Genomics

Contribution of Tumor Heterogeneity in a New Animal Model of CNS Tumors

Fuyi Chen1, Albert J. Becker2, and Joseph J. LoTurco1

Abstract
The etiology of central nervous system (CNS) tumor heterogeneity is unclear. To clarify this issue, a novel animal model was developed of glioma and atypical teratoid/rhabdoid-like tumor (ATRT) produced in rats by nonviral cellular transgenesis initiated in utero. This model system affords the opportunity for directed oncogene expression, clonal labeling, and addition of tumor-modifying transgenes. By directing HRasV12 and AKT transgene expression in different cell populations with promoters that are active ubiquitously (CAG promoter), astrocyte-selective (glial fibrillary acidic protein promoter), or oligodendrocyte-selective (myelin basic protein promoter) we generated glioblastoma multiforme and anaplastic oligoastrocytoma, respectively. Importantly, the glioblastoma multiforme and anaplastic oligoastrocytoma tumors were distinguishable at both the cellular and molecular level. Furthermore, proneural basic helix-loop-helix (bHLH) transcription factors, Neurog2 (NEUROG2) or NeuroD1, were expressed along with HRasV12 and AKT in neocortical radial glia, leading to the formation of highly lethal ATRT like tumors. This study establishes a unique model in which determinants of CNS tumor diversity can be parsed out and reveals that both mutation and expression of neurogenic bHLH transcription factors contribute to CNS tumor diversity.

Implications: A novel CNS tumor model reveals that oncogenic events occurring in disparate cell types and/or molecular contexts lead to different tumor types; these findings shed light on the sources of brain tumor heterogeneity. Mol Cancer Res; 12(5); 742–53. ©2014 AACR.

Introduction
Tumors of the central nervous system (CNS) have significant intra- and intertumor heterogeneity in terms of cellular composition, cellular proliferation, invasiveness, and epigenetic status (1, 2). Integrative large-scale gene expression analysis of 200 glioblastoma multiforme tumors revealed four subtypes: proneural, neural, classical, and mesenchymal (1). A more recent glioblastoma multiforme classification based on a combination of epigenetics, copy-number variation, gene expression, and genetic mutations has led to identification of as many as six glioblastoma multiforme subgroups (2). In addition, there are at least two prognostic subgroups of pediatric glioblastoma multiforme based on association with or without the aberrantly active Ras/AKT pathway (3, 4). Heterogeneity in glioblastoma multiforme tumors may be responsible for differential response to therapeutic interventions (1), and understanding the etiology of tumor heterogeneity in animal models may become increasingly important to design therapeutic strategies effective at targeting molecularly defined tumor subtypes.

Several animal models of brain tumor, including xenograft models, genetically engineered mouse models (GEMM), and models using virus-mediated somatic cell transgenesis have been used to address important aspects of CNS tumor biology. For example, GEMM-harboring mutations found in human gliomas have been used to assess tumorigenic effects of individual genes and mutations and to reveal the tumor cell-of-origin in some models (5, 6). Endogenous cell populations have been induced to form gliomas by both viral and nonviral somatic transgenesis of oncogenes or deletion of tumor suppressor genes in specific target cell populations (7–17). For example, oligodendrocyte precursor cells (OPC) can be the cell-of-origin for adult glioma (5, 6, 13) with OPC-originated glioma matching the human proneural subgroup of glioblastoma multiforme (6, 18). Friedmann-Morvinski and colleagues found that RNA interference (RNAi) knockdown of NF1 and p53 expression in either astrocytes glial fibrillary acidic protein (GFAP+) or neurons (Synl+) induced formation of mesenchymal glioblastoma multiforme subtypes, whereas the same RNAi in nestin-positive neural progenitors induced neural glioblastoma multiforme subtypes (16). To more fully explore causes of tumor diversity in cerebral cortex, we have developed an animal model in which multiple transgenes can be expressed in selected cell populations at different times in forebrain development.
DNA transposon systems have been previously adapted to nonviral somatic transgenesis in studies of neocortical development (19, 20), cellular reprogramming (21), and to generate animal models of glioma (17). In utero electroporation is a relatively efficient method to deliver multiple combinations of transgenes into neuronal and glial progenitors in the developing forebrain in utero (22). When combined with a DNA transposon system including cell type–specific promoters, it becomes possible to introduce multiple transgenes, including oncogenes, in different populations of cells (19, 20, 23, 24). The active Ras pathway (25) and the phosphoinositide 3-kinase/AKT pathway (9, 26) have been frequently found in patients with human glioma and used in various glioma animal models (9, 10, 12, 15). In the model described here, we introduced HRasV12 and AKT in cell populations in the radial glia lineage to ask whether diversity in glioma is linked to the population of cells induced to express oncogenic transgenes. We also tested whether addition of neurogenic transcription factors to the starting progenitor population modifies tumor type. We show by several measures, including morbidity, histology, developmental time course, tumor clonal pattern, and molecular signature, that diverse tumors are generated when oncogene expression is directed into different cell populations. Moreover, expression of the basic helix-loop-helix (bHLH) transcription factors Ngn2 or NeuroD1 along with HRasV12 and AKT results in atypical teratoid/rhabdoid-like tumor (ATRT), a pediatric tumor type rarely produced in existing tumor models.

Materials and Methods

Plasmids
A system of piggyBac transposon donor and helper plasmids were produced and used in this study. To make the donor plasmids PBCAG-HRasV12 and PBCAG-AKT, HRasV12 was PCR amplified from pTomo (15; Addgene plasmid 26292), human AKT was amplified from 1036 pcDNA3 Myr HA Akt1, (26; Addgene plasmid 9008), respectively, and inserted into the EcoRI/NotI sites of pPBCAG-enhanced green fluorescent protein (eGFP) (19). For construction of PBGFAP-HRasV12/AKT, PBMBP-HRasV12/AKT, HRasV12, and AKT were inserted into EcoRI/NotI sites of PBGFAP-GFP and PBMBP-GFP, respectively (19). For the bHLH donor plasmids PBCAG-Ngn2 and PBCAG-NeuroD1, human Neurogenin2 cDNA clone (IMAGE ID 5247719), human Neuronal Differentiation 1 cDNA (IMAGE ID 3873419) were purchased from Open Biosys tem, PCR amplified and inserted into PBCAG-eGFP EcoRI/NotI sites. CAG-Ngn2 was made by inserting Ngn2 coding sequence into EcoRI/NotI sites of pCAG-eGFP.

Animals

Pregnant Wistar rats were obtained from Charles River Laboratories, Inc. and maintained at the University of Connecticut (Storrs, CT) vivarium on a 12-hour light cycle and fed ad libitum. Animal gestational ages were determined and confirmed during surgery. Both male and female subjects were used for tumor induction. For the purpose of constructing survival curves (Figs. 2A and 3A), rats were removed from the experiment for humane purposes when they became unable to eat or drink. At the end of the 40-week survival experiment, percentage of survival for each preceding week was calculated and percentage of survival curves was generated. For tumors induced by PBCAG-Ngn2/ PBCAG-HRasV12/AKT and PBCAG-NeuroD1/PBCAG- HRasV12/AKT, survival rates were also calculated for each day. To statistically compare survival rates, a log-rank test, Breslow test, and Tarone–Ware test were performed using SPSS (IBM SPSS statistics 21). All procedures and experimental approaches were approved by the University of Connecticut Institutional Animal Care and Use Committee.

In utero electroporation

In utero electroporation was performed as previously described (19, 20, 27). Electroporation was performed at embryonic day 14 or 15 (E14 or E15) and gestation age was confirmed during surgery. All plasmids were used at the final concentration of 1.0 μg/μL except PBCAG-cyan fluorescent protein (CFP) and GLAST-PBase were used at the final concentration of 2.0 μg/μL.

Image acquisition and 3D reconstruction

Multicolor imaging was performed as described using a Zeiss Axio imager M2 microscope with Apotome with 488/546/350-nm filter cubes and the X-Cite series 120Q light source (20). All the images were further processed in Adobe Photoshop CS3 software. For three-dimensional (3D) reconstruction, P27 Wistar rats were deeply anesthetized with isoflurane and perfused transcardially with 4% paraformaldehyde/PBS (4% PFA). Samples were fixed overnight in 4% PFA and sectioned at 65-μm thickness on a vibratome (Leica VT 1000S). Sections were mounted onto microscope slides in sequential order, all aligned in the same dorsal–ventral and left–right arrangement. Images were acquired with Stereo Investigator (Microbrightfield). After imaging acquisition, outlines of the cerebral hemisphere and tumor clones were traced using color-coded contours for the hemisphere and each clone. Then traced images were transferred to Neurolucida Explorer (Microbrightfield), in which several contours from sequential sections were appended to one another, and organized and stacked in order. 3D model was created using Neurolucida Explorer software 3D visualization option to visualize the clonality of selected clones of tumor cells.

Immunohistochemistry

Animals were deeply anesthetized with isoflurane and perfused transcardially with 4% paraformaldehyde/PBS (4% PFA). Samples were post fixed overnight in 4% PFA and sectioned at 65-μm thickness on a vibratome (Leica VT 1000S). Sections were processed as free-floating sections and stained with GFAP (Cell Signaling Technology; 3670X), CC1 (Calbiochem; OP80), and NG2 (Chemicon; AB5320) antibodies. Images were acquired and processed as previous described (19, 20).
For histologic analysis, immunohistochemistry and hematoxylin and eosin staining were carried out on paraffin-embedded 4-μm sections as described in (28, 29) using GFAP (DAKO; Z0334), MAP2c (Sigma; M4403), vimentin (DAKO; M0725), Synaptophysin (DAKO; M0776), Cytokeratin (BMA Medicals; T-1302), Actin (DAKO; M0851), epithelial membrane antigen (DAKO; M0613) antibody.

**Tumor cell culture**

Tumors were dissected from PBCAG-HRasV12/AKT–transfected animals at the age P21. After dissection, tumor tissues were chopped and trypsinized into single-cell suspension. Then cells were cultured in a serum-free medium consisting of Dulbecco’s Modified Eagle Medium with l-glutamine, sodium pyruvate, B-27, N-2, basic fibroblast growth factor, and EGF (Invitrogen). Two days later, tumor cell adherent cultures were passaged into neurosphere suspension cultures at a density of 10 cells/μL and allowed to grow for 3 days to form tumor spheres.

**RNA extraction, cDNA synthesis, and qRT-PCR**

Animals ages P21 to P27 were deeply anesthetized with isoflurane. Brains were quickly removed on ice. Tumors were identified on the brain surface, removed, and then chopped into cubes. Tissue from the opposite hemisphere that was tumor free was used as control. RNA extraction was performed using the Ambion RNAqueous Kit (Invitrogen) according to the manufacturer’s instructions. cDNA synthesis was performed using the Transcripter First Strand cDNA synthesis Kit (Roche) following manufacturer’s protocol. Quantitative real-time PCR (qRT-PCR) was performed using Fast SYBR Green master mix (Applied Biosystem). Primer sequences are listed in Supplementary Table S1. Triplicates were included from each sample. Fold of regulation for each of the 24 genes probed was calculated using the ΔΔCt method. More specifically, the Ct values obtained from tumor and control brain RNA samples are directly normalized to a housekeeping gene (GAPDH). ΔΔCt is the difference in the ΔCt values between the tumor and control samples. The fold change in expression of the gene of interest between the tumor and control brain is then equal to 2^ΔΔCt. Unsupervised hierarchical clustering was performed on fold of regulation for each of the 24 genes probed and a heat map was generated using the clustergram command in the MATLAB bioinformatics toolbox (MathWorks). Other statistical analyses were performed using KaleidaGraph version 4.0 (SynergySoftware 2006). A confidence interval of 95% (P < 0.05) was required for values to be considered statistically significant. All data are presented as means and SEM.

**Results**

**A model of glioblastoma multiforme by piggyBac-mediated somatic transgenesis**

We developed a nonviral somatic cell transgenesis approach to produce tumors from endogenous rat neural progenitors in vivo. HRasV12 and AKT transgenes were introduced into embryonic neocortical neural progenitors, also known as radial glial progenitors, by in utero electroporation of the lateral ventricles of embryonic rats. For nonviral cellular transgenesis, we produced a system of plasmids consisting of a helper plasmid that expresses the piggyBac transposase in radial glial progenitors via the glutamate aspartate transporter (GLAST) promoter (GLAST-PBase) and thereby drives transposon integration into the genomes of radial glia progenitors, and a set of donor plasmids containing transgenes flanked by transposon inverted terminal repeats (ITR) to be integrated into the genome by piggyBac transposase activity. The donor plasmids included HRasV12 and AKT under the control of one of three different promoters [CAG, myelin basic protein (MBP), or GFAP; Fig. 1A] and a multicolor system of donor plasmids (20) with the CAG promoter driving expression of eGFP, monomeric red fluorescent protein (mRFP), or CFP to create multicolor clonal labeling (Fig. 1A; refs. 20, 30). Electroporation of this system of plasmids unilaterally into the lateral cerebral ventricles of E14–E15 rat embryos (Fig. 1A) invariably resulted by 21 days after birth in the formation of large unilateral tumors (Fig. 2F). Tumors were composed of both regions of uniformly colored cells indicating significant regionalized clonal expansion (Fig. 1C and D), and regions containing a mixture of differently colored cells indicating clonal mixing (Fig. 1F). In addition to tumors, streams of cells typically of one or two different colors were found distal to the tumor core. These streams were frequently in directionally oriented chains suggesting migration and invasion into surrounding neural tissue (Fig. 1E). Survival experiments showed that tumor-bearing animals began dying at 3 weeks of age and the rate of death seemed stable by 31 weeks after birth for the CAG donor plasmid–treated animals (Fig. 2E). Tumors were confirmed in all electroporated animals (30 of 30).

Histologically, tumors induced by HRasV12 and AKT driven by the CAG donor plasmids were composed of fibrillary and gemistocytic elements (Supplementary Fig. S1A). The tumors diffusely infiltrated neighboring tissues (Supplementary Fig. S1B) and had cells with highly pleomorphic cytologic appearance, and increased mitotic activity indicative of malignancy (Supplementary Fig. S1C). Tumor cells frequently invaded the subarachnoidal space (Fig. 2G), and tumors had areas of necrosis indicative of high malignancy (Fig. 2H arrow). Tumors seemed by P7 and necrotic areas were found as early as at P4. Immunohistochemical analysis revealed that tumor cells showed strong positivity for GFAP (Fig. 2I), vimentin (Fig. 2J), and mitogen-activated protein 2 (MAP2; Fig. 2K). Some neurons entrapped within tumors were positive for synaptophysin, whereas the bulk of the tumors did not show synaptophysin positivity. Interestingly, we did not observe vascular proliferation in the rat tumors produced by the CAG donor plasmids, but observed vascular proliferation in tumors induced by all other plasmid combinations used in this study. Moreover, when cultured in neurosphere suspension medium, tumor cells form tumor spheres (Supplementary Fig. S2). In sum, the piggyBac transposon system effectively produces a tumor model resembling human glioblastoma multiforme (WHO grade 4).
MBP and GFAP promoter–directed oncogene expression produces distinct tumor types

The binary piggyBac system allows for directing insertion of transgenes in one population of cells (i.e., radial glia neural progenitors), but then delayed expression of oncogene expression in later-generated cell types in the lineage. This feature allowed us to introduce oncogenes into neural progenitors at the embryonic lateral ventricle surfaces, but then have their expression turned on later, primarily in either astrocytes, or oligodendrocytes. For this experiment, we constructed donor plasmids in which HRasV12 and AKT expression was controlled by either a mouse GFAP promoter fragment (19; PBGFAP-HRasV12/AKT) or a rat MBP promoter fragment (31; PBMBP-HRasV12/AKT). We first assessed the activity of the mouse GFAP and rat MBP promoters using GFP as a reporter. We found that the GFAP promoter resulted in 69.3% C63% astrocytes, 19.4% C62% neurons, 7.1% C62% oligodendrocytes, and 4.3% C60.7% oligodendrocyte precursor cells. In contrast, the MBP promoter construct (PBMBP-eGFP/mRFP) labeled 57.0% C62% oligodendrocytes, 16.2% C60.7% astrocytes, 19.3% C62% neurons, and 7.5% C60.7% oligodendrocyte precursor cells (Fig. 2C and D). One-way ANOVA showed a significant difference between GFAP and MBP promoter–labeled astrocytes (P = 6.91E-05) and oligodendrocytes (P = 2.69E-05). A significant difference was also found between NG2 cells labeled by GFAP and MBP promoters (one-way ANOVA, P = 0.021172), but no significant difference was found between the fraction of labeled neurons (one-way ANOVA, P = 0.989884). The differences in the cell populations labeled by the MBP and GFAP promoter fragments are consistent with enriched targeting of oligodendrocytes and astrocytes, respectively; however, each promoter was also capable of labeling some fraction of each cell type.

We next compared and contrasted tumors produced by the donor plasmids containing either the GFAP or MBP promoters driving oncogene expression. For both donor plasmids, tumors were found in all animals and 65.2% (15 of 23) of animals died by 40 weeks (Fig. 2E and F). A log-rank test showed no significant difference in survival among animals bearing tumors induced by GFAP, MBP, or CAG donor plasmids (log-rank test; P = 0.194 between

Figure 1. Multicolor rat glioma. A, schematic representation of piggyBac IUE approach and plasmids used. In utero electroporation was performed between E14 and E15 with plasmids listed. We used the multicolor piggyBac system to clonally label cells, oncogene donor plasmids to induce tumors, and both donor and conventional episomal plasmids encoding transcription factors to evaluate their role in gliomagenesis. After the animals were born, tumor assessment was performed at different postnatal ages. B, representative images of a P22 rat brain hemisphere transfected with multicolor piggyBac plasmids and PBCAG-HRasV12/ATK donor plasmids. Necrosis is labeled with N. C, overlay image showing large regions of tumor cells were uniformly labeled with mRFP. D, overlay image showing other regions of tumor were uniformly labeled with eGFP. E, streams of tumor cells invading tissue adjacent to tumors. F, region of the tumor containing a mixture of red- and green-labeled transformed cells indicating regions of clonal mixing. G, 3D reconstruction of 5 tumor clones in a P27 rat transfected with PBGFAP-HRasV12/ATK. CT, cortex; CC, corpus callosum. Scale bar, 500 μm in B and 100 μm in C, D, E, and F.
Figure 2. Distinct tumor types are induced by different donor plasmids. A, mouse GFAP promoter fragment–labeled astrocytes. B, rat MBP promoter fragment–labeled oligodendrocytes. C, quantification of proportions of labeled cells by GFAP and MBP promoter fragments (one-way ANOVA; **P < 0.05; ***P < 0.01; NS, no significance difference). D, representative images of neuron, astrocyte, oligodendrocyte, and NG2 cell. E and F, Kaplan–Meier survival curves and representative appearance of tumor-bearing brains. The log-rank test showed no difference in survival across all groups. G to K, characterization of tumors induced by PBCAG-HRasV12/AKT. G, subarachnoidal spread of tumor cells (H&E). H, necrosis (black arrow, H&E). I to K, tumor cells with fibrillary processes were positive for GFAP, vimentin, and MAP2. L to P, characterization of tumors induced by PBGFAP-HRasV12/AKT. L, overview of H&E staining. Black arrow, vessels. M, necrosis (black arrow). N to P, GFAP, vimentin, and MAP2–positive processes. Q to U, characterization of tumors induced by PBMBP-HRasV12/AKT. Q, prominent oligodendroglial components (H&E). R, round cell appearance of tumor cell (inset) and mitosis (black arrow, H&E). S, GFAP–positive astroglial process. T, oligodendroglial cells were vimentin-negative, whereas astroglial elements were vimentin-positive. U, MAP2–positive oligodendroglial component (black arrow, inset) and MAP2–positive astrocytes component (gray arrow). Scale bar, 200 μm in A, B, L–N, P, Q, and S; 100 μm in G, I–K; 50 μm in H, O, R, and T; 20 μm in D; and 10 μm in inset in R and U.
CAG and GFAP; \( P = 0.554 \) between GFAP and MBP donor plasmid–transfected animals. For comparison between CAG and MBP donor plasmid–transfected animals, log-rank test, \( P = 0.0933 \); Breslow test, \( P = 0.121 \); Tarone–Ware test, \( P = 0.106 \). A histopathologic analysis of tumors in both GFAP and MBP promoter conditions indicated frequent mitotic figures (Supplementary Fig. S1D, Fig. 2R, black arrow), similar to the CAG promoter condition described above, but also showed vascular proliferates consistent with highly malignant tumor types. Tumors were observed in 23 of 23 animals assessed for tumors 14 to 280 days after birth. In addition, GFAP donor plasmid–induced tumors, unlike the MBP plasmids, resulted in tumors with prominent necrotic foci frequently surrounded by "pseudopalisading" tumor cells (Fig. 2M, arrow). Immunohistologic assessment of tumors induced by GFAP donor plasmids indicated process-rich astroglial cells with delicate processes positive for GFAP, vimentin, and MAP2 (Fig. 2N–P). We conclude based on the histopathologic findings that the GFAP donor plasmid–induced tumors are malignant astroglial tumors resembling human glioblastoma multiforme (WHO grade 4). Although similar in many ways to tumors induced by the CAG donor plasmid, the multicolor clonal analysis indicated that the GFAP donor plasmids resulted in tumors with larger clonal territories than the CAG donor plasmids. Similarly, the tumors in the GFAP donor plasmid condition showed more clonally associated invasion and expansion into striatum than did tumors in the CAG donor plasmid condition.

In contrast with the GFAP and CAG donor plasmid–induced tumors, the MBP donor plasmid–induced tumors contained a mixture of astroglial and oligodendroglial components, and lacked prominent necrotic foci and pseudopalisading cell arrangements typical of the GFAP donor plasmid–induced tumors (Fig. 2M). In some areas of MBP donor plasmid–induced tumors, there were processes with GFAP and vimentin positivity (Fig. 2S and T), but these areas were less frequently observed than in the GFAP donor plasmid–induced tumors. In addition to the small astroglial cell fraction, the MBP donor plasmid–induced tumors contained prominent "honeycomb"-like cell clusters (Fig. 2Q) with round isomorphic nuclei located centrally to perinuclear halos (Fig. 2R, inset): a feature resembling cytologic characteristics of human oligodendroglioma. Cells were largely negative for vimentin (Fig. 2T), but positive for MAP2, an immunostaining pattern consistent with mixed oligodendroglial and astroglial components. MAP2 immunopositivity showed strong perinuclear cytoplasmic staining without significant process labeling (Fig. 2U, black arrow, inset) typical of oligodendroglia. Some MAP2-stained cells in MBP donor plasmid–induced tumors displayed bi- or multipolar processes typical of astroglial cells (Fig. 2U, gray arrow). In sum, the histologic and immunohistochemical patterns observed in the tumors induced by MBP donor plasmids were distinct from those observed with the GFAP and CAG donor plasmids, and are most similar to human anaplastic oligoastrocytoma (WHO grade 3).

Expression of bHLH transcription factors modifies tumor type

Next, we addressed whether addition of neurogenic transcription factors, Neurogenin 2 (Ngn2) and Neuronal Differentiation 1 (NeuroD1), known to alter the fates of neural progenitors (32) would change tumor phenotype. We chose Ngn2 and NeuroD1 because they have been previously shown to block the differentiation of neural progenitors into astrocytes and to promote differentiation into neurons (32). We hypothesized that transcription factors with neuron promoting effects would either inhibit tumor formation, or result in the formation of tumors distinct from CAG donor plasmid–induced tumors. Indeed, expression of either Ngn2 or NeuroD1 along with the CAG donor plasmids resulted in a distinct tumor type that we never observed with any of the three other tumor-inducing plasmid combinations. Furthermore, animals transfected with the Ngn2- or NeuroD1-modifying plasmids showed earlier death and higher rates of death than the other tumorigenic plasmid conditions (Fig. 3A). Log-rank tests showed animals transfected with Ngn2- or NeuroD1-modifying plasmids had significantly shorter survivals (\( P < 0.0001 \)) than animals transfected with CAG, GFAP, or MBP donor plasmids (Fig. 3A).

Upon postmortem analysis of cerebral tumors in Ngn2 and NeuroD1 donor plasmid conditions, we encountered large cerebral tumors with highly irregular surfaces by 14 days of age (Fig. 3B). Histologically, these large tumors contained cells that were poorly differentiated and showed regions of extensive necrosis and proliferative features. The tumors contained prominent rhabdoid cellular elements demonstrated in H&E sections (black arrow in Fig. 3H and L). The overall immunohistochemical profile indicated teratoid characteristics. Cells had characteristic "capping"-type expression patterns of vimentin (black arrow in Fig. 3I), and some cells were strongly positive for actin (Fig. 3I). There was also focal expression of epithelial membrane antigen (Fig. 3K). We also did not observe significant expression of cytookeratin (Lu-5 epitope). Nuclear expression of integrase interactor 1 (INI1; hSNF5/SMARCB1) was preserved in cells in these tumors. Although poorly differentiated, the tumors showed regions of focal expression of astroglial GFAP (Supplementary Figs. S4B and S5C) and vimentin–positive processes (Fig. 3I and M) as well as scattered positivity for the neuronal markers MAP2 (Supplementary Figs. S4D and S5B) and synaptophysin (Supplementary Fig. S4C). Intriguingly, synaptophysin positivity marked areas and clusters of tumor cells with cytologically neuronal features. The non-organoid distribution of respective elements argues against entrapped neurons. MAP2c expression seemed to reflect dendritic neuronal structures. In sum, the tumors induced by combining Ngn2 or NeuroD1 donor plasmids with the CAG donor plasmid histologically most resembled atypical teratoid rhabdoid tumor (ATRT) like tumors.

Hertwig and colleagues (33) have reported that a panel of 7 signature genes can be used in error-free classification of three CNS tumors: glioma, primitive neuroectodermal tumor (PNET), and ATRT. We therefore assessed the
expression of this panel of 7 genes in the tumors induced by HRasV12/AKT in combination with Ngn2. We found significant upregulation in the expression of 6 out of 7 genes in the tumors induced by HRasV12/AKT in and Ngn2 (Supplementary Fig. S6). Among the 6 upregulated genes, SPP1, which has been suggested as a diagnostic marker to distinguish ATRT from PNET and medulloblastoma (34) and as grade indicator of glioma (35), showed the greatest upregulation (6.6-fold). The pattern of gene upregulation combined with the histologic features suggests that the tumors induced by HRasV12/AKT in combination with Ngn2 or NeuroD1 are ATRT-like tumors.

As the bHLH transfection conditions resulted in a unique and highly lethal tumor, we next addressed whether this apparent phenotypic transformation from glioblastoma multiforme to ATRT-like tumors was due to a transient...
expression of bHLH factors in radial glia progenitors or to expression in tumor cells. The piggyBac transposon system allows for gating whether a transgene is integrated into a transfected cells or whether it remains episomal and is thus lost in cells that undergo subsequent proliferation (22). We substituted the Ngn2 donor plasmids used above with a plasmid, CAG-Ngn2, in which the CAG-Ngn2 transgene is not flanked by ITR sequences and so is not subject to transposase-mediated genomic integration (22). As a result, transgene expression from these episomal plasmids is lost in 1 to 2 cell divisions (22, 23). Addition of episomal CAG-Ngn2 was sufficient to produce tumors (Supplementary Fig. S3; Fig. 3C–E) with clusters of tumor cells expressing epithelia membrane antigen (Fig. 3F) and isolated cells expressing cytokeratin (Lu-5 epitope; Fig. 3G). Nuclear INI1 was also preserved (data not shown). Thus, transient expression of Ngn2 in radial glial progenitors and their immediate progeny are sufficient to produce ATRT like tumors.

Distinct developmental patterns of tumors

The four tumor-inducing conditions described above (CAG donor plasmid, GFAP donor plasmid, MBP donor plasmid, and CAG donor plasmid with a bHLH transcription factor modifying donor plasmid) produced histologically distinct tumors when assessed at similar postnatal developmental time points. We next sought to address whether these differences might be reflected in differences in the developmental time course of each tumor type. As shown in Fig. 4A–E, we found that the CAG donor plasmid condition resulted in very early signs of abnormal cell proliferation with large aggregates of fluorescently labeled cells invading striatum and the ventricular and subventricular zones of neocortex by the day of birth (P0) in all animals.

examined (3 of 3). In contrast, aggregates of proliferative cells were not apparent in either the GFAP (0 of 3) or MBP donor plasmid (0 of 3) condition in the first few days after birth (P2–P3; Fig. 4F and K), but instead normally differentiating neurons were apparent a few days after birth with only scattered cells outside of the neuronal cortical plate. By the end of the first postnatal week, masses of aberrantly proliferating cells seemed in both the MBP and GFAP donor plasmid conditions (Fig. 4G and L; 3 of 3 animals in MBP, GFAP donor–transfected animals, respectively). These masses were often of a single clonally labeled color and were present in areas centered within white matter and the subpial zone (Fig. 4G and L). The time course of tumor growth was similar in the MBP and GFAP donor plasmid conditions; however, as indicated in the histopathologic analysis, the morphology of cells in the two conditions differed in morphology by P21 (Fig. 4H–J and M–O). In spite of the delayed appearance of large tumors in the GFAP and MBP donor plasmid conditions relative to the CAG donor plasmid, the size of tumors in the MBP and GFAP donor plasmid conditions by P21 reached sizes larger than those in the CAG donor plasmid condition (Fig. 4C, H, and M). Finally, consistent with the early lethality and highly aggressive nature of the tumors induced by CAG donor plasmids with addition of Ngn2-modifying plasmid, the tumors in this condition (Fig. 4P–U) were obvious by the day of birth (4 of 4) and expanded rapidly into very large tumors with street-like necroses invading the entire cerebral hemisphere by 3 weeks after birth (7 of 7). The developmental patterns observed are consistent with when the promoters expressing the oncogenes are most active; the ubiquitous CAG promoter is strongly active early in progenitors, whereas the GFAP and MBP promoters are most active later in the lineage when glial cells begin differentiating.

Gene expression differences in tumor types

The Cancer Genome Atlas research network identified four subtypes of glioblastoma multiforme subtypes by gene expression profiles and gene mutation (1). Through this analysis, a group of 24 signature genes was identified that could be used to categorize the four subtypes (1). We therefore used expression levels of these 24 signature genes to further assess whether the four tumour patterns produced in our study could be distinguished based on the expression of this set of genes. To do this, we used q-PCR and unsupervised hierarchical clustering analysis to compare and categorize the tumors produced by the four conditions. We found, consistent with the agreement in histopathologic analysis, that the tumors produced by the CAG and GFAP donor plasmids clustered together in terms of expression of the 24 genes (Fig. 5). The tumors that were most distinct in histopathology, those produced by the MBF donor plasmids and modified by the Ngn2 donor plasmids, differed in gene expression patterns relative both to each other and to the CAG and GFAP donor plasmid conditions (Fig. 5). Epidermal growth factor receptor (EGFR) is frequently amplified and overexpressed in human glioblastoma multiformes; however, EGFR transcript was not prominent in all tumor types. This might be because Ras and AKT are downstream factors of the EGFR pathway (15). We also found that p53 was neither mutated nor deleted (data not shown), and p53 transcript showed about 2-fold of upregulation. Thus, the heterogeneity we find in tumors produced by differing promoter conditions and modifying transcription factor expression is also reflected in different gene expression patterns.

Discussion

Two, not mutually exclusive, explanations of tumor heterogeneity have been proposed (36). Overwhelming experimental and clinical evidence shows that different genetic mutations result in different tumor types including different CNS tumor types (37). For example, Hertwig and colleagues (33) showed that infection of postnatal mouse neural stem cells with viruses containing V12HRAS or c-MYC could result in formation of 3 different tumor types depending upon the combination and sequence in which oncogenes were introduced. Similarly, Jacques and colleagues (38) showed that different combinations of conditional genetic deletions in p53, retinoblastoma, and PTEN in mouse subventricular zone neural stem cells could induce formation of either PNET or glioma. Evidence for differing cell types being a source of CNS tumor heterogeneity has also been found (39–42). For example, transduction of mutationally stabilized N-Myc into neural stem cells from perinatal murine cerebellum and brain stem resulted in formation of medulloblastoma/primitive neuroectodermal tumors, whereas N-Myc transduced into neural stem cells (NSCs) isolated from forebrain resulted in diffuse glioma tumors (41). As different neural progenitor types have different gene expression profiles that may modify tumor cell differentiation, it seems likely that both cell-of-origin and differences in gene mutation contribute to tumor-type diversity in the CNS (36). Our study indicates that in the developing neocortex, cell populations of origin can contribute to tumor diversity, and moreover, that expression of nononcogenic transcription factors expressed in the same population can also modify tumor type.

Although pediatric glioblastoma multiforme shares histologic similarities with adult glioblastoma multiforme, they are now thought to be different entities with different molecular characteristics (2, 43). It has been shown, for example, that pediatric glioblastoma multiformes display a spectrum of copy-number variations distinct from adult glioblastoma multiforme (44). Integrated molecular profiling experiments also reveal differences between pediatric and adult glioblastoma multiforme. For example, isocitrate dehydrogenase (IDH) hotspot mutations are frequently found in adult high-grade glioma but not in pediatric tumors (2, 43). In addition, whereas PDGFRA is the predominant target of focal amplification in pediatric high-grade glioma, in adult glioblastoma, EGFR is the most common target (43). Moreover, histone H3.3 mutations are frequently found in pediatric glioblastoma multiforme, whereas they are absent in adult glioblastoma multiforme (2, 45). The cell-of-origin
pediatric glioblastoma multiforme is unknown and might be different from adult glioblastoma multiforme as well. We have used the CAG donor plasmids to induce glioblastoma multiforme, which seemed as early as P7 in the rat and shows necrotic areas as early as at P4, a developmental time period in the precocial rat that corresponds to the neonatal period in human. Future experiments will be needed to determine whether the early-arising tumors modeled in this system are more similar to pediatric or adult glioblastoma multiforme in their molecular identity and cell-of-origin.

Our study indicates that the cell of mutation for both ATRT like tumors and glioblastoma multiforme can be radial glial cells of embryonic neocortex. The in utero electroporation method we used targets radial glia and radial neural progenitor cells that line the lateral ventricles of the lateral forebrain. This population of progenitors at the ventricular surface of E14/15 rat forebrain contains sub-populations of progenitors capable of generating neurons of different types, and primarily glia or primarily neurons (23). We used the ubiquitous CAG donor plasmid to induce expression of HRasV12 and AKT immediately in the radial progenitor population. Ngn2 or NeuroD1 expressed by the same immediately active promoter was sufficient to change the tumor type generated. The tumor difference was apparent as early as the day of birth, approximately 1 week after induced gene expression. We also found that transient expression of Ngn2 (by a nondonor episomal plasmid) in radial glia was sufficient to produce ATRTs. The effectiveness of transient Ngn2 expression, in combination with previous findings that Ngn2 or NeuroD1 expressed in...
glioma induces cell death and neuronal differentiation without changing tumor type (46, 47), supports the idea that Ngn2 and NeuroD1 act in early-stage radial progenitors to change the type of tumor generated. We have coexpressed Ngn2 with GFAP donor plasmids and found that the resulted tumors were similar to tumors induced by GFAP donor plasmids alone. But we did find rhabdoidal cells in the resulted tumors (data not shown). However, the results have to be interpreted with caution because the mouse GFAP promoter fragment is also active in radial glia. In the future, combining Ngn2 with MBP donor plasmids may distinguish the time of action of Ngn2.

Loss of function in the INI1 gene is believed to be a significant cause of ATRT in humans (48). INI1 knockout mice develop tumors but these do not seem to be ATRT (49). Hertwig and colleagues (33) showed that transplantation of NSC/neural progenitor cells (NPCs) serially infected with c-MYC and V12HRAS could generate ATRTs with gene expression profiles similar to human ATRT, suggesting that in rodent models additional mutation types can lead to ATRT. Similarly, we demonstrated in this study that ATRT like tumors are generated by HRasV12 and AKT transfection of neocortex radial glia, but only when coexpressed with the bHLH transcription factors Ngn2 or NeuroD1, two genes that on their own are nononcogenic. This underscores the strong possibility that tumor-type diversity may be influenced not only by the cell of mutation but also by the specific molecular context present in that cell.

Our current results also show that the GFAP and MBP promoter–active populations in the radial glia lineage generate tumors with different molecular and histologic features. Interestingly, all cell types are labeled by the GFAP and MBP donor plasmids, but yet the tumor types generated were consistently different in histology and molecular signature. This may suggest that tumor diversity is determined by the predominant cell type in a population that undergoes transformation. By mixing the GFAP and MBP donor plasmids in future experiments and tracking their clonal expansion, we may be able to distinguish whether one tumor cell population is dominant over the other.

Several attributes of piggyBac transposon system demonstrated here make this model potentially useful for a variety of novel applications in tumor biology. The main advantage of the piggyBac IUE method is that it allows for introduction of multiple transgenes. In this study, for example, we simultaneously introduced a transposase helper plasmid to target stable transgenesis in GLAST-positive cells, two oncogene-expressing donor plasmids with their own promoters, 3 fluorescent reporter genes (Figs. 1, 2, and 3), and a transcription factor. The high coexpression efficiency allowed us to direct expression in different subpopulations in sequence and to introduce a clonal labeling method and a modifying transcription factor. This functionality should make this approach a useful platform for screening potential modifiers of tumor development and for determining further how genetic modifiers alter tumor development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: F. Chen, J.J. LoTurco

Development of methodology: F. Chen, J.J. LoTurco

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Chen, A.J. Becker, J.J. LoTurco

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Chen, A.J. Becker, J.J. LoTurco

Writing, review, and/or revision of the manuscript: F. Chen, A.J. Becker, J.J. LoTurco

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.J. LoTurco

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References

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Fuyi Chen, Albert J. Becker and Joseph J. LoTurco


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