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Original Research

Pioneering models of pediatric brain tumors

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ABSTRACT

Among children and adolescents in the United States (0 to 19 years old), brain and other central nervous system tumors are the second most common types of cancers, surpassed in incidence only by leukemias. Despite significant progress in the diagnosis and treatment modalities, brain cancer remains the leading cause of death in the pediatric population. There is an obvious unfulfilled need to streamline the therapeutic strategies and improve survival for these patients. For that purpose, preclinical models play a pivotal role. Numerous models are currently used in pediatric brain tumor research, including genetically engineered mouse models, patient-derived xenografts and cell lines, and newer models that utilize novel technologies such as genome engineering and organoids. Furthermore, extensive studies by the Children's Brain Tumor Network (CBTN) researchers and others have revealed multiomic landscapes of variable pediatric brain tumors. Combined with such integrative data, these novel technologies have enabled numerous applicable models. Genome engineering, including CRISPR/Cas9, expanded the flexibility of modeling. Models generated through genome engineering enabled studying particular genetic alterations in clean isogenic backgrounds, facilitating the dissection of functional mechanisms of those mutations in tumor biology. Organoids have been applied to study tumor-to-tumor-microenvironment interactions and to address developmental aspects of tumorigenesis, which is essential in some pediatric brain tumors. Other modalities, such as humanized mouse models, could potentially be applied to pediatric brain tumors. In addition to current valuable models, such novel models are anticipated to expedite functional tumor biology study and establish effective therapeutics for pediatric brain tumors.

Introduction

Despite an overall improvement in terms of survival for children and adolescents with cancers over the past decades, brain and other central nervous system (CNS) tumors maintain a dismal prognosis [1,2]. Pediatric brain tumors are the most common types of solid cancers in children and the leading cause of death [3]. In adolescents (ages 15 to 19 years), cancers of the brain and other CNS tumors surpass leukemias as the most common type of cancer in general. The incidence of pediatric brain tumors differs among different geographical areas, with the highest rates reported in the United States [4]. In 2020, a Central Brain Tumor Registry of the United States report estimated that the average annual age-adjusted incidence rate of CNS tumors among children aged 0 to 14 was 5.83 per 100,000 individuals. The annual age-adjusted mortality rate of CNS tumors in this age group was determined to be 0.71 per 100,000, with brain tumors being the number one cause of cancer death among 0 to 14 years of age [5].

Pediatric brain tumors are a diverse and continuously branching category of tumors. The extensive research and progress over the past decades in imaging, molecular diagnostics, surgical techniques, and tailored therapy demonstrated that histological characteristics are insufficient to define different brain tumor entities, as even histologically similar tumors harbor distinct molecular features and consequently have different prognoses and therapeutic responses. Starting in 2016, the World Health Organization (WHO) incorporated molecular diagnosis criteria to characterize different brain and CNS tumors, opening a new chapter in the overall management of this class of disease [6]. This classification marked the transition from traditional diagnostic approaches based on histologic/microscopic findings and immunohistochemistry (IHC) to the newer platforms for molecular diagnosis based on tumor genomics. Furthermore, the molecular diversity and the variability in treatment response based on the genetic architecture of pediatric tumors led to the development of the first WHO Classification of Pediatric Tumors in 2021. The classification has a multilayered approach that includes morphological criteria, IHC, and molecular characteristics [7]. Except for a limited number of genetic predisposition syndromes, pediatric brain tumors are considered sporadic events. The genes associated with these familial brain tumor predisposition syndromes include *NF1* for neurofibromatosis type 1, *NF2* for neurofibromatosis type 2, *TP53* for Li-Fraumeni syndrome, *PTCH1* for Gorlin syndrome, and

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SMARCB1 for rhabdoid tumor predisposition syndrome [8]. The genetic backgrounds of sporadic pediatric brain tumors have been extensively studied, in addition to these familial tumor predisposition syndromes [9]. For example, medulloblastomas are molecularly subclassified into four groups driven by different mutations of genes, including *CTNNB1, PTCH1, MYC*, and *MYCN* [10]. Diffuse intrinsic pontine glioma, one of the deadliest pediatric brain tumors, was also genetically subclassified, with the most common group driven by histone H3-K27M mutation [11]. More recently, an extensive work by researchers of the Children's Brain Tumor Network and others revealed integrative genomic and proteomic landscapes of a wide variety of pediatric brain tumors, including low-grade glioma, ependymoma, high-grade glioma, medulloblastoma, ganglioglioma, craniopharyngioma, and atypical teratoid rhabdoid tumor [12].

However, the prognoses of many pediatric brain tumors are dismal. For example, in the case of diffuse midline gliomas with histone H3-K27M mutation, half of the children survive less than one year from diagnosis, and only 10% survive two years from diagnosis, making it one of the deadliest types of cancers [2]. To understand the tumor biology in these huge varieties of tumors and to test potential therapeutics, preclinical pediatric brain tumor models are essential. Integrating a tremendous amount of knowledge obtained from previous research, including the genomic and proteomic landscape of different pediatric brain tumors, into preclinical models is essential to understanding cancer biology and establishing more effective treatment for each tumor. In this review, we will summarize and discuss the current strategies used to model different types of pediatric brain tumors, including the canonical and widely utilized models, as well as explore the more recently developed models using cutting-edge technologies such as genome engineering and organoids. We will further discuss the potentials these models could have in narrowing the gap between preclinical findings and clinical translation in pediatric brain tumor research.

Modalities for modeling pediatric brain tumors

Next generation sequencing and large-scale multiomic studies, including single-cell analyses have shifted the research paradigm and the overall understanding of childhood brain tumors, especially with regard to tumor heterogeneity and the associations between tumorigenesis and neural development [12–18]. The most common and widely studied pediatric brain tumors are gliomas and medulloblastomas. For example, instead of looking at histologically similar tumors as single entities, different tumor groups have been shown to include multiple subgroups, each with distinct patterns of genetic alterations, clinical behavior, and in some cases, cellular origin [10,11]. Widely used pediatric brain tumor models include genetically engineered mouse models (GEMMs), and xenografts (cell line-based and patient-derived) [19]. *In vitro* studies are useful tools to explore the underlying biological mechanisms of tumor cells, and can identify the genetic and epigenetic changes in cancer cells that contribute to tumor initiation and progression, as well as to predict the response and resistance to different treatments [20]. The major models used for *in vitro* research are mouse and human derived cell lines, neurospheres, and tumor stem cells. There are over 60 pediatric brain cancer cell lines largely used that cover a vast majority of the pediatric tumor types [21–23]. Biologically relevant tumor cell lines have the potential to enhance the success of exploratory drug discovery studies, especially in the light of more large scale comprehensive initiatives such as the Therapeutically Applicable Research to Generate Effective Treatments initiative, that aims to identify the one cell line or cell lines that best represents a specific cancer subtype [24,25]. A more recent and promising new avenue of *in vitro* modeling is the three-dimensional (3D) growth. For neuroblastoma, 3D prototypes better recapitulate the tumor physiology compared to two-dimensional (2D) cultures [26]. An emerging promising cell-derived tool for preclinical therapeutic research are organoid cultures, specifically cerebral organoids [27]. Organoids are used to study physiological processes closely resembling endogenous cell organization and organ architecture. Ogawa *et al.* demonstrated that a cerebral organoid could recapitulate glioblastoma development via clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) [28]. Bian *et al.* introduced multiple combinations of genetic alterations using transposon and CRISPR/Cas9 to model varieties of tumors, including pediatric glioblastoma, medulloblastoma, atypical teratoid rhabdoid tumor (AT/RT), and CNS primitive neuroectodermal tumor (CNS-PNET) [29]. *In vivo* models are still the first line platforms for drug safety and efficacy questions to support human testing. Many models are used in different fields, and by far for pediatric brain research, the most utilized organism is the mouse. Like *in vitro* models, *in vivo* systems have both advantages and limitations, all of which require consideration when designing preclinical studies. As previously mentioned, the two major categories of murine models used in pediatric brain tumor studies are patient-derived xenografts (PDXs) and GEMMs. We will review these different platforms of models for pediatric gliomas and medulloblastomas, which have been extensively studied, as well as other pediatric brain tumors.

Modeling pediatric gliomas

Gliomas are the most common brain tumor type in children [3]. The subtypes of gliomas that are commonly diagnosed in the pediatric population are astrocytoma, brain stem gliomas, ependymoma, and optic nerve gliomas. Pediatric gliomas are a heterogeneous category of tumors and they pose a great challenge for therapy. Based on their histological grade, gliomas can be subdivided into low-grade gliomas and high-grade gliomas; the latter tumors include the distinct subtype of Diffuse Midline Glioma H3-K27 altered, that is usually treated as a separate category given its molecular features, clinical presentation, and prognosis. The latest WHO Classification of Tumors of the CNS shed light on integrated diagnosis for brain tumor classification with an emphasis on molecular criteria [7,30]. The common pathways that are affected in pediatric gliomas are cell proliferation, mitosis, and neo angiogenesis pathways, such as MAPK, EGFR, and VEGF pathways. The most common altered genes in pediatric gliomas are *BRAF, TP53*, histone H3, *FGFR*, and *MYB/MYBL1* [31–42].

In vitro models

In vitro models remain key tools to explore the underlying biological mechanisms of tumor development, to identify the genetic and epigenetic changes in cancer cells, and are very useful tools to predict the response and resistance of cancers to different treatments. The basic *in vitro* models utilized in pediatric brain tumors include mouse or human-derived cell lines and primary cells such as tumor stem cells or neurosphere cultures. The most critical feature for reliable *in vitro* models is the ability of the respective cell lines to recapitulate the genetic characteristics of the primary tumors from which they were derived or which they're purposed to model. The major advantage that these established glioma cell lines offer is the fact that they have defined molecular characteristics, offering reliable, subtype-specific, large scale, easily reproducible information with regards to drug sensitivity, and are useful in identifying novel therapeutic targets. However, the major disadvantages of these models are the fact that they fail to recapitulate tumor heterogeneity [20], and they cannot incorporate the tumor microenvironment that drives a critical part of tumor biology. Chemically induced brain tumors have been historically used to study gliomas and other tumor types. The most used methods to generate brain tumor formation are N-nitrosurea and carcinogenic viruses such as RSV-1 and human adenovirus [22]. The cell lines isolated from brain tumors induced in mice and rats have been largely used and these include RG2, BT7C, CNS1, C6, and 9L [43–51]. These cell lines in general have been passaged for long time. The genomic deviation from the original tumors, and phenotypic homogeneity to some extent are major drawbacks of these models [52,53]. Patient-derived cell lines are the models that overcome

these rodent cell lines, with which inter- and intra-tumor heterogeneity can be studied [54,55]. The Brain Tumor Resource Lab (BTRL) offers a very useful and up-to-date platform that provides patient- derived cell lines and mouse models with a focus on studying pediatric brain tumors [56]. The platform offers 18 characterized high-grade glioma cell lines. PBT04-FHTC, PBT05-FHT are *MYCN* amplified, *ID2* amplified cell lines, while GBM-511FHTC and GBM-110FHTC have *CDKN2A* alteration with the latter harboring the *BRAF* mutation [56,57]. The Childhood Cancer Repository it's another major cancer cell line bank and one of the highly utilized stable high grade glioma cell lines is CHLA-200 that harbors the *MYC* mutation. For diffuse midline glioma and diffuse hemispheric gliomas with histone H3 alterations, there are a series of largely used stable cell lines, most of which harbor different histone mutations. SF7761, SF8628, PED8, PED17, PED36, HSJ-DIPG017, SU-DIPG-VI, VUMC-DIPGA, and JHH-DIPG-1 have H3.3 K27M mutation; VUMC-DIPG-B, and SU-DIPG-IV have H3.1 K27M; and GBM002 has H3-G34R mutation [23,58-60]. Despite the usefulness and practicality of these stable cell lines, there are some disadvantages that ought to be addressed, such as the high passage number that drives inherent phenotypic changes [52], the 2D culture conditions fail to recapitulate the actual tumor 3D architecture, and the lack of heterogeneity of the cell populations, which is another important characteristic of *in vivo* tumors. To some extent, neurosphere cultures are superior when it comes to the above-mentioned limitations, given the serum-free culture media and the maintenance of tumor heterogeneity, making them more attractive models, although they lack tumor to tumor microenvironment interactions [61–64]. Neurosphere cultures, due to the serum-free culturing environment favor the growth of glioma stem cells (GSC), defined by their tumor-initiating capacity following serial transplantation, self-renewal, and the ability to recapitulate tumor heterogeneity. Neurospheres have also been used for *in vivo* engraftment, and have been shown to successfully recapitulate the pathways of the original tumor behavior [65]. However, while extremely important in the overall tumor biology, GSC represent only a minority of the tumor cell populations; therefore, by favoring the selective growth of these cells, neurosphere cultures fall short in representing the more differentiated cancer cell populations that constitute the bulk of tumors in patients.

Patient-derived xenograft models

While *in vitro* models remain valuable, cost-efficient, versatile tools for high throughput drug screening and discovery of novel cancer biomarkers, *in vivo* pediatric brain tumor models that closely recapitulate the tumor molecular and histopathological features remain the gold standard in research of pediatric brain tumors [16,66]. The two major lines of *in vivo* models are PDX and GEMMs. Cancer cell lines derived from patient tumors or animal models can be engrafted into model animals to generate xenograft models. The patient samples can be engrafted with or without previous culture *in vitro*. The major advantage of the method is that it retains the molecular architecture of the original tumors making them more representative models than *in vitro* models [52,67]. Xenograft models are generally established in immunocompromised rodents, with various strains used. BALB/c mice and severe combined immunodeficient mice [9], as well as Rag1-deficient mice are the commonly used strains. These models, however, have different susceptibilities based on their genetic background. For example, BALB/c mice are notoriously sensitive to radiation, making them unsuitable for radiation efficiency studies [68]. The immunodeficient models have the obvious disadvantage of abolishing the interactions between tumor and tumor immune microenvironment, which play essential roles in shaping tumor biology [69]. A potential way of addressing this limitation is a more recent and promising model in which immunodeficient mice receive human bone marrow to reconstitute a human immune response. These humanized mice provide an opportunity to recapitulate human brain tumors and test the efficiency of various immunotherapies [70–72]. This model addresses the immune microenvironment and tumor heterogeneity issues that fall short with the canonical models with the potential for better drug response prediction. The limitations of these models, like with any murine model, is that they are expensive and technically complicated by the risk of xenogeneic graft-versus-host responses; however, they are a step closer to recapitulating human cancers in animals [73].

Based on the mode of implantation, PDX can be subdivided as orthotopic or intracranial engraftment and heterotopic or subcutaneous engraftment. Arguably, intracranial models are superior in replicating the brain tumor microenvironment, however, subcutaneous models were shown to recapitulate the histopathological features and stromal development as accurately as intracranial models. There are several useful pediatric brain tumor xenograft repositories that offer a very useful, in some cases subtype specific, repertoire of PDX. St. Jude Children's Research Hospital has 37 novel patient-derived orthotopic xenograft (PDOX) models generated from pediatric brain tumor patients complemented by readily available analyses of histopathology, whole-genome and whole-exome sequencing, RNA-sequencing, and DNA methylation arrays [74]. This repository covers the main molecular subtypes including H3.3 K27M and H3.1 K27M mutations. This repository, along with the BTRL resource and other large scale, broadly available repositories, offers a very useful study platform for pediatric brain tumor research. The Children's Oncology Group ACNS02B3 study, characterized 30 patient-derived orthotopic xenograft models and seven cell lines representing 14 molecular subgroups of pediatric brain tumors. PDOX models were found to be representative of the tumors found in patients in terms of histology, IHC, gene expression, DNA methylation, copy number, and mutational profiles. *In vivo* drug sensitivity of targeted therapeutics was associated with distinct molecular tumor subgroups and specific genetic alterations [57]. The unprecedented initiative proved the usefulness of PDX models as a key resource for pediatric brain tumor research. There are, however, limitations in PDX models as well, as it's the case with virtually any models. Again, the main disadvantage with immunodeficient mice is the inability to study the tumor to immune system interactions; another limitation is the fact that not any given passaged cell lines or patient sample can successfully be engrafted. A debatable aspect of PDX, is the degree to which the engrafted tumors recapitulate and maintain the stromal characteristics of patient samples with multiple passages as multiple passages select the most aggressive cells, depleting the characteristic heterogeneity of the tumors. Another consideration with PDX is its inability of recapitulating the early tumorigenesis, since the engrafted cells have already reached their malignant potential, therefore the model is not suitable to study the initial tumorigenesis mechanisms [52].

Genetically engineered mouse models and viral delivery models

The other major types of *in vivo* models used in pediatric glioma studies are GEMMs and viral delivery models. GEMMs recapitulate the early stages of tumor initiation in animals with native immune system and intact blood brain barrier and undisrupted microenvironment [75]. This makes GEMMs the first line models for tumor mechanism and drug discovery studies. Most mouse models of glioma are generated by altering key signaling pathways disrupted in human gliomas, including Ras, EGFR, Akt, Rb, PTEN, NF1 and PDGF pathways [76,77]. Some of the early GEMMs for pediatric gliomas used the tumor suppressor *Nf1* and *Trp53* mutations. The modeling strategy uses crossbreeding of mice with different genetic backgrounds resulting in the final combined knockout of *Nf1* and *Trp53*, that spontaneously develop brain tumors over a period of 13 months for some of the models. While the crossbreeding is laborious and tumor formation latency is long, the model shows a range of astrocytoma stages, from low-grade astrocytoma to glioblastoma, with accurate histopathological features [78,79]. A derivative model from the *Nf1/Trp53* knockout background was obtained by introducing CNS heterozygosity of *Pten* into the *Nf1/p53* astrocytoma model. Resulting mice had accelerated morbidity, shortened survival, and full penetrance

of high-grade astrocytomas. Haploinsufficiency of *Pten* accelerated formation of grade 3 astrocytomas, whereas loss of *Pten* heterozygosity and Akt activation with subsequent progression into grade 4 tumors [78]. One of the precautions that are involved with these models is the targeting of "general" tumor suppressor, such as *Ttp53*, which is not a glioma specific event and mandates the exclusion of the scenario that the formed brain lesions are potential metastases. An elegant solution for the non-selective silencing of tumor suppressors came with the introduction of conditional or inducible conditional knockout, in which the target gene can be edited in a tissue specific and/or time-dependent way. The most common method to make conditional knockout mice is the Cre-loxP system. To achieve precise temporal specificity in the Cre-loxP system, Cre can be fused with a hormone responsive element, and induced by the exogenous inducers tamoxifen or tetracycline [80]. Another transgenic mouse model uses the glial fibrillary acidic protein (*GFAP*) promoter to express oncogenic V12Ha-*Ras* in astrocytes. Activation of the p21-ras signaling pathway from aberrantly expressed receptors promotes the growth of malignant human astrocytomas. These transgenic astrocytomas are pathologically like human astrocytomas, with a high mitotic index, nuclear pleomorphism, infiltration, necrosis, and increased vascularity. The transgenic tumor model exhibits additional molecular features associated with human astrocytomas, including a decreased or absent expression of p16, p19, and PTEN as well as overexpression of EGFR, MDM2, and CDK4. Cytogenetic analysis revealed consistent clonal aneuploidies of chromosomal regions syntenic with comparable loci altered in human astrocytomas [81–83]. More recently, Larson *et al.* established a meticulous model of H3-K27M glioma with inducible H3.3 K27M cooperated with activating *PDGFRA* mutation and *Trp53* loss and successfully dissected the mechanisms how the oncohistone transform neural stem cells (NSCs) [84]. Viral delivery models bypass the laborious and expensive steps of crossbreeding of traditional GEMMs. With the establishment of the versatile replication-competent avian sarcoma-leukosis virus long terminal repeat with splice acceptor/tumor virus A (RCAS/t-va) RCAS/t-va virus mediated gene delivery model Cre recombinase is delivered to somatic cells to establish non-germline GEMMs [85]. RCAS/t-va marked a new era in brain tumor modeling in general, offering valuable tools for the pediatric CNS tumor study. The RCAS virus is selectively delivered into cells *via* binding to its specific cell surface receptor t-va. T-va is only expressed in avian cells, but mammalian cells can gain the expression through genetic engineering. RACS/t-va based GEMMs have some advantages over Cre-loxP based models. The virus penetrance into receptor expressing cells is low, so only a small fraction of cells can acquire the expression of target genes. This makes the model closer to actual tumor initiation where only a small number of transforming cells are key players in tumor initiation. Genetically engineered mammalian cells that express the t-va receptor for the virus can get multiple RCAS infections simultaneously or sequentially, which makes this model suitable to study the effect of multiple genes on tumorigenesis in a very precise fashion. There are several established pediatric brain tumor models using the RCAS platform. PDGFB overexpression in Nestin expressing cells of the neonatal brainstem, along with *Ink4a-ARF* deletion, leads to brain stem gliomas formation [86–91]. More recently, pediatric gliomas were successfully induced by using *in utero* electroporation (IUE) [92,93]. The method uses a targeted delivery of oncogenic plasmids into the developing mouse embryonal brain. Successfully targeted mice develop fully penetrant brainstem gliomas with different latencies, histological and molecular features, based on the plasmids delivered [94]. These models are particularly useful for modeling gliomas of the brain stem and H3K27M mutant gliomas – given the anatomical location and the young age of the patients who present with these tumors that make it particularly suitable. Patel *et al.* successfully generated diffuse brain stem gliomas by delivering a combination of *PDGFB* ligand along with dominant negative *Trp53* (DNp53) and either H3.3^{K27M}or H3.3^{WT}. In the IUE models by Pathania *et al.*, transposable H3.3^{K27M} and *Trp53* loss using a non-transposable gRNA/Cas9 targeting the *Trp53* locus (K27M-P, 2-hit), were sufficient to induce tumorigenesis in either hindbrain or cortex [95]. Another IUE model established by Miklja *et al.* uses the simultaneous delivery of three oncogenic plasmids: DNp53, H3-K27M and *PDGFRA*^{D842V} on a PiggyBac base [96]. Another genetically driven model uses blastocyst *in utero* injection. The model, however, has the inherent limitations of extensive and complex cross breeding and can be technically challenging for large scale experiments. These models can be useful to study the early stages of tumorigenesis and to study driver events that lead to tumorigenesis [97]. Transposon mediated delivery is a versatile platform that allows plasmid delivery into cells [98–101]. The Sleeping Beauty transposon delivery model is widely used in adult and pediatric gliomas modeling. While the system is fast and offers inducible, controllable targeted gene expression, there is some evidence of off-target effects induced by the carrier system itself, namely it was suggested that the construct might influence gene expression [102]. Pediatric high grade gliomas have successfully been induced by using transposon-mediated integration of plasmid DNA into cells of the subventricular zone of neonatal mouse brain [101,103-105]. This model system is particularly useful in identifying novel genetic tumor drivers and the effect of different genetic alteration on tumor phenotype [106,107].

Genome-engineered stem cell-based and organoid models

Genome engineering using CRISPR/Cas9 contributed to the flexibility of tumor modeling with the feasible introduction of bona fide genetic alterations observed in patient tumors [108]. Single-cell RNA-sequencing of adult glioblastoma models derived from human induced pluripotent stem cells (iPSCs) demonstrated that these models recapitulate inter- and intra-tumor heterogeneity observed in glioblastoma patient samples [109]. This approach is essential in pediatric brain tumor research as well. Chen *et al.* presented Notch activation as a shared characteristic in H3-K27M and G34R cells using astrocytes introduced with H3.3 K27M and G34R mutations using CRISPR/Cas9 [34]. Further, the effect of Notch activation was reversible upon editing H3-K27M back to wildtype [110]. The strength of these models includes that they can facilitate investigations on particular genes or genetic alterations in otherwise isogenic clean backgrounds. Haag *et al.* introduced the H3.3 K27M mutation in human iPSCs and found that the H3.3 K27M mutation drives tumorigenesis in NSCs, but not in oligodendrocyte precursor cells [111]. Similarly, Funato *et al.* introduced H3.3 G34R mutation into human embryonic stem cells and demonstrated intriguing findings, where this particular mutation can transform forebrain precursor cells, but not hindbrain precursor cells [112]. Genome engineering technologies have accelerated the accumulation of such insightful knowledge in the field of pediatric brain tumor research.

Modeling medulloblastomas and other pediatric brain tumors

The current hypothesis with regards to the development of embryonal tumors in the CNS is that tumor formation starts in embryonic or fetal cells that remain in the brain after birth [113]. The different types of CNS embryonal tumors include medulloblastomas and non-medulloblastoma embryonal tumors. The latter include embryonal tumors with multilayered rosettes, medulloblastomas, CNS neuroblastoma, CNS ganglioneuroblastomas, CNS embryonal tumors not otherwise specified, CNS embryonal tumors with rhabdoid features. CNS AT/RT is also a type of embryonal tumor, but it is treated differently than other childhood CNS embryonal tumors. Medulloblastoma is the most common embryonal tumor of the CNS. Compared to pediatric high-grade gliomas, medulloblastoma has a better prognosis. The 5-year survival rate for this this type of tumor is over 70% [5]. The most recent 2021 WHO Classification of Tumors of the CNS stratified medulloblastoma into four molecular subgroups with four and eight further subgroups for SHH and non-WNT/non-SHH medulloblastoma, respectively. The WNT group is characterized by the activation of the WNT/β-catenin signaling pathway and has the best prognosis. The SHH group

is characterized by deregulation of the SHH signaling pathway and has an intermediate prognosis while Group 3 and Group 4 are less characterized subtypes. There are several established cell lines and PDX cell lines frequently used in the medulloblastoma research field. The different groups of medulloblastoma have been also modeled *in vivo* either by GEMMs or by orthotopic transplantation of modified mouse cerebellar progenitor cells engineered to overexpress oncogenes and/or to inactivate tumor suppressors. More recently spheroids and organoid-derived models of human medulloblastoma subgroups have been reported. Unlike glioblastoma, primary cultures from patient samples can be maintained for only very short term in medulloblastoma and are not used as frequently [114–116].

In vitro models

There are over 40 medulloblastoma cell lines, the majority of which have been well characterized. Most cell lines pertain to the aggressive Group 3 medulloblastomas with *MYC* amplification. These established human tumor lines are versatile and easy to grow in culture as monolayers or spheres in stem cell conditions, and are commonly used to assess the effect of drug treatments in preclinical trials. However, molecular analysis by next generation sequencing has revealed that they do not always faithfully recapitulate primary tumors and in some cases these lines have acquired additional mutations and/or have partially lost genetic material [117]. Around half SHH medulloblastoma cell lines have mutations in *TP53*, and almost all Group 3 cell lines bear *MYC* amplification, while only a small part of SHH and Group 3 medulloblastoma patients typically have these mutations. This points to the limitations of these models, where these *in vitro* models transform through serial passages.

MED5R is the most utilized cell line for the WNT subgroup, and DAOY, UW228, UW426 are the ones for the SHH subtype. There are a larger number of available cell line models for the Group 3: D341Med, D384Med, D425Med, and D458. These are widely available cell lines, as well as the cells that are available through the BTRL platform, including Med-411FHTC, Med-2112FHTC. All the Group 3 cell lines have MYC amplifications. For the Group 4, CHLA-01-MED, CHLA-01R-MED are available through ATCC [23,118-126]. Given the limitations mentioned above of traditional *in vitro* models, organoids and other 3D culturing models are additional potential platforms to study medulloblastoma. 3D spheroid models have been shown to be more reflective of human disease and outcomes by mimicking tumor biology and drug response [114,115].

Patient-derived xenograft models

GEMMs of medulloblastoma are precise platforms that prove valuable tools for *in vivo* testing but fail to recapitulate the accentuated heterogeneity or microenvironment of human tumors. PDX models address these limitations and have become increasingly prevalent in preclinical research. PDXs are generated by implanting tissues from medulloblastoma patients' tumors into immunocompromised mice. In the case of acute PDX, during the initial processing of the tumor sample, there are no intermediate *in vitro* steps, eliminating the risk of culturing related genetic changes [75]. The most utilized approach of medulloblastoma PDXs is orthotopic engraftment, expanding tumors intracranially *in vivo*. The presence of stromal environmental components and the heterogeneity of the tumor cell population provide a significant advantage, particularly concerning preclinical evaluation of small molecules or other interventions [122,127]. Single-cell RNA-sequencing of PDX models continues to provide insights into tumor evolution, while analysis of the genetic and epigenetic landscape reveals new insights into tumorigenesis and progression. PDXs from patient tumors have limitations in terms of the amount of available material and variable engraftment rates [128]. As with any xenograft, implantation can disrupt the cell–matrix interactions and the blood brain barrier and lack of tumor initiation. In recent years, biobanks have been established allowing the availability of 15 PDXs, including one WNT, four SHH, seven Group 3, and three Group 4. Five out of the seven Group 3 PDXs harbor *MYC* amplification. The establishment rate for medulloblastoma was around 35% [75,129]. When compared to subcutaneous grafting, orthotopic engraftment in the cerebellum may be more efficient and might allow a better grafting efficiency for less aggressive tumors. Important information is provided on these PDXs including transcriptomic and whole-exome sequencing data.

Genetically engineered mouse models and viral delivery models

In a similar fashion to pediatric gliomas, the major directions of *in vivo* modeling for medulloblastomas are GEMMs and PDX models. GEMMs for medulloblastoma are generated by recapitulating precise genetic alterations seen in patient samples [130,131]. The Patched 1 model ($Ptch1^{+/-}$) is the first mouse model of medulloblastoma, and has been extensively used to assess the role of genes that drive tumorigenesis in the SHH subtype [132]. Since the establishment of this model, many conditional and inducible knockout models have been developed, enabling precise temporal and spatial gene manipulations with different combinations of $Ptch1^{+/-}$, $Trp53^{-/-}$, and $Cdkn2c^{-/-}$ [133–138]. GEMMs of medulloblastoma can also be obtained through somatic gene transfer using polyethylenimine-mediated transfection and IUE. Kawauchi *et al.* introduced DNp53 and Myc plasmids in embryonal brains through IUE and established models of group 3 medulloblastoma [139], and Forget *et al.* established group 4 medulloblastoma models through overexpression of constitutively active SRC and DNp53 [A Forget *et al,* PMID: 30205043]. As another example of medulloblastoma models, Zuckermann *et al.* introduced somatic CRISPR/Cas9 deletion of *Ptch1* by IUE into wild type E13.5 mouse embryos ("CRISPR-Ptch1") and successfully obtained SHH medulloblastoma with complete penetrance by 16 weeks of age [140].

The RCAS/t-va system is used for medulloblastoma modeling as well [130,141-143]. This robust system relies on the use of an avian retroviral vector, RCAS, to target gene expression to neuronal progenitors in transgenic mice in which the *Nestin* gene promoter drives expression of the viral receptor. Several SHH models were developed by infection with RCAS virus expressing *Shh*, alone or in combination with genes that include *MYCN*, activated *Akt, HGF, WIP1, BCL2*, all of which accelerate the onset of SHH medulloblastoma. GEMMs of Group 3 medulloblastoma were also generated through overexpression of *Myc* and *Bcl2* in addition to RCAS-Shh [134,141,144]. Another approach uses mutagenesis with the Sleeping Beauty transposon in Nestin-Cre mice with the backgrounds of *Trp53* mutation *Pten* knockout, where SHH or Group 3/Group 4 medulloblastomas were induced [106].

Genome-engineered stem cell-based and organoid models

NSCs have been utilized to assess the role of potential drivers of medulloblastoma, including *Myc* and *Gfi1*. Transplantation of NSCs that overexpressed *Myc* alone, or with oncogene *Gfi1* or *Gfi1b* into the cerebella of immunocompromised mice induced Group 3 medulloblastoma. This approach uses retroviral or lentiviral vectors that can conditionally express or repress genes of interest to modify mouse neuronal progenitors or human iPSC-derived NSCs. Marked progenitors are then implanted into the cerebellum of naïve immunocompromised mice or of naïve syngeneic animals, giving rise to tumors consistent with Group 3 based on histopathological and molecular analyses [145–147]. Huang *et al.* differentiated iPSCs derived from patients with Gorlin syndrome, a tumor predisposition syndrome caused by mutations in *PTCH1*, into neuroepithelial stem cells. In this model, CRISPR/Cas9 disruption of *GSE1*, which is commonly co-mutated in adult medulloblastoma, resulted in accelerated tumorigenesis, suggesting *GSE1* as a candidate tumor suppressor in medulloblastoma [148]. As discussed above, organoid models serve as robust *ex vivo* models for pediatric brain tumors [29]. Ballabio *et al.* overexpressed *Otx2* and *c-Myc* in cerebellar organoids, con-

Table 1
Major *in vivo* models utilized for pediatric brain tumor research. Summary of preclinical models utilized for research on pediatric gliomas, medulloblastomas, and other pediatric brain tumors.

SOME OF THE MAJOR *IN VIVO* MODELS UTILIZED FOR PEDIATRIC BRAIN TUMOR RESEARCH						
TUMOR	MODEL SYSTEM	GENETIC BACKGROUND VARIATIONS	PATHWAYS	REFERENCE	BENEFITS	LIMITATION
GLIOMAS	RCAS-TVA viral delivery model	Nestin-t-va; RCAS *Kras* and *Akt*	RAS; AKT	[90]	• Highly controllable model systems • Versatile • Can recapitulate early stages of tumor formation • Tend to be well characterized • Are largely available compared to newer models	• Mouse analogue evaluation only – not suitable for the study of human immunotherapies • Can be laborious and expensive -some models require extensive cross-breeding • Potential side effects of the system itself
		Nestin-t-va; *Pten* $^{fl/fl}$; RCAS *Kras*; RCAS Cre	PTEN; RAS	[91]		
		Nestin-t-va; *Trp53* $^{fl/fl}$; RCAS-*Pdgfb*; RCAS Cre	P53 PDGFB	[88]		
		Gfap-t-va; *Trp53* $^{fl/fl}$; RCAS-*Pdgfb*; RCAS Cre				
		Nestin-t-va; *Ink4a/Arf* $^{-/-}$	PDGFB Ink4a-Arf AKT	[86,89]		
	GEMM	*Trp53* $^{-/-}$; *Nf1* $^{fl/fl}$; GFAP-Cre	P53 NF1 RAS	[77]		
		Trp53 $^{+/-}$; *Nf1* $^{+/fl}$; *Pten* $^{fl/+}$; GFAP-Cre	P53 PTEN NF1	[78]		
		Gfap-V12*Ha-ras*; Gfap-V12*Ha-ras* Gfap-*EGFRvIII*	RAS ERBB	[81–83]		
		H3K27M; *Trp53*cKO	H3 P53	[84]		
	Transposon delivery models and IUE	*Erbb* ^{V664E} Piggybac transposon	ERBB	[76]		
		Hras/Kras-G12V Piggybac transposon	RAS	[76]		
		PDGFRA ^{D842V}- Piggybac transposon	PDGFR	[76]		
		H3K27M-DNP53	H3 P53	[95]		
		H3K27M-DNP53- *PDGFRA* ^{D842V}	H3 P53 PDGFRA	[96]		
	Genome engineered stem cell-based/ ex vivo organoids	*HRAS* ^{G12V} and *TP53* mutated H3-K27M H3-G34R	RAS P53 H3	[111,112]	• Inexpensive and easy to use • Can be used for immune studies	• Extensive genome editing can result in off-target effects
	PDX	Various backgounds	Various genetic alterations	[52]	• Maintain original tumor features • Can be passaged indefinitely • Useful for large scale experiments	• The immunocompromised status of the host makes them unsuitable for immune studies • Repeated passaging favor the selection of the more aggressive clones
MEDULLOBLASTOMA	RCAS/t-va viral delivery model	Nestin-t-va; RCAS-*Shh* + *N-Myc*	SHH Myc	[130]	• Highly controllable model systems • Versatile • Can recapitulate early stages of tumor formation • Tend to be well characterized • Are largely available compared to newer models	• Mouse analogue evaluation only – not suitable for the study of human immunotherapies • Can be laborious and expensive -some models require extensive cross-breeding • Potential side effects of the system itself
		Nestin-t-va; RCAS-*Shh*	SHH	[130]		
		Nestin-t-va *Trp53*$^{-/-}$ RCAS-*Myc*	GROUP3 Myc P53	[130]		

(*continued on next page*)

Table 1 (*continued*)

SOME OF THE MAJOR *IN VIVO* MODELS UTILIZED FOR PEDIATRIC BRAIN TUMOR RESEARCH						
TUMOR	MODEL SYSTEM	GENETIC BACKGROUND VARIATIONS	PATHWAYS	REFERENCE	BENEFITS	LIMITATION
		Nestin-t-va RCAS-*Myc*, RCAS-*Bcl2*	GROUP3 Myc	[130]		
	GEMM	BlbP-Cre+/-:*Ctnnb1*$^{+/lo}$xex3-*Trp53*$^{flx/flx}$	WNT	[131]		
		Ptch1 $^{+/-}$; *Math1*-Cre *Ptch1* $^{+/-}$; *Gfap*-Cre *Ptch1* $^{+/-:}$ *Trp53* -/- *Ptch1* $^{+/-}$; *Ink4c* $^{-/-}$ *Ptch1* $^{+/-}$; *Kip1* $^{-/-}$ *Ptch1* $^{+/-}$; *Ptch2* $^{-/-}$	SHH	[132–136]		
		Trp53 $^{-/-}$; *Pten* $^{-/-}$	P53 PTEN SHH	[137,138]		
	Transposon delivery models and IUE	Co-electroporation of Myc and trp53DN into embryonic cerebellar progenitor cells Co-electroporation of SRC-CA and DNp53 into E13.5 developing cerebella	*Group 3* Myc P53 *Group 4* P53	[139]		
	Genome engineered stem cell-based/ ex vivo organoids	*MYCN* transduction *DDX3X* mutation *GSE1* loss	MYC	[148]	• can provide information with regards to genetic causation • relatively easy to manipulate • suitable for large scale experiments	• risk of off target effects inherent with genome editing • clonal selection favors the more aggressive cells
	PDX	Various genetic backgrounds	*Variable*	[56,57]	• Maintain original tumor features • Can be passaged indefinitely • Useful for large scale experiments	• The immunocompromised status of the host makes them unsuitable for immune studies • Repeated passaging favor the selection of the more aggressive clones

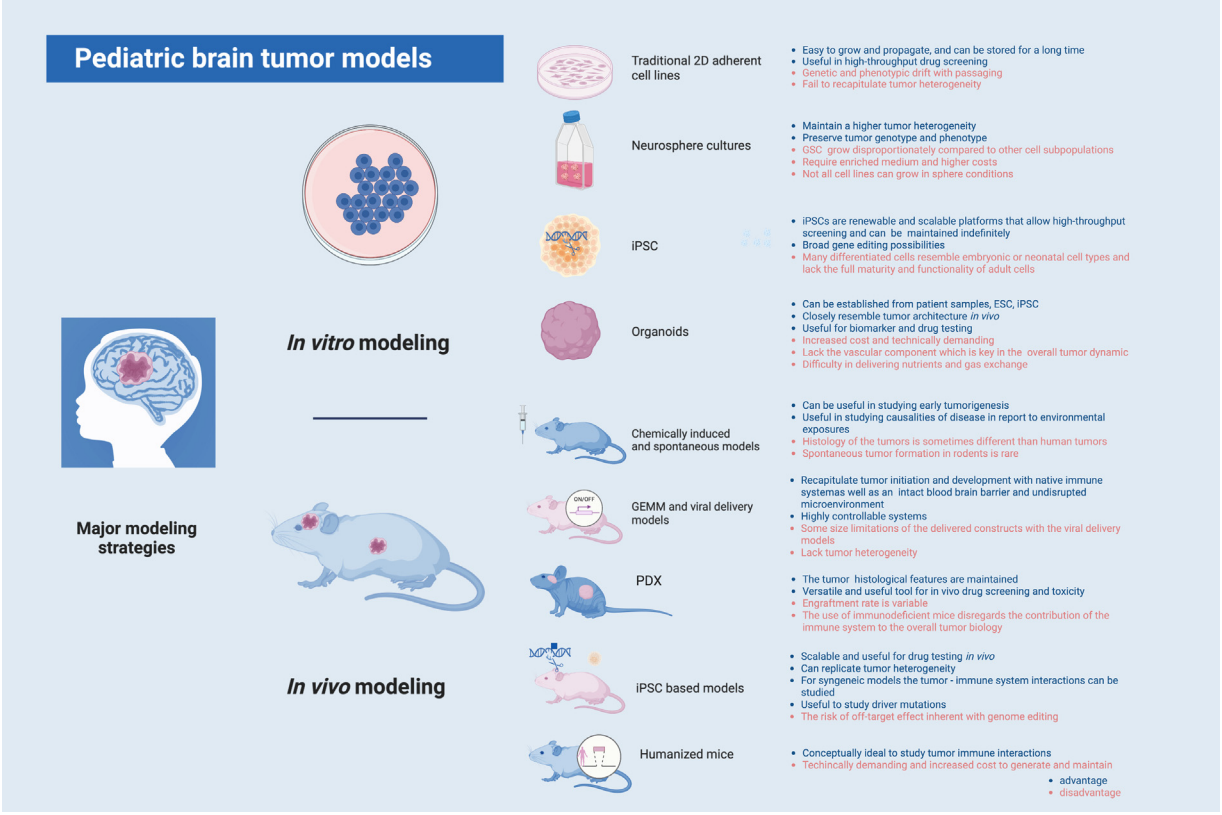

Figure 1. Graphical summary. Summary of modeling modalities utilized for research on pediatric brain tumors.

firmed that these models recapitulated the formation of Group 3 medulloblastoma, and further evaluated the efficacy of Tazemetostat in their *ex vivo* models [114]. Organoid models with or without genome engineering technologies can serve as a robust platform for modeling tumorigenesis and preclinical drug testing. Similar approaches have been taken for modeling other pediatric brain tumors. For example, Terada *et al.* knockout *SMARCB1* in combination with *TP53* in human iPSCs and generated models resembling AT/RT [149]. Further, Parisian *et al.* induced *SMARCB1* knockdown during the differentiation of cerebral organoids and found that there is a narrow developmental window where *SMARCB1* loss could take effect for the transformation of the neural lineage cells [150]. This suggests that organoid models are useful not only for preclinical testing but also for dissecting the tumor biology in the context of neural development, which will shed light on the research on pediatric brain tumors, especially embryonal tumors [151].

Conclusions

There is no doubt that a wide variety of preclinical models of pediatric brain tumors have contributed to our understanding of the tumor biology of these difficult-to-treat tumors and to therapeutic development. PDX models or cell lines derived from them can serve as a robust platform for testing therapeutic efficacy with their feasibility in handling and their nature of preserved inter- and intra-tumor heterogeneity, which should always be taken into account when considering cancer treatment. GEMMs and viral delivery models are essential in dissecting functions of particular genes and genetic alterations in tumor biology. The variety of these mouse models is still expanding based on the accumulated knowledge about the multiomic landscape of pediatric brain tumors. Genome engineering technologies have expanded the flexibility of tumor modeling and contributed to tumor models with authentic pathobiology observed in patients, enabling examination of gene functions in isogenic backgrounds. Organoid models paved the way to investigate interactions between tumor and tumor microenvironment and further dissect tumorigenesis in the context of neural development, which is a critical aspect in the research of pediatric brain tumors. There are novel approaches that can be practically applied to pediatric brain tumor models, including humanized mouse models. Multiple options of models became available and can be chosen depending on the purpose of the research (Table 1, Figure 1). Considering the rarity of each tumor type as a nature of pediatric brain tumors, integrative and collaborative efforts, together with such available clinical models are necessary to develop novel and effective treatment. As an example, CBTN has established more than 80 preclinical models and the largest multiomic dataset derived from patient tumors and those models [152], which will lead to thorough biological understanding of each tumor. In these contexts, such robust tumor models established so far and addition of novel ones will continue to contribute to the accelerated discovery of effective therapeutics for pediatric brain tumor patients.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Florina-Nicoleta Grigore: Conceptualization, Writing – original draft. **Serena Johanna Yang:** Writing – original draft, Writing – review & editing. **Clark C. Chen:** Supervision. **Tomoyuki Koga:** Conceptualization, Supervision, Writing – review & editing.

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