Cancer Cell
Article

PLAGL2 Regulates Wnt Signaling to Impede Differentiation in Neural Stem Cells and Gliomas

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DOI 10.1016/j.ccr.2010.03.020

SUMMARY

A hallmark feature of glioblastoma is its strong self-renewal potential and immature differentiation state, which contributes to its plasticity and therapeutic resistance. Here, integrated genomic and biological analyses identified *PLAGL2* as a potent protooncogene targeted for amplification/gain in malignant gliomas. Enhanced PLAGL2 expression strongly suppresses neural stem cell (NSC) and glioma-initiating cell differentiation while promoting their self-renewal capacity upon differentiation induction. Transcriptome analysis revealed that these differentiation-suppressive activities are attributable in part to PLAGL2 modulation of Wnt/β-catenin signaling. Inhibition of Wnt signaling partially restores PLAGL2-expressing NSC differentiation capacity. The identification of *PLAGL2* as a glioma oncogene highlights the importance of a growing class of cancer genes functioning to impart stem cell-like characteristics in malignant cells.

INTRODUCTION

Malignant gliomas, the most common primary brain tumors in adults, are associated with an extremely high rate of morbidity and mortality (Furnari et al., 2007). In its most aggressive form, glioblastoma (GBM) has an average survival of 1 year and characteristic features of diffuse invasion, intense apoptosis resistance and necrosis, robust angiogenesis, and a varied so-called multiforme histological profile suggestive of developmental plasticity. It is well known that malignant gliomas are heterogeneous both in their cell composition and in the relative abundance of cells capable of propagating tumor cells, albeit the underlying mechanism remains poorly understood (Furnari et al., 2007; Rich and Eyler, 2008; Vescovi et al., 2006). The recent identification of a subpopulation of tumor cells, designated as glioma stem cell or glioma-initiating cells (GICs), with strong tumor repopulating potential has illuminated a potential basis for the intense plasticity and heterogeneous nature of this disease (Bao et al., 2006; Calabrese et al., 2007; Hemmati et al., 2003; Lee et al., 2006; Piccirillo et al., 2006; Singh et al., 2004; Son et al., 2009).

Significance

The identification of tumor-initiating cells with stem cell-like properties in diverse cancers, including glioblastoma (GBM), represents an important conceptual advance with therapeutic implications. There is increasing recognition that elucidation of the molecular factors governing self-renewal and differentiation of the glioma-initiating cells will advance our understanding of glioma pathogenesis and biology. Such insights—at the intersection of stem cell and cancer biology—may provide new points for therapeutic intervention. Here, the identification of *PLAGL2* as an amplified gene in human GBM with oncogenic activity and capacity to regulate Wnt signaling highlights the contribution of differentiation defect in gliomagenesis and points to differentiation pathways as promising targets for malignant glioma treatment.

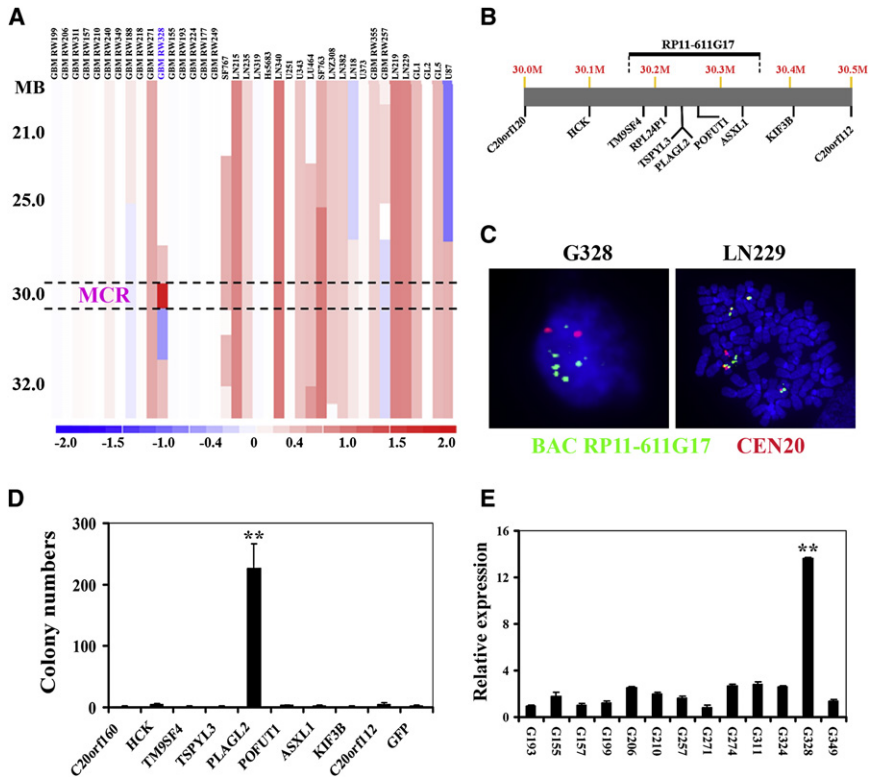

Figure 1. Genomic and Functional Characterization of 20q11.21 Amplification in GBM

(A) Array CGH heat map detailing *PLAGL2* amplification at 20q11.21 in GBM tumor specimens and cell lines. Regions of genomic amplification and deletion are denoted in red and blue, respectively. Mbs denote the position on chromosome 20 in megabases.

(B) Schematic of the resident genes within minimal common region of the 20q11.21 amplicon and the chromosomal region spanned by the FISH probe used in (C) are denoted.

(C) FISH of representative GBM specimen and cell line using a probe within the amplified genomic region labeled with FITC (green) and centromere reference probe labeled with Cy3 (red).

(D) Quantification of soft agar colony formation of the nine resident ORFs within the 20q11.21 amplicon in *Ink4a/Arf* $^{-/-}$; *Pten* $^{-/-}$ astrocytes. Data is shown as mean ± SD from duplicate cultures of three independent experiments. **p < 0.001 by two-tail t test.

(E) PLAGL2 showed copy number-driven expression in the GBM specimen with 20q11.21 amplification by qRT-PCR. Results are normalized with β-actin expression and shown as mean ± SD. Data are from two independent experiments with triplicates. **p < 0.001 by two-tail t test. See also Figure S1.

These GICs share certain features of normal neural stem cells (NSCs) including the expression of neural progenitor markers, long term self-renewal capacity, and partial multi-lineage differentiation potential. However, unlike the normal NSCs that follow the developmental hierarchy and differentiate inevitably into replication-arrested mature cells (Alvarez-Buylla et al., 2001; Gage, 2000; Temple, 2001), the GICs exhibit anomalous developmental programs that enable escape from terminal differentiation cues and preserve self-renewal state (Jackson et al., 2006; Ricci-Vitiani et al., 2007; Sanai et al., 2005). Notably, restoration of their differentiation capacities can drastically reduce GIC tumorigenic potential, supporting the idea that maintenance of an aberrant differentiation state can contribute to glioma pathogenesis (Jackson et al., 2006; Lee et al., 2008; Ricci-Vitiani et al., 2007; Zheng et al., 2008).

GBM possesses a highly rearranged genome. High-resolution genome-scale analysis of such has uncovered myriad somatic alterations on the genomic and epigenetic levels, which presumably harbor GBM-related oncogenes or tumor suppressors. To identify these events, we previously had performed high resolution, oligo-based array comparative genomic hybridization (CGH) profiling of 18 pathologically verified primary GBM specimens and 20 established glioma cell lines (Wiedemeyer et al., 2008). Using the nonheuristic genome topography scan (GTS) algorithm, we further identified and ranked the signature genomic events known previously for GBM (e.g., EGFR amplification or CDKN2A deletion) as well as many previously uncharacterized alterations based on their amplitude, width, and recurrence of a copy number alteration (Wiedemeyer et al., 2008). Here we carried an in-depth study on one of the uncharacterized amplified/gained regions that is localized at chromosome 20q11.21.

RESULTS

PLAGL2 Is Targeted for Amplification and Overexpression

GTS analysis of the array CGH profiles of 18 pathologically verified primary GBM specimens and 20 glioma cell lines identified chromosome 20q11.21 as a region of amplification or gain (Figure 1A; Wiedemeyer et al., 2008). These profiles delimited a 500 Kb minimal common region of amplification encompassing seven characterized genes (*HCK*, *TM9SF4*, *TSPYL3*, *PLAGL2*, *POFUT1*, *ASXL1*, and *KIF3B*), two conserved open reading frames (ORFs) (*c20orf120* and *c20orf112*), and one pseudogene (*RPL24P1*) (Figure 1B). Regional amplifications/gains were further confirmed in the primary tumor (#G328) and cell lines (LN229) by fluorescence in situ hybridization (FISH) using a probe within the 20q11.21 amplicon (Figure 1C). Subsequent analysis of the TCGA data confirmed increased copy number of a broad region at 20q11.21 in 35 of 238 (14.7%) human GBM array CGH profiles (http://tcga-data.nci.nih.gov/tcga/findArchives.htm). The potential cancer relevance of this region was further suggested by its amplification/gain in approximately 40% of primary colorectal cancer specimens and cells lines (Figure S1, available online; Martin et al., 2007).

To determine the oncogenic potential of all nine genes residing within this amplicon, we first assessed their ability to promote anchorage-independent growth. In these studies, we used freshly isolated primary murine astrocytes doubly null for *Ink4a/Arf* and

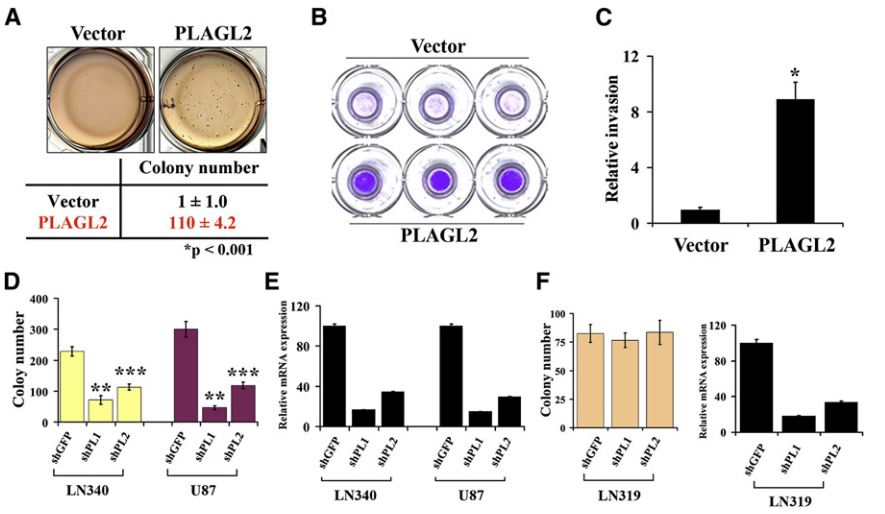

Figure 2. PLAGL2 Promotes Cellular Transformation and Invasion

(A) Ectopic PLAGL2 expression enhanced anchorage-independent growth in human glioma LN215 cells.

(B) PLAGL2 expression in LN215 cells promoted cell invasion through matrix protein in a Boyden chamber. Representative images of invasion assay showing empty vector control and PLAGL2-expressing LN215 cells after 16 hr induction with 10% FBS followed by crystal violet (0.2%) staining.

(C) Quantification of (B).

(D) Knockdown of PLAGL2 expression in LN340 and U87 glioma cells decreased soft agar colony formation.

(E) Efficiency of PLAGL2 knockdown using shRNAs in (D).

(F) Knockdown of PLAGL2 expression in LN319 glioma cells resulted in modest effect on its colony formation activity. The PLAGL2 knockdown efficiencies in (E) and (F) were measured by qRT-PCR and are presented here after normalization with β-actin expression. Data shown in (A)–(F) are the mean ± SD from at least two independent experiments with triplicates. *$p < 0.001$, **$p < 0.005$, and ***$p < 0.01$. See also Figure S2.

Pten, two signature mutations present in human primary GBM (Cancer Genomes Atlas Research Network, 2008; Parsons et al., 2008). Notably, these *Ink4a/Arf*$^{-/-}$ *Pten*$^{-/-}$ astrocytes do not exhibit anchorage-independent growth unless transduced with additional oncogenes such as the mutant epidermal growth factor receptor EGFRvIII (data not shown). In this "sensitized" glioma-relevant background, the above nine ORFs were placed in the pLenti6-V5-DEST expression vector and introduced individually into the *Ink4a/Arf* $^{-/-}$ *Pten* $^{-/-}$ astrocytes. Of the nine cancer gene candidates, only PLAGL2 induced colony formation in the semisolid media (Figure 1D). Consistent with PLAGL2 as the target in this amplicon, quantitative real-time reverse transcriptase PCR (qRT-PCR) revealed that PLAGL2 showed gene copy number-driven expression in the primary GBM specimen (#G328) with the 20q11.21 amplification (Figure 1E). Together, these data suggest that *PLAGL2* functions as an oncogene that is targeted for amplification/gain and overexpression in a subset of human GBM and colorectal cancer cases.

PLAGL2 Promotes Cell Transformation In Vitro and Tumorigenesis In Vivo

PLAGL2 (pleiomorphic adenoma gene like 2), a putative C_2H_2 zinc finger transcription factor, was initially identified through structural homology to its family member PLAG1, a protooncogene frequently rearranged and overexpressed in pleiomorphic salivary gland adenomas and lipoblastomas (Hensen et al., 2002; Kas et al., 1997, 1998). Aberrant PLAGL2 expression has recently been implicated in human acute myeloid leukemia (Landrette et al., 2005). However, the biological functions of PLAGL2, including whether and how it is involved in tumorigenesis, still remain largely unknown. To substantiate the cancer relevance of PLAGL2, we assayed the oncogenic activity of PLAGL2 in several cell-based systems using both gain- and loss-of-function strategies. Consistent with the murine *Ink4a/Arf*$^{-/-}$ *Pten*$^{-/-}$ astrocytes findings, enforced PLAGL2 expression also conferred markedly increased anchorage-independent growth of murine *p53*$^{-/-}$ astrocytes (Figure S2A) and human glioma cell lines including LN215, A172, Hs683, LNZ308, SF767, and U343 (Figure 2A and Figure S2A). In addition, we assessed the impact of PLAGL2 on invasive activity, a hallmark feature of malignant gliomas (Furnari et al., 2007). Using the modified Boyden chamber assay, enforced PLAGL2 expression in both human LN215 cells (Figures 2B and 2C) and primary murine *Ink4a/Arf*$^{-/-}$ *Pten*$^{-/-}$ as well as *p53*$^{-/-}$ astrocytes (Figures S2B and S2C) significantly enhanced their invasive capacity through matrigel. Conversely, shRNA-mediated suppression of PLAGL2 reduced anchorage-independent growth of LN340 and U87 cells—two glioma lines with 20q11.21 amplification and relatively high PLAGL2 expression levels (Figures 2D and 2E). Of note, suppressing PLAGL2 expression in the LN319 glioma line lacking 20q11.21 amplification and with low PLAGL2 expression failed to impact on anchorage-independent growth (Figure 2F), suggesting that PLAGL2 may serve a tumor maintenance role in those gliomas with *PLAGL2* gene amplification and high expression.

In line with the colorectal cancer genomic observation, PLAGL2-expressing immortalized rat intestinal epithelial cells (IEC6) exhibited oncogenic properties of (a) growth in an anchorage-independent manner (Figure S2D), (b) anoikis resistance on ultra-low cluster plates (Figure S2E), and (c) robust tumor growth with metastatic potential within 5 weeks after tail vein injection. In contrast, IEC6 vector control cells failed to produce illness within 3 months of observation (Figure S2F). On the basis of these functional studies, we conclude that *PLAGL2* is a potent oncogene that is targeted for amplification/gain in a subset of colorectal cancers.

PLAGL2 Promotes Renewal of NSC/Progenitor Cells by Inhibiting Their Differentiation

While enforced PLAGL2 expression in human LN215 glioma cells or primary mouse *Ink4a/Arf*$^{-/-}$ *Pten*$^{-/-}$ astrocytes had a notably minimal impact on cellular proliferation, these cells adopted a rounded, less attached, refractile appearance reminiscent of stem/progenitor cells (Figure S3). Correspondingly, these PLAGL2-expressing LN215 cells showed increased expression of the neural stem/progenitor marker Nestin (Figure S3C). These

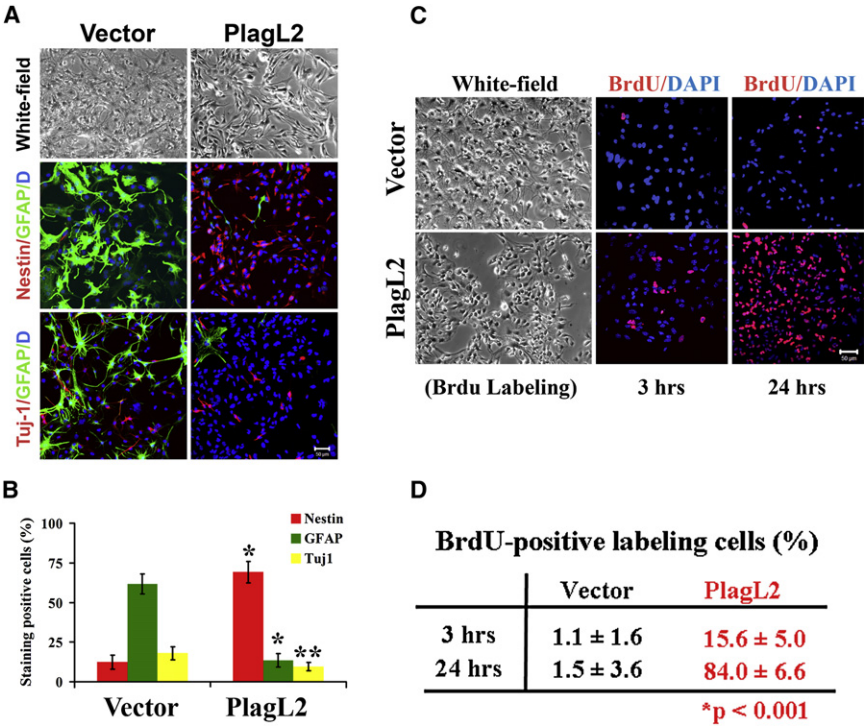

Figure 3. PlagL2 Promotes NSC/Progenitor Cell Proliferation by Attenuating Its Differentiation

(A) The multi-linage differentiation induced by FBS was markedly diminished in PlagL2-expressing $p53^{-/-}$ NSCs. Representative immunofluorescence staining of empty vector control and PlagL2-expressing NSCs cultured in the presence of 1% FBS (without EGF and bFGF) for 5 days.

(B) Histograms show the percentage of Nestin-, GFAP-, and Tuj1-positive cells after 1% FBS induction in (A).

(C) PlagL2 sustained NSC proliferation under differentiation induction. Representative immunostaining of empty vector control and PlagL2-expressing $p53^{-/-}$ NSCs cultured for 5 days in 1% FBS (without EGF and bFGF) and labeled with BrdU (10 μg/ml) for either 3 or 24 hr, respectively.

(D) Quantification of the BrdU-positive cells in (C). Data shown in (B) and (D) are the mean ± SD. Results are representative of three independent experiments. *$p < 0.001$ and **$p < 0.01$ by two-tail t test. Scale bars represent 50 μm. See also Figure S3.

findings prompted us to speculate that PLAGL2 may exert its oncogenic impact in part via promoting a stem/progenitor cell-like state. To test this hypothesis, we first used murine $p53^{-/-}$ NSCs as a model system as it affords a primary cell system and allows for analysis in the context of p53 mutation, one of the most frequent alterations in human primary GBM (Cancer Genomes Atlas Research Network, 2008; Parsons et al., 2008). This $p53^{-/-}$ NSC model system (and confirmatory studies in wild-type as well as $Ink4a/Arf^{-/-}$ $Pten^{-/-}$ NSCs) was used to explore PLAGL2-directed cellular and molecular activities analogous to those of our previous studies (Zheng et al., 2008).

When maintained in the serum-free NSC proliferation media supplemented with growth factors EGF and bFGF, both vector control and PlagL2-expressing cells registered roughly the same NSC/progenitor cell marker patterns such as Nestin expression and showed minimal differences in proliferation as measured by BrdU incorporation rate (data not shown). Upon exposure to differentiation media (neural basal media with 1% fetal bovine serum), as expected, the $p53^{-/-}$ NSC vector control cultures rapidly adopted a flattened cell morphology, drastically diminished Nestin expression, and reciprocally increased GFAP (astrocytic marker) or Tuj1 (early neuronal lineage marker) positive staining. In contrast, PlagL2-expressing $p53^{-/-}$ NSCs retained their stem/progenitor-like morphology and showed feeble expression of mature lineage markers; 70% of PlagL2-expressing cells retained Nestin expression compared with 5% of control cells (Figures 3A and 3B). Additionally, we failed to observe cell-cycle exit that accompanies differentiation in the PlagL2-expressing cells as evidenced by their robust BrdU incorporation following either a 3 or 24 hr BrdU pulse (3 hr: 1.6 ± 1.1% versus 15.6 ± 5.0%; 24 hr: 1.5 ± 1.6% versus 84.0 ± 6.6%) (Figure 3C, $p < 0.001$). Similar results were also observed using wild-type and $Ink4a/Arf^{-/-}$ $Pten^{-/-}$ mouse NSC cultures (Figures S4A and S4B).

To confirm further the impaired differentiation phenotype, we used additional differentiation protocols. We first used the bone morphogenetic protein (BMP)-mediated differentiation assay, which is known for its function to activate SMAD1/5/8 and elicit astroglial differentiation of NSC cultures (Lim et al., 2000; Panchision and McKay, 2002). As expected, BMP2 treatment arrested cell proliferation and stimulated rapid differentiation of the majority of vector control $p53^{-/-}$ NSCs (77%–80%) into GFAP- and S-100-positive astroglial lineage cells (Figures 4A and 4B); in contrast, over 70% of PlagL2-expressing NSCs retained strong Nestin expression and showed minimal increase of GFAP- and S-100-positive cells. Accordingly, compared to 4% of vector control cells, which incorporated BrdU following BMP treatment, 61% of PlagL2-expressing cells were stained for BrdU (Figure 4C). Analogous differentiation findings were obtained for $p53^{-/-}$ NSC cultures maintained in neural basal media without growth factors (Figure S4C). In addition, we confirmed that BMP2 induced strong activation of SMAD1/5/8 components in both vector control and PlagL2-expressing NSCs (Figure 4D), indicating the impaired differentiation phenotype is not attributable to suboptimal activation of the BMP signaling pathway in the PlagL2-expressing NSCs.

Progressive differentiation of normal stem/progenitor cells is associated with loss of self-renewal capacity, while cancer cells with high tumorigenic potential typically exhibit strong self-renewal activity with impaired differentiation (Beachy et al., 2004; Buick et al., 1979; Dick, 2008; Reya et al., 2001; Sanai et al., 2005). We thus tested whether PlagL2 also affects NSC self-renewal activity as measured by neurosphere formation. When cultured at low density (100 cells/ml) in the NSC

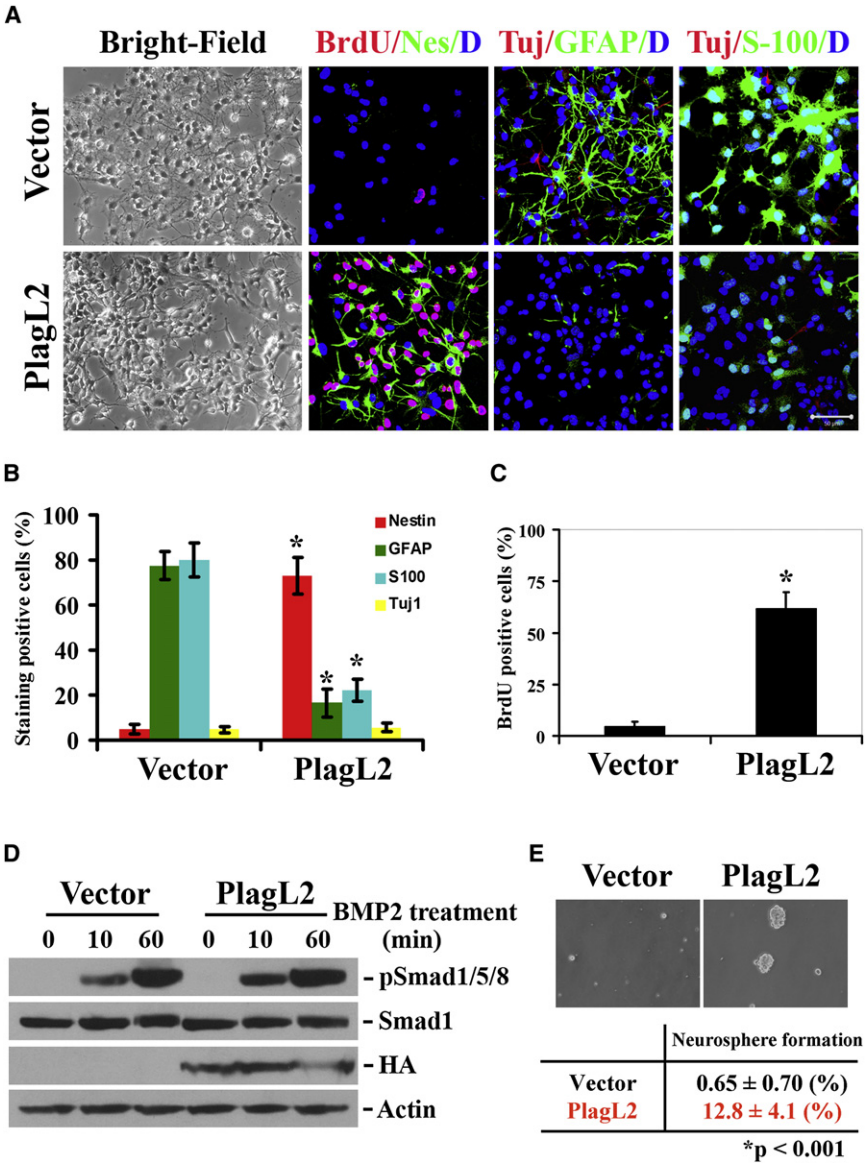

Figure 4. PlagL2 Blocks BMP2-Mediated Differentiation and Maintains NSC/Progenitor Proliferation and Self-Renewal

(A) BMP2-mediated astroglial differentiation was abrogated in PlagL2-expressing *p53*$^{-/-}$ NSCs. Representative immunofluorescence staining of empty vector control and PlagL2-expressing NSCs cultured in the presence of BMP2 (50 ng/ml) for 5 days with EGF (20 ng/ml) and bFGF (10 ng/ml) and labeled with BrdU (10 μg/ml) for 3 hr. Nes, nestin; Tuj, Tuj1; D, DAPI. Scale bars represent 50 μm.

(B) Histograms show the percentage of the Nestin-, GFAP-, and Tuj1-positive cells after BMP2 treatment in (A).

(C) Quantification of the BrdU-positive cells in (A). Data shown in (B) and (C) are the mean ± SEM. Results are representative of three independent experiments with triplicate cultures. *p < 0.001 by two-tail t test.

(D) Western blot analysis of pSmad1/5/8 in empty vector control and PlagL2-expressing *p53*$^{-/-}$ NSCs. Cells were stimulated with BMP2 (50 ng/ml) for 10 and 60 min. pSmad1/5/8 and Smad1 indicated serine phosphorylated Smad and total Smad levels, respectively. HA denotes HA-PlagL2.

(E) PlagL2 preserved NSC self-renewal under BMP2 treatment. A total of 200 empty vector control or PlagL2-expressing *p53*$^{-/-}$ NSCs were cultured in 6 well ultra-low attachment plates for 10 days in the presence of BMP2 (50 ng/ml), EGF (20 ng/ml), and bFGF (10 ng/ml). (Top) Representative images of neurospheres formed; (Bottom) quantification of the neurosphere formation assays. Data shown in (E) are mean ± SEM from two independent experiments with duplicate cultures. *p < 0.001 by two-tail t test. See also Figure S4.

proliferation medium with EGF and bFGF, the PlagL2-expressing *p53*$^{-/-}$ NSCs yielded only slightly more multipotent neurospheres (17.0 ± 3.7% for PLAGL2-expressing NSCs versus 13.8 ± 3.1% for vector control NSCs; p = 0.032) and modestly higher proliferation rates to those of control *p53*$^{-/-}$ NSCs (data not shown). Following BMP2 treatment, while less than 1% of vector control *p53*$^{-/-}$ NSCs formed neurospheres, PlagL2-expressing *p53*$^{-/-}$ NSCs readily formed neurospheres (0.65 ± 0.7% versus 12.8 ± 4.1%; p < 0.001) (Figure 4E). Together, these data establish that enforced PlagL2 expression impedes NSC differentiation and preserves their stem cell features such as self-renewal potential and proliferation under differentiation induction.

PLAGL2 Promotes Gliomagenesis and Maintenance of GIC "Stemness" upon Differentiation

The impact of PLAGL2 on NSC renewal and differentiation, coupled with its transforming activity on primary astrocytes as well as immortalized intestinal epithelial cells, prompted us to assess whether PLAGL2 influences the stem cell-like properties of human glioma cells. These stem-like properties are thought to reside in the GIC subpopulation, which manifest by their self-renewal, partial multi-lineage differentiation capacities, and superior tumorigenic potential in orthotopic models (Bao et al., 2006; Lee et al., 2006; Piccirillo et al., 2006; Singh et al., 2004; Son et al., 2009). To assess such properties, we used early passage, patient-derived GICs that, under serum-free conditions, retain phenotypes and genotypes more closely mirroring primary tumor profiles as compared to serum-cultured established glioma cell lines that have largely lost their developmental identities (Galli et al., 2004; Lee et al., 2006; Pollard et al., 2009).

Using the GIC model system, we observed that PLAGL2-transduced GICs (PLAGL2 GBM-1) versus vector-transduced GICs (control GBM-1) displayed a 4.3-fold increase (8.11% versus 1.89%) of CD133-positive subpopulation; CD133 is a surface progenitor marker associated with human GICs (Bao et al., 2006; Lee et al., 2006; Piccirillo et al., 2006; Singh et al., 2004) (Figure 5A and Figure S5A). Similar results were obtained

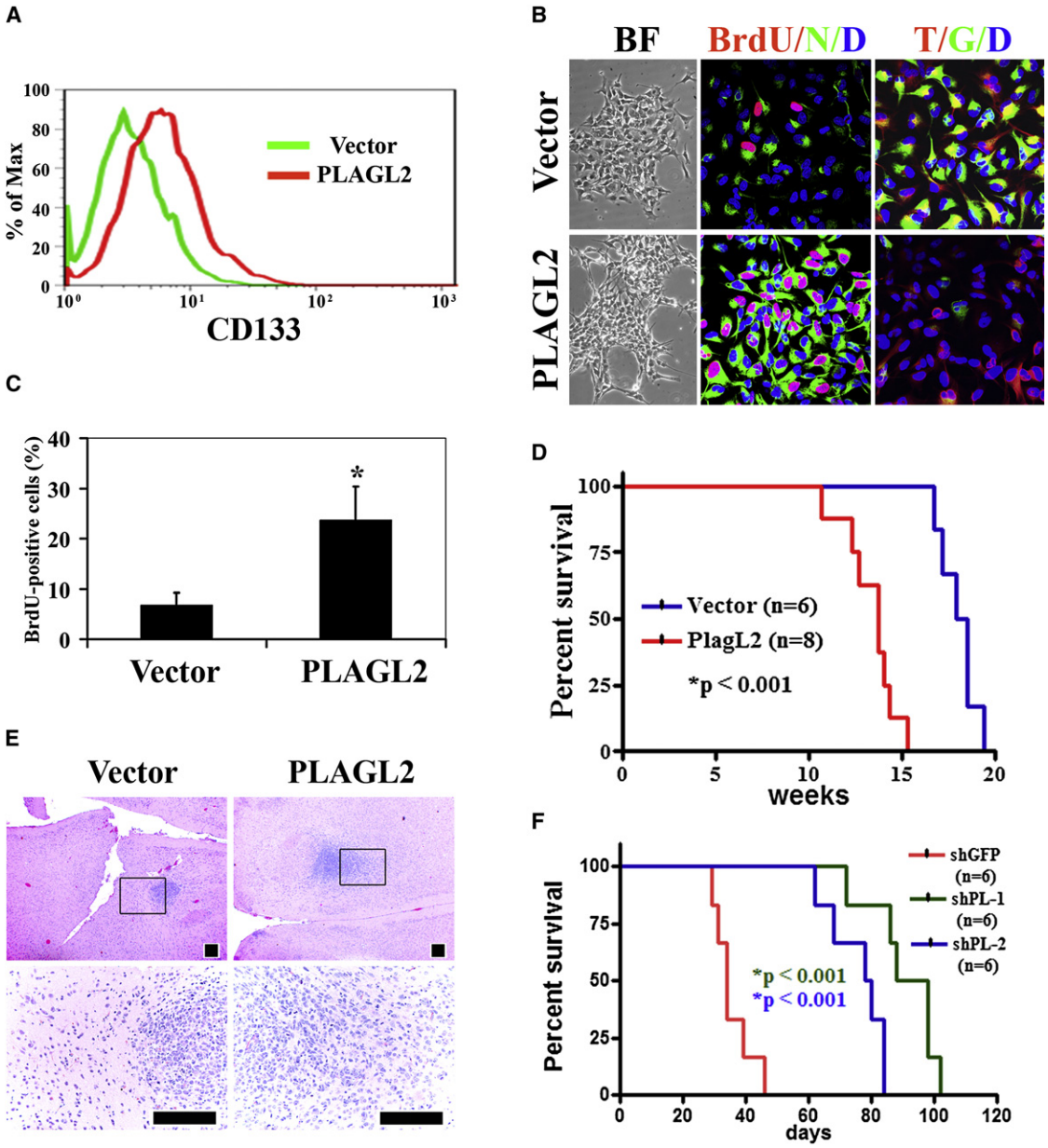

Figure 5. PLAGL2 Modulates GIC Differentiation State and Promotes Gliomagenesis

(A) FACS analysis of CD133-staining empty vector control (green) versus PLAGL2-expressing GBM-1 cells (red) cultured in the NSC proliferation medium with EGF and bFGF.

(B) PLAGL2 attenuated GIC differentiation and sustained their proliferation. Representative immunostaining of empty vector control and PLAGL2-expressing GBM-1 cells cultured in the presence of 2% FBS (without EGF and bFGF) and labeled with BrdU (10 μg/ml) for 3 hr. BF, bright-field; N, nestin; T, Tuj1; G, GFAP; D, DAPI.

(C) Quantification of the BrdU-positive cells in (B). Data is shown as mean ± SD, representative of three independent experiments with duplicate cultures. *p = 0.003 by two-tail t test.

(D) Overexpression of PLAGL2 promoted gliomagenesis. Survival of animals intracranially grafted with empty vector control (n = 6) and PLAGL2-expressing (n = 8) GBM-1 cells. *p < 0.001 by log rank test.

(E) PLAGL2 promoted glioma cell invasion. Representative H&E staining of early tumors derived from empty vector control and PLAGL2-expressing GBM-1 cells at 50 days. Scale bars represent 50 μm.

(F) Reduction of PLAGL2 expression through shRNA in GICs represses their tumorigenic potency. Survival of animals injected with GBM-3 cells infected with shGFP, shPL-1, or shPL-2 (n = 6 for each). *p < 0.001 by log rank test. See also Figure S5.

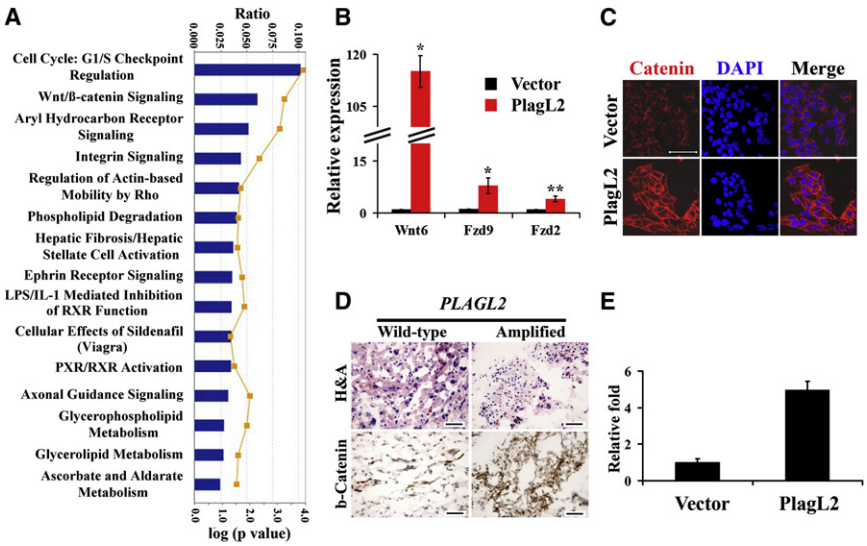

Figure 6. PlagL2 Activates Wnt/β-Catenin Pathway in NSC/Progenitor Cells

(A) Ingenuity pathway analysis of PlagL2-directed transcriptome showed enrichment of the canonical Wnt/β-catenin pathways.

(B) Wnt6, Fzd2, and Fzd9 were significantly upregulated in the PlagL2-expressing $p53^{-/-}$ NSCs quantified by qRT-PCR. Data are shown as mean ± SD after normalization with β-actin expression. The results are from triplicates of two independent experiments. *p < 0.005 and p** < 0.001 by two-tail t test.

(C) PlagL2 upregulated β-catenin levels in NSCs upon differentiation. Representative immunostaining against β-catenin of empty vector control and PlagL2-expressing NSCs cultured for 24 hr in the presence of 1% FBS (without EGF and bFGF). Scale bar represents 50 μm.

(D) Immunohistochemical staining of H&E and β-catenin of control (G199) and PLAGL2 amplified (G328) human GBM specimens. Scale bars represent 50 μm.

(E) TCF reporter activity was assessed using the β-catenin-responsive TOPflash reporter in empty vector control and PlagL2-expressing $p53^{-/-}$ NSCs. Data shown is mean ± SD from two independent experiments with triplicates. *p < 0.001 by two-tail t test. See also Figure S6.

in two other independently derived GIC lines (Figures S5A and S5B). When cultured under differentiation-inducing conditions, PLAGL2-expressing GICs retained robust Nestin expression and substantially higher cell proliferation relative to the control cells as measured by a 1 hr BrdU incorporation assay (6.8 ± 2.5% versus 23.7 ± 6.7%; p = 0.003) (Figures 5B and 5C). Moreover, the in vivo tumorigenic assay indicated that the PLAGL2-GICs (GBM-1) displayed enhanced tumorigenic potential. Compared to the control mice grafted intracranially with 5000 vector control GBM-1 that survived about 4 to 5 months (Figure 5D), the mice injected with PLAGL2-GBM-1 often developed diffusively infiltrating gliomas within 2 to 3 months and died at a median of 13.2 weeks. Notably, when examined at early time points after implantation, unlike vector control GICs that generated well circumscribed lesions with a clear boundary, the PLAGL2-GIC tumors were substantially more invasive with diffusively infiltrating tumor cells in the surrounding brain parenchyma (Figure 5E). These in vivo findings are consistent with the above in vitro Boyden chamber assay indicating that high PLAGL2 expression promotes glioma cell invasion. Conversely, shRNA-mediated silencing of endogenous PLAGL2 expression in a human GIC line (GBM-3) with relatively higher PLAGL2 expression substantially decreased its in vivo tumorigenicity (Figure 5F and Figure S5C). Together, these findings support the hypothesis that PLAGL2 functions to maintain stemness of the GICs in the context of differentiation cues and enhances their tumorigenicity and cellular invasion potential.

PLAGL2 Activates Wnt/β-Catenin Pathway in NSC/Progenitor Cells

PLAGL2 is a Zinc finger transcription factor localized in the nucleus (Kas et al., 1998). In an attempt to elucidate the molecular mechanisms underlying PlagL2's impact on murine $p53^{-/-}$ NSC and human GIC differentiation, we performed transcriptome comparisons of PlagL2-transduced and vector control $p53^{-/-}$ NSCs following the differentiation induction. As early as 24 hr after induction, approximately 400 genes exhibited significant differential expression. Ingenuity pathway analysis of the PlagL2-directed transcriptome identified "cell-cycle G1/S checkpoint control" as the most enriched canonical pathway (Figure 6A), consistent with the higher proliferation observed in the PlagL2-expressing NSCs and GICs under differentiation induction. Notably, the Wnt/β-catenin cascade emerged as the second most enriched canonical pathway. qRT-PCR further confirmed that mRNA transcripts of multiple Wnt pathway components, including the Wnt6 ligand and Fzd9 and Fzd2 receptors, were substantially upregulated in PlagL2-expressing NSCs (Figure 6B). Consistently, upregulation of Wnt6, Fzd9, and Fzd2 was also observed in PlagL2-expressing astrocytes as well as the primary GBM clinical sample (G328) with PLAGL2 amplification (Figures S6A and S6B).

Wnt/β-catenin signaling plays an important role in maintaining stem cells of various lineages including NSC/progenitor cells, and aberrant activation of the Wnt/β-catenin pathway leads to deregulated growth and cancer (Chenn and Walsh, 2002; Clevers, 2006; Kalani et al., 2008). Thus, our unbiased genome-scale analysis and confirmatory findings raised the possibility that PlagL2's function of suppressing NSC/progenitor cell differentiation might be mediated in part through activation of the Wnt/β-catenin pathway. A large body of previous work has established that the canonical Wnt signaling pathway executes its actions through the regulated degradation of the transcriptional coactivator β-catenin (Clevers, 2006; He et al., 2004; Logan and Nusse, 2004). In the absence of Wnt stimulation, β-catenin is phosphorylated by GSK3 within the Axin complex and then targeted for rapid degradation by the proteasome (Aberle et al., 1997; Amit et al., 2002; Liu et al., 2002). On the contrary, stimulation by Wnt inhibits β-catenin phosphorylation and promotes stability.

Consistent with PlagL2-induced activation of Wnt receptors and ligands in NSC/progenitor cells, immunofluorescence staining revealed a significantly increased β-catenin accumulation within the PlagL2-NSC/progenitor cells as compared to the vector control cells (Figure 6C). In parallel, immunohistochemistry

analysis detected strong β-catenin staining in both malignant glioma patient samples with *PLAGL2* amplification (Figure 6D) and the orthotopic transplants of PLAGL2-GICs (Figure S6C), reinforcing the role of PLAGL2 on Wnt/β-catenin pathway activation. Moreover, introduction of PlagL2 into murine NSC/progenitor cells and astrocytes enhanced their TCF-dependent TOPflash reporter activity (Figure 6E and Figure S6D). Similar results were observed using HEK293T and human glioma LN215 cells (Figures S6E and S6F). Collectively, these data indicate that PLAGL2 can activate the canonical Wnt/β-catenin signaling cascade.

Inhibition of Wnt Signaling Attenuates PLAGL2's Activity on NSC/Progenitor Cell Differentiation

Numerous studies have documented the importance of Wnt proteins and its canonical signaling surrogates in the maintenance of stem cells in different tissues (Adachi et al., 2007; Chenn and Walsh, 2002; Grigoryan et al., 2008; Nusse, 2008; Pinto and Clevers, 2005; Zechner et al., 2003). Aberrant activation of Wnt/β-catenin signaling tips the homeostatic balance toward pathological states including malignant transformation. Consistently, we found that introduction of a stabilized form of β-catenin into the mouse $p53^{-/-}$ NSC/progenitor cells attenuates their differentiation capacity while enhancing proliferation (Figure S7A). This established paradigm, coupled with the above genomic analysis, prompted us to test whether PlagL2 acts through the Wnt/β-catenin pathway to maintain the progenitor state of normal and neoplastic stem cells under differentiation-inducing conditions.

We first investigated whether suppression of Wnt/β-catenin signaling through Dickkopf-1 (DKK1) can attenuate PlagL2's function on NSC/progenitor differentiation. DKK1 is a secreted Wnt inhibitor, which functions through direct binding to the Wnt coreceptor Lrp5/6 and thus disrupts Wnt-induced canonical Wnt/β-catenin pathway activation (Mao et al., 2001; Semenov et al., 2001). Since our data indicated that PlagL2 activates Wnt/β-catenin cascade on upstream signaling events through upregulating ligand and receptor expression, one would predict that introduction of DKK1 should suppress PlagL2-mediated Wnt pathway activation. Indeed, while enforced PlagL2 expression in NSC/progenitor cells significantly increased the TOPflash reporter activities as compared to the control cells, further cotransfection with DKK1 largely reversed the stimulatory effect of PlagL2 on the reporter activity (Figures 7A). Moreover, introduction of DKK1 into PlagL2-expressing NSC/progenitor cells partially restored their differentiation potential as demonstrated by the decreased progenitor marker (Nestin) staining, increased lineage marker (GFAP/Tuj1) expression, and reduced cell proliferation (Figures 7B and 7C). Similar results were obtained when shRNAs against Wnt6 or Fzd2 were introduced into PlagL2-expressing NSC/progenitor cells (Figures 7D and 7E and Figure S7B).

DISCUSSION

Cancers arise from disturbances of critical cellular functions impacting self-renewal, differentiation, survival, and proliferation (Al-Hajj and Clarke, 2004; Hanahan and Weinberg, 2000; Reya et al., 2001) and such disturbances are often driven by genomic aberrations in cancer cells. In the current study, integrative genomic and functional analyses led to the identification and validation of PLAGL2 as a potent oncoprotein frequently targeted for copy number gain/amplification in human malignant gliomas and colon cancers. Using both normal and neoplastic NSC/progenitor cells as model systems, we demonstrated that ectopic PLAGL2 expression greatly enhances NSC/progenitor cell self-renewal and proliferation upon differentiation induction, suggesting that PLAGL2 executes its oncogenic activities through regulating the state of cellular differentiation. Additionally, we have provided evidence that this function of PLAGL2 is at least partially attributable to its capacity to activate Wnt/β-catenin signaling.

It has become increasingly clear that many cancers are heterogeneous, reminiscent of normal tissue renewal with stem cells at the apex of this hierarchy. The identification of clonogenic tumor-initiating cells with stem-like features across many different tumor types has supported the existence of a cellular hierarchy in tumors (Al-Hajj et al., 2003; Bonnet and Dick, 1997; Hemmati et al., 2003; O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Singh et al., 2004; Taylor et al., 2005). These tumor-initiating cells represent a reservoir of self-sustaining cells with potent proliferative activity, differentiation resistance, and tumorigenic potential (Clarke et al., 2006; Dick, 2008; Gilbertson and Rich, 2007; Reya et al., 2001). Therefore, a relative arrest in differentiation, such as seen in the PLAGL2-overexpressing GICs, could contribute to malignant glioma progression through maintenance and expansion of the GIC pool under conditions that would otherwise lead to their differentiation. Alternatively, the differentiation-resistant state promoted by elevated PLAGL2 expression could potentially lead to the accrual of self-renewing glioma cells in which cell proliferation and differentiation are uncoupled and therefore will allow a plurality of glioma cells to assume indefinite proliferative capacity as suggested by the "blocked differentiation" model of cancer (Buick et al., 1979). Support for this view derives from the observations that PLAGL2 overexpression leads to decreased astroglial differentiation, enhanced renewal in NSCs under differentiation induction conditions, increased CD133+ subpopulations, and enhanced GIC tumorigenicity in vivo.

We provide further evidence that enhanced PLAGL2 expression contributes to the progression of a subset of human malignant gliomas through regulating their differentiation and renewal. Unlike normal NSCs that undergo progressive differentiation along a developmental hierarchy into lineage-restricted progenitors and then their mature progenies (Alvarez-Buylla et al., 2001; Gage, 2000; Temple, 2001), human malignant glioma cells generally show minimal terminal differentiation traits and instead exhibit robust expression of progenitor markers such as Nestin (Tohyama et al., 1992). Furthermore, the self-renewal capacity of the isolated GICs tends to correlate with their tumor grade (Singh et al., 2003), supporting the idea that "differentiation arrest" represents a common feature of malignant glioma progression. Like *PLAGL2*, some oncogenes can function to interfere with normal stem cell differentiation, *MYC* being a notable example, and such oncogenes also affect glioma cell differentiation (Ben-Porath et al., 2008; Bruggeman et al., 2007; Molofsky et al., 2003; Shachaf et al., 2004). However, PLAGL2's function on differentiation does not appear to use the MYC axis as

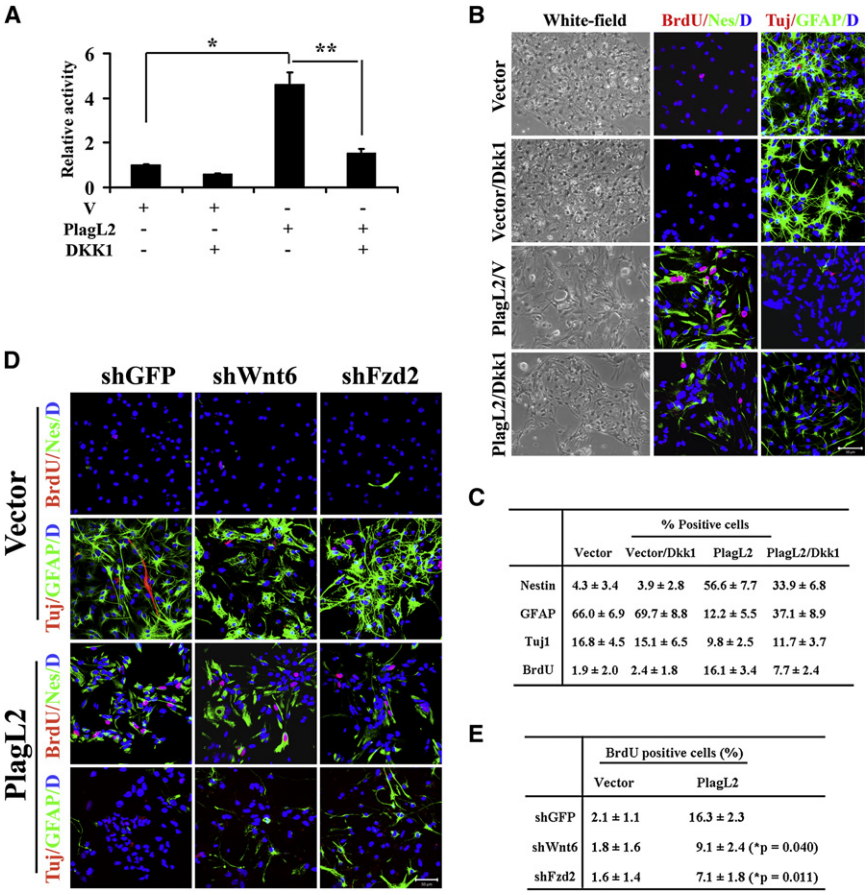

Figure 7. Inhibition of Wnt Signaling Sensitizes PlagL2-Expressing NSCs to Differentiation

(A) DKK1 attenuated PlagL2-induced Wnt signaling activation. Vector control or DKK1 constructs were cotransfected with the TOPflash reporter construct into vector control or PlagL2-expressing *p53*$^{-/-}$ NSCs. Forty-eight hours after transfection and culture in NSC proliferation medium with EGF (20 ng/ml) and bFGF (10 ng/ml), the cells were transferred to culture in the presence of 1% FBS (without EGF and bFGF) and TOPflash assays were performed. Data shown are mean ± SEM from two independent experiments performed in triplicate. *$p < 0.001$ and **$p < 0.01$ by two-tail t test.

(B) DKK1 reduced PlagL2's activity on NSC differentiation. Representative immunofluorescence staining of vector, DKK1/vector, PlagL2-expressing, and DKK1/PlagL2-expressing *p53*$^{-/-}$ NSCs cultured in the presence of 1% FBS (without EGF and bFGF) for 5 days and labeled with BrdU (10 μg/ml) for 3 hr. Nes, nestin; Tuj, Tuj1; D, DAPI. Scale bars represent 50 μm.

(C) Quantification of the percentage of the Nestin-, GFAP-, Tuj1-, and BrdU-positive cells in (B). Data shown are mean ± SEM from two independent experiments with duplicate. **$p < 0.01$ and ***$p = 0.016$ by two-tail t test.

(D) Wnt6 and Fzd2 knockdown partially restored differentiation of PlagL2-expressing NSCs. Representative immunofluorescence images from empty vector or PlagL2-expressing *p53*$^{-/-}$ NSCs infected with control (shGFP) or shRNA against Wnt6 and Fzd2 lentivirus. The cells were immunostained with the indicated antibodies after culture in differentiation medium (neural basal media + 1% FBS, without EGF and bFGF) for 5 days and labeling with BrdU (10 μg/ml) for 3 hr.

(E) Quantification of the percentage of BrdU-positive cells in (D). Data were shown as mean ± SD from two independent experiments with duplicate cultures. See also Figure S7.

suggested by the minimal impact of PLAGL2 on MYC protein expression (data not shown), suggesting that multiple pathways can be commandeered to sustain self-renewal of GICs in the face of differentiation cues. Consistent with this notion, the BMP-BMPR signaling system that controls the activity of normal brain stem cells can function as a key inhibitory regulator of GICs by regulating their differentiation status (Bao et al., 2006; Calabrese et al., 2007; Hemmati et al., 2003; Lee et al., 2006; Piccirillo et al., 2006; Singh et al., 2004; Son et al., 2009). For example, dysregulated BMP pathway with epigenetically silenced BMP receptor 1B (*BMPR1B*) expression has been demonstrated to contribute to the tumorigenicity of a subset of malignant gliomas through blocking their GIC differentiation (Lee et al., 2008). Forced BMPR1B expression in these GICs restored their differentiation potential and substantially decreased their tumorigenic potential, reinforcing further the view that targeting these differentiation control pathways may provide novel treatment avenues for malignant gliomas.

The canonical Wnt cascade is a critical regulator of stem cell renewal and differentiation in many tissues (Clevers, 2006; Nusse, 2008; Reya and Clevers, 2005). During normal brain development, Wnt signaling is required for controlling the size of the self-renewing NSC/progenitor population (Kalani et al., 2008). Moreover, while attenuation of Wnt signaling causes a reduction in the NSC/progenitor compartment, its activation through β-catenin can increase the cycling and expansion of neural precursors (Chenn and Walsh, 2002; Zechner et al., 2003), raising the possibility that abnormal Wnt pathway activation may promote NSC/progenitor transformation. Indeed, uncontrolled Wnt pathway activation through mutations in APC (Huang et al., 2000), β-catenin (Zurawel et al., 1998), or axin (Baeza et al., 2003; Dahmen et al., 2001) has been found in a subgroup of medulloblastomas, a pediatric brain tumor in the cerebellum. Although these specific mutations appear to be rare in gliomas, the level of β-catenin expression appears to be related to the histological grade in a subset of gliomas (Utsuki et al., 2002). Sustained β-catenin activation independent of the mutations has also been demonstrated in a subset of breast, ovarian, and pancreatic cancers (Bafico et al., 2004; Wang et al., 2009). In the present study, our findings that PLAGL2 functions to activate Wnt/β-catenin pathway while repression of Wnt activation in PLAGL2-expressing NSCs sensitize them

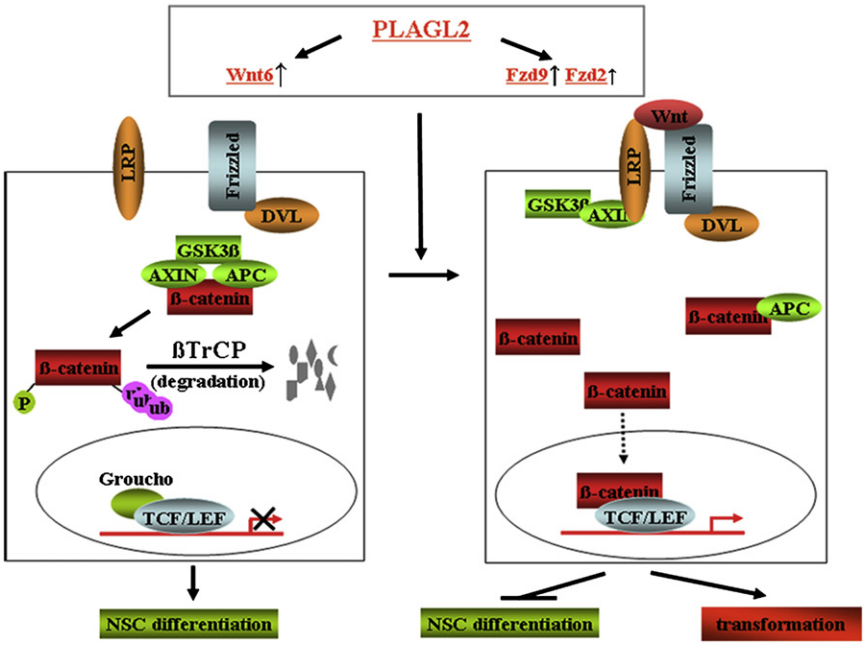

Figure 8. Model of PlagL2's Function on Wnt/β-Catenin Signaling Activation and NSC Differentiation

to differentiation further support Wnt signaling as a key contributor to the malignant behavior of at least a subset of malignant gliomas.

Overall, these data support a model in which PLAGL2 functions primarily to affect gliomagenesis via its impact on differentiation and self-renewal (Figure 8). In the unstimulated NSC/progenitor cells lacking PLAGL2 overexpression, the Wnt receptor complexes are not bound by ligands, allowing the axin/APC/GSK3β complex to phosphorylate β-catenin and target it for rapid degradation. When abnormally overexpressed, PLAGL2 activates Wnt ligand and receptor transcription, resulting in the disruption of the axin/APC/GSK3β complex, β-catenin stabilization, and its subsequent activation. The activated β-catenin is then transferred into the nucleus and activates its downstream effectors, which functionally contribute to PLAGL2-mediated differentiation arrest and self-renewal maintenance under differentiation induction. However, it is worth noting that attenuation of Wnt signaling in PlagL2-expressing NSCs through either DKK1 overexpression (Figures 7B and 7C) or Wnt6/Fzd2 knockdown (Figures 7D and 7E) only partially reverses PlagL2-mediated NSC/progenitor differentiation suppression, suggesting that the combined actions of PLAGL2 at multiple points in the pathway are needed for full effort and/or that other differentiation/renewal pathways contribute to regulation of differentiation processes in gliomas.

In summary, aberrant expression of PLAGL2 can function to sustain normal and neoplastic NSC/progenitor cell renewal and proliferation under differentiation induction conditions, findings consistent with the prevailing view that differentiation induction restrains tumor cell self-renewal and tumorigenic potential (Pierce and Speers, 1988). Indeed, the differentiation therapy has contributed significantly to the management of leukemia (Wang and Chen, 2008). In this regard, one would predict that a more detailed understanding of the pathways governing self-renewal and/or differentiation of NSCs and, by extension, GICs would provide productive entry points for novel drug development efforts in malignant gliomas and other cancer types.

EXPERIMENTAL PROCEDURES

Cell Lines and Human Samples

The human glioma cell lines LN215, LN229, A172, Hs683, LN319, LN340, LNZ308, SF767, U87, and U343 and the human embryonic kidney cell line HEK293T were purchased from American Type Culture Collection. Frozen tumor specimens and human GIC lines were obtained from the Memorial Sloan-Kettering Cancer Center tumor bank. All tumor specimens were collected after obtaining written informed consent preoperatively. This study was approved by the Institutional Review Boards of the Memorial Sloan-Kettering Cancer Center and Dana-Farber Cancer Institute. The GICs were generated as described previously (Bao et al., 2006; Lee et al., 2006; Piccirillo et al., 2006; Singh et al., 2004) without sorting and maintained as tumor spheres using low-attachment plates in NeuroCult NS-A proliferation media (human; Stem Cell Technologies) with growth factors. The common genomic and genetic mutations of the human glioma cell lines and GICs are included in Table S1.

Mice and Orthotopic Transplants

Primary murine *p53*$^{-/-}$ and *Ink4a/Arf*$^{-/-}$ *Pten*$^{-/-}$ astrocytes were isolated from 5-day-old pups as previously described (Bachoo et al., 2002) and maintained in DMEM containing 10% FBS. Primary murine wild-type, *p53*$^{-/-}$, and *Ink4a/Arf*$^{-/-}$ *Pten*$^{-/-}$ NSCs were isolated from the SVZ regions of 1-month-old mice as previously described (Rietze and Reynolds, 2006; Zheng et al., 2008) and maintained in NeuroCult NS-A proliferation media (mouse; Stem Cell Technologies) with growth factors. The orthotopic transplantation was performed as previously described (Zheng et al., 2008). Animals were followed daily for development of neurological deficits. All mice experiments were performed with the approval of the Harvard and Dana-Farber Cancer Institute Institutional Animal Care and Use Committee.

Antibodies for Immunofluorescence, Immunohistochemistry, and Western Blot Analysis

The following antibodies were used: GFAP (Dako and BD Biosciences), human nestin, mouse nestin (Chemicon), Tuj-1 (Covance), S-100 (Lab Vision), PS-Smad1/5/8, Smad1 (Cell Signaling), CD133 (Miltenyi Biotech), BrdU (Abcam), HA (Covance), β-catenin, β-actin (Santa Cruz Biotechnology), and EGFR (Upstate).

Immunostaining

Cells were seeded on polyornithine (Sigma-Aldrich)- and fibronectin (Sigma-Aldrich)-coated coverslips and cultured within the indicated media. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100 in PBS and blocked with 1% BSA. Subsequently, samples were incubated with indicated primary antibodies overnight at 4°C and corresponding Alexa Fluor-conjugated secondary antibodies (Invitrogen) 1 hr at room temperature and mounted with mounting medium with DAPI (Vector). Microscopic images were obtained with a Zeiss LSM 510 confocal microscope in the Harvard NeuroDiscovery Center optical imaging core, using constant exposure times for each channel in individual experiments.

Cell Invasion and Anchorage-Independent Growth Assays

Cell invasion assays were performed in duplicate or triplicate in a Boyden chamber of 24 well inserts coated with matrix proteins as per the manufacturer's protocol (BD Biosciences). Cells (2×10^5) were washed and then seeded in serum-free DMEM (Invitrogen). The chemoattractant used was DMEM + 10% FBS. After 18 hr incubation and removal of the noninvading cells, the chambers were stained with 0.5% Crystal Violet (Sigma-Aldrich) for 20 min and the invading cells were counted. Anchorage-independent growth assays were performed in duplicate or triplicate in 6 well plates. 10^4 of indicated cells per well were seeded in DMEM + 10% FBS containing 0.4% low-melting agarose on the top of bottom agar containing 1% low-melting agarose DMEM + 10% FBS. After 14–21 days, colonies were stained with iodonitrotetrazoliumchloride (Sigma-Aldrich) and counted.

NSC Self-Renewal Assay

NSC self-renewal capacity was measured by plating 100 cells/ml into 6 or 12 well ultra-low cluster plates in NSC proliferation media containing growth factors (EGF 20 ng/ml and bFGF 10 ng/ml; Sigma-Aldrich) with or without BMP2 (50 ng/ml; R&D Systems). The number of neurospheres that formed subsequently per well was quantified after 8 (no BMP2) or 14 days (with BMP2) and relative sphere formation was plotted versus indicated control.

Expression Profiling and Ingenuity Pathway Analysis

mRNA expression profiling was performed at the Dana-Farber Microarray Core facility using the Mouse Genome 430 2.0 Array (Affymetrix). Student's t test was used to identify differentially expressed genes according to expression profiles. The ingenuity pathway analysis application was performed to identify canonical pathways that were most significant to the functionally related genes according to the manufacturer's instruction (Ingenuity Systems). In brief, differentially expressed genes with threshold $p \leq 0.05$ were mapped into Ingenuity Pathways Analysis database as input. Complete profiles are deposited on the GEO website under super series accession number GSE21143.

Statistical Analysis

Tumor-free survivals were analyzed using Graphpad Prism4. Statistical analyses were performed using nonparametric Mann-Whitney test. Comparisons of cell growth, self-renewal, and differentiation were performed using the unpaired Student's t test. For all experiments with error bars, standard deviation was calculated to indicate the variation within each experiment and data, and values represent mean ± SEM.

shRNA Sequences

See Supplemental Experimental Procedures for siRNA and shRNA sequences.

ACCESSION NUMBERS

Complete microarray data are deposited on the GEO website under super series accession number GSE21143.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2010.03.020.

ACKNOWLEDGMENTS

We thank S. Zhou and S. Jiang for excellent mouse husbandry and care. H.Z. was supported by the Helen Hay Whitney Foundation. H.Y. is supported by the American Brain Tumor Association. J.H.P was supported by the Damon Runyon Cancer Research Foundation. R.W. is supported by a Mildred Scheel Fellowship (Deutsche Krebshilfe), and S.N.Q. is supported by a fellowship from the Canadian Institutes of Health Research. Grant support comes from the Goldhirsh Foundation (R.A.D.), the Ben and Catherine Ivy Foundation (W.C.H., L.C., and R.A.D.), the Multiple Myeloma Research Foundation (Y.A.W.), and National Institutes of Health grants RO1CA99041 (L.C.), 5P01CA95616 (W.C.H., L.C., and R.A.D.), 1RC2CA148268-01 (W.C.H., L.C., and R.A.D.), and 1RC2CA148222-01 (R.A.D.). R.A.D. is an American Cancer Society Research Professor and is supported by the Robert A. and Renee E. Belfer Foundation for Innovative Cancer Science.

Received: October 29, 2009
Revised: February 26, 2010
Accepted: April 8, 2010
Published: May 17, 2010

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