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Mouse Models of Experimental Glioblastoma

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Abstract: Glioblastoma is one of the most common malignant brain tumors. It has poor prognosis: the survival rate is 14–15 months, even with treatment by surgery, radiation, and chemotherapy. To develop more efficacious therapies, it is essential to generate preclinical mouse models that enable mechanistic studies. Multiple murine glioblastoma models have been generated, each with distinct advantages and disadvantages. The traditional Cre-LoxP system specifically targets glioblastoma-related genes but requires extended experimental timelines. CRISPR-Cas9 methods require less time to generate mouse models, yet the off-target effects lead to variable glioblastoma phenotypes. Transposon-based insertional mutagenesis models can intercept and promote transcription but has strict limitation of insertional transgene size. Allograft cell line injection into immunocompetent mice prevents immune rejection but fails to recapitulate various features of human glioblastoma. Intracranial injection of patient-derived xenograft cell lines into immunocompromised mice preserves features of human glioblastoma but does not allow the study of immune cell function in preclinical immunotherapeutic approaches. Finally, humanized mouse models offer the potential to analyze the human adaptive immune response but not the innate

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immune response. This chapter outlines the major experimental glioblastoma models currently employed and the therapeutic approaches that can be tested.

Keywords: Cre-LoxP glioblastoma model; CRISPR/Cas9 glioblastoma model; mouse models of glioblastoma; transgenic glioblastoma model; transplant glioblastoma model

INTRODUCTION

In 2016, WHO integrated histological and molecular parameters to define the main gliomas types in place of the previous criteria taking into account only histology (1). Under the new criteria, based on histological features, gliomas are classified into four grades. Grade I is the slow growing, less malignant tumors; grade IV is the rapidly growing, highly malignant tumors (2). Glioblastoma is the most aggressive and invasive undifferentiated tumor type and has been designated Grade IV by WHO (3, 4).

Based on the molecular feature of whether there is isocitrate dehydrogenase (*IDH*) mutation, glioblastomas are mainly classified as *IDH*-wildtype and *IDH*-mutant (3, 5–7). *IDH* mutant glioblastomas are molecularly, biologically, and clinically different from *IDH* wild-type ones (5), which is important for glioblastoma biology and heterogeneity (8, 9). Clinically, primary glioblastoma cases are more related to *IDH*-wild type; secondary GBM cases are more related to *IDH*-mutant type; approximately 75% of patients with secondary glioblastoma have *IDH* mutation (10).

Pertaining to molecular gene expression studies, there are three major genetic pathways related to glioblastoma formation: (i) inactivation of the *p53* pathways accounts for 87% of glioblastomas; (ii) inactive retinoblastoma (*RB*) tumor suppressor pathways account for 77% of glioblastomas; and (iii) amplification and mutation of receptor tyrosine kinase (*RTK*) genes and activation of the phosphatidylinositol-3-OH kinase (*PI3K*) pathways account for 88% of glioblastomas (11, 12).

Finally, *in vivo* experiments have demonstrated that most GBM tumors exhibit deregulation and mutations of genes in the *p53*, *RB* and *RTK/RAS/PI3K* pathways (13–16). Clinical therapies targeting these pathways are being developed, but the treatments have not been successful (17–20) due to inefficient blood brain barrier penetration, inter-tumor heterogeneity and other compensatory/redundant signaling pathways. To better understand those pathways and their interplay, there is a need for models that reflect the glioblastoma tumor microenvironment (TME), because current *in vitro* models are not able to recapitulate this. Traditionally, 2D monolayer cell lines cultured in serum-containing medium do not reflect the heterogeneity of human tumors, and hence do not resemble clinical tumor development. Newer approaches using 3D spheres brain cell culture, such as glioma stem-like cell culture, in serum-free medium can reflect better genetic background of the tumor and maintain some phenotypic heterogeneity. However, long-term culture results in the clonal selection and genetic drift. Furthermore, *in vitro* cell culture does not model human immune cells. This limits exploration of factors regulating tumor-host interactions and immune control (21–23).

Therefore, it is essential to have animal models that properly reflect the glioblastoma TME so that the glioblastoma biology can be precisely analyzed, which allows for the evaluation of potential treatments, immune therapies and identifying the therapy targets.

MOUSE MODELS OF GLIOBLASTOMA

Currently, there are four major strategies for generating glioblastoma mouse models: spontaneous, transgenic, transplant, and humanized (Table 1).

Spontaneous glioblastoma mouse models

Spontaneous mouse glioblastoma tumors are rare (24). Therefore, setting up the spontaneous glioblastoma mouse model would require a large number of mice to observe. Slye, Holmes and Wells found only 3 spontaneous glioblastomata out of 11,188 mouse brains (25). To increase the efficiency and speed of spontaneous tumor generation, chemical or viral induction methods were used. The first successful induced brain tumor was developed in 1939 with intracranial implantation of 20-methylcholanthrene into C3H mice subarachnoid by Seligman and Shear, which led to gliomas and meningeal fibrosarcomas (26). Even though chemical-induced glioblastoma models are now outdated, several mouse cell lines established from those tumors have been later used for allograft implantation mouse models (27–31). Rous sarcoma virus has been used to induce mouse glioblastoma since the 1960s (32–34). However, virus-induced tumors had incomplete tumor penetrance (35, 36). The special maintenance requirement of the virus and the virus-induced mice dramatically increases the cost. In recent years, engineered viruses as vectors for transgenic genes are now used to generate mouse glioblastoma models. Both retrovirus and lentivirus have been applied this way, as will be discussed in the following section.

Transgenic glioblastoma mouse models

Currently, the main systems used for transgenic mouse models are the Cre-LoxP system, transposon-based system, CRISPR/cas9 system, and virus vectors delivery system. These systems can be used in both germline and somatic transgenic mice (37). The common goal of generating mouse glioblastoma models is increasing activity through overexpression of oncogenes such as *p21-RAS, PI3K, EGFR, CDK4* and *MDM2*, or decreasing activity by mutating tumor suppressor genes, such as *Pten, p53, CDKN2A* and *RB* (34). Generally, germline transgenic mouse models are generated by first introducing defined DNA alterations in germline cells, then using breeding strategies to obtain the gene related to the experiment by serial breeding from the founder mouse (38, 39). In somatic transgenic mouse models, tumors are initiated by directly implanting the induced transgenic cells, RNA, sh-RNA or engineered virus vectors into specific brain regions (15, 40–43). In recent years, these techniques have been combined to generate more precisely targeted mouse models for research. Fluorescence protein, luciferase reporter, or other tags such as human influenza hemagglutinin are tagged to the transgenic

TABLE 1 **Major experimental glioblastoma mouse models**

Model Types	Turnaround Time	Advantages	Disadvantages	Citation
Spontaneous mouse models	Variable	Reflects tumor development and progression	Need a large numbers of mice	
Spontaneous				(24, 25)
Chemical induced				(26)
Virus induced				(32, 33)
Transgenic mouse models		Can determine the molecular genetics alteration	Cannot completely reflect the phenotypic heterogeneity.	
Cre-LoxP system	10–14 months			
Traditional Cre-LoxP system		Stable, accurate	Only spatially control, led longer time needed for development.	(15, 46)
Tamoxifen inducible system		Temporal control		(51, 52)
Tet/Dox inducible system		Temporal control		(56, 57)
Transposon system	6–8 months	Less generation time		
SB system				(41, 73)
PB system				(75)
CRISPR/Cas9 system	5–7 months	Less expensive, faster, easy to introduce.	Off- target	(92, 94)
Viral vector delivery system	Days	Faster development	Vector has insertion limitation <2.5Kb	(90, 103)

Table continued on following page

TABLE 1 Major experimental glioblastoma mouse models (Continued)

Model Types	Turnaround Time	Advantages	Disadvantages	Citation
Transplant mouse models				
Allograft transplant models	21–30 days	Can model the immunity and immunotherapy of mouse experimental glioblastoma.	Murine immune response	(109, 115)
Xenograft transplant models	21–30 days	Reflects the genetic and phenotypical feature of original human tumor	Mouse is devoid of murine or human immune system	(117, 118)
Humanized mouse models			Cannot completely reflect human response	
Hematopoietic stem cells (HSCs) humanized mice	10–12 weeks	Provide fully competent human immune system		(156)
Human microbiota-associated (HMA) humanized mice	3 weeks	Avoid the impact on immune system by gut microbiota composition		(164, 165)

genes so that tracing glioblastoma growth in the mouse models, labeling targeted cells and tissue, and observing microenvironment diffusion and the immune cell response for therapy can be more convenient under the microscope (44).

Cre-LoxP transgenic glioblastoma mouse models

Most glioblastoma mouse models have used the Cre-LoxP system to target tumor genes in the specific brain tissue of interest (Figure 1A, B) (45). This system provides deep insight into the genetic drivers of glioblastoma and highlights the genetic differences between primary and secondary glioblastomas (13, 15, 46). Previously, most mouse models were generated by breeding two transgenic mice strains: a Cre-driver mouse strain which has Cre recombinase with a promoter and a LoxP floxed mouse strain that has LoxP floxed critical exons of the target gene (Figure 1C) (47). By breeding the two strains together, the system deletes the floxed region and inactivates the gene in desired tissues; the target gene remains functional in all other tissues. On average, it takes 12–18 months to obtain the desired transgenic mice. Therefore, induction of LoxP sites via Cre recombinase viruses—such as adenovirus and lentivirus—has been used to shorten the experimental timeline and generate more complex yet easy to obtain transgenic mouse models (14, 15, 37, 48).

Both of the above strategies have been applied in testing *p53* and *PTEN* function in GFAP positive glioblastoma tissues as demonstrated by the following studies. Zheng et al. generated *p53* and *PTEN* double knock-out mice targeted specifically to astrocytes by using *GFAP-Cre*$^{+}$ mice interbred with *P53*$^{flox/flox}$*;Pten*$^{flox/+}$ mice. From these, 66% of the tumors were anaplastic astrocytomas and 34% were glioblastomas (13). Their model indicated that the loss of *p53* and *PTEN* would regulate *Myc* levels and in turn control NSCs self-renewal and differentiation (13, 46). Jacques et al. demonstrated another method for generating transgenic mice that target GFAP positive cells: they used adenovirus expressing Cre recombinase (Adeno-Cre or Adeno GFAP-Cre) injected into mice that have conditional alleles flanked by LoxP sites of *RB, p53*, and *PTEN*, to ablate *RB/p53*, *RB/p53/PTEN*, or *PTEN/p53* in adult mice stem/progenitor cells. Their result indicates that initial deletion of *RB/p53* or *RB/p53/PTEN* are relevant to glioblastoma pathogenesis, and that *RB* loss is important in driving the phenotype of primitive neuroectodermal tumors (14). Friedmann-Morvinski et al. performed stereotaxic injection of Cre-inducible lentiviral vectors *shNF1-shp53* or *H-RasV12-shp53* into GFAP-Cre mice to induce *p53* deficiency in GFAP positive cells such as astrocytes. They identified that loss of *NF1* leads to increased *RAS* mitogenic signaling and increased cell proliferation, while the loss of functional *p53* induces genomic instability for glioblastoma tumorigenesis (15, 37).

Cre-LoxP has been a popular system for generating transgenic mouse for years, it can only spatially but not temporally control the tumorigenesis (49, 50). In addition, knock-out or overexpression of some critical genes may lead to early embryo lethality (51, 52). To overcome this shortcoming and accurately control the timing of tumor generation, traditional Cre-LoxP system has been modified so that it can be temporally induced by exogenous inducer tamoxifen (TAM) or tetracycline/doxycycline (Tet/Dox), making the gene expression transient and reversible (16, 53, 54).

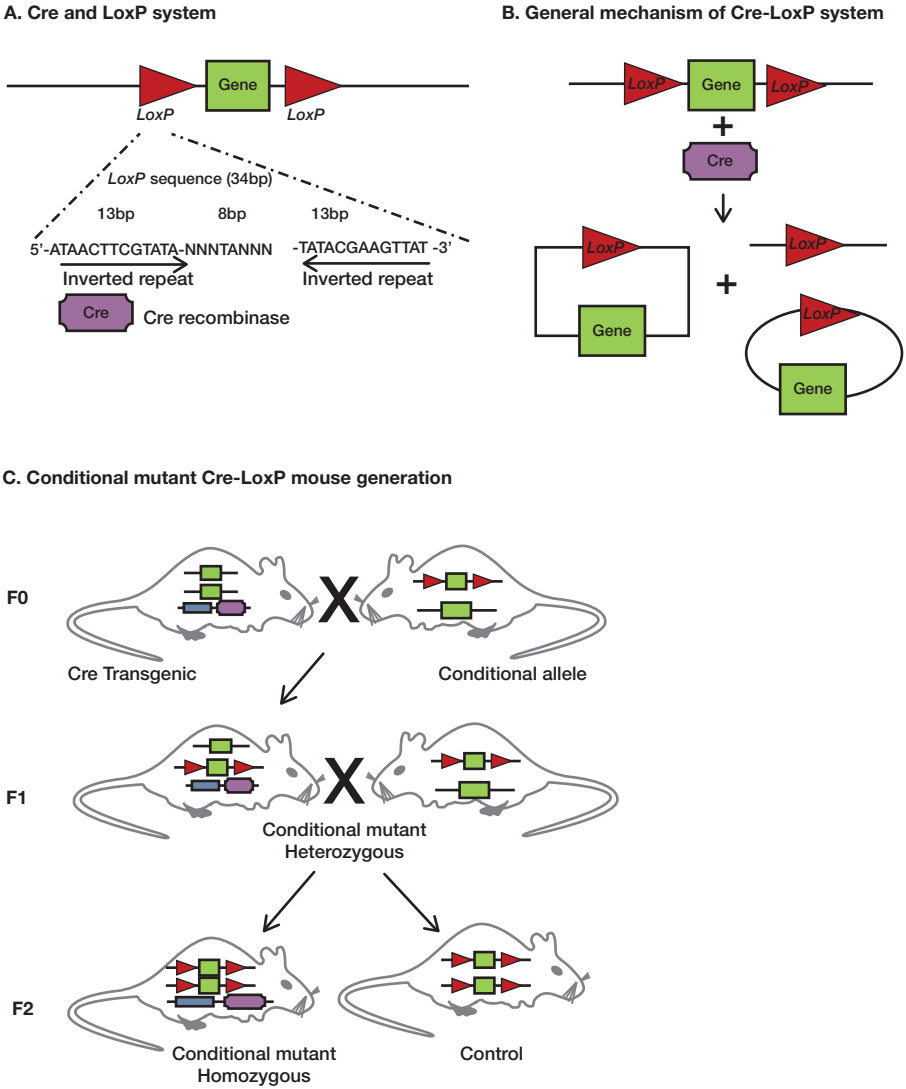

Figure 1. General Cre-LoxP deletion system. **(A)** Cre and LoxP system; 34-bp LoxP sequence consisting of two 13-bp inverted and palindromic repeats and 8 bp of core sequences; Cre recombinase is a 38-kDa DNA recombinase. **(B)** General mechanism of Cre-LoxP system; Cre recombinase recognizes the specific DNA fragment sequences between the two repeated LoxP sites and mediates site-specific deletion of DNA to create two pieces of DNA. **(C)** Traditional Cre-LoxP mouse generation;two strains of mutant mice are bred to generate Cre-LoxP mice: a Cre-driver mouse strain which has Cre recombinase with a promoter, and a LoxP floxed mouse strain that has LoxP floxed critical exons of the target gene; breeding these two strains together generates heterozygous F1 founder mice; F1 mice then breed with LoxP mice again for the F2 homozygotes.

Inducible Cre-LoxP transgenic glioblastoma mouse models

There are two widely used inducible Cre-LoxP systems. One is TAM inducible Cre-LoxP system (CreER^{TM}-LoxP system) (Figure 2 A, B) (53). Cre recombinase is fused to estrogen receptor (ER) to prevent CreER^{TM} from entering the nucleus and driving the floxed LoxP sites to delete the target transgenic DNA. When TAM, an ER agonist, is administered into the CreER^{TM}-LoxP system, it binds to the ER and initiates the translocation of CreER into the nucleus, where it can recombine with the floxed LoxP target exon of the DNA. Thus, it can control the timing of gene expression or inactivation and be used to overcome the limitation of Cre-LoxP system where some loss/gain of gene functions would lead to the lethality of mouse in embryo stage or early young (51, 52). CreER^{TM} is Cre recombinase fused to one mutated human ER. This CreER^{TM}-LoxP system needs a higher TAM dosage for induction. To avoid the potential side effects of high TAM levels, CreER^{T2} was generated. It consists of Cre recombinase fused to a triple mutant form of the human ER. Thus, only 1/10 of the TAM dosage required for the CreER^{TM} system is needed to activate CreER^{T2} (55).

To generate CreER^{TM}-LoxP inducible germline transgenic mouse models, two independent strains of mice are required. One strain expresses CreER^{TM} controlled by a cell-specific promotor. The other expresses floxed LoxP sites. The two strains of mice are bred together to generate the double transgenic mice. Adding TAM to the mice's food or drinking water permits spatiotemporal control of the target gene expression. This method efficiently avoids early embryos lethality. *IDH1* knock-in mice died perinatally if crossed *IDH1*$^{fl(R132H)/+}$ mice with *Nes*-Cre mice (51, 52). Bardella et al. successfully generated live *Nes*-CreER^{T2}; *IDH1*$^{fl(R132H)/+}$ knock-in mice by crossing *IDH1*$^{fl(R132H)/+}$ mice with the TAM-inducible *Nes*-CreER^{T2} mice. At 5–6 weeks of age, TAM induction was performed for 5 consecutive days to successfully obtain *R132H* knock-in mice. This mouse model demonstrates that overexpression of *IDH1* mutation in mouse brain subventricular zone (SVZ) cells contributed to glioblastoma formation through *Myc* and *Wnt* pathways activation, telomere pathway activation, and DNA hypermethylation (51).

The CreER^{TM}-LoxP system is extremely versatile due to the ease of gene expression control it provides. For example, brain progenitor cell specific inducible Cre mice *Ascl1*-CreER^{TM}, *NG2*-CreER^{TM}, and *Nes*-CreER^{T2} were crossed with knock-out or conditional knock-out *NF1*, *p53*, and *PTEN* mice to generate double transgenic CreER^{T2} floxed LoxP mice. Then the mice were induced by TAM at 4–8 weeks of age, and the timely control of *NF1*, *p53*, and *PTEN* knock-out in specific cells expressing *Ascl1*, *NG2*, and *Nes* allowed for identification of central nervous system cell lineages contributing to glioblastoma (8, 54).

The other widely used inducible Cre-LoxP system is Tet inducible Cre-LoxP system (Figure 2 C, D). Dox is an analog medicine to Tet. Since Dox is more efficient in controlling the Tet receptor, researchers use Dox more than Tet in this system. Thus, the system is also called the Dox inducible Cre-LoxP system. There are two types of Tet/Dox inducible systems: Tet/Dox-on and Tet/Dox-off, depending on whether the system uses reverse tetracycline-controlled transactivator (rtTA) or tetracycline-controlled transactivator (tTA). In Tet-on systems, addition of Tet induces gene expression. In Tet-off systems, the desired gene is expressed in the absence of Tet (38, 47). Both the Tet-on and Tet-off systems are

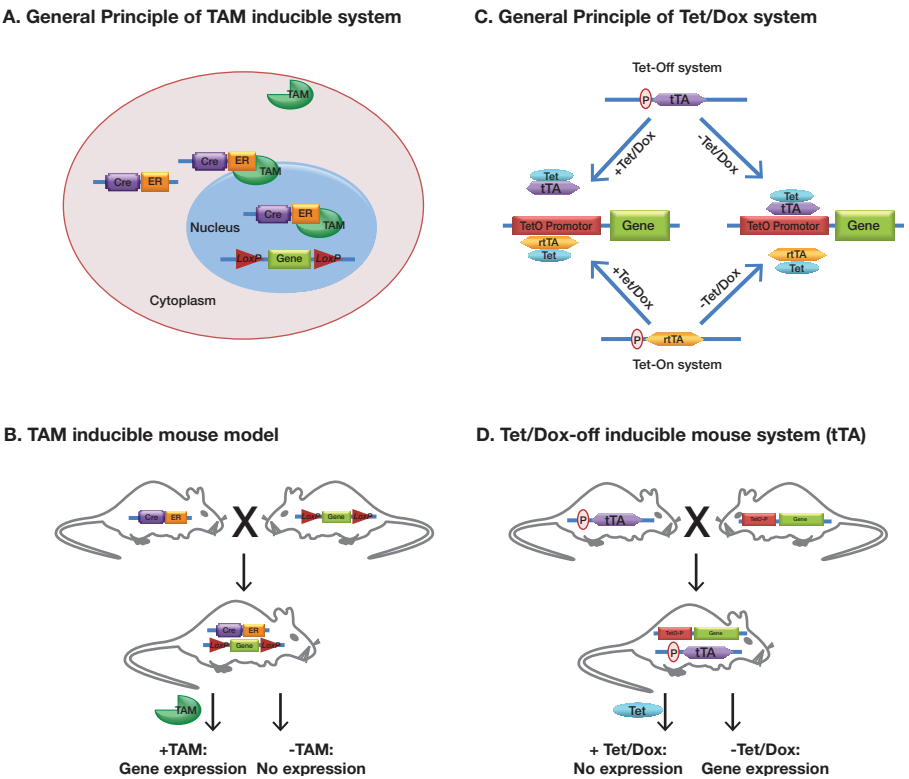

Figure 2. The Cre-LoxP inducible system. A. General Principle of TAM inducible system; in TAM inducible system, Cre is ligated to the ER which stays in the cytoplasm until the administration of TAM; when TAM is administered, CreER binds to the estrogen receptor and initiates the translocation of CreER into the nucleus, where it recombinase with the floxed LoxP target exon of the DNA. **B.** Tamoxifen inducible mouse model; two independent mouse strains, one strain expressing CreER, the other expressing two LoxP sites with or without a stop code, are bred together to generate double transgenic mice; adding TAM to food or drinking water of the double transgenic mice permits in vivo spatiotemporal control of the target gene expression. **C.** General principle of Tet/Dox system; two types of Tet/Dox general inducible systems: Tet-on and Tet-off. In Tet-on systems, rtTA is expressed; in the absence of Tet/Dox, inactivated rtTA cannot bind to TetO sequence of Cre gene, so Cre is not expressed; after Tet/Dox administration, activated rtTA binds to TetO promoter of Cre to induce Cre expression, which activates the Cre-LoxP system; In the Tet-off system, tTA is expressed; in the absence of Tet/Dox, activated tTA can bind to TetO sequence of Cre and induce Cre expression; after Tet/Dox administration, tTA is inactivated; inactivated rTA cannot bind to TetO promoter, therefore Cre expression is inhibited. **D.** Tet/Dox-off inducible mouse system (tTA); two independent strains of transgenic mice are needed: one strain requires tTA expression, the other strain requires the expression of the mutant gene of interest is controlled by TetO promotor with Cre expression; these two strains of mice are bred together to generate double-transgenic mice; by adding Tet/Dox to food or drinking water of the double transgenic mice allows the *in vivo* target gene to express spatiotemporally.

used widely to spatiotemporally control tumor generation in transgenic mouse models (16, 40).

Similar to the TAM inducible transgenic mouse model, the Tet/Dox inducible model requires two independent strains of transgenic mice. One strain is the transactivator requiring rtTA or tTA expression under the control of a specific promotor. The other is the responder requiring that the expression of the mutant gene of interest is controlled by TetO promotor with Cre expression. These two strains are bred together to generate the desired double-transgenic mice. Then Tet/Dox is added to food or drinking water of the double transgenic mice to allow the target gene to express spatiotemporally (56, 57). Although Tet/Dox inducible Cre-LoxP system can flexibly control the timing of transgenic expression, one shortcoming is the leakiness of rtTA, which can result in undesired transcription of the target genes. This is because rtTA has some affinity for TetO sequences even in the absence of Tet (58). In addition, the potential side effects from high doses of Tet/Dox are also unknown. To avoid these limitations, mutagenized rtTA^{2S} were generated to increase Dox sensitivity, allowing it to function at Dox concentration 10 times lower than rtTA (59).

Transposons-based transgenic glioblastoma mouse models

Transposons were first identified more than 50 years ago (60). Transposons can move from one genomic location to another through "cut-and-paste" mechanisms (Figure 3 A) (61, 62). Sleeping beauty (SB) and PiggyBac (PB) are two widely used transposases that have been successful in establishing functional mutagenesis *in vivo* and *in vitro*. SB transposase inserts a transposon into a TA dinucleotide base pair sequence in the recipient DNA, while PB transposase inserts a transposon into a TTAA dinucleotide base pair sequence (63). PB integration sites are mainly localized near transcriptional start sites (TSSs), CpG islands, and DNaseI hypersensitive sites. In contrast, SB integrations are more randomly distributed, so the PB system can perform more efficient stable gene transfer than the SB system (64–66). SB and PB transposon systems have been used in both germline and somatic cells of transgenic mice (Figure 3 B, C) (45, 60, 65, 67–71).

In germline transposon models, two mouse strains, one that expresses the transposase and one that carries transposons with gene trap cassettes are needed to breed the desired mice in multiple generations (60, 69, 72). For example, *Rosa26-LSL-SB11* (SBase) mice which had conditional floxed-stop SB transposase allele knocked in are bred with *T2/Onc2,3* mice which had mutant SB transposon to generate heterozygous mice that expressed SB transposase *T2/Onc2,3/+; SBase/+*. Then these mice interbred to produce homozygotes for later experiments (73).

The transposons model can also be used in the context of somatic cell mouse models. Virus SB *transposase* system was used to overcome the shortcoming of transient expression of polyethylenimine/plasmid DNA (PEI/DNA) (74). Thus, it was able to deliver *shRNA-p53* with seven other combinations to identify the functions of the oncogenes in different glioblastoma formation pathways. This technique enabled rapid production of different genetically engineered mouse strains and sped up the preclinical drug screening for glioblastomas (70, 75, 76).

The SB/PB transposase system can also avoid embryonic lethality in mice (77). For example, *ATRX* mutation, together with mutation of *p53* and point mutation of histone *H3.3* variant, occurred in 31% of primary glioblastoma in pediatric

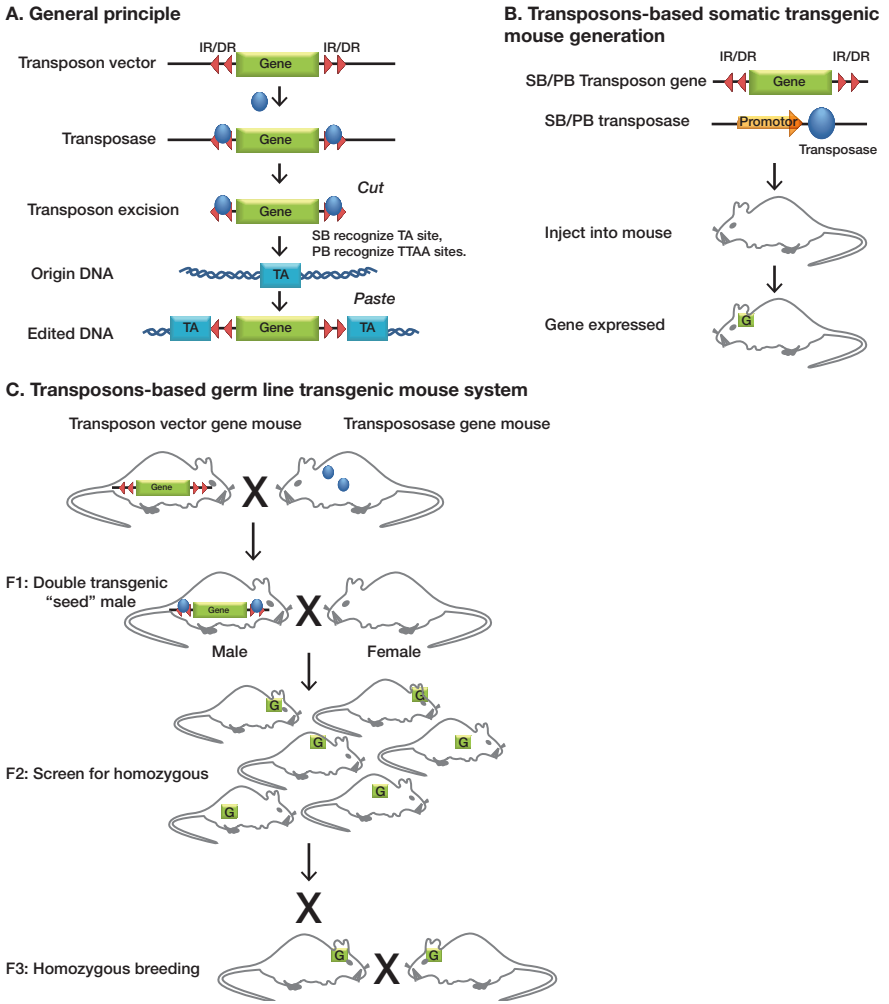

Figure 3. The Transposons-based system. A. General principle of transposon-based system; the transposon-based system includes two parts: a transposon vector containing DNA sequence that is flanked by inverted repeat/direct terminal repeat (IR/DR) sequences, and the transposase enzyme responsible for excision and reintegration of the transposon under the control of a promoter; when transposon vector and transposase are present together, a "cut-and-paste" transposition reaction occurs; the transposon is excised from its original location and re-integrated to a new location within the genome. *SB* and *PB* are two different transposases. *SB* transposase inserts a transposon into a TA dinucleotide base pair sequences, *PB* transposase inserts a transposon into a TTAA dinucleotide base pair sequences. **B.** Transposon-based somatic cell transgenic mouse system; to generate the somatic cell transgenic mouse model, two plasmids are injected together into mice to cause mutations in specifically targeted cells; the transposon insertion sites are detected using PCR screening. **C.** Transposon-based germ line cell transgenic mouse system; to generate the germ line cell transgenic mouse model, two mouse strains are required: one strain carries the transposons vector gene, and the other carries the transposase gene; these two mouse strains are bred to generate the F1 generation of double transgenic mice; F1 males are crossed with wild-type females to segregate the different insertion events in their sperm cells, generating F2 in the process; then the F2 mice are screened to select the ones with the desired mutant allele, and these mice are crossed together to generate F3 homozygous mice.

patients (78–80). However, knock-out of *ATRX* resulted in embryonic lethality in mice (75, 77); the zygotes never grew beyond the 4-cell stage (75). Koschmann et al. (41) used SB transposase system to develop somatically mutant *ATRX* in mice to overcome this limitation. They injected combined plasmids encoding SB transposase/firefly luciferase, *shRNA-p53*, and *NRAS*, with or without *shRNA-ATRX*, into the lateral ventricle of neonatal mice to generate *ATRX* deficiency, *p53* loss, and *NRAS* overexpression mouse model (41). But Pathania et al. (75) using SB system by combining $H3.3^{K27M}$-SBase with *ATRX/p53* knock-down constructs injected in neonatal mice, could not induce tumor. Then this group injected combined plasmid produced by PBase system: a transposable ***sh***RNA against *ATRX* together with $H3.3^{K27M}$, and a plasmid knock-down *p53* with CRISP/Cas9, into E12.5-E13.5 embryos to generate the desired mouse model (75, 76). In short, both Koschmann and Pathania tried to use *SB* system to generate glioblastoma models via double knock-out *ATRX* and *p53*. Koschmann et al. succeeded through the SB system in neonatal stage, while Pathania et al. failed with the SB system in neonatal stage but succeeded with the PB system in embryo stage. This suggests that the somatic transgenic stage may be more critical for the lethal genes than the choice of method.

In the SB transposon system, there is only 40–50% chance that the excised transposon integration would occur in the genome. Additionally, because the number of transposons integrated in the genome decreases over time, a large number of transposable elements are required (81). PB demonstrated the highest efficiency and stability in gene transfer (64, 82, 83). Even though SB insertional mutagenesis system is more random and less efficient, it can integrate transposons up to 10 kb in size (84), making it capable of delivering around 80% of human cDNAs (85). In contrast, the PB system can only insert cDNAs approximately 2.4 kb in size (86).

CRISPR/Cas9 transgenic glioblastoma mouse models

CRISPR/Cas9 is an RNA guided nuclease which is involved in prokaryotic immune systems (87–89). It has been used extensively to generate cancer models through genetic editing, providing a fast, inexpensive, and simple method to identify and study genetic determinants of cancer. CRISPR/Cas9 mouse models can be generated by injecting Cas9 mRNA with one or multiple single guide RNAs (sgRNA) directly into mouse somatic cells or germline embryos, which creates precise genomic edits at specific loci (Figure 4 A, B) (90). Depending on the type of DNA repair that took place, two kinds of mouse genome modifications will occur: constitutive knock-out tumor suppressor genes through non-homologous end joining and knock-in oncogenes through homologous recombination (91–93). The whole process takes around 2–3 months, which is much faster than the Cre-LoxP system.

Although the CRISPR/Cas9 system can be used in both somatic and germline cells, researchers are more likely to use it to edit somatic cells in transgenic or wild type mice. Plasmids targeting specific genes are first edited by the CRISPR/Cas9 system, then injected into germline transgenic mouse models generated from Cre-LoxP and other transgenic systems to create more accurate and precise knock-out or knock-in mouse models (44, 92). By injecting plasmids modified by the CRISPR/Cas9 system *in utero* at Embryo stage E13.5 days, researchers generated

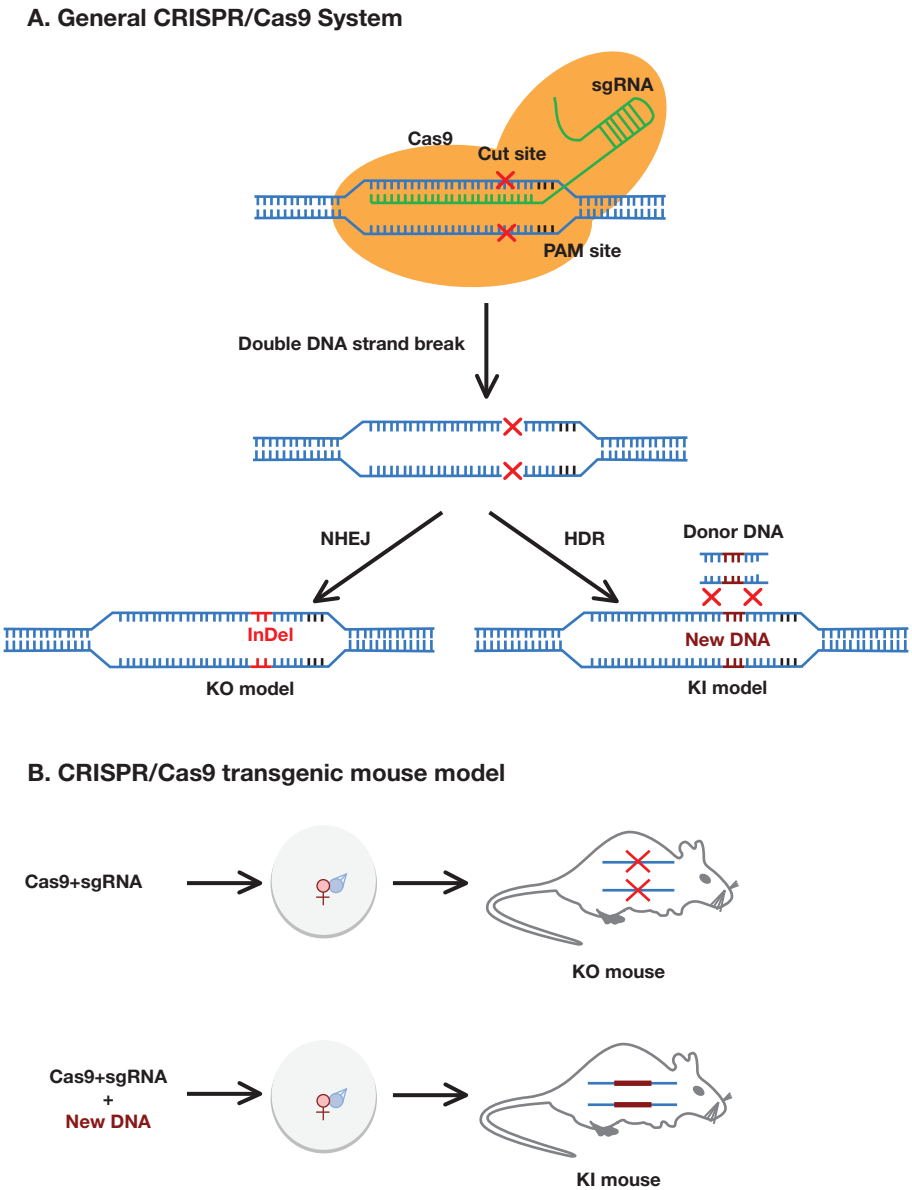

Figure 4. The CRISPR/Cas9 System. A. General CRISPR/Cas9 system; in the general CRISPR/Cas9 system, Cas9/sgRNA complex recognizes the complementary 20-nucleotide genomic sequence with a downstream protospacer-adjacent motif (PAM) sequence; it cuts three nucleotides upstream of the PAM sequence to induce double-strand DNA breaks (DSBs); the DSBs are then repaired through two major mechanisms: NHEJ pathway which is usually for knock-out genes and HDR pathway which is usually for knock- in genes. **B.** CRISPR/Cas9 transgenic mouse model; CRISPR/Cas9 germline transgenic mouse models are generated by injecting Cas9 mRNA with one or multiple single guide RNAs (sgRNA) directly into mouse germline embryos; two kinds of mouse genome modifications will occur: constitutive knock-out tumor suppressor genes through NHEJ and knock-in oncogenes through HDR.

Ptch1, p53 double loss mouse model, instead of using the traditional method of breeding *Ptch1*$^{+/-}$ mice with *p53*-null mice (92, 94). The highly aggressive glioma developed in a short period of time in all mice, and the tumors produced via CRISPR/Cas9 are mostly similar to tumors produced in germline transgenic mice (92).

With continued development, gene editing techniques are now used more often in combination for glioblastoma mouse models. Chen et al. combined the CRISPR/Cas9 system and *PB* transposase lineage labeling to induce somatic mutations in NPCs. They used *PB* transposase system producing GFP or RFP signal which can label the lineage of CRISPR-targeted progenitors *in vivo*. At the same time, they used CRISPR/Cas9 constructs containing sgRNAs targeting *NF1, PTEN*, and *p53*, alone or in combination to generate *NF1, PTEN,* and *p53* deletion in somatic cells. In this way, they demonstrated that CRISPR/Cas9 combined with *PB* transposase lineage labeling is a convenient way to produce unique tumors caused by somatic mutation in neural progenitors (44). The CRISPR/Cas9 system is a fast method to provide versatile gene editing, making it extremely useful. However, one major limitation of this nuclease technology is the non-specific and off-target cutting of DNA sequences. Because the Cas9 nuclease randomly cuts within the sequence and can target some slightly different sequences, some undesired mutations may occur, which could significantly affect the phenotype of the generated mouse models (95).

Viral vector delivery system glioblastoma mouse models

Viral vector delivery is another approach that can modify multiple genes to generate somatic transgenic mouse models (37, 96–98). Several types of virus vectors can be used to deliver transgenic or mutant genes, including adenovirus, adeno-associated virus, lentivirus, retrovirus, etc. The main difference between lentiviruses and retroviruses is that lentiviruses are capable of infecting non-dividing and actively dividing cell types, whereas retroviruses can only infect mitotically actively dividing cell types. This means lentiviruses can infect a greater variety of cell types than retroviruses (99). Combining the lentivirus transfection-induced model with targeted conditional knock-out/knock-in transgenic mouse models makes it more convenient to study the pathways that drive glioblastomas (90, 100, 101). Lentivirus engineered to co-express the TAM induced CreER^{T2} along with *PDGFB* and GFP protein can spatially and temporally control the deletion of the floxed genes in specific cells as well as easily track the transduced cells. The glioblastoma penetrance of this model was as high as 88.5% (102).

The most widely used retrovirus induction system is the RCAS-TVA delivery system (42, 102). This approach uses replication-competent avian sarcoma-leukosis retrovirus (RCAS) vectors to target cells that are engineered to express cell surface receptor TVA (a receptor for the avian leukosis viruses (ALV) envelope glycoprotein) (7). RCAS-TVA transgenic mouse models are created by injecting RCAS vectors directly into mouse brain that expresses the RCAS receptor TVA (90, 103, 104). Combining the RCAS-TVA system with Cre-loxP and other transgenic systems provides a versatile method for producing glioblastoma mouse models containing different types of proliferating cells targeting different tissues (105). To generate glioblastomas, viruses were injected into different locations in

the brains of wild type mice or mice with various deleted tumor suppressors to overexpress different types of oncogenes in *Nes* and *GFAP* positive cells (43, 97, 104). These studies provided a new way to quickly establish mouse models so that the therapeutic responses of gliomas can be simultaneously compared (97). Glioblastoma mouse models can also be generated by combining RCAS-TVA system with CRISPR-Cas9 transgenic gene editing system to somatically delete tumor suppressor genes *p53, Cdk2a*, and *PTEN* in neural stem cells (NSCs) *in vivo* (90). This RCAS/TVA/Cas9 system is extremely versatile and accurate for somatic gene editing *in vivo* (90), which can help identify the various tumor-inducing factors of different glioblastoma types. One limitation of the RCAS-TVA system is that it requires the specific TVA-transgenic mouse strains. In addition, RCAS vector has a 2.5Kb DNA insert restriction. Genes of larger size cannot be inserted into the RCAS vector (7).

Transgenic mouse models are useful for observing specific genetic alterations involved in glioblastoma initiation and progression, but it is still uncertain whether the gene changes involved in these models truly mirror the tumor progression events in human glioblastomas. Most of the time, transgenic mouse tumors have specific gene mutations in specific cell types such that those tumors are more uniform and cannot completely reflect the phenotypic heterogeneity of human glioblastomas. To accurately reflect the heterogeneity of glioblastomas, mouse models have been created by combining several techniques to generate multiple complex genetic edits. In addition, tumor heterogeneity can also be maintained via transplantation of tumor specimen into mouse models (44, 90, 92, 97, 106).

TRANSPLANT GLIOBLASTOMA MOUSE MODELS

Besides gene ablation mechanisms, immune "escape" mechanisms may also play an important role in glioblastoma development (107). Even if more is known about the gene mutations related to glioblastoma, effective treatment is still difficult due to the microenvironment which can include immune suppressive cells, such as brain microglial and macrophages (23, 108). The brain tumor cells can escape from the immune cell surveillance, which facilitates glioblastoma aggression and can potentially induce drug resistance. Thus, understanding the function of the immune system in the glioblastoma microenvironment is most important for developing immune therapy for glioblastomas. Transplant models provide a natural tumor growth environment and have good control over tumor site and size, making them highly reproducible and excellent for tumor immunology studies and preclinical immunotherapy studies.

Transplantation of tumor cells into mice can rapidly generate experimental glioblastoma model for studying tumor biology and examining therapeutic methods. Many types of biological materials can be transplanted into mice brain by intracranial implantation (31, 109) or subcutaneous injection (110) techniques. This includes engineered murine tumor cells,such as GL261; engineered virus vaccines; Cre-LoxP, TAM/Tet/Dox induced tumor cells; and cancer cells/tissues from primary patient tumors (PDX). The injection can be done at either embryo stage or post-neonatal stage to induce experimental glioblastoma (7, 109, 111–113). Adult immunocompetent mice fail to tolerate the human-specific tumor

microenvironment (TME) features, while embryonic (E12.5) mice can be engrafted. The embryonic stage injection induces experimental glioblastoma that invade the mouse brain and exhibit the complex intact TME with vasculature, astrocytes, and immune cell infiltration (111, 114).

There are two types of transplant models: the allograft transplant model, which involves implanting tumor cells from the same species, such as mouse GL261 cell lines implanted into mouse brain (30, 112, 115, 116); and the xenograft transplant model, which involves implanting tumor cells cultured from different species, such as human glioblastoma cell lines implanted into mouse brain (117, 118). There are two techniques for the transplantation: stereotactic intracranial injection and subcutaneous injection. Intracranial injection is a more preferable approach used because it directly introduces glioma cells into the brain, where the tumor can develop under the naturally occurring immune environment to model glioblastoma progression and infiltration. Subcutaneous implantation lacks these characteristics (119).

Allograft transplant mouse models

Allograft transplant mouse models are usually produced in immunocompetent mice, which offers the intact immune system and same tissue context, and thus avoid immune rejection. The cell lines used in allograft mouse model include GL261, GL26, CT-2A, P560, and 4C8. The GL261, GL26, and CT-2A cell lines were generated from carcinogens, including N-ethylnitrosourea and 20-methylcholanthrene induced into C57BL/6 mice. P560 was from spontaneous VM/Dk mouse models. And 4C8 was from B6D2F1 mouse models (120, 121). These cell lines have their own characteristic immune markers that make them suitable for different studies (Table 2) (122–127). Among these cell lines, GL261 is the most widely used for many immunotherapy and gene therapy studies (128, 129). This cell line shares several characteristics with human glioblastomas (129–132). Histologically, GL261 tumors show features of ependymoblastoma (130). Immunologically, GL261 expresses high levels of major histocompatibility complex class I (MHC I) as well as MHC II, B7–1, and B7–2, CD31, CXC chemokine receptor 4 (CXCR4) (129, 131). Genetically, GL261 shares many gene mutations with human glioblastomas, including *RAS* oncogene and *p53* tumor suppressor gene point mutations (129, 132). In general, when 1×10^5 GL261 tumor cells are injected into C57BL/6 mouse brain in 2–4 μl, around 70% of mice will develop glioblastomas and survive for about 3–4 weeks (30, 109, 112).

Allograft transplant models have been used to study the immune mechanism for radiation therapy, immune checkpoint therapy, vascular endothelial growth factor (VEGF) therapy and vaccine therapy. Whole brain radiation therapy (WBRT) is one of the therapies tested in the GL261 model. Although WBRT itself has minimal advantage in terms of survival, this approach up-regulates β2-microglobulin expression in GL261 glioblastomas *in vivo* and *in vitro*, thus increasing $CD8^+$ T cell mediated antitumor immune response. When WBRT is combined with vaccine treatment, the long-term survival increased 40–80% (116). Immune checkpoint anti-PD-1 immunotherapy with radiation is another treatment that showed promise when tested in the GL261 mouse model by inducing activation and expansion in cytotoxic $CD8^+$ T cells. It can also allow the body

TABLE 2 Summary of allograft transplant cell lines

Cell Line	Established year	Mouse Strain	Histology	I.C. Cell numbers	Tumor initial time(pid)	Immune Characteristics	Citations
CT-2A	1992	C57BL/6	Anaplastic astrocytoma	8×10^4	15–20	Express Cd133, Oct, Nestin	(122, 172)
GL26	1969	C57BL/6	Ependymoblastoma	2×10^4	31	Express MHC I, lack MHC II	(123, 124, 173)
GL261	1970	C57BL/6	Ependymoblastoma	1×10^5	24–25	Express vimentin, c-Myc. K-ras mutation, p53 mutation, High levels of MHC I. Less MHC II, B7-1, and B7-2,	(174–176)
P560	1980	VM	Anaplastic astrocytomas	1×10^4	26	Have MHC I, Lack MHC II	(127, 177, 178)
4C8		B6D2F1	Oligodendrocytic astrocytes	1×10^4	51	Express MHC I and II	(125, 126)

to maintain long-term immunologic memory (116, 133). VEGF mediates angiogenesis, and its expression is highly correlated with malignant glioblastoma grade (134). GL261 mouse models have been used to test anti-VEGF combined with vaccination immune therapy. The therapy could significantly delay tumor progression and extend survival period, providing a foundation for further evaluation of the effects of antiangiogenic therapy in the context of endogenous or vaccine-induced inflammatory responses (112).

In addition to wild type C57BL/6J mice, GL261 has also been transplanted into C57BL/6J background transgenic mice to further study the different factors or mutated genes involved in glioblastomas. The Cre-LoxP system has been used to specifically knock-out H-2K^{b} or H-2D^{b} in targeted dendritic cells and macrophages in glioblastoma mouse models to study the role of each cell type in the activation of CD8^{+} T cells in response to these central nervous system immunological challenges. The role of each cell type in generating the CD8^{+} T cell responses was different. MHC I H2-K^{b} or H-2D^{b} antigen presentation by dendritic cells and macrophages in these model systems is non-redundant (30, 31, 115).

Xenograft transplant glioblastoma mouse models

Even though mouse GL261 glioblastomas have characteristics highly similar to human glioblastomas, the model cannot replicate the human immune system. Some studies have also shown that the GL261 cell line has genetically drifted and accumulated mutations (135). To reflect the human glioblastoma immune microenvironment, the xenograft transplant mouse model has been established.

Xenograft models are generated by transplanting human glioblastoma cells lines or fresh tissue into immunocompromised mice to induce glioblastomas. Hence this is also called patient-derived xenograft (PDX). This model maintains the genetic and the histological features of the primary tumor from glioblastoma patients. The cell lines or fresh tissue from glioblastoma patients share some similar genetic changes, such as mutation of *p53* tumor suppressor gene and *PTEN* gene, loss of *p14^{Arf}* and *p16*, and overexpression of *AKT* due to *PI3K/AKT* pathway up-regulation (136, 137). However, different cell lines or tissues have significant differences in histopathological characteristics. This results in the histology of human glioblastomas being highly variable. Multiple cell lines are being used in xenograft model, such as SF-7761, glioblastoma12, Hs683, etc. (118, 138, 139). Because the culture conditions in serial generation affect tumor cell phenotype and heterogeneity (140–142), researchers tend to implant freshly isolated tumor cells or tissue fragments without culture or only culturing for a short time (34, 143). Injecting fresh human glioblastoma tumor specimen provides the most direct attempt to capture important features of human glioblastoma without any *in vitro* selection or contact with serum.

Xenograft transplants use immunocompromised mouse strains. The most popular strains are: nude mice, severe combined immunodeficient mice (SCID), non-obese diabetic mice (NOD), non-obese diabetic severe combined immunodeficiency (NOD/SCID), NOD/SCID/interleukin-2 receptor gamma chain (IL2Rγ)^{null} (NOG/NSG), NOD/SCID/Jak3(Janus kinase 3)^{null} (NOJ), and recombination-activating gene 2 knock-out serial mice (Rag2^{null}), BALB/c Rag-2^{null}/IL2Rγ^{null} (BRG), Rag-2^{null}/Jak3^{null} (BRJ), BALB/c Nude Rag-2/Jak3 (Nude R/J) (Table 3). These strains are deficient in different immune cells, and this incomplete immune

TABLE 3 Summary of frequently used immunocompromised mice

Mouse Strain	Full name	Immune Characteristics
Nude mice	Athymic nude mice	No thymus, No T cells
SCID	Severe Combined Immunodeficient Mice	No T cells, no B cells
SCID/Beige	Severe Combined Immunodeficient Mice/ Beige	No T cells, no B cells, severe reduced NK cells
NOD	Non-obese diabetic mice	Pancreatic no T cells, impaired NK cells, macrophages and dendritic cells
NOD/SCID	Non-obese diabetic Severe combined immunodeficiency	No T cells, no B cells, impaired NK cells, macrophages and dendritic cells
NOG/NSG	NOD/SCID/interleukin-2 receptor gamma chain(IL2Rγ)^{null}	No T cells, no B cells, no NK cells, impaired macrophages and dendritic cells
NOJ	NOD/SCID/Jak3(Janus kinase 3)^{null}	No T cells, no B cells, no NK cells, impaired macrophages and dendritic cells
BRG	BALB/c Rag-2^{null}/IL2Rγ^{null}	No T cells, no B cells, no NK cells
BRJ	Rag-2^{null}/Jak3^{null}	No T cells, no B cells, no NK cells
Nude R/J	BALB/c Nude Rag-2/Jak3	No T cells, no B cells, no NK cells

cell depletion affects the transplant success rate (144, 145). Recently, NSG mice have been used more for PDX research because this strain has depleted interleukin-2 (IL-2) receptor gamma. IL-2 receptor gamma signaling pathway is essential for many types of hematopoietic differentiation, so the absence of this receptor causes a dysfunction in innate immunity such as NK cells. These characteristics make NSG mice an effective model for xenograft transplant of primary tumor tissues or cells (117, 146).

The mechanism of the many preclinical treatments has been tested using this model. Several human glioblastoma cell lines including wild type *H3.3* cell lines (SF9402, SF9427, SF9012 and GBM43) and *H3.3*^{K27M} mutant cell lines (SF8628, SF7761) were transplanted into female athymic nude mice to analyze the effect of GSK J4 treatment for *H3.3*^{K27M} -mutant cell *in vivo* and *in vitro*. The results demonstrated that GSKJ4 could reverse *H3.3*^{K27M} demethylation to serve as a therapeutic strategy for lethal pediatric glioblastomas (118). Temozolomide (TMZ), which induces cell cycle arrest at G_2/M and eventually leads to apoptosis, is an agent for chemotherapy used to treat glioblastoma (147, 148). TMZ is effective for some GBM cell lines in PDX models, such as Hs683 and U87, but not for T98G and U373 (138, 149, 150). The results obtained across different cell lines, suggest variability in glioblastoma characteristics and their role in responsiveness to TMZ. The mechanism of the viable response is not clear. Some studies showed that the resistance of GBM cell lines to TMZ therapy may due to level of methylated enzyme O6-methylguanine-DNA methyltransferase (MGMT). High levels of

methylated MGMT promotor showed more response to TMZ (151). But Dr. Egana et al. was not able to demonstrate MGMT methylation could influence patient survival in Glioblastoma. While combining TMZ with bevacizumab, an antiangiogenic antibody targeting *VEGF*, increased the survival of glioblastoma mice (152).

Xenograft transplant mouse models can preserve the genetic and histological complexity of the primary glioblastomas, but this model differs from patient tumors in many ways. Immunocompromised mice xenograft models do not have an intact immune system and lack the human tumor microenvironment. In addition, a high rate of copy number variations occurs in serial *in vivo* passaged xenografts, and the murine stroma can also gradually take over (34, 153). Xenografts with tissue directly from patients may be better than xenografts with cells that have been expanded *in vitro* (154), but the differences of immune system between human and immunocompromised mice means that PDX models may not accurately reflect the biological nature of glioblastoma in patients, which is a disadvantage when it comes to preclinical drug studies and chemotherapeutic drug studies. Therefore, it is imperative to find a mouse model that can investigate human glioblastoma development and immunotherapy efficiency in human TME with intact immune system.

Humanized mouse models

To obtain mouse models with fully competent human immune systems, which enable researchers to examine the interaction between the tumor, immune system, and microbiome for patient preclinical therapy, humanized mice have been generated. These mice have been extensively used for discovering effective immunotherapeutic agents and their combinations (155). Several types of humanized mouse models have been generated, such as PDX human hematopoietic stem cells (HSCs) humanized mice and human microbiota-associated (HMA) humanized mice (156–158). To create PDX HSCs humanized mice, scientists inject human peripheral blood mononuclear cells (hu-PBMCs) or HSCs, or specific HSCs such as Hu-CD34$^+$ HSCs (hu-CD34$^+$) directly into immunodeficient mice after 50–250 cGy whole body irradiation (117, 156). The success of the humanization process is that mice have more than 25% human CD45$^+$ cells in their peripheral blood. Hu-PBMCs mice develop T cells and B cells. It is a model for research on compounds for T cell immune modulation and graft rejection. Hu-CD34$^+$ humanized mice develop almost all human stem cell lines, including T cells, monocytes, macrophages, mast cells, myeloid (SGM3) cells, NK cells (IL-15), and dendritic cells. It is a more advantageous *in vivo* model for long-term studies in the fields of human immune cell biology, immuno-oncology, and infectious disease. Jackson lab provides several types of mouse model for different study purposes. The most popular humanized immunocompromised mice strains are NOD. *Cg-Prkdc*^{scid} *Il2rg*^{tm1Wjl} Tg(CMV IL-3, CSF2, KITLG)1Eav/MloySzJ (NSG-SGM3), and NOD,B6.SCID *Il2rγ*$^{-/-}$ *Kit*$^{W41/W41}$ (NBSGW) mice (NBSGW) (117). NSG-SGM3 mice delete IL-2, but express human IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF) and stem cell factor. These factors enable the stable transplant of human HSCs for humanization (117, 159). NBSGW mice carry c-Kit mutation to support the transplantation of HSCs without irradiation because c-Kit plays a role in cell survival, proliferation, and differentiation (160). A humanized mouse can also be generated by a conditional knock-in/out of a

specific human gene or a piece of genomic sequence to precisely target a certain tissue, such as SRG-15 mice with knock-in human SIRPA and IL-15 to develop the innate lymphoid cell subsets and NK cells (161); MHC NOG-dKO mice with double knock-out MHC class I and II in NOG mice (162); and NSG-SGM3-BLT mice which involves implanting human fetal liver and thymus fragments as well as hematopoietic stem cells into immunocompromised NSG-SGM3 and NOD/SCID mice (163).

In addition to the PDX HSCs humanized mice, recently scientists also tried to generate HMA humanized mouse models since research has shown that the gut microbiome is linked to some immune-mediated and metabolic pathologies such as obesity, type 2 diabetes, and cancer (163–166). Recent studies found that balance of commensal microorganisms is important for cancer etiology and that gut microbiota can impact the treatment for cancers (167). Although mouse and human share 85% similar genomes, they have significant differences in gut microbiota composition. Around 85% of mouse gut bacteria are not found in human (168). Considering the relationship of gut microbiota composition and cancer development, scientists generated HMA models to avoid the impact on immune system by gut microbiota composition. HMA is established by using microbiota transplantation to transplant human fecal microbiota to germ-free mice (169).

Several studies have used the HMA model (164, 165). After successfully establishing the HMA mouse model, GL261 glioma cells were intracranially transplanted to set up glioblastoma HMA model for studying the response to anti-PD-1 or anti-PD-1 combined with TMZ treatments. The mice that survived longer have higher IFN-γ and higher $CD8^+$/Treg ratio than those that survived shorter. This difference in treatment response was due to the difference in the microbiomes from different patients (164).

Humanized mouse models by themselves or combined with transgenic mouse models highlight a new way to investigate the relationship between glioblastoma development, human immune system, and human microbiota system. It also provides a new platform to study the anticancer immune response for specific immunotherapeutic interventions. However, xenograft PDX humanized mouse models still have challenges due to host innate immune response in immunocompromised mice to the engraft of human cells/tissues, limited lifespan of the mice, incomplete human immune function, and poor lymphoid architecture (155, 170). The HMA humanized models also have many biological and technical problems. Whether the human donor microbiomes are successfully transplanted into germ-free mice and whether this model is reproducible still needs confirmation. The mucus properties of germ-free mice are different from conventional mice, which may not completely reflect the human response (169, 171). In addition, the transplant procedure may destroy tumor tissue architecture. Therefore, more research is needed to determine whether transplant mouse models are suitable for glioblastoma studies.

CONCLUSION

Mouse models are extremely useful for studying the biology of glioblastoma. Scientists use mouse models suitable for their experiments to gain insight into mechanisms and factors concerning tumor molecular processes, tumor

progression microenvironment, and immune and preclinical therapeutics. Spontaneously induced tumors better reflect the natural tumor growth and immune environment change; transgenic mouse systems focus on the targeted genes and pathways for tumor progression; and transplant models are better for tumor immune therapy studies. From Cre-LoxP germline transgenic mouse models to virus vector transgenic somatic transgenic mouse models, many cutting-edge technologies are combined to create combinations of gene mutations that reflect the complexity of glioblastoma in human. This will help in identifying more genotype-specific susceptibilities of human glioblastoma types, manipulating the human glioblastoma epigenome, developing glioblastoma gene therapy and immune therapy in humans, and eventually enabling more personalized, genotype, and phenotype-based treatments for glioblastoma patients in the future.

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