ANTICANCER RESEARCH *41*: 5343-5353 (2021)
doi:10.21873/anticanres.15347

Review

Large Animal Models of Glioma: Current Status and Future Prospects

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Abstract. *Enhanced understanding of the molecular features of glioma has led to an expansion of murine glioma models and successful preclinical studies. However, clinical trials continue to have a high cost, extended production time, and low proportion of success. Studies in large-animal models of various cancer types have emerged to bridge the translational gap between in vitro and in vivo animal studies and human clinical trials. The anatomy and physiology of large animals are of more direct relevance to human disease, allowing for more rigorous testing of treatments such as surgical resection and adjuvant therapy in glioma. The recent generation of multiple porcine glioma models supports their use in high-throughput preclinical studies. The demonstration of spontaneous glioblastoma formation in canines further provides a unique avenue for the study of de novo glioma. The aim of this review was to outline the current status of large animal models of glioma and their value as a transitional step between rodent models and human clinical trials.*

Advances in molecular technology have yielded an array of genetically engineered mouse models that reproduce the various features of glioblastoma (1). The strides made in these small animal (rodent) models have improved our understanding of this near-universally fatal disease and have led to many successes in drug therapies. These successes, however, have not been mirrored in human clinical trials, as made evident by less than 8% of recent cancer drugs proceeding beyond phase I (2). While a positive trend in the number of phase II clinical trials for glioblastoma exists, the converse is true of phase III results (3, 4). Clinical trials are resource-intense, with approved cancer therapies now lasting around 8 years and costing 1.2 billion dollars to develop (5). Indeed, the average development time from phase II to phase III is estimated to be 7.2 years in glioblastoma clinical trials (6). Since the approval of temozolomide in 2005 for the treatment of glioblastoma, there have been no approved therapies that improve overall survival (7, 8). The intensive nature and limited success of clinical trials highlight the need for more representative preclinical animal glioblastoma models. The present review explores the emerging evidence for reliable and reproducible large animal models of glioblastoma.

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Key Words: Glioma, glioblastoma, large animal, pig, dog, non-human primate, review.

Historical Perspectives

The role of animal models in glioblastoma research, and other cancer types, has largely been twofold: One, to better understand the molecular events leading to tumorigenesis, and two, to study the effectiveness of existing and new treatment strategies. Mice have been the primary organism of preclinical animal cancer modeling, and improved outcomes in patients with cancer have been due, at least in part, to their versatility for subsequent therapeutic development. However, as we enter the next era of therapeutic development, the

limitations of murine cancer models have sparked a search for alternative animal models, including large animal models. Large animal models overcome many of the anatomic and physiological limitations of modeling human disease in rodents. While large animal models are not intended to supplant all aspects of rodent research, they may serve as a transitional step in therapeutic development that better replicates human physiology and thus improves success in clinical trials (Figure 1) (9). The laboratory facilities required for genetic manipulation of these large animal models are not yet on par with those for mouse models; however, a growing interest in the field is expected to lead to expansion of their applicability and use (9). Common large-animal models include porcine (pig) and canine (dog) models, and, less commonly, non-human primate (NHP) models.

Limitations of Murine Cancer Models

The advantages of murine models are well documented and contributed to their establishment as the dominant preclinical animal cancer model. The small size of each organism is ideal for handling and care, lending to lower overall maintenance costs and a limited burden on individual labs and institutional housing centers (10). The rapid speed of reproduction coupled with large litter sizes is favorable for breeding and maintenance of genetically engineered mice populations (10). In addition, extensive characterization of the mouse genome has allowed for both ease and precision of genetic manipulation (10). Current molecular technology permits the spatial and temporal control of tumor formation and progression in mice, leading to tumors that better recapitulate their human counterparts (11). Logistically, mice have been an optimal organism for modeling the genetic and physiological features of cancer, but the significant difference between mice and humans contributes to their translational limitations.

On first observation, there are evident anatomical and physiological differences between humans and mice, most apparent of which is size, with humans growing to be 3,000-fold larger than mice (12). The size difference is magnified for the brain, given the fact that the human brain is over 100 times larger in weight and more than 1,000 times larger in surface area and number of neurons (13, 14). This limitation is evident, for example, in the study of optic glioma in a mouse model of neurofibromatosis type 1, where the optic nerve is smaller than a grain of rice, thus limiting the use of imaging and surgical techniques (9). Further, the mouse brain is lissencephalic, lacking the gyration and cortical development characteristic of humans and other large animals (15). Lastly, functional aspects arising from neural network phenomena, such as seizures or cognitive dysfunction, cannot be modeled in animals where the networks of interest are absent or not easily comparable with humans (16, 17). Thus, in the study of glioblastoma, well known for its infiltration of the brain parenchyma, critical anatomical differences in the organ of origin impose potentially confounding factors in preclinical investigation.

On average, humans live 30-50 times longer than mice and are known to undergo 10^5 more cell divisions, thus harboring an increased risk of neoplastic transformation relative to mice (12). The short lifespan of mice further limits the development of certain types of cancer or highly penetrant cancer associated with loss of heterozygosity mutations (12, 18). Mice also exhibit significantly higher metabolic rates than humans, posing additional challenges to pharmacodynamic and pharmacokinetic studies (12). While drug doses can be extrapolated between mice and humans, in cancer chemotherapies with narrow therapeutic indices, the ambiguity can further contribute to limitations for phase I trials (19). In humans, the blood–brain barrier (BBB) is a major obstacle for the delivery of drugs to the central nervous system (7, 20). An increased proportion of neocortical astrocytes, pericyte heterogeneity, and differences in vascular anatomy between humans and mice present additional challenges to preclinical modeling (21-23). With over 80% of the mouse genome directly correlated with human orthologs, there are genetic correlates to many of the driver mutations of human cancer (24). However, there are well-documented examples of mutation resulting in different phenotypes in mice and humans. Loss of heterozygosity in the APC gene leads to invasive carcinoma in the human intestine while in mice it is associated with intestinal polyps with limited infiltration (25, 26). Similarly, knockout Brca1/2 mice display no cancer while this mutation is highly correlated with human breast and ovarian cancer (27).

While an extensive review of mouse models of glioma is outside the scope of this review, glioma modeling has largely been accomplished by chemically induced syngeneic or autologous transplants, human glioma xenografts, and genetically engineered models (1, 28, 29). As future cancer chemotherapies are set to be targeted to specific molecular profiles, there is an increased need for animal cancer models that faithfully and reliably recapitulate human cancer.

Large Animal Models of Glioblastoma

Large-animal cancer models offer significant anatomical, physiological, and genetic advantages to preclinical cancer modeling. Porcine models have a long history in biomedical research, ranging from insulin production to surgical technique development, and are arguably the most evolved and versatile of the large-animal glioma models (30). The pig brain is gyrencephalic, better mirroring the convoluted surface of the human cortex, and better recapitulating tumor infiltration, drug delivery, and drug diffusion within cortical structures (Figure 2) (13, 15, 31-34). The size of pigs also offers significant benefits for high-resolution imaging of the brain (13, 32). Pigs also have a relatively large litter capacity, with up to 20 offspring per year (15). In addition, pigs pose fewer ethical concerns than their canine or NHP counterparts, in part because their behavior

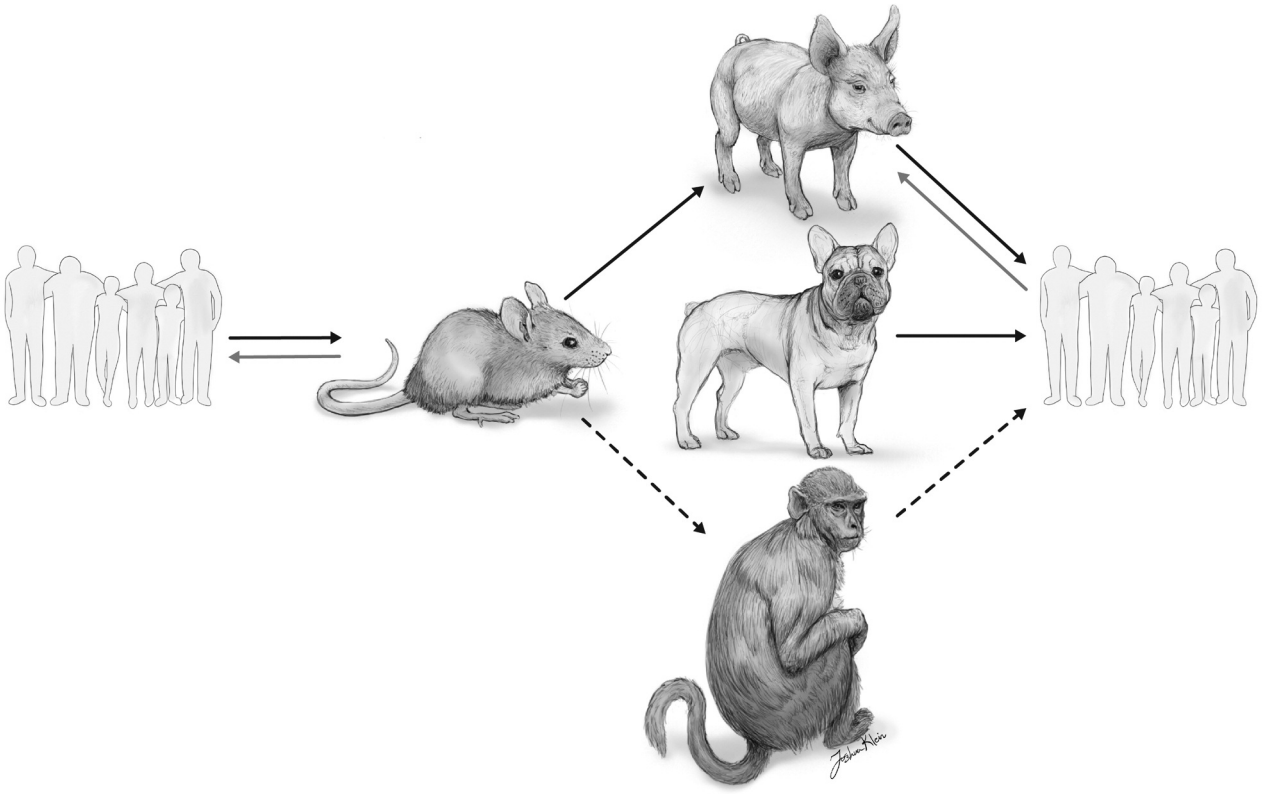

Figure 1. *Proposed workflow for translational glioma research between rodent models, large animal models, and human clinical trials.*

repertoire is more limited. Canines have demonstrated spontaneous formation of glioblastoma, thus offering a unique opportunity to study glioma in the absence of exogenous manipulative factors (35). NHPs are phylogenetically closer to humans and provide a high degree of similarity in terms of brain anatomy and molecular drivers of glioma-genesis (36). A discussion of NHP models is included for completeness; however, ethical considerations have limited their use as preclinical cancer models in many countries.

Porcine models. Domestic or agricultural pig breeds have long been used based on their low price and wide availability for common breeds such as the Landrace, Yorkshire, and Duroc (15). However, at mature size, pigs of these breeds weigh over 300 kg, posing a challenge for laboratory maintenance. To overcome these limitations, minipigs such as the Yucatan, Göttingen, and Sinclair breeds have been employed (15). With a maximum size ranging from 35-90 kg, minipig models better compare in body weight to humans and are generally favored over agricultural breeds (15). Sequencing of the genome of the domestic pig, Sus scrofa, was completed in 2012, thus expanding its applicability to biomedical and cancer research (37-40). Since then, further studies have provided the genome sequences of several laboratory pig breeds, with a profound impact on the development of genetically engineered porcine models (41, 42). More continuous and complete mapping of the porcine genome, recognition of homologous disease-linked

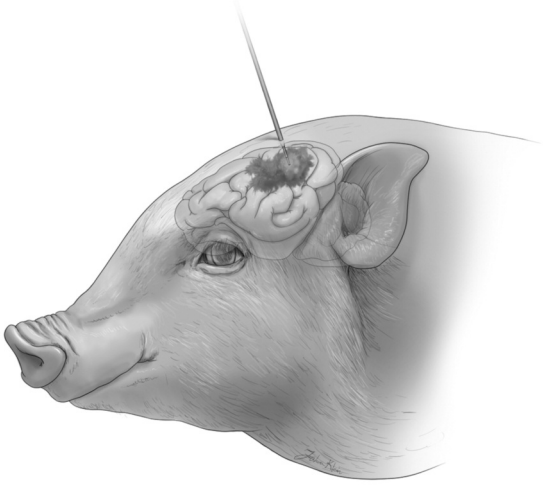

Figure 2. *Porcine brain anatomy bears greater similarity to the human brain, allowing for advanced treatment approaches, ranging from surgery to convection-enhanced delivery.*

Table I. *Porcine models of glioma.*

Type of model	Pig species	Source	Tumor formation, n	Macroscopic tumor	Histopathological tumor	Reference
CLX	Landrace	U87	14/15	Yes	Yes	Selek *et al.* (2014) (47)
		G6	1/5	Yes	Yes	Selek *et al.* (2014) (47)
	Yucatan minipig	U87	8/9	Yes	Yes	Khoshnevis *et al.* (2017) (48)
			5/5	Yes	Yes	Khoshnevis *et al.* (2020) (49)
GEM	Göttingen minipig	Lentiviral vector	6/6	Yes	Yes	Tora *et al.* (2020) (50)

CLX: Cell-line xenograft; GEM: genetically engineered model.

single nucleotide polymorphisms between humans and pigs, and awareness of reduced population genetic variation in pigs have further advanced these models (30, 43, 44). Comparative analysis of the pig and human genomes revealed similarities in epigenetic regulation and gene-transcription profiles, another supporting factor for modeling human genetic diseases (45). Indeed, successful genetic engineering has developed pig models of Huntington's disease, melanoma, neurofibromatosis type 1, hepatocellular carcinoma, and adenomatous polyposis (37-40, 46). Thus, it is reasonable to assume glioma might be modeled in the same fashion.

To date, there are several useful porcine models for glioma (Table I). As laboratories make the appropriate accommodations for larger animals, it is anticipated that this list will continue to expand. Like humans, tumor formation in pigs is rare, and a large gap in our understanding of the natural formation of cancer in pigs is attributed to the limited lifespan that domestic breeds have before commercial utilization (9). As a result, there is no current evidence of spontaneous glioma formation in pigs.

Two distinct pig models of glioma have been developed using human cell-line xenografts (CLX). Human glioma cell lines are derived from serially maintained cultures of prior resected human glioma and are commercially available. Cell lines commonly used in xenograft models include U87, U251, T98G, and A172 (28, 29, 51). Application of glioma CLX in pigs was first achieved when Selek *et al.* developed the first large-animal model of glioma (47). Utilizing 3-month-old Landrace pigs, a total of 21 animals were transplanted with U87MG (or U87) cells or G6 tumor stem cells. Given that CLX are prone to acute rejection due to immunological cross-reactivity of host immunity to human cells, a prerequisite condition is an immunosuppressed host. In mice, this has been accomplished *via* genetically engineered immunodeficiency (52). While a pig equivalent of this model does not yet exist, Selek *et al.* achieved sufficient immunosuppression through oral cyclosporine (47). Of note, genetically engineered pig hearts with α1,3-galactosyltransferase knockout and transgenic expression of human CD46 and thrombomodulin displayed reduced immunogenicity for cardiac xenotransplantation, pointing to emerging work on this front (53). Fourteen out of fifteen U87MG recipient pigs showed macroscopic tumor formation and neurological symptoms within 30 days. Magnetic resonance imaging (MRI) of the U87MG tumors were characterized by regions of brain iso-intensity on T1- and hyperintensity of T2-weighted images, consistent with human glioma (47). On histopathological analysis, the tumors displayed increased cellularity, angiogenesis, infiltration of normal brain parenchyma, and a degree of pseudo-palisading necrosis (47). On immunohistochemistry, tumor tissue stained positively for expression of glial fibrillary acidic protein (GFAP), consistent with an astrocytic character of the tumor (54, 55).

Soon after, Khoshnevis *et al.* induced the formation of glioblastoma in the Yucatan minipig *via* CLX of U87 cells (48). By 28 days post-induction, eight out of nine pigs displayed macroscopic tumor formation visible by computed tomography, with masses that were histopathologically indistinguishable from human undifferentiated glioma (48). One immediate application of CLX models is the ability to evaluate drug-delivery methods. Using the Yucatan minipig U87 glioblastoma model, stereotactically delivered intratumoral ^{165}Ho-siloxane was used to assess the feasibility of microbrachytherapy, the injection of microspheres of concentrated radioactive agents, to treat glioblastoma (49). The lower average size of the Yucatan minipigs facilitated animal maintenance, lower doses of cyclosporine, and use of stereotactic frames (48). The ability to demonstrate successful glioma formation in two laboratory pig species represents a replicable model for future glioma xenograft studies.

While xenograft models allow for the assessment of tumor drug response, they pose several limitations (56). The requirement for an immunocompromised host alters the natural tumor–immune relationship and hinders the development of immunological therapy. While the BBB provides partial immunological isolation from the rest of the organism, a detailed characterization of the pig immune system and its impact on xenograft models have not been achieved (57, 58). Further, the loss of intratumoral heterogeneity arising from serial cell culture leads to cell lines that lack the genotypic and phenotypic heterogeneity of

de novo glioma (59). Lastly, the use of established cancer cells limits study of the events leading to tumor formation and progression.

Recently, Tora *et al*. developed the first genetically engineered glioma model in pigs *via* lentiviral-induced formation of high-grade spinal cord glioma (50). In rodents, retroviral expression of platelet-derived growth factor-β, HRAS-G12V, shRNA-p53 resulted in glioma formation (60-62). Employing a similar strategy, Göttingen minipigs were injected with lentiviral vectors expressing platelet-derived growth factor-β, constitutively active HRAS, and shRNA-p53 (50). MRI of the spinal cord mass demonstrated radiological characteristics similar to those of xenografted glioma models (47, 50). Lesions confirmed as high-grade glioma on histopathological analysis demonstrated invasion of white and gray matter, high cellularity, elevated Ki-67 expression, astrocytic morphology, and immunohistochemical evidence of GFAP and oligodendrocyte transcription factor 2 (OLIG2) expression (50). Lentiviral induction of a glioma model with multiple genetic abnormalities can be used to model the genotypic heterogeneity of glioma, allowing for the study of several common driver mutations (63). A critical advantage of a genetically engineered model is the use of an immunocompetent organism, which permits investigation of the tumor-immune relationship and the testing of immunotherapies. Application of CRISPR/Cas9 and Cre recombinase-loxP mutational methods to pigs offers another means of recapitulating the molecular features of human glioma (18, 42, 64).

Canine models. Since the 1960s, canine models have been utilized to study cancer progression and treatment. The earliest canine studies induced malignancy in mongrel dogs using the Rous sarcoma virus or live avian sarcoma virus (65, 66). This created a replicable model for canine malignancies, especially astrocytoma, that could be used for treatment evaluation (65). The canine tumors created through viral induction could then be resected, frozen, reanimated, and implanted in a different mongrel dog, thus amounting to a transplantable canine cancer model (66). The transplanted canine model exhibited a 93% tumor growth probability (66). Krisht *et al*. expanded this work by implanting tumors grown in nude mice derived from canine gliosarcoma cells into five immunosuppressed adult mongrel dogs, of which four developed cavernous gliosarcomas (67). While these models displayed high penetrance for tumor formation, they did not recapitulate the spontaneous tumors observed in humans. However, dogs spontaneously develop many cancer types including osteosarcoma, lymphoma, melanoma, and glioma (35). These spontaneous tumors can help to bridge the translation of findings from engineered cancer treated in murine and *in vitro* models to human clinical trials (5). In 2015, the National Cancer Institute's Comparative Brain Tumor Consortium was created to guide the development of new treatments for human brain tumor patients through the evaluation of canine brain tumors (68).

As in human patients, glioma is the most common primary malignant brain tumor of canines (69). Brachycephalic breeds, including Boston terriers and boxers, exhibit an elevated genetic predisposition for glioma formation and are often included in studies of these tumors (69). Canine glioblastomas display a similar histological pattern as human primary glioblastoma, including GFAP/vimentin expression, pseudo-palisading necrosis, increased angiogenesis, microscopic invasion, hypercellularity, and inflammation (Figure 3) (69, 70). A comparison of the genomic profiles of canine and human glioma by Amin *et al*. revealed similarities in driver mutations, the timing of mutations, and epigenetic patterns of canine glioma and pediatric glioma (71). Dogs harboring brain tumors can receive treatment with maximal surgical resection followed by adjuvant therapy. As with humans, survival for dogs with glioma is dependent on the extent of resection, with longer survival for dogs with gross total resection as compared to those with subtotal or no resection (72). Canines with glioma exhibit 1-to 2-month survival without treatment (69). Thus, they can be used to test therapies before human trials (Figure 1). Dickinson *et al*. utilized spontaneous canine gliomas to evaluate convection-enhanced delivery of liposomal irinotecan, a topoisomerase I inhibitor, and gadoteridol in nine dogs with grade 2 or 3 gliomas (73); 88% of those tumors exhibited a post-treatment decrease in volume and demonstrated the importance of monitoring with MRI while performing convection-enhanced delivery (73). The ability of minicells containing doxorubicin to cross the BBB and selectively infect glioma tumor cells while sparing systemic toxicity has been evaluated in dogs with spontaneous glioma (74). This led to a phase I human clinical trial in Australia and the USA (75). Application of the workflow from mice to a large-animal model was seen with pharmacological testing of nanoparticle delivery of immune-modulatory microRNA to a canine model following successful trials in mice (76).

The principal benefit of studying spontaneous canine tumors is their ability for significant, natural tumor growth during a reasonable time period and the feasibility of treating in a multistep fashion under conditions similar to human tumors (73). Another value of the canine model is the heterogeneity that mimics that seen in human patients. There is inter-patient heterogeneity, as dogs of varying ages and breeds develop different types (i.e., oligodendroglioma, astrocytoma) and grades of glioma in diverse brain locations, each having different immunological and genetic profiles (77, 78). There is also intra-patient heterogeneity, as spontaneous canine glioblastoma exhibits pleomorphic cells, necrosis, vascular proliferation, and pseudo-palisading that is seen in human glioblastoma but not in murine xenografts (77). While spontaneous canine gliomas are useful during advanced stages of testing for novel therapies, they are not as useful for the characterization of the effect of single mutations on glioma-genesis, which is better accomplished in genetically

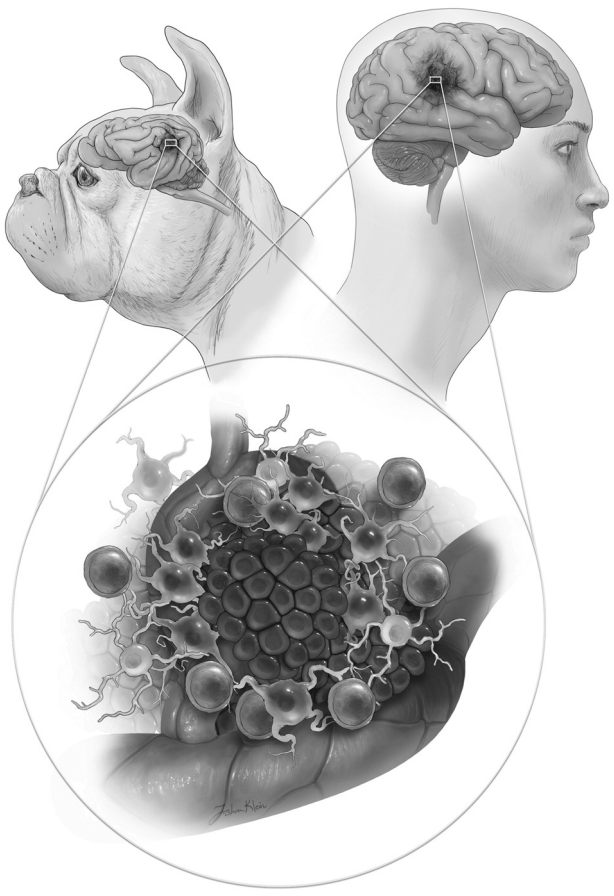

Figure 3. *Histopathological and immune microenvironment similarities between de novo canine and human glioma open opportunities for immunological studies that are not feasible with other animal models.*

engineered mouse models (77). Another limitation of these models is the genetic makeup of common companion dogs. Some outbred dogs, such as those with a mixed background, better replicate the diverse genetic background characteristic of humans, while inbred dogs often lack genetic diversity (5). Experimental sample size can also be a limitation for these studies given that only 12,000 dogs will develop detectable brain tumors annually in the USA and a minimum of 18-20 has been proposed as the sample size necessary for testing in canine clinical trials (77). The limited numbers available and the requirement for reagents suitable for canines limit the feasibility of these studies (79). An additional limitation for natural history and treatment studies is the practice of early euthanasia at different stages after symptoms develop (72, 79). The performance of neurological examinations by veterinary neurologists combined with serial MRI monitoring can allow objective quantification of progression-free survival, which unlike overall survival is not affected by euthanasia (80).

Non-human primate models. NHPs are attractive models for studying human disease because they are the most physiologically, anatomically, and genetically similar animals to humans, and therefore may best recapitulate tumor behavior and therapeutic responsiveness (36, 81). Indeed, there is a near 1:1 homology between NHP and human genomes (36). Nevertheless, studies in NHP models of cancer have been limited primarily to case reports and series of incidentally discovered tumors (82-85). One case series studied radiation-induced glioblastoma in *Macaca mulatta* (rhesus macaque) (86, 87). Lonser *et al*. demonstrated that after fractionated whole-brain radiotherapy (3,500 cGy over 2 weeks), a majority of *M. mulatta* developed glioblastoma between 2.9 and 8.3 years after radiotherapy (86). These tumors exhibited histological and genomic characteristics similar to *de novo* human glioblastoma (86). In a follow-up study, tumors in these animals with multiple glioblastomas likely arose from distinct precursor cells (87). Furthermore, primates that did not develop glioblastoma had histological evidence of atypical tumor precursor cells without common glioblastoma biomarkers (87). Together, these studies demonstrated that it was possible to model radiation-induced glioblastoma in *M. mulatta* after whole-brain radiation despite a highly variable time range of tumorigenesis (86, 87). The long latency period before apparent tumorigenesis may limit its utility to inform clinical trials. Nevertheless, this model can prove useful to study the pharmacokinetics and pharmacodynamics of potential glioma chemotherapies (88). However, BBB integrity may not recapitulate drug cerebrospinal fluid penetration in the disrupted blood–tumor barrier typical of glioma (20).

A lentiviral-induced glioblastoma model in Tupaia belangeri (tree shrew) was achieved *via* lentiviral-mediated overexpression of *H-Ras* and silencing of *Tp53* and is characterized by mesenchymal subtype glioblastoma (89). This glioblastoma displayed classic histological features of high-grade gliomas together with greater genetic similarity to human glioblastoma as compared to mesenchymal mouse-derived glioblastoma (89). Notably, there was a 100% success rate of glioblastoma formation and onset of neurological symptoms as early as 1 month after lentiviral injection (89). The phylogenetic classification of *T. belangeri* has been debated, with evidence supporting a closer association with primates than previously thought and other studies reporting the contrary (90, 91). Nonetheless, it provides another possible model for large-animal glioma modeling.

NHP models are limited by the associated ethical considerations. It has been argued that the study of NHPs is justified and necessary due to their homology with humans, while others view it as unnecessary due to the lack of significant scientific discovery arising from use of these models (92). The primary consideration when initiating research with NHPs is the potential for benefit to humans. However, as with

most novel scientific approaches and with hypothesis-driven research, the value of the research is not *a priori* predictable. The debate and controversy around NHP research have led to the reduction of NHP use in the United States and European Union (93, 94). With now widely available genome-editing technologies, the limited progress made in glioblastoma has raised interest in their use in NHPs (95).

Conclusion

As the need for effective glioma treatments remains unmet, attention has been turned to the development of more informative preclinical animal cancer models. In recent years, reproducible models of glioma have been developed in pigs using human xenografted glioma cell lines and lentiviral vector induction. Canines are shown to spontaneously develop glioblastoma, however, the relative rarity of this occurrence and lack of experimental reproducibility hinders its use. The use of NHPs, while likely to be most genotypically and phenotypically similar to humans, is limited by ethical considerations. Thus, based on the current evidence, porcine glioma models appear to be the most developed and promising of the preclinical large-animal models. The study of xenograft and genetically engineered porcine models offer a potential tool of investigation to overcome the poor translational outcomes of human clinical trials.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

Authors' Contributions

Conceptualization, TYEA; Data curation, WHH, MNP, CEB, TYEA; Writing original draft preparation, WHH, MNP, CEB; Writing review and editing, WHH, MNP, CEB, ASH, AD, KGA, SGA, RTB, AAC, RMB, BEM, JMP, TYEA. All Authors have read and agreed to the published version of the article.

Acknowledgements

The Authors would like to thank Josh Klein for the illustration of the figures.

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Received September 7, 2021
Revised September 25, 2021
Accepted September 29, 2021