

Genome Engineering Evolves Brain Tumor Modeling

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Abstract

Genome engineering using programmable nucleases such as transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeat-associated protein nine facilitated the introduction of genetic alterations at specific genomic sites in various cell types. These tools have been applied to cancer modeling to understand the pathogenic effects of the growing catalog of mutations found in human cancers. Pertaining to brain tumors, neural progenitor cells derived from human induced pluripotent stem cells (iPSCs) engineered with different combinations of genetic driver mutations observed in distinct molecular subtypes of glioblastomas, the most common form of primary brain cancer in adults, give rise to brain tumors when engrafted orthotopically in mice. These glioblastoma models recapitulate the transcriptomic signature of each molecular subtype and authentically resemble pathobiology of glioblastoma, including inter- and intra-tumor heterogeneity, chromosomal aberrations, and extrachromosomal DNA amplifications. Similar engineering with genetic mutations found in medulloblastoma and atypical teratoid rhabdoid tumors in iPSCs have led to genetically trackable models that bear clinical relevance to these pediatric brain tumors. These models have contributed to improved comprehension of the genetic causation of tumorigenesis and offered a novel platform for therapeutic discovery. Studied in the context of three-dimensional cerebral organoids, these models have aided in the study of tumor invasion as well as therapeutic responses. In summary, modeling brain tumors through genome engineering enables not only the establishment of authentic tumor avatars driven by bona fide genetic mutations observed in patient samples but also facilitates functional investigations of particular genetic alterations in an otherwise isogenic background.

Keywords: brain tumor, CRISPR/Cas9, genome engineering, glioma, modeling

Introduction

The development of genome engineering technologies using programmable nucleases, including zinc finger nucleases (ZFN),¹ transcription activator-like effector nuclease (TALEN),²⁻⁴ and cluster regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein nine (Cas9) system^{5,6} have aided in the generation of genetically engineered murine models previously unattainable through conventional homologous recombination-mediated gene-targeting.⁷ When applied to induced pluripotent stem cells (iPSCs),⁸ these technologies have transformed the field of disease modeling.⁹ For example, Reinhardt *et al.*¹⁰

generated iPSCs from patients with Parkinson's disease harboring *LRRK2* mutations as well as normal controls, and then either corrected or introduced the mutations using ZFN genome editing. The mutated or mutation-corrected iPSCs were then differentiated to dopaminergic neurons, revealing that mutant *LRRK2* induced ERK activation leading to dopaminergic neurodegeneration.

Such approaches through combinations of genome engineering and stem cell technologies have paved the way for sophisticated cancer models driven by pertinent mutations uncovered in human clinical tumor specimens. As an example, Heckl *et al.*¹¹ modified *Nf1*, *Ezh2*, *Dnmt3a*, *Tet2*, and *Runx1* in mouse hematopoietic stem cells to model acute myeloid leukemia. In other studies, colon organoids⁷ from human intestinal crypt stem cells introduced with different combinations of genetic alterations in *APC*, *SMAD4*, *TP53*, *KRAS*, and *PIK3CA* genes, which are commonly affected in colorectal cancers, were

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Table 1 Brain tumor models generated through genome engineering

| Authors (year) | Modified genes | Modalities | Species | Materials | Tumors modeled |
|--|---|----------------------------|---------|-------------------------|---------------------------------------|
| Duan <i>et al.</i> ⁴¹⁾ (2015) | <i>PTEN</i> | TALEN | Human | ESCs | GBM |
| Zuckermann <i>et al.</i> ⁶¹⁾ (2015) | <i>Ptch1, Trp53, Pten, Nf1</i> | CRISPR/Cas9 | Mouse | Embryonic brains | Medulloblastoma, GBM |
| Bian <i>et al.</i> ⁵⁶⁾ (2018) | <i>MYC, CDKN2A, CDKN2B, EGFR, NF1, PTEN, TP53</i> | CRISPR/Cas9, SB-transposon | Human | Cerebral organoids | GBM, CNS-PNET |
| Ogawa <i>et al.</i> ⁵⁷⁾ (2018) | <i>TP53, HRAS</i> | CRISPR/Cas9 | Human | Cerebral organoids | GBM (mesenchymal subtype) |
| Huang <i>et al.</i> ⁴³⁾ (2019) | <i>GSE1, KDM3B</i> | CRISPR/Cas9 | Human | NESCs (Gorlin syndrome) | Medulloblastoma (SHH subtype) |
| Terada <i>et al.</i> ⁴⁵⁾ (2019) | <i>TP53, SMARCB1</i> | CRISPR/Cas9 | Human | iPSCs | AT/RT |
| Koga <i>et al.</i> ⁴⁷⁾ (2020) | <i>PTEN, NF1, TP53, PDGFRA</i> | CRISPR/Cas9 | Human | iPSCs | GBM (mesenchymal, proneural subtypes) |
| Yu <i>et al.</i> ⁶³⁾ (2020) | <i>Trp53, Nf1, Pten, Pik3ca</i> | CRISPR/Cas9 PB-transposon | Mouse | Embryonic brains | GBM |

AT/RT: atypical teratoid rhabdoid tumor, CNS-PNET: central nervous system primitive neuroectodermal tumor, CRISPR: clusters of regularly interspaced short palindromic repeats, ESC: embryonic stem cell, GBM: glioblastoma, iPSC: induced pluripotent stem cell, NESc: neuroepithelial stem cell, PB: PiggyBac, SB: Sleeping Beauty, SHH: Sonic Hedgehog, TALEN: transcription activator-like effector nuclease.

generated.^{12,13)} These organoid models harbored features of colorectal cancers such as aneuploidy, formed tumors *in vivo* upon xenotransplantation, revealed genetic alterations underlying invasion, and accurately predicted drug responses.¹⁴⁾

Applications of genome editing techniques have led to paradigm shifts in the modeling of adult and pediatric brain cancers. Prior to these efforts, disease modeling relied heavily on genetically engineered mouse (GEM) models, human astrocyte-derived models, and patient-derived xenografts (PDX). While the utility of these models is undisputed, each harbor intrinsic limitations that restrict the interpretation of human relevance or generalizability. In this article, we review advances in brain tumor modeling through genome engineering (Table 1) and discuss the relative merits of this approach to previously available models.

Non-genome Engineering-derived Brain Tumor Models

The earlier models of brain cancer include tumors that formed in rat or murine brains treated with DNA damaging mutagens. Rat C6 glioma cell line, which was induced by exposure to methylnitrosourea,^{15,16)} produces glioma-like tumors when injected in rat brains¹⁷⁾ and has been frequently utilized as a syngeneic model in glioma research.¹⁸⁾ Similar efforts using murine models exposed to methylcholanthrene have led to the generation of the GL261, and CT-2A glioblastoma cell lines.¹⁹⁻²¹⁾ While these

syngeneic models aid in the investigations of tumor immune response, they harbor increased mutational burden and exaggerated immune response relative to those observed in human disease.^{22,23)} Moreover, these tumors tend to form a well-defined mass rather than the invasive histology seen in human gliomas.²⁴⁾

The second class of brain cancer models involves tumors that arose consequent to the introduction of transgenes. Danks *et al.* introduced SV40 T antigen under control of glial fibrillary acidic protein promoter and succeeded in transforming mouse astrocytes in 1995.²⁵⁾ Later, Holland *et al.*^{26,27)} introduced oncogenes such as *EGFR* and *CDK4* by somatic cell gene delivery using replication-competent avian leukosis virus splice acceptor (RCAS) viral vectors and their receptor, *Tva*, to generate glioma models. Importantly, the RCAS-*Tva* system has also been utilized to model pediatric brain tumors such as medulloblastoma.²⁸⁾ Different models of glioblastomas, the most aggressive form of gliomas, have been generated through the introduction of oncogenes such as *Src*, *K-ras*, *H-ras*, *PDGFB*, and *EGFRvIII*.^{29,30)} These GEM models have been fundamental in dissecting molecular mechanisms underlying genetic carcinogenesis. However, whether insights derived from these studies are pertinent to human disease remains an open question. Pertinent to this concern, there are significant discrepancies in experiments where drugs were simultaneously tested against human and murine models of glioblastoma.³¹⁾ These therapeutic differences can extend orders of magnitude.

Human cell models are essential to address such biological differences presented by murine platforms. Rich *et al.*³²⁾ and Sonoda *et al.*³³⁾ engineered human astrocytes with combinations of *TERT* and *HRAS* expression and inhibition of the TP53 pathway by simian virus 40 (SV40) T antigen or by human papillomavirus (HPV) E6 and E7 and succeeded in establishing high-grade glioma models. These models enable investigations focused on gliomagenic mechanisms in the context of human cells. To the extent that viral expression of SV40 and HPV is not found in most human glioblastomas,³⁴⁾ it remains unclear whether the physiology of these tumors is clinically relevant. Additionally, there are many features of clinical glioblastoma which have not been carefully scrutinized in these models, including inter- and intra-tumoral heterogeneity. As other examples, various monolayer cell lines derived from human gliomas were established in serum-containing media.³⁵⁾ These models are easy to expand for experimental use, but again lack typical histological features such as heterogeneity and *in vivo* invasive potential and are not ideal as some studies suggest genomic deviation from the original patient sample.^{24,36)}

PDX models overcome disadvantages of established monolayer cell lines by maintaining original phenotypes observed in clinical samples upon orthotopic engraftment, thus enabling studies on inter- and intra-tumoral heterogeneity,^{37,38)} and effects of targeted therapies.³⁹⁾ However, the heterogeneity of the PDX models serves as a double-edged sword, making experimental standardization difficult due to vast variability in background mutations present in each clinical sample.

In summary, valuable insights have been gained through various brain tumor models. Undoubtedly, they will continue to be utilized for multiple purposes. It is crucial, however, to keep in mind the various caveats associated with these different models. As genome engineering technologies emerge, these new tools may offer answers in addressing these limitations.

Brain Tumor Models Derived from Genome-Engineered Human Stem Cells

Using TALEN-mediated homologous recombination to delete *PTEN*, a tumor suppressor gene affected in 36% of glioblastoma patients,⁴⁰⁾ Duan *et al.* generated glioma models from human embryonic stem cells (ESCs) differentiated to neural stem cells (NSCs). When engrafted in immunocompromised mice, these *PTEN*-null NSCs formed neoplastic lesions and presented sensitivity to mitomycin C. Transcriptomically, the *PTEN*-null NSCs showed differential expression of PAX7 compared with

wild-type control, which was validated in the Cancer Genome Atlas (TCGA) dataset.⁴¹⁾ This was the first model to show that disruption of a glioblastoma-associated tumor suppressor leads to the reprogramming of human NSCs toward a cancer stem cell-like phenotype. However, *PTEN* alterations are seldomly observed solely by themselves in human glioblastomas and are almost always accompanied by other oncogenic events.⁴²⁾

Later, Huang *et al.*⁴³⁾ generated neuroepithelial cells (NECs) from iPSCs derived from patients with Gorlin syndrome, a tumor predisposition syndrome caused by mutations in *PTCH1*, which is associated with an increased risk of medulloblastoma. In their study, CRISPR/Cas9 disruption of *GSE1*, which is commonly co-mutated in adult medulloblastoma, resulted in accelerated tumorigenesis. Interestingly, the tumors obtained by engraftment of *GSE1* knockout NECs into the cerebellum of mice clustered closer to the Sonic Hedgehog (SHH) subtype of medulloblastoma driven by SHH pathway activation that occurs due to disruption of *PTCH1*. As an example of another brain tumor model, Terada *et al.* disrupted *SMARCB1*, which is recurrently affected in atypical teratoid rhabdoid tumors (AT/RT),⁴⁴⁾ to model this malignant pediatric brain cancer.⁴⁵⁾ This study presented a potential use of brain tumor cells derived from genetically engineered human iPSCs for drug screening.

More recently, our group established glioblastoma models by introducing different combinations of genetic alterations observed in different molecular subtypes of glioblastoma^{42,46)} into human iPSCs.⁴⁷⁾ In this study, neural progenitor cells (NPCs) were differentiated from iPSCs harboring CRISPR/Cas9-induced combinatory alterations of *PTEN/NF1* and *TP53/PDGFR*, which are commonly observed in mesenchymal and proneural glioblastoma molecular subtypes, respectively. Here, gene-edited NPCs gave rise to GBM-like tumors upon orthotopic engraftments in immunocompromised animals. The tumors were confirmed to have histological features of glioblastoma by meticulous pathological assessment, and presented the transcriptomic signatures of mesenchymal and proneural subtypes, respectively. Our study proved that introducing different combinations of driver genetic alterations in cells with isogenic backgrounds results in tumor models presenting distinct phenotypes. Furthermore, the single-cell RNA sequencing analyses revealed that these genetically engineered human iPSC-derived models presented inter- and intra-tumor heterogeneity as observed in patient samples.⁴⁸⁾ Importantly, these models showed prominent chromosomal abnormality accompanied with extrachromosomal DNA amplifications, which are commonly seen in glioblastoma samples.⁴⁹⁻⁵²⁾ As the

Table 2 Advantages and limitations of genome-engineered brain tumor models from different platforms

| Platforms | Authors | Advantages | Limitations |
|--------------------------|---|---|---|
| Human stem cells | Duan <i>et al.</i> ⁴¹⁾ Huang <i>et al.</i> ⁴³⁾ Terada <i>et al.</i> ⁴⁵⁾ Koga <i>et al.</i> ⁴⁷⁾ | Feasibility in experimental standardization in isogenic background Enabling human tumor biology investigation | Restrictions in the assessment of immune environment |
| Human cerebral organoids | Ogawa <i>et al.</i> ⁵⁷⁾ Huang <i>et al.</i> ⁴³⁾ | Enabling limited use of animals. Feasibility in the assessment of tumor–tumor microenvironment interactions in three-dimensional context <i>in vitro</i> | Restrictions in the assessment of immune environment Lack of physiological backgrounds e.g., blood vessels |
| Mouse embryonic brains | Zuckermann <i>et al.</i> ⁶¹⁾ Yu <i>et al.</i> ⁶³⁾ | Spontaneous tumor formation in syngeneic backgrounds enabling assessment of immune interactions | Restrictions in the interpretation of human relevance. Technical hurdles of <i>in utero</i> electroporation. |

tumor cells derived from these *in vivo* models grew in sphere condition *in vitro* and formed secondary tumors upon re-engraftments, these models were suitable for testing of drug sensitivity and assessments of longitudinal tumor evolution.⁴⁷⁾

Such varieties of brain tumor models show significant potential of modeling numerous types of brain tumors driven by different genetic drivers through the introduction of defined alterations in isogenic human backgrounds, facilitated by the power of genome engineering. Limitations include a lack of immune components due to engraftment of these models in immunocompromised animals (Table 2).

Brain Tumor Models in Genome-Engineered Human Cerebral Organoids

As shown in the studies of colorectal cancer models in colon organoids, tissue organoids are potential tools for modeling and investigating cancers in three-dimensional contexts.^{12,13)} In the field of neuroscience, Lancaster *et al.* established the methods of generating cerebral organoids from human pluripotent stem cells.^{53,54)} Lincous *et al.* generated glioblastoma organoid models by combining patient-derived glioma stem cells and cerebral organoids derived from human ESCs and proved that such models serve as a robust tool to investigate biological behaviors of glioblastoma invasion.⁵⁵⁾

Bian *et al.*⁵⁶⁾ and Ogawa *et al.*⁵⁷⁾ introduced genome engineering into cerebral organoids to model brain tumors *in vitro*. Bian *et al.* introduced genetic edits at the early stages of the cerebral organoid formation using combinations of the Sleeping Beauty (SB) transposon system⁵⁸⁻⁶⁰⁾ to insert multiple copies of oncogenes, thus mimicking their overexpression, and CRISPR/Cas9 for disruption of tumor suppressor

genes. Organoids electroporated with the combinations of constructs of *MYC*^{OE} (OE indicates overexpression), *CDKN2A*^{-/-}/*CDKN2B*^{-/-}/*EGFR*^{OE}/*EGFRvIII*^{OE}, *NF1*^{-/-}/*PTEN*^{-/-}/*TP53*^{-/-}, and *EGFRvIII*^{OE}/*CDKN2A*^{-/-}/*PTEN*^{-/-} each resulted in overgrowth of electroporated cells indicating neoplastic transformation. Organoids with *MYC*^{OE} presented transcriptomic signatures of central nervous system primitive neuroectodermal tumors. The other combinations were associated with transcriptome signatures seen in human glioblastomas and exhibited distinct drug sensitivity *in vivo*, suggesting the potential use of these models for future drug screening.⁵⁶⁾ In another model, Ogawa *et al.*⁵⁷⁾ introduced a cassette of *HRAS*^{G12V} and a fluorescent protein, tdTomato, at the *TP53* locus in cerebral organoids using CRISPR/Cas9 to overexpress a mutant *HRAS* while disrupting *TP53*. Overgrowth of transformed tdTomato-positive cells suggested neoplastic transformation. The transformed cells in these organoid models were transplantable to the brains of immunocompromised mice and cerebral organoids as well.

In sum, cerebral organoid brain tumor models may offer opportunities for multiplex genome engineering and provide a novel platform for drug screening *in vitro*. These models afford opportunities for *in vitro* investigations on interactions between tumor cells and brain microenvironment, which cannot be done using conventional *in vitro* models, although cerebral organoids still lack some physiological components such as an immune microenvironment and blood vessels (Table 2).

Spontaneous Mouse Brain Tumor Models Using Genome Engineering

To overcome the laborious and time-consuming processes of generating GEM models, Zuckermann

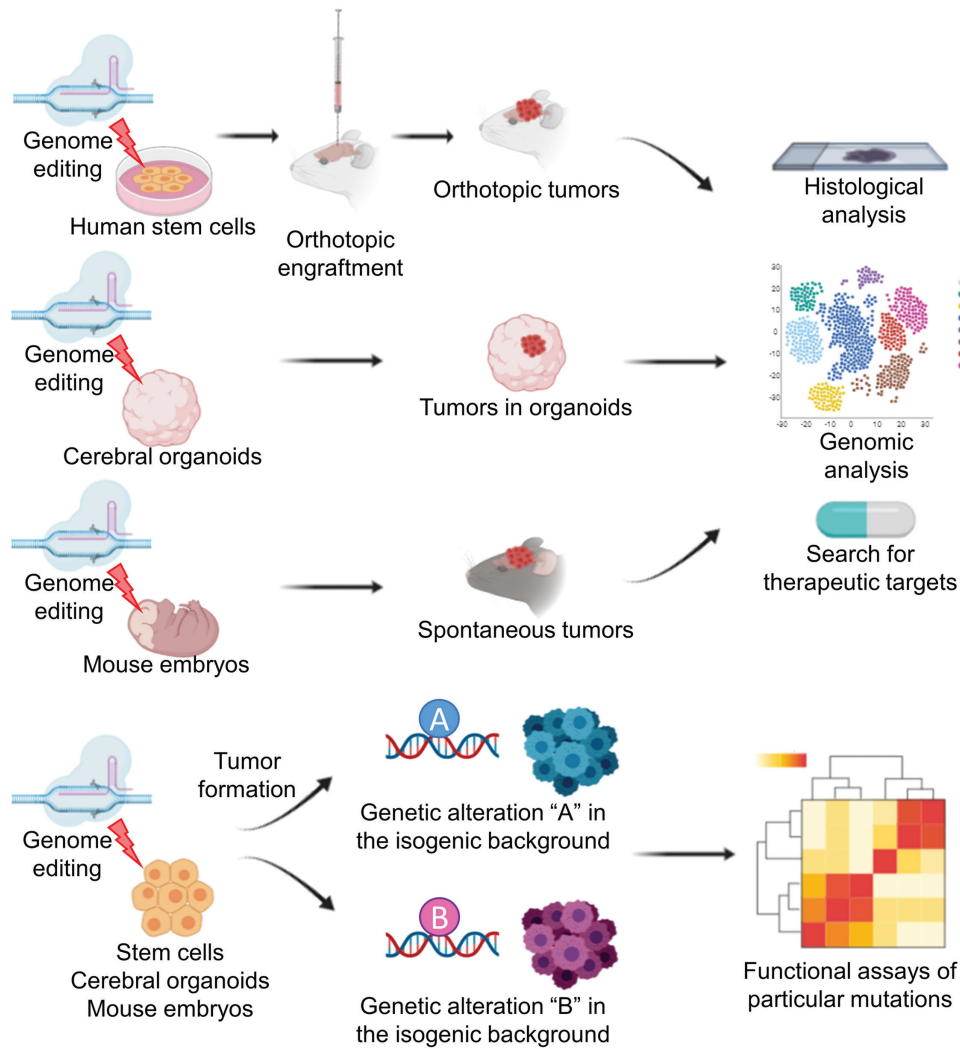


Fig. 1 Overview of brain tumor modeling using genome engineering. Introducing brain-tumor-associated mutations into human stem cells, cerebral organoids, or mouse embryonic brains give rise to various brain tumor models that can be utilized for histological, genomic analyses, and search for novel therapeutic targets. These models allow comparative functional assessment of particular genetic alterations in otherwise isogenic backgrounds.

et al.,⁶¹⁾ using an *in utero* electroporation technique of mouse embryonic brains, developed a spontaneous mouse brain tumor model by introducing Cas9 and small guide RNAs expressing plasmids to target various genes. *In utero* electroporation of CRISPR constructs targeting *Ptch1* resulted in high tumor formation efficiency. The transcriptome of these tumor models clustered together with a previously published medulloblastoma GEM model with *Ptch1* alterations. They further tested different combinations of target genes to model glioblastoma and confirmed the combination of CRISPR constructs targeting *Trp53*, *Nf1*, and *Pten* generate glioblastoma-like

tumors in eight out of eight animals. Similarly, Yu *et al.* induced mouse *in vivo* brain tumors by *in utero* electroporation of CRISPR/Cas9 constructs targeting *Trp53* and *Nf1* together with PiggyBac⁶²⁾ transposable vectors harboring different variants of *Pik3ca* mutations.⁶³⁾

These models generated through *in utero* genome engineering proved that this approach is an efficient way to establish *in vivo* syngeneic tumor models with potentially numerous combinations of genetic alterations, although limitations of this approach include technical challenges in manipulating embryos *in utero* (Table 2).

Functional Analyses of Genetic Alterations in Isogenic Backgrounds

One of the benefits of utilizing genome engineering for tumor modeling is the feasibility of introducing designed genetic alterations into any materials such as human stem cells, cerebral organoids, and mouse embryonic brains for downstream applications (Fig. 1). Such efficient genomic modifications enable functional testing of specific genetic alterations in isogenic backgrounds (Fig. 1). As proved in our models and others, different combinations of genetic alterations introduced in these isogenic platforms result in distinct phenotypes of brain tumors.^{47,61} Huang *et al.* analyzed tumorigenic functions of co-occurring mutations in conjunction with *PTCH1* alterations found in adult medulloblastoma patients. Among those co-mutated genes, *GSE1* and *KDM3B* were disrupted using CRISPR/Cas9 in an isogenic background of NESCs derived from Gorlin syndrome patients, which showed that alterations in *GSE1*, but not *KDM3B*, accelerate tumorigenesis.⁴³ This study effectively utilized genome engineering tools to validate the tumorigenic function of potential driver mutations whose roles in particular tumor formation were previously unknown. Yu *et al.* efficiently screened 27 variants of *Pik3ca* mutations in the background of *Trp53* and *Nf1* knockout and showed that C420R and H1047R mutations of this gene result in hyperexcitability of the surrounding brain.⁶³

As shown, introducing genome engineering into brain tumor modeling enables efficient investigations of genetic functions of mutations associated with tumorigenesis and tumor progression. When applied in the context of synthetic lethality, these models have the potential to accelerate the development of precision medicine as it pertains to brain tumor treatment.

Conclusion

The available literature suggests the feasibility and utility of genome engineering as a tool to model the mutations uncovered through the interrogation of human brain tumor specimens. The approach is flexible and can be applied to stem cells, organoids, and through *in utero* electroporation. In these contexts, genome engineering has enabled next-generation brain tumor models that should contribute to the accelerated discovery of effective therapeutics for brain tumor patients.

Conflicts of Interest Disclosure

The authors declare no conflict of interest.

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