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Cell

Mosaic Analysis with Double Markers Reveals Tumor Cell of Origin in Glioma

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SUMMARY

Cancer cell of origin is difficult to identify by analyzing cells within terminal stage tumors, whose identity could be concealed by the acquired plasticity. Thus, an ideal approach to identify the cell of origin is to analyze proliferative abnormalities in distinct lineages prior to malignancy. Here, we use mosaic analysis with double markers (MADM) in mice to model gliomagenesis by initiating concurrent *p53/Nf1* mutations sporadically in neural stem cells (NSCs). Surprisingly, MADM-based lineage tracing revealed significant aberrant growth prior to malignancy only in oligodendrocyte precursor cells (OPCs), but not in any other NSC-derived lineages or NSCs themselves. Upon tumor formation, phenotypic and transcriptome analyses of tumor cells revealed salient OPC features. Finally, introducing the same *p53/Nf1* mutations directly into OPCs consistently led to gliomagenesis. Our findings suggest OPCs as the cell of origin in this model, even when initial mutations occur in NSCs, and highlight the importance of analyzing premalignant stages to identify the cancer cell of origin.

INTRODUCTION

Cancer is a disease of genetic mosaicism because cancerous cells harbor genetic mutations that are absent in normal cells within the same individual. In familial cancer patients, even though initial mutations exist in every cell, in most cases, only specific cell types can progress into malignancy. Those cell types are called cancer cell of origin. Such a cell type-specific susceptibility implies the existence of a permissive or even synergizing signaling context in the cell of origin for particular genetic mutations to cause cancer formation. Therefore, identification of the cancer cell of origin would provide critical insights for understanding tumorigenic mechanisms and for designing rational therapeutic strategies.

Despite such importance, identification of the cell of origin for most cancers has been a daunting task (Visvader, 2011). The reliability of revealing cell identity solely based on molecular and cellular analyses of late stage tumors is often confounded not only by infiltrated bystander cells, but also by the acquired plasticity often found in terminal cancerous cells. To circumvent these issues, genetically engineered mouse models have been widely used to determine the tumorigenic potential of a specific cell type by initiating mutations with a cell type-specific Cre transgene. However, it is critical to note that cells initially acquiring mutations (cell of mutation) may not be the cell of origin. When mutations are introduced in stem/progenitor cells, it is extremely difficult to distinguish whether initial mutant cells directly transform or whether they merely pass on mutations to more restricted progeny that then transform. In the latter scenario, the mutated stem/progenitor cell is simply the cell of mutation, and the transforming progeny is the actual cell of origin (Visvader, 2011).

The cell of origin for malignant glioma, a type of deadly brain cancer, remains controversial. Successful isolation of tumor cells with stem cell features (known as cancer stem cells) from human gliomas (Singh et al., 2004) implies neural stem cells (NSCs) as the cell of origin. However, such NSC-like features of malignant glioma cells could be acquired during transformation rather than reflect the nature of the original cell type (Visvader, 2011). Further evidence supporting the NSC origin of glioma was obtained from mouse genetic studies. For example, the inactivation of tumor suppressor genes (TSGs) *p53* and *neurofibromatosis 1* (*NF1*) or the expression of a mutant form of *p53* in NSCs consistently led to glioma formation in mouse models, and the physical locations of tumors appeared to associate with the subventricular zone (SVZ), where adult NSCs reside (Alcantara Llaguno et al., 2009; Wang et al., 2009; Zhu et al., 2005). However, other studies suggest that NSC-derived progeny such as astrocytes or oligodendrocyte precursor cells (OPCs) might directly transform (Bachoo et al., 2002; Lindberg

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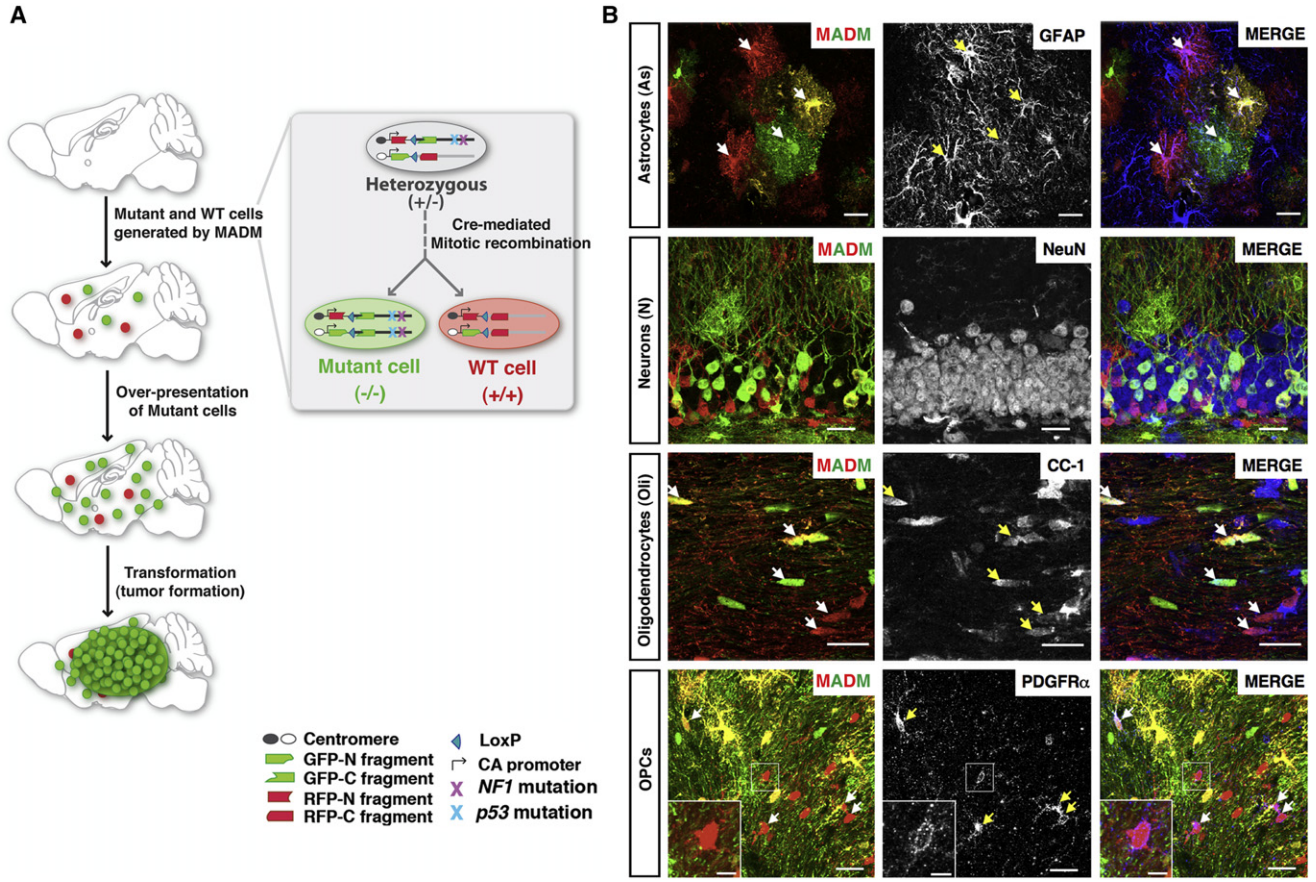

Figure 1. MADM-Based Glioma Model Allows Phenotypic Analysis at Single-Cell Resolution

(A) Scheme of MADM-based glioma modeling. Inset illustrates how MADM concurrently mutates and labels cells (for full details, see Figure S1; Hippenmeyer et al., 2010; Zong et al., 2005).

(B) Representative confocal images show *hGFAP-Cre*-induced MADM labeling of four NSC-derived cell types in a 2-month-old WT-MADM mouse. Arrows point to MADM-labeled cells expressing corresponding markers. Scale bars, 20 μm; inset, 5 μm.

See also Figure S1.

et al., 2009; Persson et al., 2010). This unresolved controversy partially stems from the distinct oncogenic mutations used in these models that make the direct comparison difficult and, more importantly, from the lack of high-resolution analyses of cellular aberrations during the transforming process.

Mosaic analysis with double markers (MADM), a mouse genetic mosaic system (Zong et al., 2005), could, in principle, be used to analyze aberrations in individual cell lineages prior to the final transformation and should thus be suitable for identifying cancer cell of origin. Via Cre/loxP-mediated mitotic interchromosomal recombination, MADM generates a small number of homozygous mutant cells, thus mimicking the sporadic loss of heterozygosity (LOH) of TSGs in human cancers (Knudson, 1971). MADM also permanently labels these mutant cells with green fluorescent protein (GFP) and their sibling wild-type (WT) cells with red fluorescent protein (RFP) within an otherwise unlabeled heterozygous mouse (Figure 1A and Figure S1A available online). The single-cell resolution benefited from the sparse labeling (0.1%–1% or much lower) (Zong et al., 2005) enables one to track mutant cells throughout the entire process of tumorigenesis. The sibling red WT cells serve as internal controls for green mutant cells, thereby greatly facilitating detailed analyses of cellular aberrations of all lineages in their native environment. In summary, MADM can provide features that are indispensable for a robust analytical paradigm to identify a cell of origin.

Here, we report the application of MADM to glioma modeling. After initiating *p53/NF1* mutations sporadically in neural stem cells (NSCs), we analyzed mutant NSCs and all of their progeny at premalignant stages. We found dramatic overexpansion and aberrant growth specifically in OPCs, but not in NSCs or other lineages. Upon tumor formation, marker staining and transcriptome analysis confirmed the OPC nature of tumor cells. Finally, introducing the same mutations into OPCs consistently led to gliomagenesis. Our findings reveal OPCs as the cell of origin for glioma even when initial *p53/NF1* mutations occur in NSCs, thus resolving the current controversy by distinguishing cancer cell of mutation from cell of origin. Importantly, although our studies focused on glioma, the analytical paradigm with MADM that is developed here could be applied to identify cellular origins for many other cancers.

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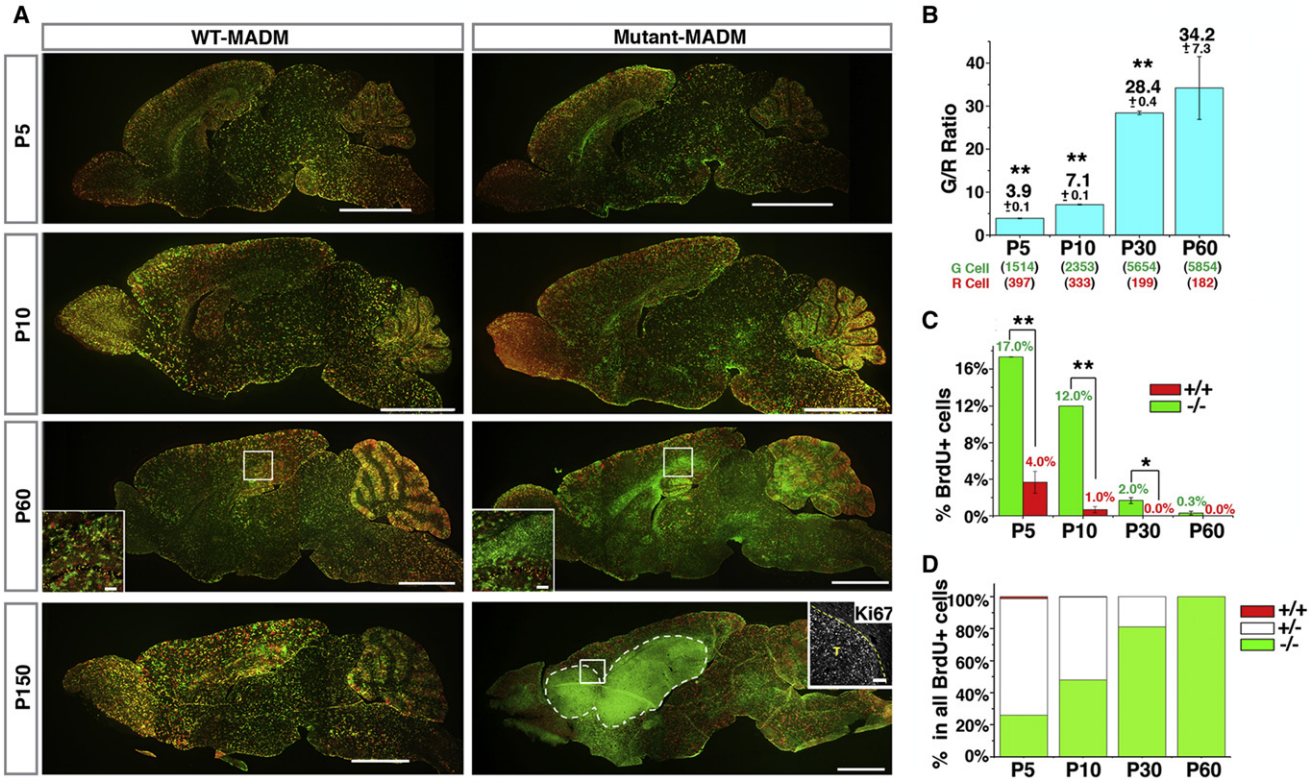

Figure 2. MADM-Mediated Sporadic Concurrent Inactivation of *p53* and *NF1* in Embryonic NSCs Reveals the Entire Process of Gliomagenesis

(A) Sagittal sections from brains of MADM mice at indicated ages. In WT-MADM mice (left column), both green and red cells are WT. In mutant-MADM mice (right column), green cells are *p53* and *NF1* double null; red cells, WT. Tumor boundary is demarcated with dashed line. Scale bars, 2 mm; insets, 100 μm. Ki67 staining shows that tumor cells are highly proliferative.

(B) Systematic quantification of G/R ratios in mutant-MADM brains from P5 to P60. Total cell numbers counted are shown in parentheses. Each number is the sum from three brains. Error bars represent standard error of the mean (SEM).

(C) The percentage of BrdU+ cells in WT and mutant cell populations in the brain parenchyma of mutant-MADM mice at indicated ages. Error bars represent SEM.

(D) The proportion of BrdU+ cells with genotypes "−/−," "+/−," and "+/+" in the brain parenchyma of mutant-MADM mice at indicated ages. "+/−" includes both double-colored and colorless BrdU+ cells.

In (C) and (D), BrdU was administered 1.5 hr prior to sacrifice. Error bars ± SEM; n = 3 mice. See Experimental Procedures for sampling scheme. *$p < 0.05$, **$p < 0.01$, paired t test.

See also Figure S2.

RESULTS

Establishment of an MADM-Based Genetic Mosaic Model for Glioma

MADM-based cancer modeling needs three prerequisites: cancer-causing gene mutation(s), MADM cassettes that reside centromerically to the prospective mutated genes, and a Cre transgene that expresses in a certain tissue or organ. To establish an MADM-based glioma model, we decided to inactivate *p53* and *NF1*, both of which are among the most frequently mutated genes in human glioma patients (McLendon et al., 2008; Parsons et al., 2008) and have been used to model glioma in mice (Reilly et al., 2000; Zhu et al., 2005). We also engineered MADM cassettes into the *Hipp11* genomic locus (Figure 1A and Figures S1A and S1B) proximal to the *p53* and *NF1* chromosomal locations on mouse Chr. 11 (Hippenmeyer et al., 2010). To induce MADM-based recombination, we chose *hGFAP*- or *Nestin-Cre* transgenes (termed *NSC-Cre* hereafter) that are expressed in both embryonic and adult NSCs (Petersen et al., 2002; Zhuo et al., 2001), thereby generating MADM-labeled cells in all NSCs-derived lineages: neurons, astrocytes, oligodendrocytes, and OPCs (Figure 1B). With the ability to identify MADM-labeled cell types, we can readily analyze the aberrant growth of GFP+ mutant cells by directly comparing to their RFP+ WT counterparts within each lineage in the same mouse brain.

MADM-Based Glioma Model Offers the Opportunity to Trace the Entire Tumorigenic Process

After recombining mutant alleles of *p53* and *NF1* with MADM alleles to generate a mutant-MADM mouse model (Figure S1C), we analyzed overall expansion of green mutant cells at different ages. In contrast to the comparable distribution of green and red cells in WT-MADM brains in which all labeled cells are wild-type (Figure 2A, left), we observed a progressive overrepresentation

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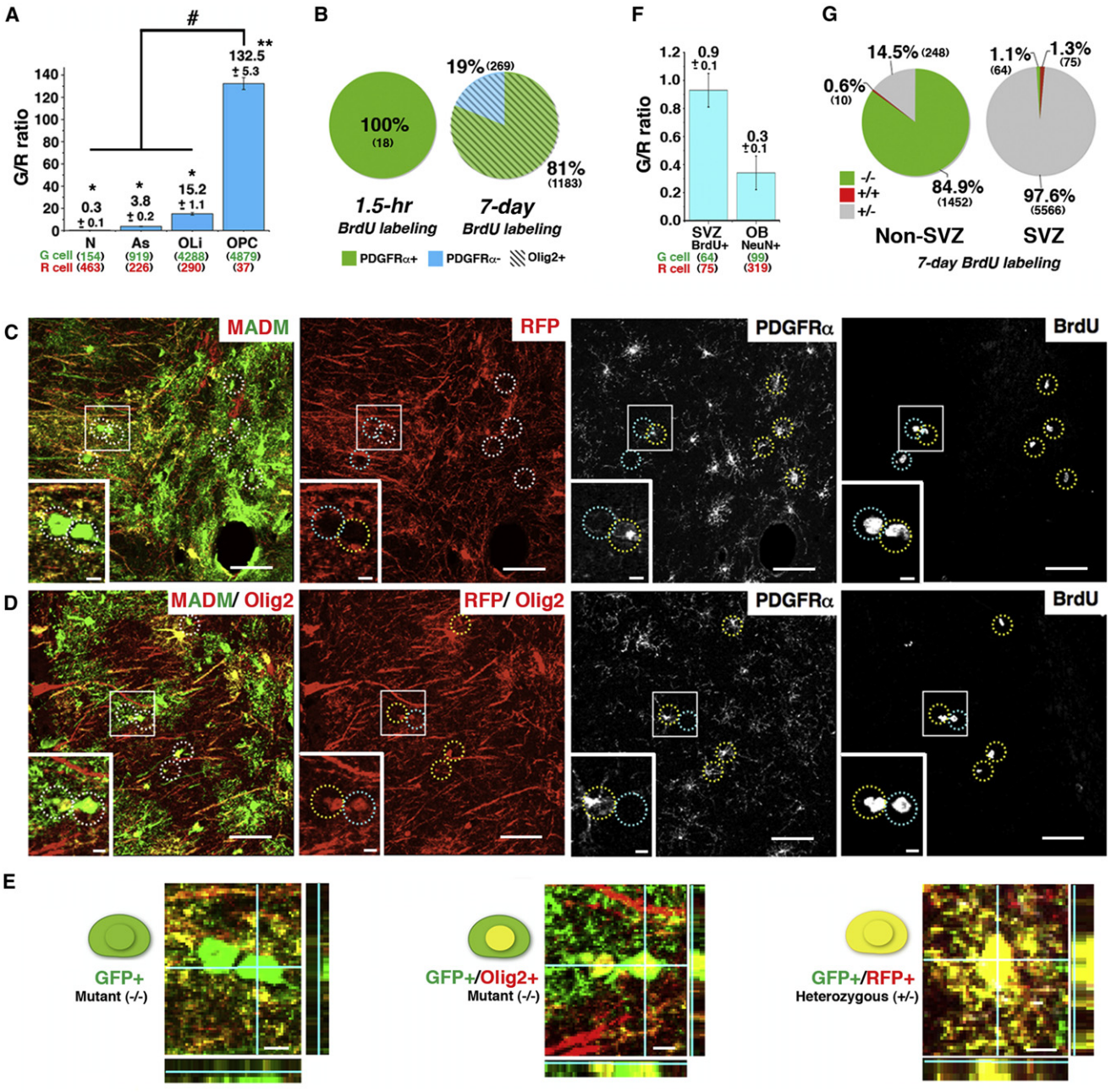

Figure 3. Analysis at a Pretransforming Stage of Gliomagenesis Suggests that OPCs Rather Than NSCs Serve as the Cell of Origin

(A) Average G/R ratios of each cell type in brain parenchyma of P60 mutant-MADM mice. *$p < 0.05$, **$p < 0.01$, paired t test. #$p < 0.0001$, one-way ANOVA. Error bars represent SEM.

(B) (Left chart) With a single BrdU injection (1.5 hr prior to sacrifice) at P60, BrdU-positive mutant (−/−) cells in the brain parenchyma consist entirely of OPCs (PDGFRα+). (Right chart) Upon BrdU administration by drinking water for 1 week, all BrdU-positive mutant (−/−) cells in the brain parenchyma belong to the oligodendrocytic lineage (Olig2+), and the majority of them are OPCs (PDGFRα+).

(C–E) "4+1" channel staining shows that all BrdU+ mutant OPCs in a P60 mutant-MADM mouse brain belong to the oligodendrocyte lineage.

(C) Without Olig2 staining, all MADM-labeled BrdU+ cells are mutant (green). Notably, some BrdU+ mutant cells are PDGFRα negative (marked with cyan circles).

(D) An adjacent section stained with Olig2 together with MADM, BrdU, and PDGFRα shows that all mutant BrdU+ cells have red nuclei, indicating positive staining of Olig2.

(E) Representative magnified confocal images show: mutant cells without Olig2 staining (left), mutant cells with Olig2 staining in red channel (middle), and heterozygous yellow cells (right). The orthogonal z axis is shown on the side of each panel. Scale bars: (C and D) 20 μm; insets, 5 μm; (E) 5 μm.

(F) G/R ratios of BrdU+ cells in the SVZ and NeuN+ cells in the olfactory bulb (OB) from P60 mutant-MADM mice. BrdU was given in drinking water for 7 days.

of green mutant cells in mutant-MADM brains from postnatal day 5 (P5) to P60 (Figure 2A, top three of the right column) and eventually the formation of GFP+ full-blown tumors at ~5 months of age (Figure 2A, bottom-right).

Because mutant (green) and wild-type (red) sibling cells originate from the same mother cell in equal numbers initially (Figure 1A), the ratio of green to red cell numbers (referred to as the G/R ratio hereafter) allows us to quantitatively evaluate the extent of mutant cell expansion. A G/R ratio equal or close to 1 indicates no growth advantage of the mutant population, whereas a G/R ratio > 1 indicates a growth advantage of mutant over WT cells (referred to as "overexpansion" hereafter). From P5 to P60, the average G/R ratio in mutant-MADM brains increased from ~4 to ~30 (Figure 2B), indicating a continuous overexpansion of mutant cells. However, the increase of G/R ratio appeared to reach a plateau between P30 and P60, suggesting that overexpansion of mutant cells had largely stopped between those ages. In addition to quantifying cell numbers, we also compared the proliferative status between mutant and WT cells with a brief BrdU pulse to label cells undergoing DNA replication. At all time points analyzed, the percentage of BrdU+ cells in the mutant population was significantly higher than that in the WT population (Figure 2C). Furthermore, whereas WT cells largely ceased to proliferate at early postnatal age (P10), some mutant cells (~0.3% among all mutant cells population) continued to divide and contributed to the entire population of BrdU+ cells in the brain parenchyma at P60 (Figure 2D). Thus, mutant cells showed not only elevated, but also prolonged proliferative capacity compared to WT cells at a stage before tumor formation (termed the "pretransforming" stage hereafter).

At later ages (4–5 months), all mutant-MADM mice developed brain tumors with strong Ki67 staining (Figure 2A, bottom-right). Tumors were invariably GFP+ (n = 28), indicating that they originated from MADM-induced mutant cells. Although transcriptome profiles of these tumors were very similar (see below), pathological analysis revealed great heterogeneity in their appearance: some showed typical astrocytic features; some showed malignant glioma features such as necrosis, multinuclear giant cells, and perivascular and perineuronal satellitosis; and most were highly anaplastic (Figure S2, Figures 4B–4D, and Figure 5D). Taken together, MADM allows us to trace mutant cells at all gliomagenic stages and provides the analytical accessibility between initial mutations and the final transformation.

Mutant OPCs Manifest Dramatic Overexpansion in Pretransforming MADM Brains

We next sought to determine the identity of glioma cell of origin by separately determining the G/R ratio of NSCs and of each NSC-derived cell type at P60, when mutant cell expansion had largely ceased but tumors were yet to arise. We predicted that, at this ostensibly dormant stage in tumorigenesis, the mutant cell type capable of transformation should manifest significant overexpansion and maintain sustained proliferative activity. We first quantified the G/R ratios of all four NSC-derived cell types (Figure 3A). The average G/R ratio from different brain regions showed slight reduction of mutant neurons and only minor expansion of mutant astrocytes and oligodendrocytes. In stark contrast, the G/R ratio of OPCs was > 130, significantly higher than those of the other three cell types (Figure 3A and Figure S3). Notably, mutant OPCs appeared to have impaired differentiation potentials to give rise to mature oligodendrocytes, as the G/R ratio of oligodendrocytes was ~10 times less than that of OPCs (Figure 3A). Moreover, brief BrdU pulses at P60 exclusively labeled mutant OPCs in brain parenchyma, although the total number was low, suggesting that mutant OPCs were progressing but were still relatively dormant at this age (Figure 3B, left). Remarkably, despite such a dramatic overrepresentation of mutant OPCs, we did not observe any pathological features in these brains by conventional pathological analysis (Table S2 and data not shown), demonstrating the capability of MADM to probe into a previously inaccessible stage during tumor development.

Mutant OPCs Constitute the Majority of the Proliferation Pool in Pretransforming MADM Brains

It seems paradoxical that we found nearly full penetrance of tumor formation in our model at 4–5 months of age (Table S2), yet dividing cells at P60 were so rare (Figure 2C and Figure 3B, left). Considering that pretransforming cells could undergo a prolonged cell cycle, we evaluated the slow-dividing cell population by extending the duration of BrdU administration and observed that 30% of all mutant OPCs were able to incorporate BrdU (Figure 3B, right, and data not shown), suggesting that many mutant OPCs were dividing slowly. We noticed that a small fraction of BrdU-positive mutant cells in the brain parenchyma did not express the OPC marker PDGFRα (Figures 3B, right, and 3C). To determine whether these were cells from other lineages or oligodendrocytes differentiated from recently divided OPCs, we costained brain sections adjacent to those shown in Figure 3C with antibodies against BrdU, PDGFRα, and Olig2, a "pan-oligo" marker that expresses in both OPCs and oligodendrocytes. Using a "4+1 channel" staining scheme (Figures 3C–3E), we found that all BrdU+, PDGFRα-negative cells examined expressed Olig2 (Figure 3D), suggesting that they were newly differentiated oligodendrocytes from initially BrdU-labeled mutant OPCs. Taken together, our detailed analysis reveals that, among all mutant cell types, OPCs are the only proliferative population in the brain parenchyma at pretransforming stages.

Although we did not observe the same extent of overexpansion in other mutant cell types as that in mutant OPCs at P60, it could be a result of increased cell death in neuronal and astrocytic lineage following the overexpansion of NSCs. Therefore, it is critical to directly analyze the proliferative activity of adult NSCs within the SVZ (Doetsch et al., 1999). After 7 days of BrdU feeding of mutant-MADM mice, we quantified all BrdU+ cells in the SVZ, which include type B, C, and A cells (Doetsch

(G) Quantification of cells with indicated genotypes among all BrdU+ cells in the non-SVZ brain parenchyma (left chart) or the SVZ (right chart) after one week of BrdU administration. n = 3 mice for all quantification in (A), (B), (F) and (G). See Figure S3 and Extended Experimental Procedures for detailed systematic sampling schemes. Error bars represent ± SEM. Total cell numbers counted in (A), (B), (F), and (G) are shown in parentheses. Each number is the sum from three brains. See also Figure S3.

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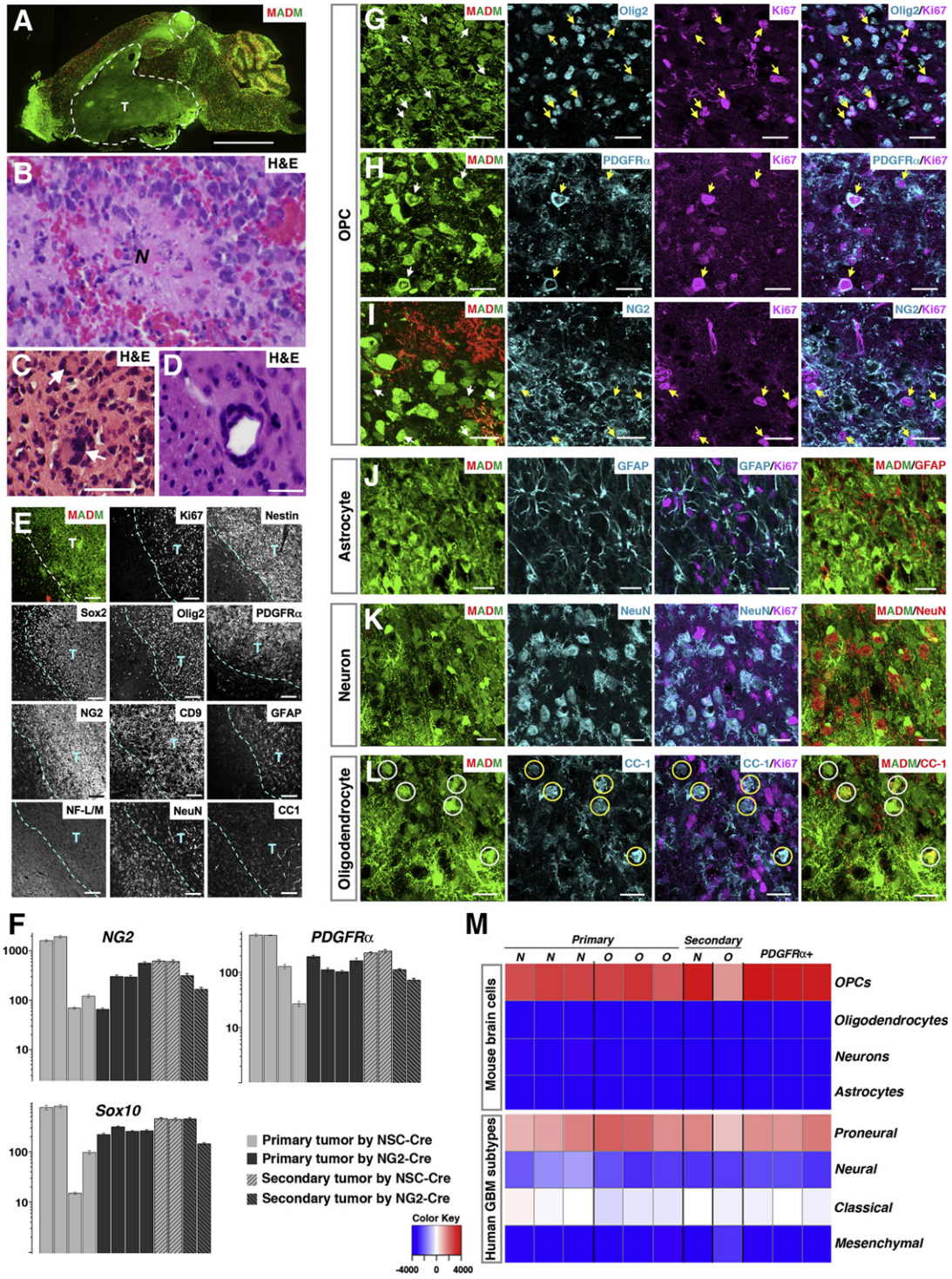

Figure 4. MADM-Generated Glioma Cells Exhibit Many OPC Features

(A) Representative image of a mutant-MADM brain carrying a GFP+ glioma.

(B–D) Adjacent H&E staining of tumor regions shows typical glioma features, including necrotic areas ("*N*" in B), multinucleated giant cells (C), and perivascular satellitosis (D). Scale bars: (A) 2 mm; (C and D) 200μm. Magnification in (B), 400×.

(E) Representative low-magnification images show elevated expression of a panel of well-established glioma markers in tumor regions. All staining was done with adjacent sections from the same tumor. Tumor boundary is demarcated by dashed lines. T, tumor mass. Scale bars, 100 μm except for CD9 staining, for which it is 50 μm.

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et al., 1999; also see Figure S6A). In stark contrast to our findings in the brain parenchyma, where 85% of BrdU-positive cells were mutant (Figure 3G, left), only ~1% of BrdU-positive cells were mutant in the SVZ (Figure 3G, right). More importantly, the G/R ratio of BrdU+ cells in adult SVZ was not significantly different from 1 ($p = 0.39$), indicating that there was no overexpansion of mutant adult NSCs (Figure 3F). Furthermore, we did not observe any overexpansion of mutant granule neurons in the olfactory bulb, which are the main progeny generated by adult NSCs (Figure 3F). Therefore, *p53* and *NF1* mutations seemed unable to enhance the proliferative activity of adult NSCs. Taken together, detailed comparison between mutant and WT cell behaviors in NSCs and all progeny lineages strongly suggests that NSCs function as the cell of mutation but fail to directly transform, whereas OPCs function as the cell of origin for glioma.

Tumor Cells in Fully Developed Gliomas Exhibit Salient OPC Features

Following the investigation at pretransforming stages, we next analyzed the expression pattern of cell-specific markers in malignant tumors generated in the MADM-glioma model (Figures 4A–4D). Under low magnification, we observed prominent enrichment of NSC markers Nestin and Sox2 and the astrocytic marker GFAP, but not neuronal or oligodendrocyte markers in the tumor region (Figure 4E). This observation is consistent with previous findings in both mouse models and human patients (Alcantara Llaguno et al., 2009; Louis et al., 2007; Wang et al., 2009; Zhu et al., 2005) and has been considered as evidence to support the NSC origin of gliomas. However, it is important to note that these tumors also showed enriched expression of OPC markers, such as Olig2, PDGFRα, NG2, CD9, and O4 (Figure 4E and data not shown). The expression of some OPC markers was further confirmed by quantitative RT-PCR from crude tumor samples (Figure 4F).

To clarify whether these markers were expressed by tumor or bystander cells, we analyzed the tumor sections at higher magnification and found that GFP+ proliferating tumor cells expressed many markers for OPCs (Figure 4G-I), but not for neurons or astrocytes (Figure 4J and 4K). In some tumors, there were a few GFP+ cells expressing oligodendrocyte marker CC-1, but not Ki67 (Figure 4L), which could be either residential cells or oligodendrocytes differentiated from tumor cells. To extend the molecular characterization of the cellular identity, we next performed global transcriptome comparison between tumor samples and all four neuroglial cell types (Cahoy et al., 2008) and confirmed that tumor cells closely resembled OPCs, but not any other neuroglial cell types (Figure 4M). Importantly, regardless of diverse pathological features (Figure S2), all tumor samples shared almost identical molecular profiles (Figure 4M and data not shown), suggesting a common cell of origin for these morphologically heterogeneous tumors.

In line with immunofluorescent staining (Figure S2 and Figure 4E), we observed elevated Nestin level in some, but not all, tumors by transcriptome and qRT-PCR analyses (data not shown). However, it is unclear whether Nestin is expressed in OPC-like tumor cells or in bystander cells. To clarify this problem, we enriched for OPC-like tumor cells by using an immunopanning method widely used to purify WT OPCs based on their surface expression of PDGFRα (Figures S4A–S4C) (Cahoy et al., 2008). The transcriptome of purified OPC-like tumor cells displayed an even higher extent of similarity to that of normal OPCs (Figure 4M), demonstrating that these cells contributed to the OPC signatures of glioma samples. qRT-PCR results showed that, in addition to consistent expression of OPC markers, purified OPC-like cells from some tumors expressed Nestin at a comparable level to NSC-derived neurospheres (Figure S4D). This finding was further confirmed by marker costaining of the tumor mass, showing that some GFP+ dividing tumor cells coexpressed both PDGFRα and Nestin (Figure S4E). Importantly, regardless of their Nestin expression levels, these panned tumor cells, when orthotopically allografted into NOD-SCID mouse brains, effectively initiated secondary tumors (Figure S4F and Table S4) that recapitulated both histological (Figures S4G–S4K) and molecular features (Figures 4F and 4M and Figure S4D) of their primary tumors. Taken together, these data demonstrate that highly proliferating tumor cells manifested salient OPC features. Although Nestin was expressed in some tumor cells, the fact that primary tumor cells with either undetectable or high levels of Nestin expression could efficiently initiate new tumors implies that Nestin expression appears irrelevant to tumorigenicity and is likely acquired during transformation. Therefore, analyses of tumor cells further support OPCs, rather than NSCs, neurons, or astrocytes, as the cell of origin for glioma in *p53*, *NF1* mutation-driven glioma model.

Localization of Earliest Neoplastic Lesions Suggests a Gray Matter Origin of Glioma

The analyses of lesion locations at early stages of transformation could provide great insights for understanding the cancer cell of origin. We first analyzed morphological or cyto-architectural changes of mutant cells in fully developed tumors, reasoning that some features could serve as landmarks of transformation, thereby helping to determine the locations of early lesions. In all malignant tumors, even in the smallest lesions identifiable by classic pathology, MADM staining revealed a prominent

(F) Quantitative RT-PCRs confirm the overexpression of OPC markers in MADM-generated glioma.

(G–L) Confocal images at high magnification show that proliferating (Ki67+) green tumor cells express markers for OPCs (G–I, pointed by arrows), but not for other cell types (J–L). The signals of cell type marker staining in the right column of (J–L) were converted to red for better examination of their colocalization with GFP. Some Ki67-negative green cells were CC1+ (circled in L). Scale bars, 20 μm.

(M) Transcriptome comparison between tumor samples and four neuroglial cell types (top four rows) and the four subtypes of human GBMs defined by TCGA (bottom four rows) with the single sample Gene Set Enrichment Analysis (ssGSEA) method. *N* and *O* represent tumor samples from mutant-MADM mice induced by *NSC-Cre* (*Nestin-Cre* or *hGFAP-Cre*) and by *NG2-Cre*, respectively. *PDGFRα+* indicates primary tumor cells enriched by anti-PDGFRα immunopanning method. Red to blue indicates significantly similar to dissimilar.

See also Figure S4.

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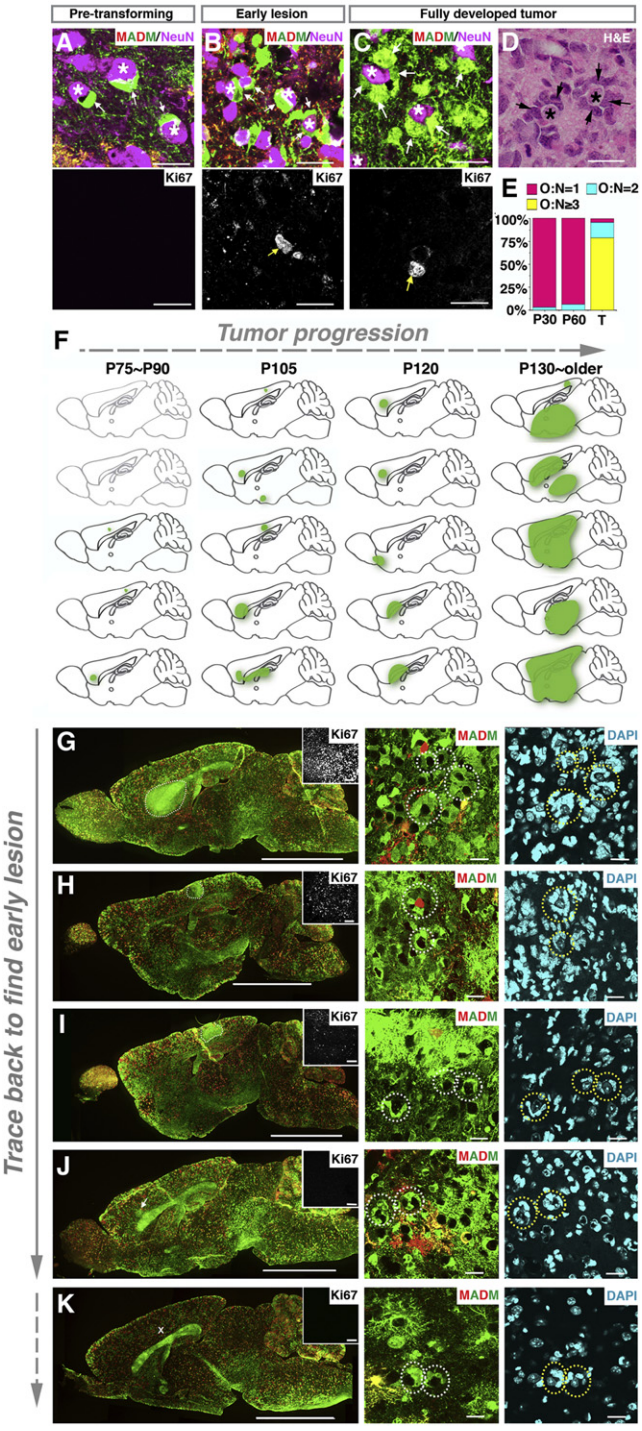

Figure 5. Spatial Analyses of Early Lesions Based on Perineuronal Cytoarchitecture as a Landmark Implicate that Gliomas Initiate at Brain Regions Away from the SVZ

(A–C) Immunofluorescent staining of mutant-MADM brains at distinct tumorigenic stages. Neuronal nuclei were stained with NeuN and are marked as "*." Arrows point to perineuronal pretransforming OPCs or tumor cells. The proliferating status of perineuronal mutant cells is shown by Ki67 staining (yellow arrows in the bottom row).

(D) H&E staining of the adjacent section of (C) shows perineuronal satellitosis.

(E) Proportion of perineuronal structures with distinct mutant OPC-to-neuron ratios (O:N) either in pretransforming MADM mutant brains (P30 and P60, n = 3 brains each) or in tumors (T, n = 4).

(F) Schematic summary of lesion sites (green spots), which are defined by O:N $\geq$ 3 perineuronal structure together with MADM labeling, Ki67 staining, and also pathology in most cases. Brains that are devoid of any detectable lesions are shown in light gray. The analysis is based on a cohort of mutant-MADM mice induced by *hGFAP-Cre*.

(G–K) Representative brain images from (F) with gliomas from medium to small sizes. Insets show Ki67 staining of the tumor regions. The glioma identity in these brains was confirmed by pathological criteria except for (K), in which the perineuronal structure of $\geq$ 3 mutant OPCs suggests that it should be a lesion at its early stage.

Scale bars: (A–D) 20 μm; (G–K) left column, 2 mm; middle and right columns, 50 μm.

See also Figure S5.

cyto-architectural feature in which multiple glioma cells wrapped around a single neuronal cell body (Figure 5C), a salient pathological feature known as perineuronal satellitosis in human glioma (Figure 5D). It should be noted that normal OPCs are often juxtaposed to neuron cell bodies (Figure S5A), implicating that perineuronal satellitosis in tumors might stem from this unique cyto-architecture of OPCs.

When we compared OPC::neuron association from pretransforming to tumor-bearing brains, we found that the number of mutant OPCs for each accompanying neuron changes from mostly one (occasionally two) prior to transformation (Figures 5A and 5E and Figure S5B) to frequently three or higher in any Ki67-positive lesions (Figures 5B, 5C, and 5E). Therefore, we used "three or more mutant OPCs per neuron" as a landmark to examine a cohort of brains for initiating lesions that cover a period from right after the dormant stage to around the time when tumors can readily be identified (Figures 5F and 5G–5K). In 2.5- to 3.5-month old mice, we observed the earliest detectable lesions in cortical gray matters (Figure 5F, P75–P90 and top of P105 column). In further developed lesions, tumor cells were more frequently found to overlap with white matter tracks and the SVZ (Figure 5F, bottom of P105, P120, and > P120 columns). This observation indicates that previous findings of glioma in white matter tracks and the SVZ (Persson et al., 2010; Zhu et al., 2005) might be the path or endpoint, rather than the starting point, of tumor cell migration and further argues against NSCs and for OPCs as the cell of origin.

OPCs Can Be Directly Transformed into Malignant Glioma by *p53/NF1* Mutations

Our results thus far point toward the following scenario for glioma development in our model: NSCs carrying *p53/NF1* mutations give rise to mutant OPCs, which multiply, progress, and eventually transform into malignancies. However, it remains unclear whether mutations must occur in NSCs or whether OPCs can be directly transformed by the same set of mutations. To address this question, we next used *NG2-Cre* that has been reported to specifically express in OPCs, but not NSCs (Komitova et al., 2009; Zhu et al., 2008), to perform MADM analysis. We first verified that *NG2-Cre* exclusively labels OPCs and oligodendrocytes, but not mature astrocytes or neurons in brain parenchyma of WT-MADM mice (Figures 6A–6D). Next, we examined the

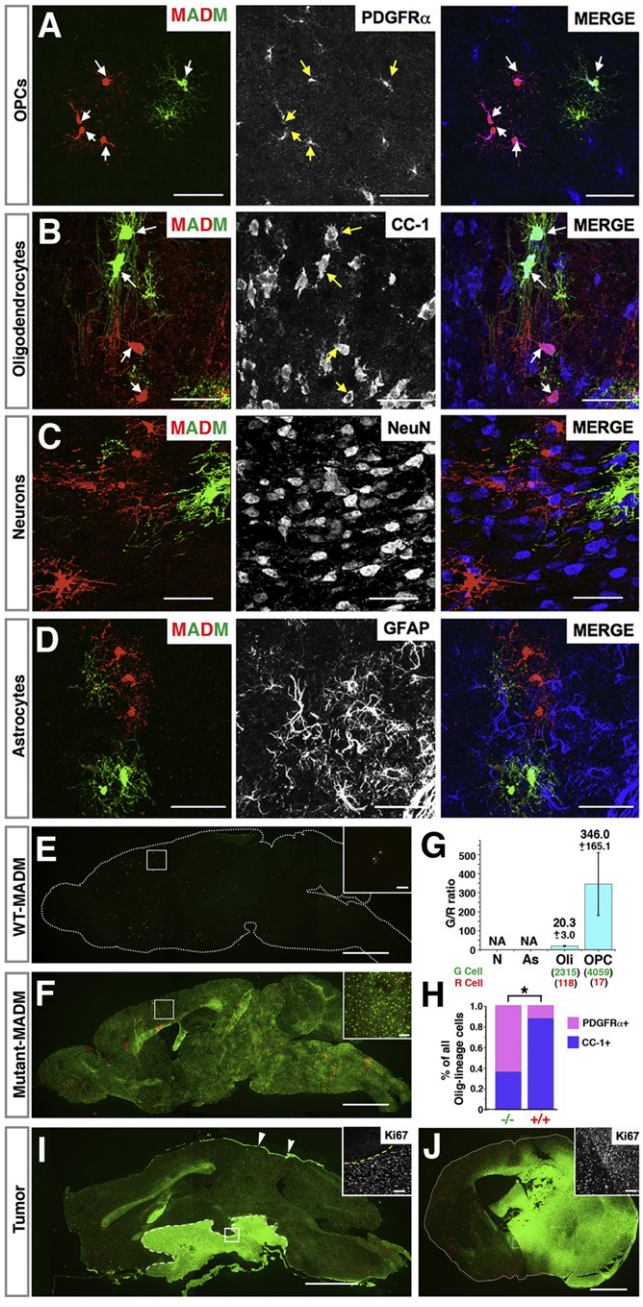

Figure 6. OPCs Can Be Directly Transformed into Malignant Glioma

(A–D) In the MADM system, NG2-Cre transgene labels OPCs and oligodendrocytes, but not astrocytes or neurons. Arrows point to MADM-labeled cells expressing indicated markers.

(E–H) Mutant OPCs overexpand at pretransforming stages. Brain sections from P60 WT-MADM (E) or mutant-MADM (F) mice induced by *NG2-Cre*.

(G) G/R ratios within each cell lineage in P60 Mutant-MADM brains induced by *NG2-Cre*. NA, not applicable. Error bars represent ± SEM. Total cell number being counted is shown in parentheses.

(H) Percentage of OPCs (PDGFRα+) versus oligodendrocytes (CC1+) within mutant and WT cell populations. n = 3 mice in (G) and (H). *$p < 0.05$, t test.

(I and J) Representative gross images of malignant glioma in *NG2-Cre*-induced mutant-MADM mice, either locating around hypothalamus with invasion into the subarachnoid space (I) or residing within the brain parenchyma (J). Arrows in (I) point to tumor cells spreading along the meninges. Ki67 staining in insets shows that tumor cells are highly proliferative. Scale bars, 2mm; inset, 100 μm. See also Figure S6.

entire SVZ of WT-MADM mice and never found any GFP- or RFP-labeled cells coexpressing GFAP (Figures S6B and S6C). Lastly, we thoroughly examined the olfactory bulbs (OB) from four WT-MADM mice and did not find any NeuN+ MADM-labeled green or red cells (Figures S6D and S6E), further supporting the absence of *NG2-Cre* expression in NSCs.

In stark contrast to the extremely sparse labeling in WT-MADM mouse brains induced by *NG2-Cre* (Figure 6E), the green mutant OPCs in mutant-MADM mice populated the entire brain (Figure 6F) and reached an average G/R ratio of more than 300 (Figure 6G) at P60. Consistent with findings from the *NSC-Cre*-based MADM model, the differentiation process of mutant OPCs was hampered (Figures 6G and 6H). These data demonstrate that introduction of *NF1* and *p53* gene mutations directly into OPCs is sufficient to drive overproliferation and to impair OPC differentiation.

After 8 months of age, we found GFP+ tumors in brains of *NG2-Cre*-induced mutant-MADM mice at nearly full penetrance (Figures 6I and 6J, Figure S7A, and Table S3). These tumors showed marker staining and transcriptome profiles indistinguishable from *NSC-Cre*-induced malignant gliomas (Figures S7C–S7H and Figures 4F and 4M). Dissociated cells from these tumors could effectively initiate secondary tumors in allografting assays (Figures S7B and S7I–S7N and Table S4), demonstrating the malignancy of these OPC-initiated tumors.

Despite their indistinguishable marker expression patterns, transcriptome profiles, and tumor-initiating ability, we observed that tumors induced by *NSC-Cre* tended to reside in the brain parenchyma, whereas those induced by *NG2-Cre* were more frequently found around the ventral hypothalamic region and often invaded into the subarachnoid space (Figure 6I, Figure 7B, and Table S3). However, tumors induced by the two Cre lines at equivalent locations showed indistinguishable pathological features (Figures 7A and 7B). Moreover, although tumor cells with subarachnoid invasion were morphologically distinct from those in the brain parenchyma, transplanting them into the brain parenchyma of NOD-SCID mice resulted in secondary tumors that were morphologically indistinguishable from those primary tumors in the brain parenchyma (Figure 7C). These data strongly suggest that the variation in tumor pathology is merely an effect of tumor location. The differences in latency and spatial distribution between *NG2-Cre*- and *NSC-Cre*-induced tumors most likely are attributed to technical reasons, such as the much lower expression level of *NG2-Cre* transgene or possible spatial biases of these Cre lines. It is also possible that these two models have distinct noncell-autonomous cues for tumor formation. For instance, NSC-Cre generates heterozygous astrocytes or neurons, whereas NG2-Cre does not, which might function as special niches to facilitate tumor formation and to bias the tumor location. Nevertheless, all lines of evidences, including marker expression patterns (Figures 4F–4L and Figure S7), transcriptome profiles (Figure 4M), tumorigenic capacity, and pathology (Figure S4, Figure 7, and Figure S7), indicate that tumors from

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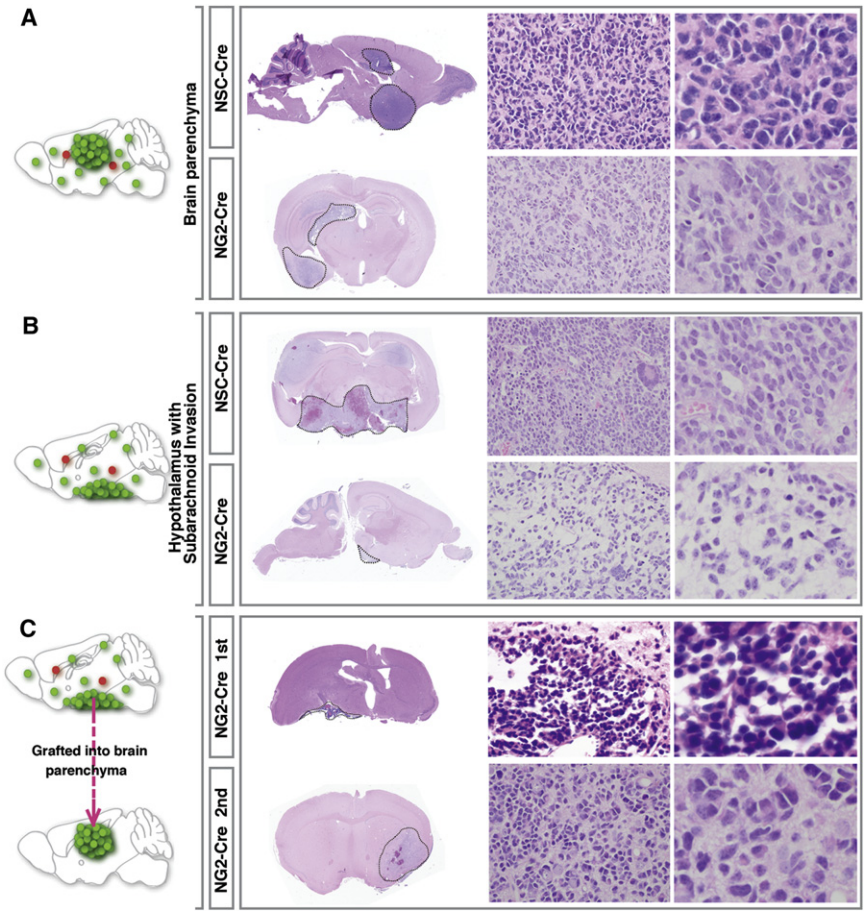

Figure 7. Comparative Pathological Analyses of *NSC*- and *NG2-Cre* Induced Tumors Suggest that Tumor Cell Morphology Is Highly Dependent on the Location Rather Than Initially Mutated Cell Types

(A and B) Regardless of the Cre lines used, tumors at the same location exhibit indistinguishable pathological features.

(C) Transplantation of tumor cells from *NG2-Cre*-induced glioma with subarachnoid invasion (top row) into the brain parenchyma of NOD/SCID mice to generate secondary tumors (bottom row). Pathological features of the primary and the secondary tumors mimic tumor features in (B) and (A), respectively.

Tumor boundaries are demarcated by dashed lines. The magnification of images in the middle columns is 400×. Images from the right column are 2.5× digital zoom-in of the corresponding middle-column ones.

both models are intrinsically identical. Therefore, our data demonstrate that OPCs can be directly transformed into malignant glioma as a result of concurrent *p53*/*NF1* mutations.

Finally, we investigated the human relevance of our studies by comparing the transcriptome profile of tumor cells in our model with the molecular signatures of human glioma samples. Among four subtypes of human malignant glioma revealed by recent studies from the Cancer Genome Atlas (TCGA) (Verhaak et al., 2010), both *NSC-Cre*- and *NG2-Cre*-initiated glioma models match well with the proneural subtype (Figure 4M), which has the poorest responses to chemo- and radiotherapies (Verhaak et al., 2010). Therapeutic strategies designed according to the intrinsic OPC nature should have promising effectiveness for proneural or even other subtypes of glioma.

DISCUSSION

In this study, we used the mouse genetic mosaic system termed MADM to track the entire tumorigenic process, with the aim of identifying the cell of origin for glioma when initial concurrent mutations of *p53* and *NF1* occur in embryonic NSCs. Taking advantage of the in vivo cellular resolution and WT internal control cells afforded by MADM, we analyzed lineage-specific cellular aberrations at premalignant stages and observed dramatic overexpansion and elevated proliferative activity in the OPC lineage but minimal abnormalities in NSCs and all other progeny lineages. Furthermore, marker staining, cyto-architecture, and transcriptome analyses all point to OPCs as the transforming cell type for malignant glioma. Finally, direct introduction of *p53*/*NF1* mutations into OPCs led to the formation of glioma that were indistinguishable from those initiated from NSCs, based on both transcriptome and pathological analyses. Therefore, our studies clarified that, for the glioma model induced by *p53* and *NF1*, though NSCs could serve as the cell of mutation, OPCs serve as the actual cell of origin.

MADM as a Tool to Distinguish Cancer Cell of Origin and Cell of Mutation

The identification of the cancer cell of origin is crucial but often controversial, even with the use of genetic modified mouse models (Visvader, 2011). Conceptually, caution must be taken that cells acquiring initial mutations (cell of mutation) may not directly transform into malignancy because their transforming potential could be manifested by their progeny cell types. For example, when *Patched* mutation was introduced into NSCs in a mouse model of medulloblastoma, malignant transformation only occurred after NSCs gave rise to lineage-restricted granular neuron precursors (GNPs) (Schüller et al., 2008; Yang et al., 2008). In this scenario, the transforming potential of NSCs (cell of mutation) is only manifested by mutant progeny GNPs, the cell of origin that possesses the conducive signaling context. Therefore, to unambiguously identify cancer cell of origin, one has to genetically dissect the transforming potentials of all progeny derived from the initial mutated cell type. This could be feasible if highly specific Cre-transgenic lines are available, such as in the case of medulloblastoma studies (Schüller et al., 2008; Yang et al., 2008). However, such a strategy cannot be

generalized to other cancer models due to the lack of specific Cre-transgenic mice in every lineage for most tissues. An alternative approach is to analyze aberrant growth in all cell lineages derived from initial mutant cells during pretransforming stages (Visvader, 2011), which is now possible with MADM-based cancer models.

Our study extends rather than contradicts previous reports. Limited by the cellular resolution at pretransforming stages, previous studies demonstrated the transforming potential of NSCs but did not clearly distinguish cell of mutation from cell of origin (Alcantara Llaguno et al., 2009; Wang et al., 2009; Zhu et al., 2005). MADM, on the other hand, offers a robust analytical paradigm for the identification of the cell of origin with both permanently GFP-labeled mutant cells and RFP-labeled WT internal control cells immediately after the initial mutations occurred. In principle, MADM could be used for all types of cancer, as long as oncogenic mutations for a particular type of cancer, a tissue-specific stem cell Cre-transgenic line, and cellular markers of each lineage within that tissue are available.

Our studies emphasize the importance of intersection between genetic mutations and the signaling context within the cell of origin. We show here that OPCs are particularly sensitive to *p53*/*NF1* mutations, whereas NSCs and other brain cell types are much less responsive. Interestingly, it has been reported that the *NF1* mutation alone can promote OPCs to proliferate in mice and zebrafish (Bennett et al., 2003; Lee et al., 2010), suggesting that NF1 plays a pivotal role in regulating OPC proliferation with conserved mechanisms across species. Such intersections should be further exploited to help understanding of the molecular mechanism of OPC transformation, which should provide critical insights for developing effective therapeutic strategies. Notably, whereas OPCs appear to be particularly responsive to *NF1* mutation, other cell types, including NSCs, could be specifically vulnerable to other genetic lesions. It would be very interesting to investigate whether different mutations could transform distinct cell of origins, which might account for the great heterogeneity of human glioma.

OPC Is an Important Glioma Cell of Origin with Underappreciated Proliferative and Plastic Potentials

One of the notions that support the prevalent view of NSCs as the glioma cell of origin is the persistent proliferative activity of NSCs in the brain during the entire life, which makes them susceptible to oncogenic mutations. Additionally, NSCs share many cellular properties with glioma cancer stem cells, such as the capability to self-renew, the potential to differentiate into multiple cell lineages, and the expression of some common cellular markers such as Nestin and Sox2. However, a large body of work has revealed that many of these features are also shared by OPCs. First, OPCs in fact represent the largest proliferative pool in the brain parenchyma for both rodents and human (Dawson et al., 2003; Geha et al., 2010). Second, OPCs at early developmental stages have been found to express many commonly used stem cell markers, including Nestin and Sox2. Third, OPCs isolated from rat optic nerve can be readily reprogrammed into a multipotent NSC-like status under in vitro conditions, which become self-renewable and can differentiate into astrocytes and neurons in addition to oligodendrocytes (Kondo and Raff, 2000). Intriguingly, OPCs from human subcortical white matter intrinsically behave like NSCs without reprogramming (Nunes et al., 2003), raising the possibility that primate OPCs are more plastic than their rodent counterparts. Taken together, the intrinsic nature of OPCs renders them great susceptibility to oncogenic mutations, which could be harnessed to devise effective therapeutic strategies.

Previously, it has been reported that the injection of PDGF-BB-expressing virus into the corpus callosum, the expression of PDGF-BB under CNPase promoter, and the overexpression of v-erbB under human S100β promoter all led to glioma formation with OPC features (Assanah et al., 2006; Lindberg et al., 2009; Persson et al., 2010). Although providing circumstantial evidence, these studies have faced challenges to unequivocally pinpoint OPCs as the cell of origin for malignant glioma (Visvader, 2011) due to noncell-autonomous effects caused by the secretion of PDGF-BB (Assanah et al., 2006; Lindberg et al., 2009) and the use of non-OPC-specific promoter to drive the initial tumorigenic event (Persson et al., 2010). By circumventing all of these caveats, our studies now firmly establish that OPCs serve as the cell of origin for malignant glioma with relevant genetic mutations. For clinical applications, intrinsic OPC properties should be fully exploited to design effective therapies for glioma, especially for the proneural subtype. For example, understanding the unique proliferative capacity of OPCs could help devise treatments to stall tumor progression; deciphering the migration mechanism of OPCs could enhance the effectiveness of surgery by preventing the infiltration of tumor cells into the entire brain; and probing into the differentiation process of OPCs could facilitate the design of differentiation therapy strategies.

The Application of MADM beyond Cancer Cell of Origin Studies

Compared to conventional models, the MADM-based cancer model bears significant advantages for exploring critical cancer biology problems beyond the cell of origin. Its reliable labeling of tumor cells at the single-cell resolution should help to distinguish bona fide tumor cells from bystander cells in a complex tumor mass, which is highly valuable for dissecting tumor architecture, tracking metastasized tumor cells, and studying tumor-niche interactions. Importantly, the availability of a built-in internal control enables quantitative comparison between mutant and WT sibling cells. Such rigorous phenotypic analyses within the same animal largely remove variations often introduced by comparing phenotypes between individual animals, thereby helping to identify subtle but important phenotypes that could be easily missed by using conventional approaches.

Using *Drosophila* genetic mosaic system, combined with the application of positive marking with gene manipulation at the mosaic level, helped to elucidate many fundamental questions in cancer biology, such as identifying important oncogenes and TSGs by forward genetic screening (Potter et al., 2001; Tapon et al., 2001) and deciphering the complicated tumorigenic processes such as metastasis (Pagliarini and Xu, 2003). Along this same principle, we expect that a further modified MADM system could address even more sophisticated problems in cancer biology beyond the capability of the current one. For instance, by replacing fluorescent reporters with a transcription factor such as tTA or rtTA to specifically manipulate gene

expression in mosaic mutant cells, one could model "second hit" events or therapeutic strategies and gain deep insights into the molecular network for cancer formation. By incorporating novel magnetic resonance imaging reporters such as H-ferritin (Genove et al., 2005) into MADM cassettes, one could noninvasively trace tumor initiation, progression and metastasis in real time. Also, by combining with transposon-based mutagenesis such as *Sleeping Beauty* (Collier et al., 2005) or *PiggyBac* (Ding et al., 2005), the MADM system should be able to perform forward genetic screening for novel recessive TSGs in mouse somatic cells without involving a large number of animals.

Concluding Remarks

In summary, our study demonstrates the importance of analyzing the entire process of tumor development for identifying cancer cell of origin. MADM-based tumor modeling can be applied in principle to any other tumor type and should help to resolve many important problems in cancer biology. Broadly, the ability to perform sporadic single-cell genetic manipulations in unambiguously labeled cells should make MADM an invaluable analytical tool for fields such as developmental biology and neuroscience that rely heavily on in vivo analyses.

EXPERIMENTAL PROCEDURES

Mouse Lines and Genotyping Methods

All animal procedures were based on animal care guidelines approved by the Institutional Animal Care and Use Committee. Mouse lines and genotyping methods used in this study can be found in the Extended Experimental Procedures.

Tissue Preparation and Histology

After anesthesia, mice were perfused with 4% ice-cold paraformaldehyde (PFA) following the standard procedure. Brains were isolated, postfixed (overnight at 4°C), cryoprotected in 30% sucrose (overnight at 4°C), and embedded into optimal cutting temperature (O.C.T.) prior to cryosectioning with a cryostat.

Imaging

All confocal images were collected by an Olympus FV-1000 upright laser confocal microscope and analyzed with Fluoview 1000 software. Adobe Photoshop CS3 was used for image processing.

Immunohistochemistry

Immunohistochemistry was performed using standard methodology. Details about antibodies and working solutions can be found in the Extended Experimental Procedures and Table S1.

Quantification

All quantification described in this work was performed based on the systematic sampling scheme described in the Extended Experimental Procedures and Figure S3.

Purification of WT OPC and OPC-like Glioma Cells

WT OPCs from P8 cortical caps or OPC-like tumor cells from fresh glioma samples were purified as previously described (Cahoy et al., 2008) with minor modification. Detailed procedures can be found in the Extended Experimental Procedures and Figure S4.

Tumor Cell Grafting

Tumor cells were suspended into Neurobasal medium with a density of 50,000 viable cells /μl. Tumor cell suspension (2–3 μl) was injected into right striata of NOD-SCID mice (JAX laboratory) as previously described (Ligon et al., 2007). Mice were monitored daily and sacrificed at the onset of neurological symptoms or once moribund or based on designed time point cohort.

Microarray and Gene Expression Analysis

44K Mouse Development Oligo Microarrays (Agilent Technologies) were used for microarray analysis. Transcriptome comparison of tumor samples with WT neuroglia cell types (Cahoy et al., 2008) or human GBM subtypes was preformed as previously described (Verhaak et al., 2010). Array data are available at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE26676. Detailed procedures are described in the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and four tables and can be found with this article online at doi:10.1016/j.cell.2011.06.014.

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