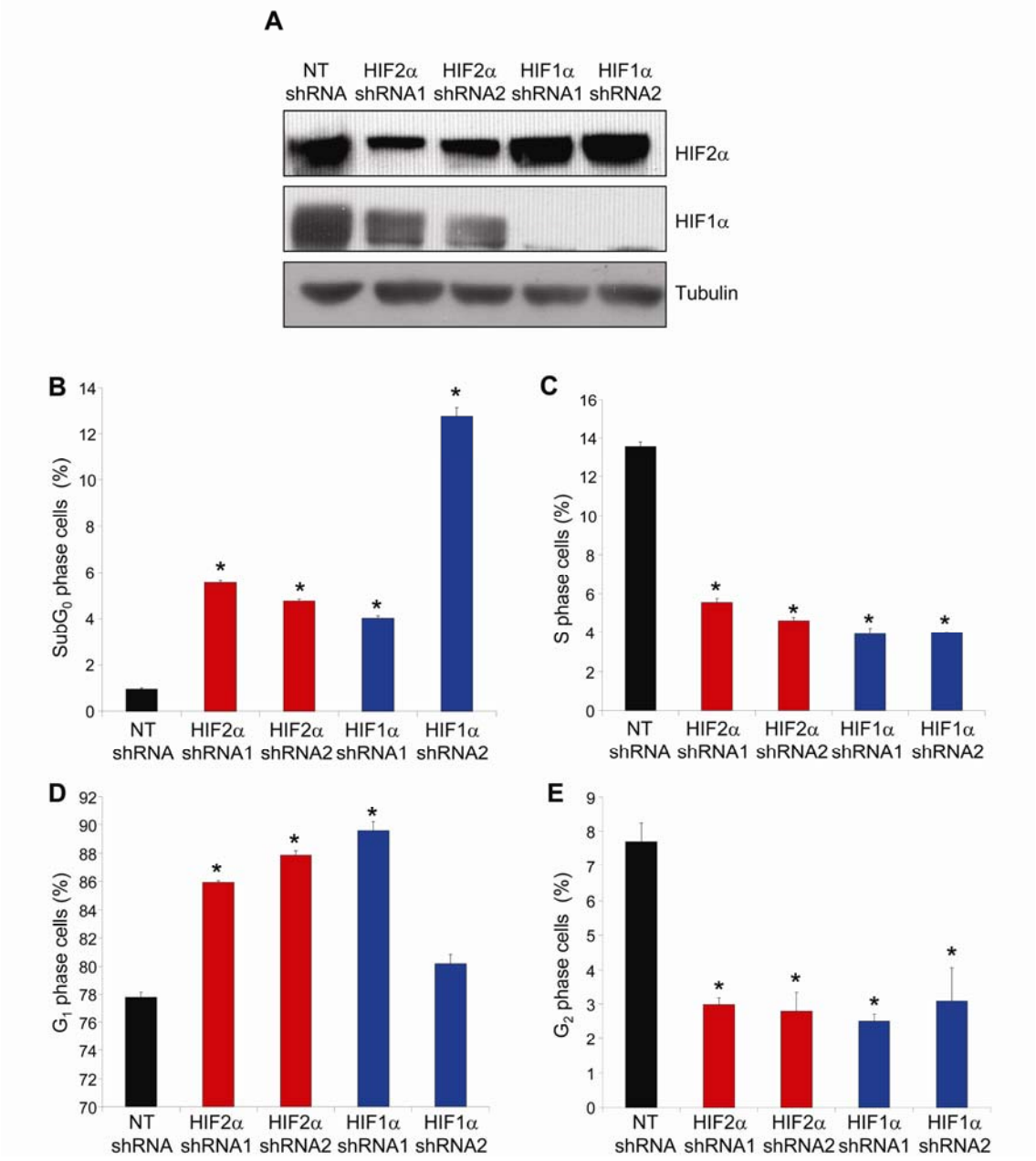

A
T3359 Glioma Nonstem
T3359 Glioma Stem
Normoxia
Hypoxia
Normoxia
Hypoxia
NT shRNA
HIF2α shRNA
HIF1α shRNA
PI
ANNEXIN V FITC


B
NT shRNA
HIF2α shRNA
HIF1α shRNA
Annexin V-positive cells (%)
100
80
60
40
20
0
*
#,*
Glioma Stem - + - +
Hypoxia - - + +

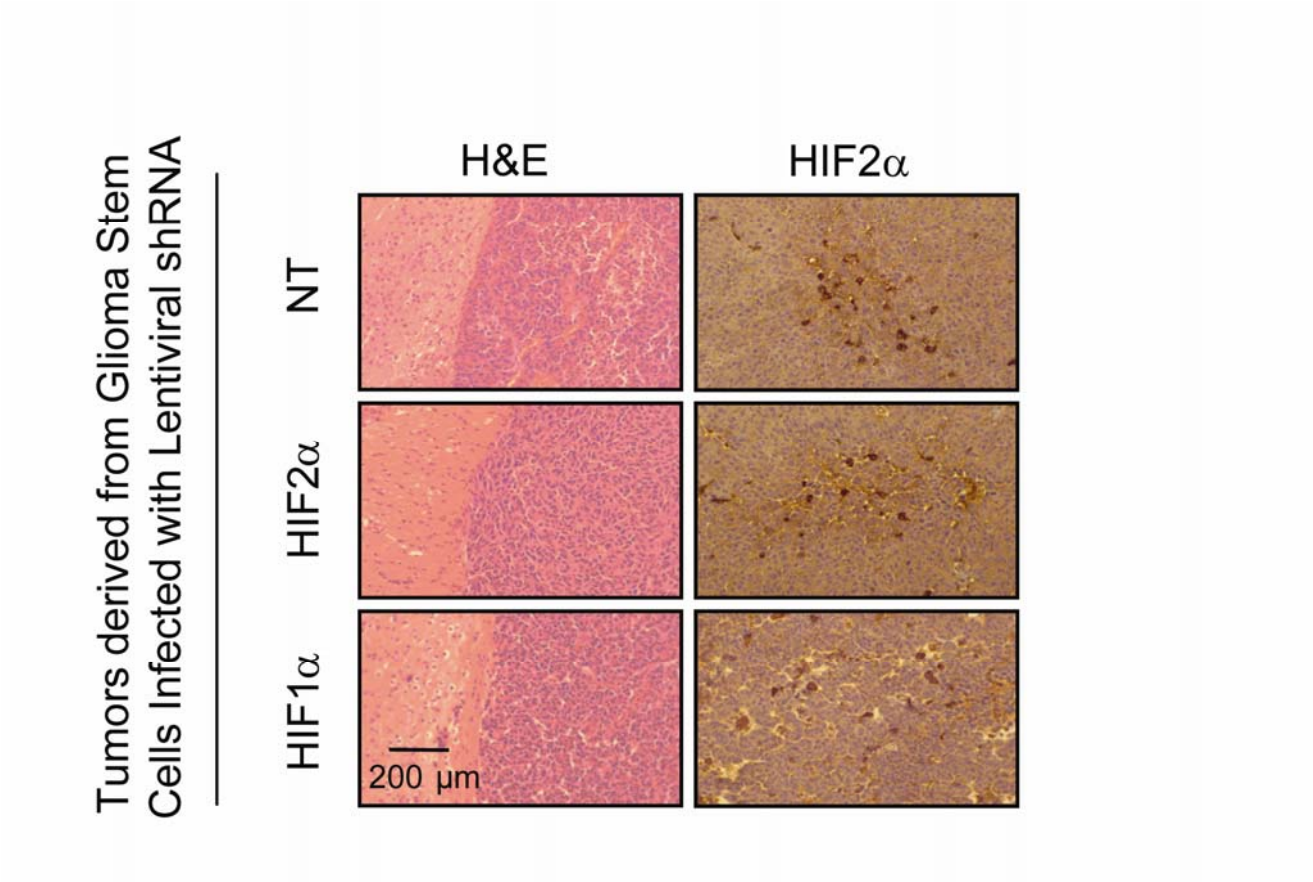
**Supplemental Figure 14. HIFα Knockdown Induced Apoptosis in Glioblastoma Cells.** (A) 200,000 lentiviral infected glioblastoma stem or nonstem cells were incubated in each well of 6-well plates for 48 hr followed by normoxia or hypoxia treatment for 24 hr. Cell apoptosis was then determined with the Annexin V Apoptosis Detection Kit (Santa Cruz, Inc). While knockdown of HIF1α lead to remarkable cell apoptosis in both glioblastoma stem and nonstem cells, knockdown of HIF2α increased apoptosis in glioblastoma stem but not nonstem glioblastoma cells isolated from a T3359 patient specimen passaged short term in immunocompromised mice. (B) Quantification of Annexin V analysis data. *, $P < 0.05$ with ANOVA comparison to nontargeting shRNA of the same cell type and hypoxia treatment. #, $P < 0.001$ with ANOVA comparison of HIF2α shRNA treated GSCs to HIF2α shRNA treated nonstem cells with identical oxygen treatment.


A
Post-FACS (hr)
0
24
T3565 Glioma Stem Annexin V+ Cells
Trypan Blue-positive Cells (%)
100
80
60
40
20
0
NT shRNA
HIF2 shRNA1
HIF2 shRNA2
HIF1 shRNA1
HIF1 shRNA2
B
NT shRNA
HIF2α shRNA1
HIF2α shRNA2
HIF1α shRNA1
HIF1α shRNA2
0 Post-FACS (hr)
24
72
108
500 μm
T3565 Glioma Stem Annexin V□ Cells
C
Relative caspase-3/7 activity
6
5
4
3
2
1
0
0
20
40
60
80
100 (hr)
Time after Annexin V Sorting
NT shRNA
HIF2α shRNA-1
HIF2α shRNA-2
HIF1α shRNA-1
HIF1α shRNA-2

**Supplemental Figure 15. HIFα Knockdown Induces Apoptosis and Blocked Neurosphere Formation in Glioma Stem Cells.** GSCs isolated from the patient sample T3565 were infected with lentivirus expressing the indicated shRNA for 72 hr. Cells were then FACS sorted using Annexin V antibody. Annexin V-positive and Annexin V-negative cells were collected and cultured separately in 24 well plates for up to 108 hr. (A) The majority of Annexin V-positive cells were dead immediately after sorting, and all of the cells were dead after 24 hr, as determined using trypan blue staining. (B) Representative images of Annexin V-negative cells cultured in the presence of puromycin to select for cells with stable shRNA integration. Annexin V-negative cells from the nontargeting group survived and formed neurospheres whereas HIFα targeted cells failed to form neurospheres. The loss of the ability to form neurospheres was more rapid in HIF2α (72 hr) than HIF1α targeted cells (108 hr), but both groups had minimal neurospheres at 108 hr after sorting. (B) Annexin V-negative cells isolated as above were plated in 96 well plates. Caspase-3/7 activity was measured after 24, 72, and 108 hr using the Caspase-Glo®-3/7 Assay Systems kit (Promega). Both HIF2α and HIF1α knockdown groups show significant increases in Caspase-3/7 activity, but HIF2α induced apoptosis at an earlier time point.


A
NT shRNA
HIF2α shRNA1
HIF2α shRNA2
HIF1α shRNA1
HIF1α shRNA2
HIF2α
HIF1α
Tubulin
B
SubG0 phase cells (%)
C
S phase cells (%)
D
G1 phase cells (%)
E
G2 phase cells (%)

**Supplemental Figure 16. HIFα Knockdown in Glioma Stem Cells Increased Apoptosis and Cell Cycle Arrest.** GSCs isolated from T3359 were infected with the indicated shRNAs. 48 hr after infection cells were fixed by ethanol and analyzed for cell cycle. (A) HIFs were successfully targeted by shRNAs. (B) The percentage of $SubG_0$ cells increases with HIF targeting. (C) The percentage of cycling, S-phase cells decreases with HIF targeting. (D) The percentage of $G_1$ arrested cells increases with HIF targeting. (E) Decreased progression through the cell cycle results in a decreased percentage of cells reaching the G2 phase of the cell cycle. *, $P < 0.01$ with ANOVA comparison to nontargeting control (NT).




**Supplemental Figure 17. Tumors Arise from Cells with Unsuccessful HIF2α Knockdown.** GSCs derived from a D456MG pediatric glioblastoma xenograft were infected with nontargeting shRNA or shRNA targeting HIF2α or HIF1α. 72 hr after infection 50,000 cells were injected into the brains of immunocompromised mice (n=4 for each of three arms). Mice were allowed to survive until the development of neurologic signs at which time animals were sacrificed and brains fixed and sectioned. Hematoxylin and eosin staining demonstrated the presence of tumors in mice exhibiting neurologic signs. Immunohistochemistry revealed the presence of HIF2α expressing cells in tumors, even those derived from GSCs infected with lentivirus expressing shRNA directed against HIF2α.