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Olig2-regulated lineage-restricted pathway controls replication competence in neural stem cells and malignant glioma

Keith L. Ligon^{1,4,*}, Emmanuelle Huillard^{1,10,*}, Shwetal Mehta^{2,*}, Santosh Kesari², Hongye Liu⁵, John A. Alberta², Robert M. Bachoo³, Michael Kane², David N. Louis⁶, Ronald A. DePinho^{3,9}, David J. Anderson⁷, Charles D. Stiles^{2,§}, and David H. Rowitch^{1,8,10,§}

¹ Department of Pediatric Oncology, Dana-Farber Cancer Institute and Harvard Medical School, 44 Binney Street, Boston, MA 02115

² Department of Cancer Biology, Dana-Farber Cancer Institute and Harvard Medical School, 44 Binney Street, Boston, MA 02115

³ Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, 44 Binney Street, Boston, MA 02115

⁴ Department of Pathology, Division of Neuropathology, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115

⁵ Informatics Program, Children's Hospital Boston, 300 Longwood Avenue, Boston, MA 02115

⁶ Pathology Service and Cancer Center, Massachusetts General Hospital, Boston, MA 02129

⁷ Division of Biology, Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA 91125

⁸ Divisions of Neonatology and Hematology-Oncology, Children's Hospital, 300 Longwood Avenue, Boston, MA 02115

⁹ Center for Applied Cancer Science, Dana-Farber Cancer Institute, Boston, MA 02115

¹⁰ Departments of Pediatrics and Neurological Surgery and the Institute for Regeneration Medicine, UCSF, 533 Parnassus Avenue, San Francisco CA 94143

Summary

Recent studies have identified stem cells in brain cancer. However, their relationship to normal CNS progenitors, including dependence on common lineage-restricted pathways, is unclear. We observe expression of the CNS-restricted transcription factor, OLIG2, in human glioma stem and progenitor cells reminiscent of Type C transit amplifying cells in germinal zones of the adult brain. *Olig2* function is required for proliferation of neural progenitors and for glioma formation in a genetically relevant murine model. Moreover, we show *p21^{WAF1/CIP1}*, a tumor suppressor and inhibitor of stem cell proliferation, is directly repressed by OLIG2 in neural progenitors and gliomas. Our findings identify an Olig2-regulated lineage-restricted pathway critical for proliferation of normal and tumorigenic CNS stem cells.

§Authors for correspondence: (e-mail: charles_stiles@dfci.harvard.edu, tele (617) 632-3512, fax (617) 632-4663; david_rowitch@dfci.harvard.edu, tele (617) 632-4201, fax (617) 632-2085).

*These authors contributed equally

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Introduction

Investigation of highly tumorigenic cellular subpopulations in adult primary tumors has revealed fundamental similarities to normal stem and progenitor cells within the same lineage (Garraway and Sellers, 2006). Such findings support the proposition that proliferation and survival of tumor cells may be dependent on the same “lineage-restricted” pathways that govern early phases of organogenesis. However, this concept remains to be functionally tested in a broad range of solid cancers, and potential interactions between such lineage-restricted regulatory factors and classical tumor suppressors are poorly understood.

From a developmental perspective, the regulatory networks controlling stem cell behavior in the vertebrate brain are relatively well characterized. Primary cancers of the brain, therefore, provide a favorable context in which to consider the conserved features of normal and tumorigenic stem cells. The most common form of primary brain cancer is the malignant glioma, “primary glioblastoma” (Astrocytoma, WHO Grade IV) (Louis et al., 2002). These uniformly fatal cancers are characterized by stereotypical combinations of genetic lesions, commonly involving amplification/activation of the epidermal growth factor receptor (EGFR) and loss-of-function mutations in the retinoblastoma (Rb) signaling axis for cell cycle control (Louis et al., 2002; Maher et al., 2001). At a histopathological level, primary glioblastomas consist of a heterogeneous mixture of cell types with some evidence of multi-lineage differentiation, recent work has identified cells with stem-like features in glioblastoma (Bao et al., 2006; Galli et al., 2004; Singh et al., 2004; Vescovi et al., 2006). The varied appearance of these tumors has led to proposals that the “cell-of-origin” is either a developmentally arrested neural stem cell or else a committed progenitor cell that has de-differentiated to a more stem-like state (Kesari and Stiles, 2006).

Two cell populations that promote glioma growth have been identified. Rapidly cycling glioma progenitor cells, which express the proliferation marker Ki67, and generally comprise less than 30 percent of tumor cells. Even less frequent within the tumor is a highly tumorigenic subpopulation of glioma stem cells identifiable by the stem/progenitor cell marker CD133 (Bao et al., 2006; Singh et al., 2004). These two replication competent cell populations invite comparisons to progenitors in the subventricular zone (SVZ) of the normal adult brain (Sanai et al., 2005). In this stem cell niche, relatively quiescent “type B” neural stem cells give rise to rapidly dividing “type C” progenitor cells (Doetsch et al., 1999).

During central nervous system (CNS) development, the bHLH transcriptional repressor proteins Olig1 and Olig2 play essential roles in the lineage specification of progenitor cells into neuronal sub-types (somatic motor neurons and forebrain cholinergic neurons) and oligodendrocytes (Furusho et al., 2006; Hack et al., 2005a; Lu et al., 2002; Mizuguchi et al., 2001; Novitsch et al., 2001; Takebayashi et al., 2002; Zhou and Anderson, 2002). At early embryonic stages, one key role of Olig2 is to maintain progenitor cells in a replication-competent state (Lee et al., 2005). The structurally related Olig1 transcription factor is required for maturation of oligodendrocyte progenitors (Arnett et al., 2004; Xin et al., 2005). Although it is co-expressed with Olig2 at early stages, Olig1 function is dispensable for specification of neurons or oligodendrocytes from replication competent progenitor cells (Lu et al., 2002; Zhou and Anderson, 2002).

Several lines of evidence suggest that the activity of Olig2 might provide a mechanistic link between growth of malignant glioma and adult neural stem cells. First, a subpopulation of type B and type C cells in the adult rodent brain express Olig2 (Bachoo et al., 2002; Hack et al., 2005a; Menn et al., 2006). Second, exposure to glioma relevant mitogens such as EGF or PDGF (Jackson et al., 2006) stimulates proliferation of Olig2+ rapidly dividing “type C” transit amplifying cells and glioma-like growths. Lastly, we and others have shown that all malignant

gliomas, irrespective of grade, express Olig2 in at least some fraction of the malignant cell population (Azzarelli et al., 2004; Bouvier et al., 2003; Ligon et al., 2004; Lu et al., 2001b; Marie et al., 2001; Ohnishi et al., 2003). Here we show that Olig2 function is required for glioma formation and that a common Olig2-dependent mechanism for cell cycle control regulates growth of normal and malignant neural progenitor cells.

Results

OLIG2 is expressed in human glioma stem/progenitor cells

To determine whether OLIG2 might have a functional role in tumorigenesis we first examined whether OLIG2 is expressed in the specific cell types capable of contributing to tumor growth. We quantitated glioma progenitor cells by their expression of the proliferation marker Ki67 or CD133, which has been proposed as a marker for glioma stem cells (Bao et al., 2006; Singh et al., 2004). Immunohistochemical (IHC) co-localization of OLIG2 and Ki67 within human glioblastoma sections shows expression of OLIG2 within at least 85% of Ki67+ glioma progenitor cells by manual counting ($n=3$ tumors) (Figure 1A). Quantitative flow cytometry of fresh surgical glioblastoma specimens ($n=11$) shows that while Ki67+ cells represent a minority of the total OLIG2+ population (~37%), the vast majority (~85%) of Ki67+ glioma progenitor cells are OLIG2+ (Figure. 1A–C). In contrast, analysis of OLIG1 expression showed less correlation with cycling glioma progenitor cells, as only 57% of Ki67+ cells expressed OLIG1 (Figure 1C).

Flow cytometry analysis of fresh human glioblastomas ($n=11$) shows that the CD133+ cells comprise only 6% of the tumor; however, almost all (98%) of these are OLIG2-positive (Figure 1D, E). In contrast, only 7% of the CD133 positive cells express Olig1 (Figure 1E). Taken together, these findings establish OLIG2 as a marker expressed in two cell types that contribute to tumor growth. However, as indicated in Figure 1C, E and summarized in Figure 1F, OLIG2 expression is not confined to glioma progenitors.

Olig2 function is required for glioma formation

To determine whether *Olig* genes are required for glioma formation we used a murine model of malignant glioma that emulates common genetic lesions underlying primary glioblastoma in humans (Bachoo et al., 2002). In this model, neural stem/progenitor cells from $p16^{Ink4a}/p19^{Arf}$ null mouse embryos are transformed with a retrovirus containing a constitutively active mutation of epidermal growth factor receptor (EGFRvIII) identified in human glioblastoma multiforme (GBM) (Rasheed et al., 1999). The resulting cells can be cultured as neurospheres capable of glioma formation with 100% penetrance when transplanted into the brains of immunocompromised (SCID) mice (Figure 2A). The tumors thus formed closely model the neuropathological and immunophenotypic features of human anaplastic astrocytoma WHO Grade III (Figure 3 and Supplemental Figure 1) including prominent expression of Olig2, rapid growth, and marked single cell infiltration of normal brain structures. In this model, tumor cells can be positively identified at single cell resolution using human specific antibodies to EGFR (hEGFR).

To remove *Olig* function in this model we generated neurospheres from compound transgenic mouse embryos that were *Ink4a/Arf* null and either wild type, heterozygous or null with respect to combined *Olig1/2* function (Zhou and Anderson, 2002) (Figure 2A). We chose to initially evaluate deletion of both *Olig* genes because both are expressed in malignant gliomas, and during development they have partially redundant functions (Lu et al., 2002; Zhou and Anderson, 2002). Analysis of survival cohorts showed that all animals implanted with *Olig1/2* wild type ($n=12$) or heterozygous ($n=25$) *Ink4a/Arf*^{-/-} EGFRvIII neurospheres developed neurological symptoms and died within 100 days post injection (dpi) (Figure 2B,

Table 1). In striking contrast, 91% of the mice injected with *Olig1/2*-null *Ink4a/Arf*^{-/-} EGFRvIII neurospheres survived without symptoms as long as 39 weeks post injection ($n = 23$) (Figure 2B, Table 1) and did not show evidence of tumor growth in the brain (see below).

These observations are consistent with requirements for unique or overlapping functions of *Olig2* and *Olig1* in glioma formation. To investigate the specific effects of *Olig2*, we infected *Olig1/2*-null *Ink4a/Arf*^{-/-} EGFRvIII neurospheres with a retrovirus encoding full-length mouse *Olig2* (Figure 2A). Exogenous production of *Olig2* expression in *Olig1/2*-null *Ink4a/Arf*^{-/-} EGFRvIII neurospheres effectively restores the tumorigenic phenotype (Figure 2C, Table 1) albeit with slightly increased latency and reduced penetrance compared to *Ink4a/Arf*^{-/-} EGFRvIII neurospheres with intact *Olig1/2* function. In addition, *Ink4a/Arf*^{-/-} EGFRvIII neurospheres derived from single knockout, *Olig1*-null donors demonstrated robust tumor formation with 87% of animals ($n=10$) developing fatal tumors by 10 weeks post injection (Figure 2B). Together, these findings demonstrate that *Olig2* function is specifically required for glioma formation. Further, they rule out the possibility that culturing of *Ink4a/Arf*^{-/-} EGFRvIII neurospheres from *Olig1/2*-null animals selects for *Olig*-independent progenitors that are fundamentally incapable of glioma formation.

Olig2 regulates proliferation and morphology of tumorigenic neurospheres

The intracranial tumors that form in this murine model of malignant glioma can be readily visualized using human specific EGFR antibodies. As indicated in Figure 3A–F and Supplemental Figure 1, these tumors closely recapitulate the neuropathological and immunophenotypic features of human anaplastic astrocytoma WHO Grade III. In contrast, histopathological analysis of *Olig1/2* null *Ink4a/Arf*^{-/-} EGFRvIII neurospheres injected animals at 70 dpi showed that these cells survive and engraft in the brain but do not form gliomas (Figure 3G, H, J, K). They were identified as scattered cells with differentiated astrocytic and neuronal morphologies immediately surrounding the injection site, but with no evidence of mass-like growth or tumor formation in nearly all (91%) cases (Table 1).

In vitro analysis to further investigate the effects of *Olig* loss-of-function indicates that *Olig1/2*-null *Ink4a/Arf*^{-/-} EGFRvIII neurospheres are capable of proliferation, albeit at a significantly lower rate than *Olig1/2*-heterozygous cells ($p < 0.001$) (Figure 4A). *Olig1/2*^{+/-} *Ink4a/Arf*^{-/-} EGFRvIII neurospheres are tri-potent (capable of expressing astrocyte, neuron and oligodendrocyte markers in differentiation conditions), whereas the *Olig1/2*^{-/-} *Ink4a/Arf*^{-/-} EGFRvIII neurospheres are bi-potent, with evidence of astrocytic and neuronal differentiation (Supplemental Figure 2), as expected based on known roles of *Olig1/2* for oligodendrocyte specification in neurosphere cultures (Zhou and Anderson, 2002). Moreover, the morphology of *Olig1/2*-null neurospheres is dramatically altered (Figure 4C, D). Relative to their wild type counterparts, the *Olig1/2*-null cells show increased adhesiveness and migration on the culture dish. Cells lacking *Olig* function are eventually unable to sustain growth as free floating spheres and proliferate instead as a partial monolayer (Figure 4D). The aberrant adhesion of *Olig*-null *Ink4a/Arf*^{-/-} EGFRvIII neurospheres is of interest since monolayer culture is one of the important external cues for differentiation of normal neurosphere cultures (Reynolds and Weiss, 1992).

Interestingly, restoration of *Olig2* activity in *Olig*-null *Ink4a/Arf*^{-/-} EGFRvIII neurospheres by retroviral transduction restores wild type growth and morphology *in vivo* (Figure 3I,L) and *in vitro* (Figure 4B,E). Therefore, *Olig2* regulates both the net proliferation and the histopathological appearance of *Ink4a/Arf*^{-/-} EGFRvIII neurospheres in the brain. Loss of *Olig2* function suppresses tumor formation and promotes a more mature and differentiated morphologic phenotype of the engrafted cells.

Olig2 regulates proliferation and morphology of normal neural stem cells

To better define the relationship between malignant and normal neural stem cells, we examined Olig expression in neural progenitor cells established from normal mice. Proliferating cells in S-phase were marked by a 2 hour pulse of bromodeoxyuridine (BrdU). Subsequent immunohistochemical analysis shows that Olig2 is expressed in the majority (85%) of the cycling cells (Figure 5). In contrast, relatively few of these cells express Olig1 (24%). The uncoupling of Olig1 and Olig2 expression in these normal neurospheres is reminiscent of that found in glioma stem cells of human GBM (Figure 1C).

Analysis of the growth rates of *Olig1/2*- or *Olig2*-null neural progenitors demonstrates a significant reduction (2.2- and 1.6-fold, respectively) in growth for both genotypes relative to heterozygous or wild type controls (Figure 5E). In addition, *Olig1/2* null neural stem cells show a marked reduction in the number of rapidly cycling cells in S phase as determined by BrdU labeling (1.6 fold reduction) (Figure 5F). Cell death (as measured by Trypan blue exclusion) is not affected by Olig status (Figure 5G). We observed that single cell suspensions of *Olig1/2*-null neurospheres are capable of self-renewal (data not shown). However, the morphologic appearance of neurospheres is clearly regulated by Olig status in a fashion similar to that noted for *Ink4a/Arf*^{-/-}EGFRvIII neurospheres (Figure 5H–J).

A computational screen for direct genetic targets of Olig2

An oppositional relationship between growth and differentiation is controlled by pRB, p53 and their downstream effector proteins (Adams and Kaelin, 1998; Cheng et al., 2000; Kippin et al., 2005; Meletis et al., 2006). Olig2 is known to function as a transcriptional repressor (Mizuguchi et al., 2001; Novitsch et al., 2001). Using a strategy summarized in Figure 6, we conducted a genome-wide computational screen for Olig2 repressible genes that are enriched for cis-acting E-box regulatory elements and might function as components of the pRB or p53 signaling networks.

Entry-level expression profiling data (Figure 6 and Supplementary Tables 1 and 2) showed, as expected, that *Olig2*-null neurospheres lacked oligodendroglial lineage gene expression signatures (e.g. PDGFR α , PLP, MBP). Some genes indicative of stem cell character (Ivanova et al., 2002; Livesey et al., 2004; Parker et al., 2005; Ramalho-Santos et al., 2002) were not significantly changed in *Olig2*-null neurospheres relative to wild type whereas others, including prominin 1/CD133, were actually more prevalent in the *Olig2*-null cells. Astrocyte specific markers were generally unaffected by Olig2 expression with the exception of Gfap which can mark both fibrous astrocytes in the brain (Bignami et al., 1972) and neural progenitor cells (Doetsch et al., 1999). Canonical neuronal marker genes showed no apparent trend, with some genes being up-regulated and others down-regulated in the absence of Olig2 (Figure 6 and Supplementary Tables 1 and 2).

Filtering the expression profiling data for Olig2-repressible, E-box enriched probe sets (Figure 6) identified four p53-inducible genes (Figure 6 and Supplemental Table 3. Three of these p53-responsive genes are poorly characterized from a functional perspective. However the fourth is *p21*^{WAF1/CIP1} (hereafter termed “*p21*”), which encodes a p53-inducible inhibitor of cell cycle progression. The p21 gene product antagonizes phosphorylation of pRB by inhibiting the activity of cyclin-dependent kinase 2 (Adams and Kaelin, 1998). We focused our subsequent studies on the relationship between Olig2 and p21 because targeted disruption of *p21* has been shown to enhance the proliferation of adult neural progenitor cells (Kippin et al., 2005).

The tumor suppressor *p21* is a direct target of Olig2 repression in normal stem cells and malignant glioma

The activity of *p21* is regulated primarily at the transcriptional level through canonical E-box DNA-binding elements in the upstream promoter/enhancer region of the gene (Gartel and Radhakrishnan, 2005) leading to the hypothesis that Olig2 might directly regulate *p21* through such sites (Figure 7A).

Quantitative PCR was used to validate the original expression profiling data implicating *p21* as an Olig2 repressible gene. As indicated in Figure 7B, *p21* expression is upregulated nearly three-fold in *Olig2*-null neurosphere cells relative to their wild type counterparts. Moreover, in a human glioma cell line (U87), ectopic Olig2 suppresses the expression of a *p21/luciferase* reporter construct (Figure 7B) while a dominant-negative Olig2 defective in binding DNA causes increased expression ($p < 0.0001$) (Figure 7C).

Chromatin immunoprecipitation (ChIP) assays show that *p21* is a direct genetic target of Olig2. For these assays a variety of controls were used to exclude adventitious interactions between Olig2 antibodies and the *p21* gene. First, we took advantage of the fact that cortical progenitor cells from E14 rat embryo do not express Olig2 unless they are exposed to FGF or Sonic hedgehog proteins (Gabay et al., 2003; Kessaris et al., 2004). As shown (Figure 7D), ChIP assays detect an FGF-dependent Olig2/*p21* interaction in these cortical progenitor cells. Second, we transfected a tamoxifen-activated Olig2 fusion protein into a human glioma cell line (HOG) that does not express endogenous Olig2. Using ChIP assays, we found that this ectopic Olig2 binds to human *p21* regulatory elements in the presence of tamoxifen but not in its absence (Figure 7E). Moreover, the ectopic Olig2 does not interact with upstream regulatory elements of two other *p21*-like CDK2 inhibitors, *p27* and *p57*, that are regulated primarily by post transcriptional mechanisms (Agrawal et al., 1996; Gartel and Radhakrishnan, 2005; Hengst and Reed, 1996; Pagano et al., 1995) (Figure 7F). Finally, we used ChIP assays to show endogenous Olig2 interacting with *p21* regulatory elements in fresh surgical isolates of human malignant gliomas (glioblastoma) but not with control IgG (Figure 7F). Collectively these data show that Olig2 interacts directly with upstream regulatory elements of *p21* and functionally can repress its expression.

Discussion

Genetic lesions that underlie malignant glioma in humans include activating mutations in the EGFR and PI-3 kinase pathways and loss-of-function mutations in tumor suppressors such as *p53* and *p16^{INK4A}/p19^{ARF}* (Barber et al., 2004; Barker et al., 1997; Duerr et al., 1998; Rasheed et al., 1997; Rasheed et al., 1999; Samuels et al., 2004; Steck et al., 1988). However, these mutations are not unique to cancers of the CNS. Lineage-restricted pathways that regulate brain tumor behavior may represent more specific therapeutic targets with little potential to affect off target cell types. We show here that a stereotypic combination of mutant *EGFR* and *Ink4a/Arf* loss, two genetic lesions commonly found in primary gliomas in humans, can evidently only cause brain tumors in the context of Olig2 function. In both normal and malignant neural stem cells, Olig2 functions as a direct repressor of the cell cycle inhibitor gene, *p21*. Together, our findings demonstrate an Olig2-regulated lineage-restricted pathway that is critical for proliferation of normal and tumorigenic CNS stem cells.

Olig2 expression is a unifying feature of normal stem cells and malignant glioma

Analogies have been drawn between formation of blood and development of the brain, a common underlying theme being the replication competent stem cell, which occasionally divides asymmetrically to give rise to committed progenitor cells (Anderson, 2001). Recent evidence indicates that the cell-of-origin for some leukemias resembles in many respects

normal hematopoietic progenitors (Krivtsov et al., 2006). This observation and other data support the emerging view that critical lineage specific determinates of cancer progenitor cell growth may be found in their normal developmental counterparts (Garraway and Sellers, 2006; Kho et al., 2004).

Although the cell of origin for malignant glioma remains obscure (Kesari and Stiles, 2006; Sanai et al., 2005), recent studies have highlighted similarities with multipotent neural stem and progenitor cells found in the developing and adult mammalian brain (Jackson et al., 2006). Highly tumorigenic subpopulations of cells in malignant gliomas have been identified that express the hematopoietic stem cell marker CD133. As is true of normal progenitor cells from the germinal zones of the embryonic and adult CNS, these tumor stem cells can be cultured as self-renewing “spheres” with multipotent characteristics (Bao et al., 2006; Galli et al., 2004; Singh et al., 2003; Yuan et al., 2004). In analogy to neural progenitor cells of the adult brain (Sanai et al., 2005), malignant glial tumors might be viewed as populations of (1) developmentally uncommitted stem cells marked by CD133, (2) rapidly cycling, transit amplifying cells marked by Ki67 that contribute significantly to tumor growth and (3) CD133-negative, non cycling progeny cells that make up the bulk of the tumor mass. We show here that all three of these tumor cell populations contain cells that are positive for OLIG2.

Analogies can be drawn between the OLIG2 expression pattern in glioma and in normal adult brains where expression is observed in (1) a subset of “type B” stem cells, (2) in transit amplifying (“type C”) progenitors of the subventricular zone (Hack et al., 2005b; Menn et al., 2006) and (3) in terminally differentiated oligodendrocytes throughout the brain. In preliminary studies (data not shown) we have noted that not all of the CD133-positive cells in our clinical specimens are positive also for Ki67. The mixture of Ki67-positive and negative cancer stem cells might suggest that the OLIG2-positive stem cells represent a transition state between the B and the transit amplifying C cell of the normal brain.

Because it is expressed in three biologically distinct cell cohorts, OLIG2 alone cannot serve as a specific marker for any functional subset of glioma cells. However, the critical role of Olig2 in murine gliomagenesis provides incentive to look for additional markers that could be used in combinatorial fashion to resolve these functional subsets in human cancers. A better resolution of the putative stem cell population marked by CD133 would be of special interest. Additional markers might also be used to better define the relationship between glioma stem cells and the type B and type C cells within the germinal zone of the adult brain.

An Olig2-dependent pathway underlies glioma formation and regulates growth of normal neural stem cells

Both normal and tumorigenic progenitor cells share an Olig2 requirement for growth. At what developmental time point might such effects be relevant? Loss of *Olig2* function in the embryonic forebrain ganglionic eminence does not result in global changes to ventricular zone proliferation or cell death. Rather, one observes a failure to develop oligodendrocytes (Lu et al., 2002; Zhou and Anderson, 2002) and subsets of neurons (Furusho et al., 2006), suggesting primarily an early role in cell fate specification. However, sustained expression of Olig2 at adult stages, in a subset of “type B” stem cells and transit amplifying (“type C”) progenitors (Menn et al., 2006) suggests additional roles for this transcription factor in late-stage proliferating progenitor cells found in the adult SVZ. We further speculate that Olig2 could also serve cell cycle regulatory roles in slowly-replicating NG2 progenitor cells that are abundant in the adult cerebral cortex (Ligon et al., 2006).

To better understand the mechanisms critical for growth of glioma we focused on the proliferating subset of cells in human GBM. Approximately 10–15% of cells in these tumors are actively dividing and of these we found that 85% expressed OLIG2. Both *OLIG1* and

OLIG2 are expressed in human glioma (Lu et al., 2001b; Marie et al., 2001). However, our findings indicate that *Olig2* function in particular is necessary for tumorigenesis in a robust murine model of primary glioma that incorporates activation of EGFR and mutation of the *Ink4a/Arf* locus. Interestingly, similar findings are obtained when *Olig2* is conditionally deleted from an independent glioma model based on mutations of the *Nf1* and *p53* loci (R. Lu and L. Parada, personal communication). Thus, the requirement for *Olig2* function may apply to glioma formation in general.

Olig2 functions as a “gateway” gene for brain tumor development

Olig2 fulfills criteria of a lineage-restricted competence factor for brain cancer and in this respect it bears striking analogy to the bHLH-LZ transcription factor, *MITF*, in melanoma (Du et al., 2004; Garraway and Sellers, 2006). First, *Olig2* function is crucial for the development of neural progenitors and progeny cells specifically in the CNS. Second, its expression is deregulated in brain cancer. Finally, its function is required for tumor formation.

However, the notion of *Olig2* as a critical lineage-restricted competence factor for brain cancer does not depend on functions as an oncogene *per se*. Although *OLIG2* ectopic expression in the T-cell lineage is associated with T-cell leukemia (Ferrando et al., 2004; Wang et al., 2000), gain-of-function studies in mice suggest it is unlikely to be sufficient for brain cancer formation (Lu et al., 2001a, KL, EH and DHR, unpublished observations). Further, our data argue against critical functions for *Olig2* in specification of stem cells. We and others (Zhou and Anderson, 2002) have noted that *Olig1/2*-null neurospheres are capable of self-renewal and differentiation into two of the three principal neural cell types. Expression of stem cell marker genes is either unaffected in *Olig2*-null neurospheres compared to controls or actually up-regulated in the *Olig2*-null cells (Figure 6 and Supplemental Table 1). However, we cannot rule out the possibility that *Olig2* co-localization in type B cells of the SVZ (Menn et al., 2006) and with nearly all CD133-positive cells in human glioma (this study) might regulate the transition from quiescent stem cell to transit amplifying progenitor cell in the context of normal development and tumorigenesis, respectively.

Our genetic data establish that *Olig* function is critical for the glioma promoting effects of mutation of the $p16^{\text{INK4A}}/p19^{\text{ARF}}$ tumor suppressor locus and resonate with recent reports that age-related competence of such neural progenitors is regulated by $p16^{\text{INK4a}}$ function (Molofsky et al., 2006). Together, these data suggest that possibility that *Olig* function is critical for several cell cycle regulatory mechanisms underlying growth and survival of both normal and tumorigenic CNS progenitors.

Molecular mechanisms of *Olig2* gateway function

The *p21* cell cycle inhibitor gene is notable for its important role in maintaining the relatively quiescent state of stem cells for both blood (Cheng et al., 2000) and brain (Kippin et al., 2005). In addition, *p53* regulation of neural stem cell growth is also mediated in part through its effects on *p21* (Meletis et al., 2006). Targeted disruption of *p21* enhances the proliferation rate of neural stem cells in the adult mammalian forebrain (Kippin et al., 2005). Our data show that the *p21* locus is subject to direct *Olig2* transcriptional repression thus providing a direct link between *Olig2* and the cell cycle regulatory apparatus in normal and gliomagenic progenitors. Collectively, these observations raise the interesting possibility that *OLIG2* function might be dispensable for tumor formation in a *p21* null context. However an alternative, more complex scenario is suggested by our computational screen, which reveals three additional *p53*-inducible genes that may serve as direct genetic targets for *Olig2* (Supplemental Table 3). The *p21* gene product does function as a tumor suppressor in knockout mice but its activity as such is rather weak. Moreover, *p21* loss-of-function mutations are infrequent in human cancers (Adams and Kaelin, 1998; Gartel and Radhakrishnan, 2005). The

laboratory and clinical data suggest that loss of p53 gene function upstream of p21 is a far more effective route to cancer. Thus OLIG2 might suppress multiple p53 targets and/or other non-p53 target genes in its role as a “gateway” gene for brain cancer.

Interestingly, primary glioblastomas are highly resistant to radiation and cytotoxic drugs despite the fact that *p53* is generally intact within these tumors. Although there are multiple routes to suppression of p53 function (Besson and Yong, 2001), our data suggest a novel, OLIG2-dependent avenue to p53 pathway antagonism which is potentially active even at early stages of gliomagenesis. One might imagine that increased *OLIG2* and decreased *p21* expression would predict more aggressive tumor and shorter survival times. However, the literature on p21 and glioma survival is ambivalent. Some studies show that *p21* expression is higher in low-grade than in high-grade astrocytomas (Kamiya and Nakazato, 2002) and that the presence of *p21* is associated with prolonged survival (Korshunov et al., 2002). Other studies indicate that higher p21 staining is associated strongly with higher histological grade ($p < 0.001$), a higher rate of proliferation ($p = 0.021$), and worse survival (Miettinen et al., 2001). Still other studies report that p21 levels have no impact at all on survival in high grade gliomas (Korshunov et al., 2002; Kraus et al., 2001). There are many pitfalls to interpreting the impact of *p21* (or any gene, for that matter) on survival in gliomas. However one obvious problem is that previous studies have monitored p21 expression in the whole tumor. It is conceivable that important prognostic insights have been hitherto obscured for this reason. It is possible that useful prognostic insights could be derived from an analysis of p21 expression in CD133/OLIG2-positive glioma cells.

We should also note that, in apparent contradistinction to our findings, it has recently been reported that ectopic OLIG2 expression retards growth of an immortalized glioma cell line, binds to promoter elements of p27 and stimulates expression of p27 mRNA and protein (Tabu et al., 2006). These observations of a linkage between OLIG2 and p27 gene transcription in a glioma cell line stand apart from a wide range of data showing that p27 is regulated primarily by post transcriptional mechanisms in other cell types (Agrawal et al., 1996; Gartel and Radhakrishnan, 2005; Hengst and Reed, 1996; Pagano et al., 1995). The p27 primers used for our ChIP assays amplify in the region identified by Tabu et al and yet we did not detect any direct OLIG2:p27 interactions in primary human tumor samples. Further, p27 expression in our OLIG2 null neurospheres was not altered. Some of the discrepancies between the data reported here and that of Tabu et al may reflect the differential properties of serum-cultured glioma cell lines versus primary neurospheres and fresh human tumors that have been noted in recent studies (Lee et al., 2006).

The Olig2-regulated lineage-restricted pathway: a potential therapeutic target in brain cancer

Brain tumors remain a major cause of cancer-related death despite advances in surgery, imaging and conventional treatment modalities (Louis et al., 2002; Maher et al., 2001). This emphasizes the need to develop novel medical strategies based on a comprehensive understanding of the biological mechanisms underlying gliomagenesis (Rich and Bigner, 2004). Recent findings defining progression from normal progenitor to cancer stem cell in leukemias have suggested that therapeutic targeting of a self-renewal program expressed in an abnormal context may be possible (Krivtsov et al., 2006). Identification of conserved transcriptional regulatory mechanisms within normal and neoplastic hematopoietic progenitor cells has facilitated diagnosis of acute myelogenous leukemia and suggested novel therapeutic avenues (Tenen, 2003). Indeed, recent studies have identified OLIG2 expression as a negative prognostic indicator in human glioblastoma (Liang et al., 2005), and our findings identify this core transcriptional regulator as an important candidate for anti-tumor therapeutics.

In preliminary experiments, stem cells derived from a primary human glioma were transduced with hairpin RNAi expression vectors or dominant negative mutation constructs targeted

towards OLIG2. Genetic knockdown of OLIG2 by these means inhibited the ability of these human glioma stem cells to form intracranial tumors in SCID mice (S.K. and C.D.S., unpublished observations). Further experiments with multiple human tumors are needed to comprehensively assess the requirement for *OLIG2* function in the settings of the various genetic lesions associated with human glioma such as *PTEN*, *INK4a/ARF* and *p53*. While transcription factors are not generally considered 'drugable' targets in anti-tumor therapy, OLIG2 activity could be inhibited at the level of expression, post-translational modification or protein-protein interactions (Kondo and Raff, 2004; Setoguchi and Kondo, 2004; Sun et al., 2003; Walensky et al., 2004). Equally, it should be possible to define downstream factors in the Olig-regulated pathway that could prove useful in this regard.

Experimental Procedures

Mouse procedures

Animal husbandry was performed according to DFCI ACUC approved protocols for all experiments reported. *Olig1-cre* and *Olig1/2* (Lu et al., 2002; Zhou and Anderson, 2002) mouse lines were used on a mixed Swiss and C57Bl6/J background. *Ink4a/Arf* mice were obtained from the NCI mouse depository on a C57Bl6/J background. Immunocompromised Icr-SCID homozygous mice were obtained from Taconic Inc. (Model #ICRSC-M). For injections, neurospheres were dissociated and resuspended in HBSS at a concentration of 100,000 viable cells/ μ l. Two μ l were injected into the right striatum as previously described (Bachoo et al., 2002). No effects of passage number were noted for all cell lines injected. Animals were placed into survival or time point cohorts and were sacrificed at the onset of neurological symptoms or once moribund.

Neurosphere cultures

Normal neural stem cells (NSC) were isolated from the ganglionic eminences of E14.5 C57Bl6/J embryos, using techniques previously described (Laywell et al., 2002). Cells were cultured in DMEM/F12 (with L-Glutamine, Invitrogen) medium containing glucose (0.3%), penicillin/streptomycin (50 μ g/mL), Apo-Transferrin (0.1mg/mL), Progesterone (20nM), sodium selenite (30nM), putrescine (60 μ M), insulin (25 μ g/mL) and 20ng/mL EGF (all reagents from Sigma). NSC were cultured on plates coated with anti-adhesive (Laywell et al., 2002) and neurospheres were passaged once a week by mechanical dissociation. Live cells were counted using a hemocytometer and trypan blue exclusion and re-plated at equivalent densities (2×10^4 cells/well) in 6-well plates.

Ink4a/Arf^{-/-}EGFRvIII neurospheres were generated from various genetic backgrounds using identical techniques. *Ink4a/Arf*^{-/-}EGFRvIII neurospheres were generated from *Ink4a/Arf*^{-/-}NSCs (on *Olig1/2*^{+/-} or *Olig1/2*^{-/-} background). These cells were subsequently infected with EGFRvIII encoding retrovirus at first passage (P1) and treated with puromycin (2 μ g/mL) 48 hours after infection. For the rescue experiment, *Ink4a/Arf*^{-/-}EGFRvIII neurospheres of various genetic backgrounds were transduced with Olig2 or GFP encoding retroviruses at P3 and selected with blasticidin (2 μ g/mL). Expression of Olig2 or GFP was confirmed by immunocytochemistry. Selection drugs were maintained at all times during *in vitro* growth. Viable *Ink4a/Arf*^{-/-}EGFRvIII cell numbers at passage were generally higher for all genetic backgrounds than normal neurospheres and no changes in growth properties were noted up to 15 passages including *Olig1/2*^{-/-} lines, which showed partial growth deficiencies. *Ink4a/Arf*^{-/-}EGFRvIII neurospheres were injected at least 2 passages after last infection. No difference in growth or tumor formation was noted upon testing of frozen neurosphere lines (1 heterozygous and 1 knockout line tested).

Determination of neurosphere growth rates were performed by counting the total viable cell numbers at time of passage over several passages (from 4 to 10 passages) and averaged to calculate mean cell number at passage. To estimate the number of proliferating cells, neurospheres were pulsed with BrdU (1 μ M) during the last 1 hour of culturing, dissociated, and processed as previously described (Kippin et al., 2005).

For Olig2 and BrdU immunostaining of neurosphere sections, neurospheres were pulsed with 1 μ M BrdU for two hours prior to fixation, washed in PBS and fixed in 4% PFA. Spheres were embedded in HistoGel (Richard Allan Scientific) subsequently paraffin embedded, and sectioned at 5 μ m. Rabbit polyclonal anti-Olig2 (1/10,000) and mouse monoclonal anti-BrdU (BD Biosciences; 1/100) were used in a standard immunohistochemistry protocol. All numbers are expressed as mean \pm s.e.m.

Retroviral vectors and virus production

Construction of the retroviral vector encoding the constitutively active mutant of EGFR (EGFRvIII) has been described (Bachoo et al., 2002). Full-length mouse Olig2 was PCR cloned and an XbaI site added via primer sequence to allow cloning into the EcoRI/XbaI sites of the pWZL-BLAST retroviral vector (Morgenstern and Land, 1990). GFP was similarly PCR cloned with primers that added EcoRI and XbaI sites to the 5' and 3' ends respectively.

Chromatin immunoprecipitation

Chromatin immunoprecipitations were performed as described by Shang et. al. (Shang et al., 2002) with the following modifications: For HOG cells stably transfected with Olig2-estrogen receptor fusion construct, tamoxifen (0.5 μ M) was added for four hours prior to the crosslinking step. Rat cortical progenitor cells were isolated from E14 embryos in serum free media as previously described (Williams et al., 1997). Proliferation and Olig2 expression were induced by addition of fibroblast growth factor 2 (30ng/ml). After four days cells were processed for chromatin immunoprecipitation. Fresh surgical isolates of human glioblastoma were snap frozen in liquid nitrogen. Frozen tissue was homogenized and fixed with formaldehyde and processed for chromatin immunoprecipitations.

The primers used for ChIP analysis were as follows: Rat p21 F(forward), AGGTGTCTAGACTCCAGATT, R (reverse) AAAATCAAGGCTTTGCTGG (Olig2 binding region). Human p21 SM19F TCCTGCAGCACGCGAGGTT, SM19R TGTGAGCAGCTGCCGAAGTC (-219 to +7, TATA box region); SM18F GGCAGGAGGCCAAAAGTCCT, SM18R GAAGCCTGTCCTCCCCGAGG (-1434 to -987, Olig-2 binding region); SM17F GAACAGGGTATGTGATCTGC, SM17R TGGTACTGAGCTTCACAATG (-2137 to -1794, near p53 binding sites); and SM29F TCTGTGAAAACATGCCAGC, SM29R TTGAAACAGGGGACCGTGTC (-4018 to -3732, far upstream region).

Luciferase Assay

The p21 promoter-luciferase reporter plasmid was constructed using 2.4kb fragment from the upstream region containing Olig2 binding sites in the human p21 promoter. The construct was cotransfected with pcDNA3.1/myc/HIS-A, mouse Olig2 expression plasmid or mouse DN-Olig2 expression plasmid using Lipofectamine 2000 (Invitrogen) in U87 cells. Cells were harvested and assayed for luciferase activity 2 days after transfection using a luciferase assay kit (Promega). Luciferase activity for each construct was normalized using the protein concentrations and analyzed using a Berthold Mithras LB940 platereader for luminescence.

Real-time PCR for p21

RNA was extracted from neural progenitor cells using TRIzol reagent (Invitrogen) and further purified using the RNeasy kit (Qiagen). One μg of total RNA was used to generate cDNA, which was then used for the quantitative PCR using pre-made TaqMan gene expression assays (Applied Biosystems) for p21 and rodent GAPDH.

Computational Screen for Olig2 Target Genes

The Affymetrix Mouse 430A2 microarray data of the murine *Olig2*-null neural stem cells (four separate lines) were analyzed in comparison with wild-type neural stem cells (three separate lines) using the non-parametric wilcoxon ranksum test. A p-value < 0.05 was considered significant. At this level of significance there were 882 probe sets corresponding to genes that were over-expressed in *Olig2*-null cells relative to their wild type counterparts. An additional 1021 probe sets corresponded to genes that were down-regulated in *Olig2*-null cells relative to wild type cells. From the long list of 882 over-expressed probe sets, we used the “MAPPER” search engine and database (Marinescu et al., 2005a; Marinescu et al., 2005b) to identify 167 probe sets that contained an E-box motif.

Flow Cytometry

Dissected tissues from 11 independent cases of glioma were placed in DMEM solution with antibiotics on ice until processing. Tissue was washed in artificial cerebrospinal fluid (CSF) and acutely dissociated as described previously (Singh et al., 2004) using a modified tissue dissociation medium containing Collagenase IV (1mg/ml; Worthington), Hyaluronidase (0.67mg/ml; Sigma), DNase I (0.4mg/ml; Worthington), kynurenic acid and N-acetylcysteine (60 $\mu\text{g}/\text{ml}$; Sigma). Cells were washed and used fresh for flow cytometry analysis or resuspended in media for culture. Trypsin was avoided due to higher proteolytic activity and potential cleavage of cell surface proteins (Anderson et al., 2002). Red blood cells were removed using lympholyte-M (Cedarlane).

Fluorescent activated cytometry was performed on a FACSCalibur machine (BD Biosciences). Antibodies used included: mouse IgG1 anti-CD133 antibody (AC133, unconjugated and PE- and APC-conjugated, Miltenyi Biotech, Auburn, CA), rabbit polyclonal Olig2 and Olig1 antibodies (Arnett et al., 2004), mouse IgG1 and anti-Ki67 (M0722, Dako, Carpinteria, CA). Cells were first stained for surface CD133 (antibody dilution 1:100) for 30 minutes at 4°C then washed (PBS with 1% human serum), fixed, and permeabilized to allow access to intracellular antigens (Cytfix/Cytoperm solution; BD Biosciences). Cells were then further incubated with antibody for intracellular antigens (dilution 1:100) for 30–90 minutes at 4°C and washed. Cells were incubated with secondary antibodies (anti-rabbit Alexa488 or Alexa610-PE, and anti-mouse IgG Cy5 or Alexa488, Molecular Probes) for 30 minutes at 4°C and washed twice before FACS analysis. Single positives and isotype stains were used as controls for compensation and gating thresholds. Data was analyzed using FLOWJO software (v6.2.1; Tree Star, Inc).

Histology Analysis, Immunohistochemistry, and *in situ* Hybridization

Histological screening of tumors was performed by serial analysis of H&E coronal sections taken at approximately 2mm intervals along the entire brain. Tumors were scored as present based on identification of a collection of atypical cells greater than 2mm in diameter, although generally tumors at 2 months exceeded 1.5cm in size and infiltrated the entire right hemisphere. Selected animals that lacked histologic evidence of tumors were also more stringently screened for tumor growth by IHC for hEGFR.

Immunohistochemistry was performed according to standard protocols (Ligon et al., 2004). A new monoclonal antibody to Olig2 was developed by standard methods. Polyclonal antibodies

to Olig1 and Olig2 were previously described (Arnett et al., 2004). Other antibodies were Ki67 (rabbit polyclonal, Novocastra, Ki67p), p21 (mouse monoclonal, SantaCruz F5), NeuN (Chemicon), TuJ1 (Sigma), hEGFR (Dako), Nestin (BD Biosciences), GFAP (Sigma) and BrdU (Chemicon). In situ hybridization was performed on human tumors as previously described (Lu et al., 2001b) using full length cDNA encoding human CD133 (obtained from OpenBiosystems Inc.).

Collection and use of fresh and discarded human tumor tissue was approved through Brigham and Women's Hospital (BWH) Institutional Review Board. After frozen section diagnosis of glioma by the attending neuropathologist, representative tissue samples were dissected. Portions of the tumors were collected in chilled media for the studies described here and other portions were allocated for paraffin embedding for histological diagnosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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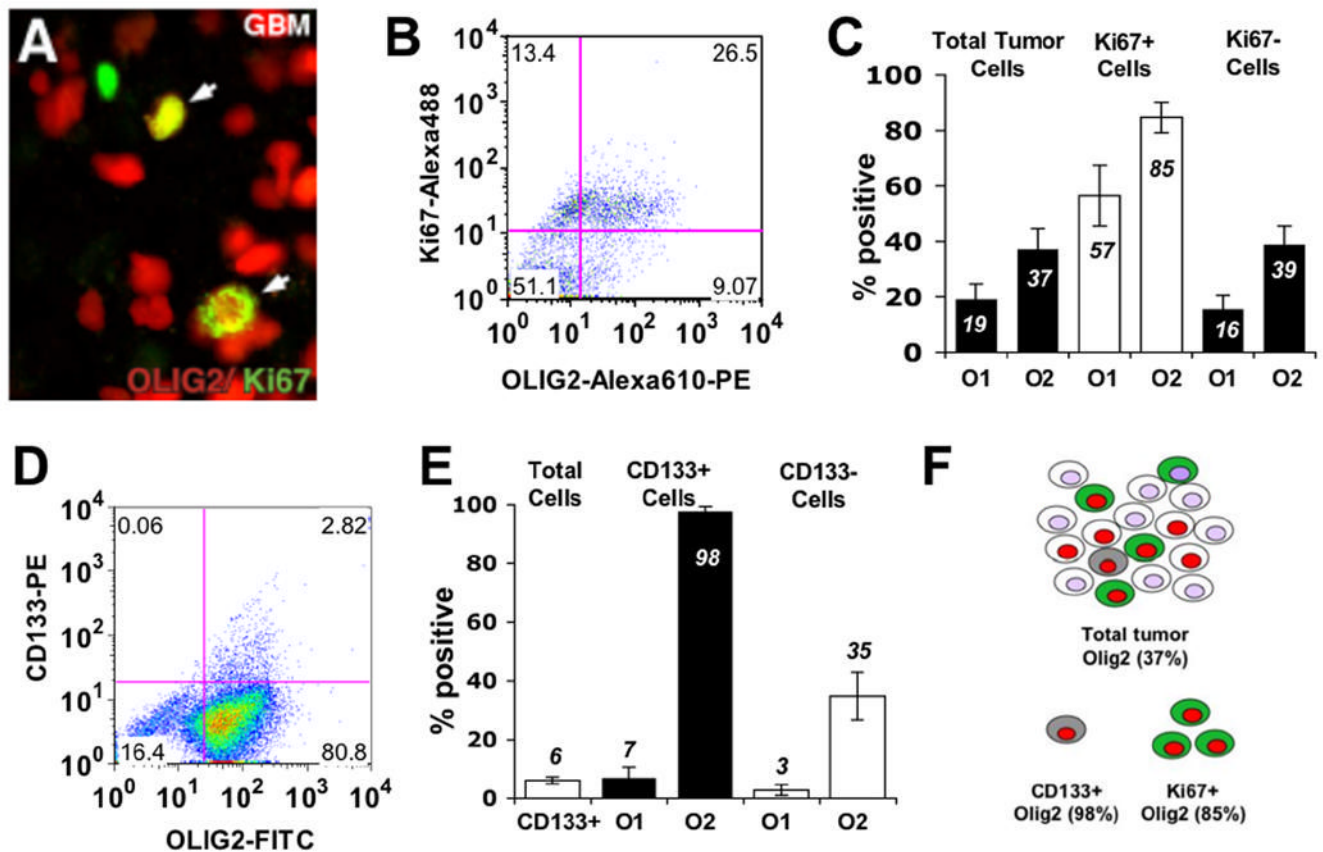


Figure 1. Human glioma progenitor cells and glioma stem cells express OLIG2

(A) Immunohistochemistry for OLIG2 (red) and the proliferation marker, Ki67 (green) in a human glioblastoma. Arrows denote co-localization of proteins (yellow) in glioma progenitor cells. (B) Scatter plot showing flow cytometric analysis of Ki67 and OLIG2 in a human glioblastoma sample. (C) Summary of flow cytometry results characterizing glioma progenitor cell populations in human glioblastomas (n=11). (D) Scatter plot showing flow cytometric analysis of CD133 and Olig2 in human glioblastoma sample. (E) Summary of flow cytometry results characterizing CD133+ putative glioma stem cell populations in fresh human GBM with respect to OLIG2 (n=11) and OLIG1 (n=4) expression. All p values < 0.001. O1=OLIG1, O2=OLIG2. Error bars indicate s.e.m. (F) Summary of the data shown in panels C and E.

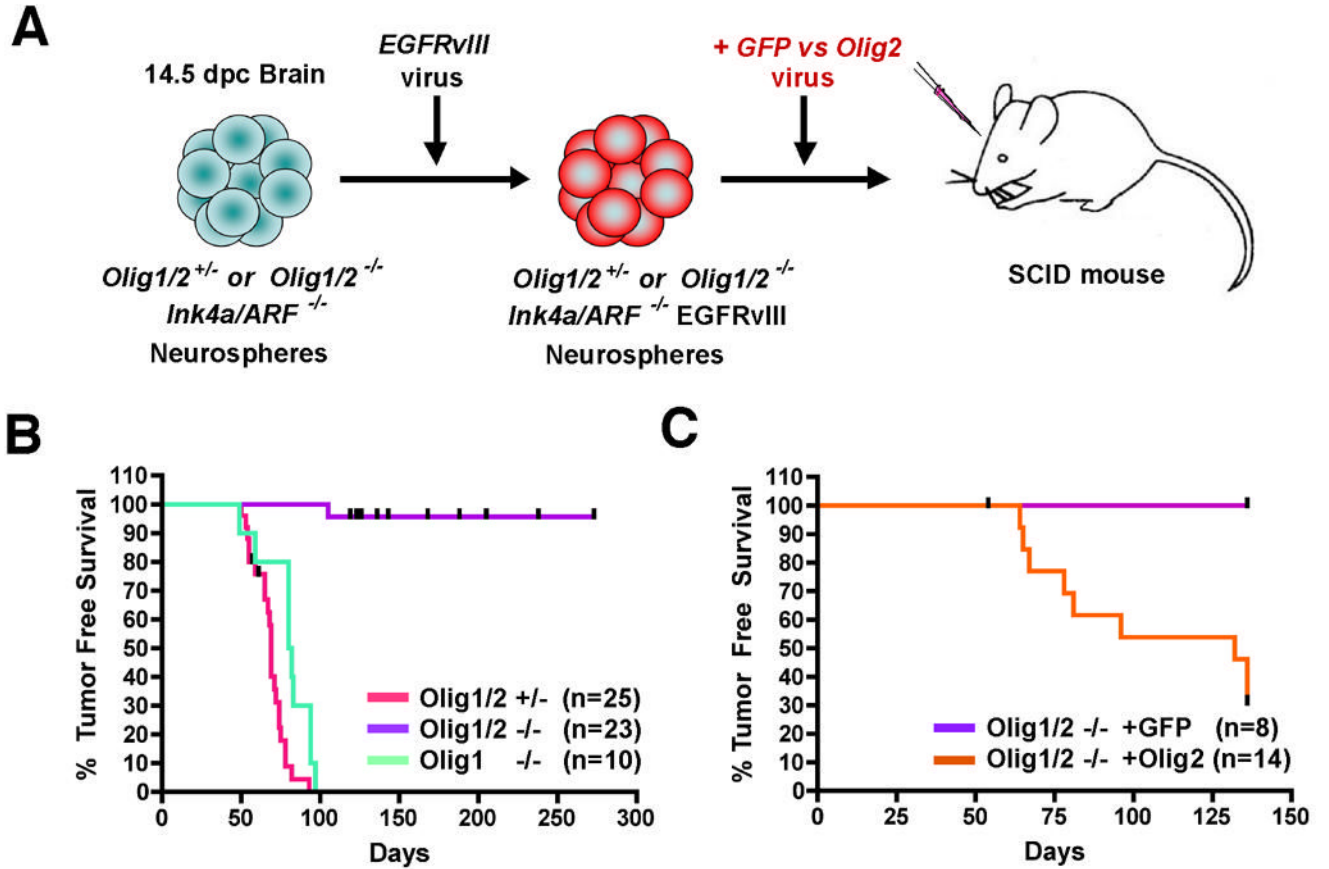


Figure 2. *Olig2* is required for glioma formation from neural stem cells

(A) Strategy for murine glioma model utilizing genetically defined neural stem cells as the cell of origin. 2×10^5 $Ink4a/Arf^{-/-}$ EGFRvIII neurosphere cells of the indicated genotypes were transplanted orthotopically into the striatum of SCID mice and animals monitored for symptoms of glioma formation. (B) Kaplan-Meier survival analysis. SCID mice injected with $Olig1/2^{+/-}$ or $Olig1^{-/-}$ $Ink4a/Arf^{-/-}$ EGFRvIII neurospheres die from tumors with short latency (median survival=69 days and 81 days, respectively) relative to mice injected with $Olig1/2$ -null $Ink4a/Arf^{-/-}$ EGFRvIII neurospheres (96% survival at 273 days). Censored animals in the $Olig1/2^{-/-}$ group (up ticks) indicate non-cancer deaths. $p < 0.01$ for $Olig1/2^{-/-}$ versus $Olig1^{-/-}$ or $Olig1/2^{+/-}$. (C) Kaplan-Meier survival analysis showing restoration of tumor phenotype in $Olig$ deficient mice by $Olig2$ encoding retrovirus (orange) (median TFS = 132 days), but not control GFP virus (purple) (100% survival at 136 days), $p < 0.01$.

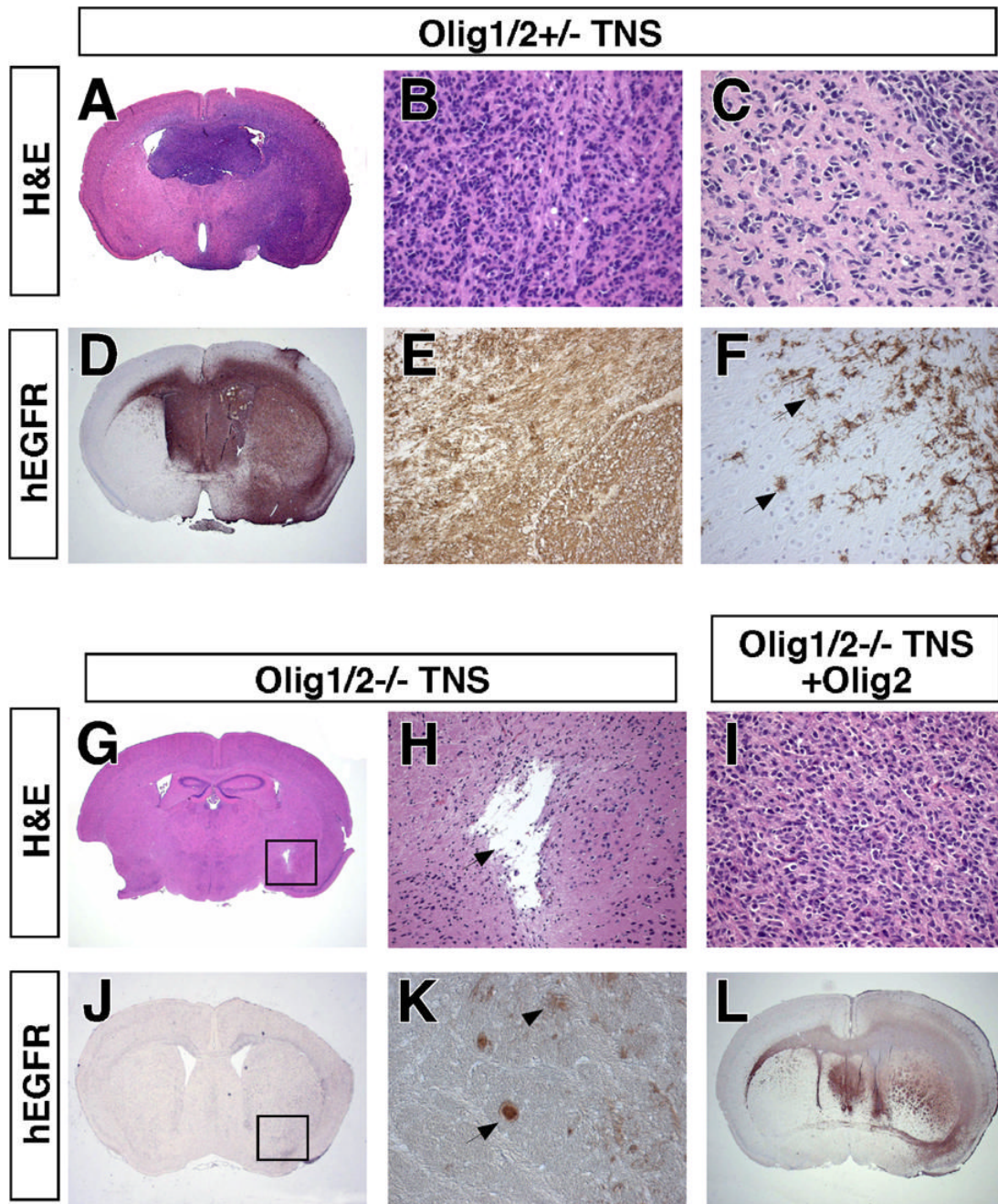


Figure 3. *Olig2* is required for glioma growth but not neurosphere engraftment in the brain
 (A, D) H&E staining and hEGFR IHC on coronal section of SCID mouse brain 100 days after injection with *Olig1/2^{+/-} Ink4a/Arf^{-/-} EGFRvIII* neurospheres demonstrating growth of malignant glioma. (B, E) Tumor histologically resembles human anaplastic astrocytoma W.H.O. grade III and uniformly expresses hEGFR. (C, F) Single cell infiltration of brain parenchyma is seen by H&E and hEGFR IHC. For additional characterization see Supplemental Figure 1. (G, J) Age matched brain injected with *Olig1/2^{-/-} TNS*. Box indicates injection site. (H) No tumor formation is detected by H&E staining at site of injection (arrow). (K) Rare surviving hEGFR+ *Olig1/2^{-/-} Ink4a/Arf^{-/-} EGFRvIII* neurospheres have morphological characteristics of astrocytes (arrowhead) and neurons (arrow). (I, L) Subsequent

infection of *Olig1/2*^{-/-} *Ink4a/Arf*^{-/-} EGFRvIII neurospheres with *Olig2* encoding retrovirus rescues formation of gliomas histologically identical to those from *Olig1/2*^{+/-} *Ink4a/Arf*^{-/-} EGFRvIII neurospheres.

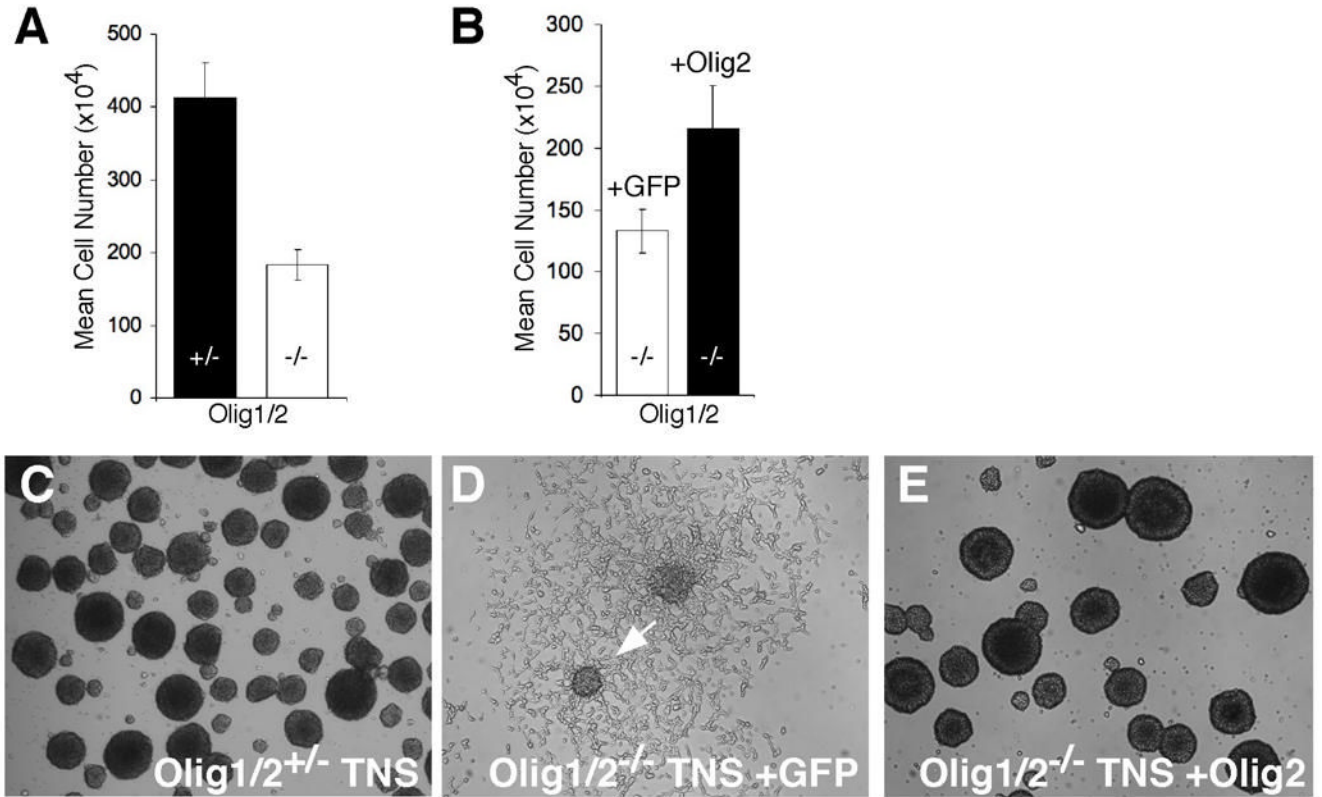


Figure 4. Olig2 regulates the growth and morphology of *Ink4a/Arf*^{-/-}EGFRvIII neurospheres (A) The mean viable cell number of *Olig1/2*^{-/-} *Ink4a/Arf*^{-/-}EGFRvIII neurospheres is significantly reduced compared to *Olig1/2*^{+/-} controls ($p < 0.001$). (B) Partial restoration of *Olig1/2*^{-/-} *Ink4a/Arf*^{-/-}EGFRvIII neurosphere growth is seen following infection with Olig2 retrovirus but not with the GFP control ($p < 0.05$). (C) Morphology of *Olig1/2*^{+/-} *Ink4a/Arf*^{-/-}EGFRvIII neurospheres (Tumorigenic Neurospheres, TNS) resembles that of normal neurospheres. (D) *Olig1/2*^{-/-} *Ink4a/Arf*^{-/-}EGFRvIII neurospheres exhibit marked adherence and migration on underlying substrate (arrow). (E) Infection of *Olig1/2*^{-/-} *Ink4a/Arf*^{-/-}EGFRvIII neurospheres with Olig2 retrovirus completely restores cells to the morphology of wild type *Ink4a/Arf*^{-/-}EGFRvIII neurospheres. All neurospheres were cultured under growth conditions in the presence of EGF.

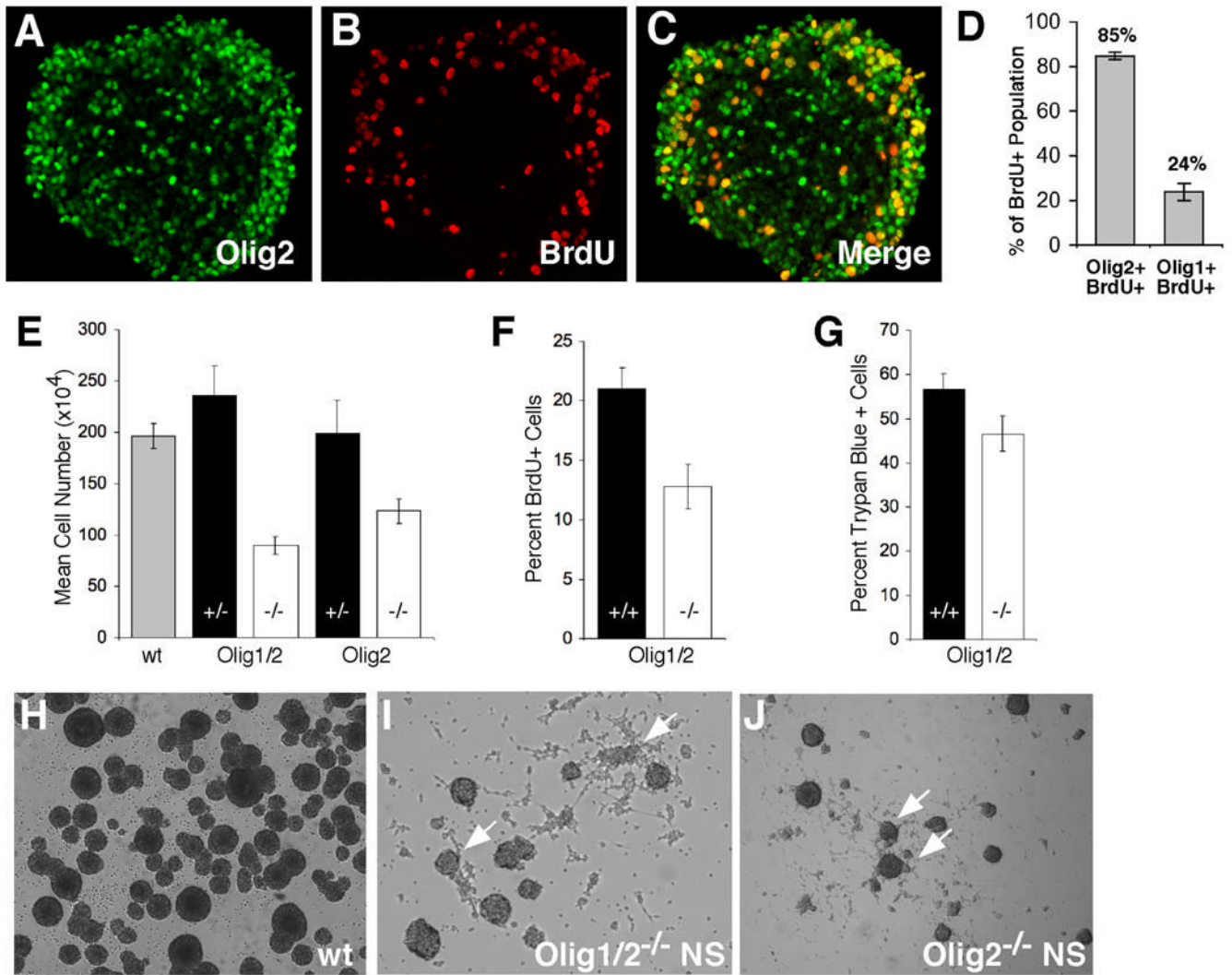


Figure 5. Olig genes regulate the growth and morphology of normal neurospheres

(A–D) Olig2 is highly expressed in undifferentiated and proliferative neurosphere cells. (A) IHC showing widespread Olig2 expression (green) in a cultured neurosphere. (B) IHC for BrdU. (C) Merged image showing strong co-localization of Olig2 and BrdU. (D) Manual quantitation of IHC co-localization results for Olig1 and Olig2 expression ($p < 0.01$). (E) The mean viable cell number at various passages for *Olig1/2*^{-/-} or *Olig2*^{-/-} neurospheres was significantly lower than wild type or heterozygous counterparts ($p < 0.001$). (F) The percentage of proliferating BrdU+ cells is significantly lower in the *Olig1/2*^{-/-} cultures relative to wild-type ($p < 0.05$). (G) Cell death measured by trypan blue staining is not significantly altered in the *Olig1/2*^{-/-} cultures compared to wild-type. (H) Typical rounded morphology of wild type neurospheres. (I, J) *Olig1/2*^{-/-} and *Olig2*^{-/-} neurospheres are reduced in numbers and size. Spheres also show extensive adherence and migration on the substrate (arrows). All neurospheres were derived from ganglionic eminences of wild type or *Olig* mutant 14.5 dpc embryos and were cultured in the presence of EGF.

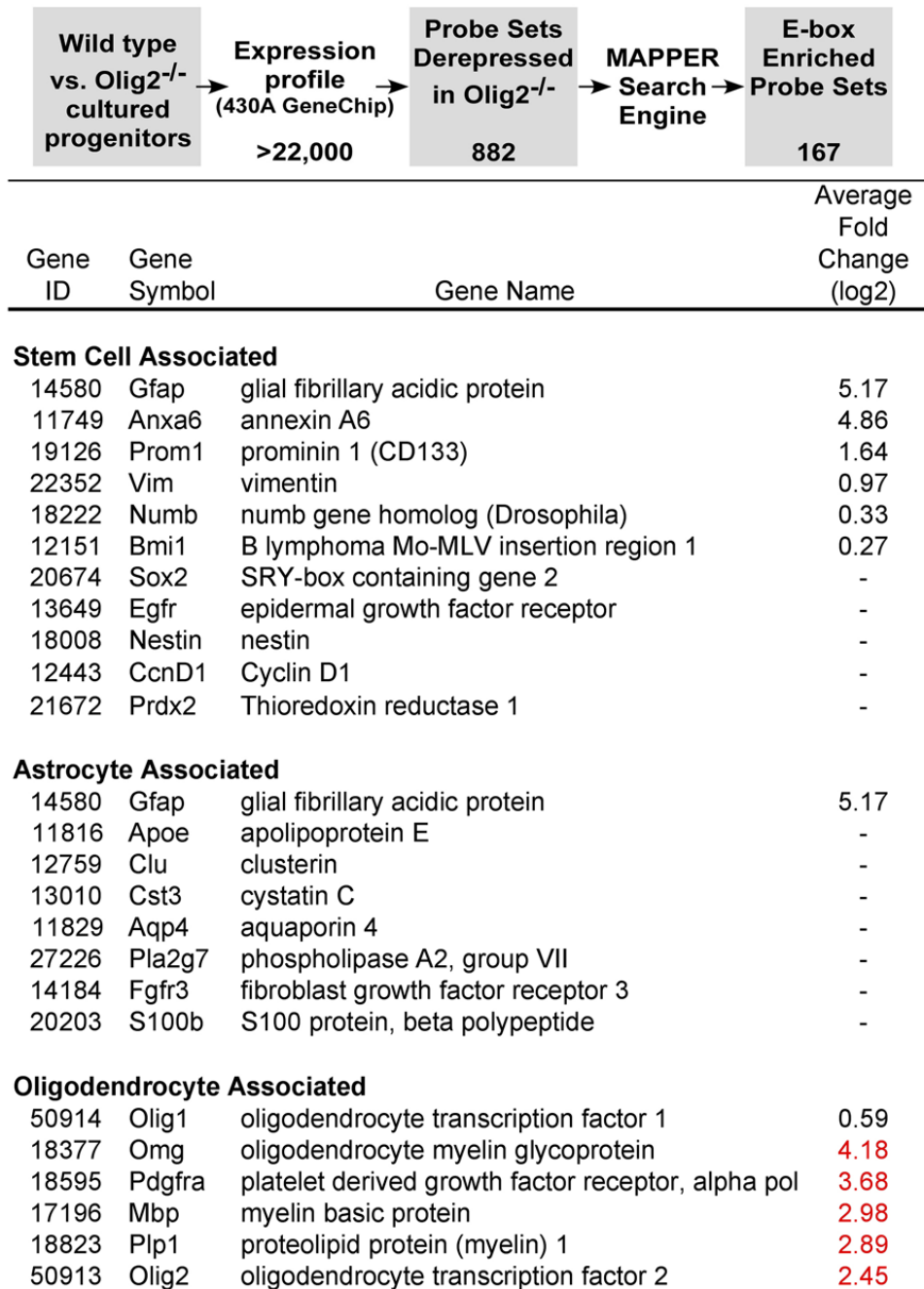


Figure 6. A computational screen for direct genetic targets of Olig2

The screen is predicated on the functions of Olig2 protein as a transcriptional repressor (Mizuguchi et al., 2001; Novitch et al., 2001) that binds to canonical E-box regulatory elements of its target genes (Lee et al., 2005). Microarray expression profiling identified 882 probe sets that were derepressed (up-regulated) in *Olig2*-null neural stem cells relative to their wild type counterparts (see Supplementary Table 1). An additional 1021 probe sets corresponded to mRNAs that were down-regulated in *Olig2*-null cells relative to wild type (Supplementary Table 2). The list of derepressed probe sets was filtered through the MAPPER tool for binding site analysis to identified 167 genes with promoters enriched for E-box control elements. Analysis of this list of potential Olig2 targets revealed the presence of *p21* and three additional

p53-inducible genes (see Supplementary Table 3). A sampling of marker genes for stem cells, oligodendrocytes, astrocytes and neurons that are either derepressed (black font), repressed (red font) or unchanged (–) in *Olig2*-null cells relative to wild type is shown.

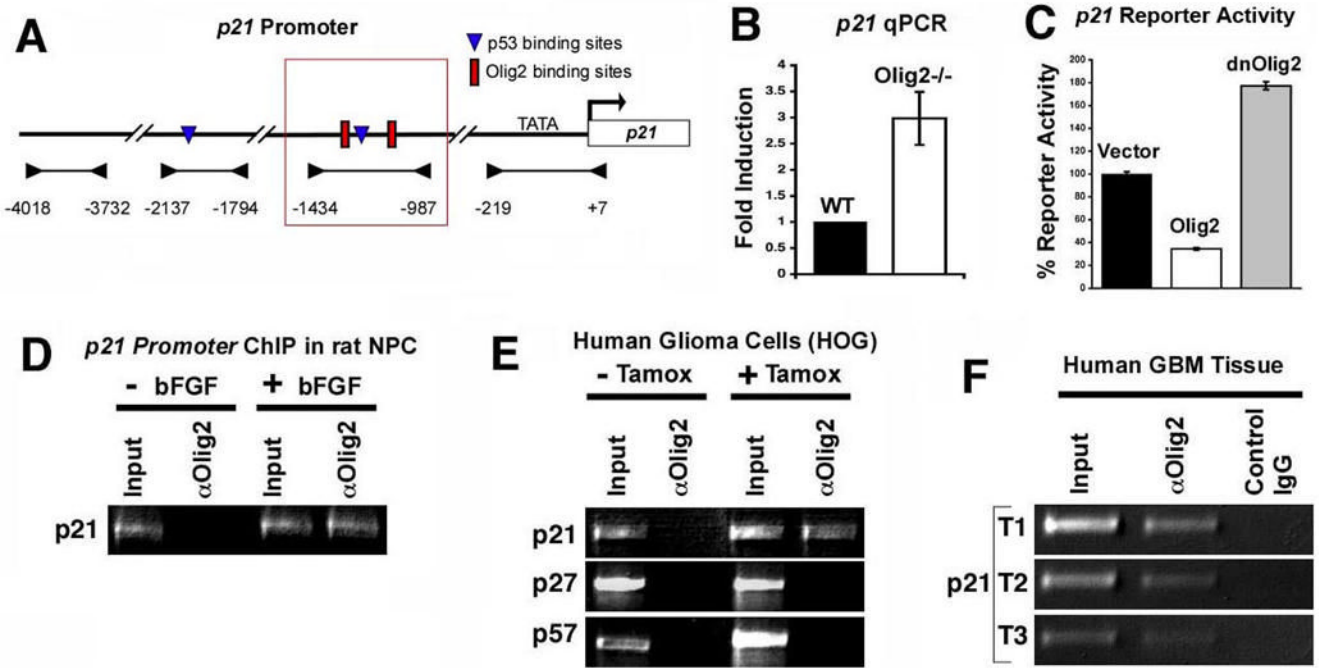


Figure 7. *p21* is a direct target of Olig2 repression in neural stem/progenitor cells and human glioblastoma

(A) Diagram of *P21* promoter region. Arrowheads indicate promoter regions analyzed by PCR in ChIP assays. E-boxes (red) and a p53 binding site (blue) are shown within the region that is bound by Olig2 (red box). (B) Quantitative PCR validates increased expression of *p21* mRNA in *Olig2*-null (n=6) versus wild type (n=3) neurospheres ($p < 0.036$). (C) *p21* luciferase reporter activity is repressed by Olig2 expression in a human GBM cell line (U87) while a dominant negative Olig2 (dnOlig2) defective in binding DNA yields increased expression (p values < 0.0001). (D) ChIP analysis using anti-Olig2 antibody to detect Olig2 binding to the *p21* promoter in E14 rat cortical progenitor cells. Olig2 binding was only detected upon induction of Olig2 expression by FGF. Input is total DNA. (E) ChIP analysis of *p21* promoter in a human glioma cell line transfected with a construct encoding a tamoxifen-activated Olig2 fusion protein. No binding to the related cell cycle inhibitors *p27* or *p57* is detected. Olig2 is detected using anti-ER antibody. (F) ChIP analysis demonstrating OLIG2 is bound to the *p21* locus *in vivo* within freshly isolated human GBM specimens (n=3, T1–T3). Mock ChIP was conducted with normal rabbit IgG as control.

Table 1**Survival data summary**

Total animal counts combine data from survival cohorts and timed endpoint cohorts. Median tumor free survival is calculated only using animals in survival cohorts. Survival was 96% at 273 days for animals injected with *Olig1/2*^{-/-} *Ink4a/Arf*^{-/-} EGFRvIII neurospheres and 100% at 136 days for those injected with *Olig1/2*^{-/-} :GFP infected *Ink4a/Arf*^{-/-} EGFRvIII neurospheres. In all experiments, the presence of tumors was scored as positive for collections of atypical cells greater than 2mm in diameter.

<i>Ink4a/Arf</i> ^{-/-} EGFRvIII Genotype	Total % Developing Tumors	# Tumor Deaths/Total # Animals	Total #Independent Lines	# Animals in Survival Cohorts	Median Tumor Free Survival (days)
<i>Olig1/2</i> +/+	100	12/12	2	12	37
<i>Olig1/2</i> +/-	100	51/51	4	25	69
<i>Olig1/2</i> -/-	9	4/45	4	23	n/d
<i>Olig1/2</i> -/-:GFP	0	0/8	2	8	n/d
<i>Olig1/2</i> -/-:Olig2	64	9/14	2	14	132
<i>Olig1</i> +/-	100	7/7	1	7	59
<i>Olig1</i> -/-	94	16/17	2	10	81