Preclinical Evaluation of Radiation and Perifosine in a Genetically and Histologically Accurate Model of Brainstem Glioma

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Abstract

Brainstem gliomas (BSG) are a rare group of central nervous system tumors that arise mostly in children and usually portend a particularly poor prognosis. We report the development of a genetically engineered mouse model of BSG using the RCAS/tv-a system and its implementation in preclinical trials. Using immunohistochemistry, we found that platelet-derived growth factor (PDGF) receptor α is overexpressed in 67% of pediatric BSGs. Based on this observation, we induced low-grade BSGs by overexpressing PDGF-B in the posterior fossa of neonatal nestin tv-a mice. To generate high-grade BSGs, we overexpressed PDGF-B in combination with Ink4a-ARF loss, given that this locus is commonly lost in high-grade pediatric BSGs. We show that the likely cells of origin for these mouse BSGs exist on the floor of the fourth ventricle and cerebral aqueduct. Irradiation of these high-grade BSGs shows that although single doses of 2, 6, and 10 Gy significantly increased the percent of terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)–positive nuclei, only 6 and 10 Gy significantly induce cell cycle arrest. Perifosine, an inhibitor of AKT signaling, significantly induced TUNEL-positive nuclei in this high-grade BSG model, but in combination with 10 Gy, it did not significantly increase the percent of TUNEL-positive nuclei relative to 10 Gy alone at 6, 24, and 72 hours. Survival analysis showed that a single dose of 10 Gy significantly prolonged survival by 27% (P = 0.0002) but perifosine did not (P = 0.92). Perifosine + 10 Gy did not result in a significantly increased survival relative to 10 Gy alone (P = 0.23). This PDGF-induced BSG model can serve as a preclinical tool for the testing of novel agents.

Introduction

Gliomas may arise anywhere in the central nervous system (CNS). An uncommon location for gliomagenesis in the CNS is the brainstem. Brainstem gliomas (BSG) develop predominantly in the pediatric population where they constitute up to 20% of childhood brain tumors and are the leading cause of death for children with brain tumors (1). According to histopathologic characteristics, BSGs are divided into high grade (WHO grades 3 and 4), which comprise ∼85% of BSGs [also called diffuse intrinsic pontine gliomas (DIPG)] if the tumor is primarily localized to the pons], with the remaining being low grade (WHO grades 1 and 2). By contrast, BSGs in adults are much less common, accounting for <2% of gliomas. Furthermore, BSGs in adults are less aggressive than those in children and are thought to be biologically different (2).

Focal radiation consisting of daily 2 Gy doses for ∼30 doses over 6 weeks is the current standard of care for these tumors—a treatment modality that unfortunately provides only temporary relief of symptoms. Despite numerous clinical investigations to date, there have been no chemotherapeutic or biological agents that have proven clearly beneficial for the treatment of high-grade BSGs in children. The median survival for these children is <1 year after diagnosis, although it has been observed that children <3 years of age may fare better (3).

There is limited understanding of the biology of BSGs in children due to limited availability of clinical specimens. As these tumors are not routinely resected and autopsy rates are quite low, such clinical specimens are extremely precious (4). The limited studies published on this entity show that these tumors have similar genetic alterations as pediatric gliomas arising in other parts of the brain but distinct from the molecular characteristics of adult gliomas (5–10). Erbb1 is amplified in a small subset of BSGs and overexpressed in a larger subset (11). Kras and AKT activation have been...
described in 60% of pediatric high-grade gliomas including several BSGs (12). p53 mutations have been described ranging from 29% to 71% of pediatric BSGs (8, 10, 13–16). The most common reported molecular alterations occur in platelet-de-
derived growth factor receptor (PDGFR), which is overexpressed in about 30% to 80% of high-grade gliomas including BSGs (17–19), and loss of expression of Ink4a-ARF, which has been described ranging from 17% to 100% of pediatric glioblasto-
mas in the brainstem (8, 16, 20).

There are two published rat models of BSG, both are allo-
graft models using cell lines derived from rat cortical gliomas that are stereotactically implanted into the brainstem. The rats develop tumors in the brainstem, but it is not clear to what extent these accurately model the histology and genetics of the human disease (21, 22). The cell lines are derived from a carcinogen-induced cortical glioma and are main-
tained in serum conditions before implantation, culture condi-
tions that are inferior to culturing in basic fibroblast growth factor and epidermal growth factor in maintaining the phenotype and genotype of primary gliomas (23). In addi-
tion, a human xenograft BSG model in rats has also been recently reported, which uses adult glioma cell lines that arose from adult cortical gliomas and are implanted into immuno-
deficient rats (24). To our knowledge, there is no genet-
ically engineered mouse model for this disease.

Here, we initially observed that PDGFRα is overexpressed in 67% of high-grade BSGs overall, including 87% of biopsy speci-
mens. We used the RCAS/tv-a system to overexpress PDGFR-B in nestin-expressing cells lining the fourth ventricle and aque-
duct. This gene transfer resulted in the formation of low-grade BSGs, whereas PDGF overexpression in conjunction with Ink4a-ARF loss in the same periventricular cells resulted in the formation of high-grade BSGs. These tumors were histo-
logically similar to diffuse pediatric BSGs in many respects. A short-term analysis of radiation therapy (RT) in this BSG mod-
el showed that higher doses of RT are more effective in induc-
ing cell cycle arrest. As the AKT pathway is thought to mediate radioresistance (25), we tested perifosine, an inhibitor of AKT signaling, alone and in combination with RT in this BSG mod-
el. Perifosine treatment significantly increased terminal deox-
ynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)–positive nuclei, but in combination with RT, perifo-
sine did not significantly increase TUNEL-positive nuclei relative to RT alone at 6, 24, or 72 hours after RT. Survival anal-
alysis showed that a single dose of 10 Gy increased survival of high-grade BSG-bearing mice by 27% (57 days versus 45 days; $P = 0.0002$), whereas perifosine monotherapy did not. Pretreat-
ment of 10 Gy RT with perifosine did not result in a signifi-
cantly increased median survival relative to 10 Gy alone (64 days versus 57 days; $P = 0.23$). In summary, our PDGF-induced BSG model is the first genetically engineered BSG mouse mod-
el that may serve as useful preclinical model for testing of nov-
el treatment regimens.

Materials and Methods

**Generation of BSGs.** To generate BSGs, we injected 1 μL (10⁵ cells) of RCAS-PDGFR-B–expressing DF1 cells into nestin

**tv-a (Ntv-a) or Ntv-aInk4a-ARF−/− mice. The genetic back-
ground of our Ntv-a mice is a mix of BALB/c, FVB, B6, 129,
and SW strains. Ink4a-ARF−/−:Ntv-a is a strain of BALB/c,
FVB, B6, and 129 strains. Greater than 20 generations of backcrossing were done to generate the lines. Injections were intracranial (2 mm posterior to the bregma at the midline position within 72 h of birth using a Hamilton syringe). Mice were monitored carefully for signs of tumor development (hydrocephalus, lethargy, head tilt). On the appearance of brain tumor symptoms, mice were euthanized with CO₂, and brain tissue was extracted, fixed in formalin, and paraffin embedded.

**Investigation of the cell origin of BSGs.** To determine the cell of origin, we injected 1 μL (10⁵ cells) of DF1 cells ex-
pressing RCAS–green fluorescent protein (GFP) or RCAS–red
fluorescent protein (RFP) into Ntv-a mice as described above. Mice were euthanized 24 h later ($n = 2$) or at 4 wk of age ($n = 6$). Their brain tissue was then extracted, postfixed for 30 min in 4% paraformaldehyde, transferred into 30% sucrose at 4°C for cryoprotection, embedded in OCT, frozen on dry ice, and stored at −80°C. Brains were sectioned in 10-μm sections and inspected with fluorescent microscope for GFP- or RFP-labeled cells.

**Treatment of BSG-bearing mice.** We used total body ir-
radiation (TBI) for all the irradiation experiments, with the ex-
ception of the survival analysis. TBI was delivered with a Gamma Cell 40 Irradiator (MDS Nordion) that delivers 106 cGy/min. For survival analysis, mice were sedated with ketame-
xine and xylazine, and irradiation of the head was done using a X-RAD 320 from Precision X-Ray at 115 cGy/min (the rest of the mouse was shielded with a lead jig). Perifosine treatment was i.p. at a dose 30 mg/kg/d (in normal saline/5% dextrose) once daily and was for a minimum of three daily doses. Combination of perifosine and RT was done as follows: perifosine was given daily for 3 d as monotherapy followed by single-dose RT. At the end of the treatment, mice were eutha-
nized, and brains were dissected and placed in formalin.

**Immunohistochemistry.** Immunohistochemistry on mouse and human formalin-fixed, paraffin-embedded sec-
tions was performed using Discovery XT (Ventana Medical Systems). Human BSG tissue was obtained from Memorial Sloan-Kettering Cancer Center, Children’s National Medical Center, Montreal Children’s Hospital, and Carolinas Health-
care System. Analysis of human BSG tissue was Institutional Review Board approved at all institutions. The following antibi-
odies were used: PDGFRα (1:100; Cell Signaling), Ki-67 (1:100 human; vector for mouse; DAKO), phospho-H3-Ser10 (1:800; Upstate), Olig-2 (1:250; Millipore), and GFAP (1:1,000; DAKO). For immunofluorescence, we used anti-RFP antibody from Rockland at 1:200 followed by Alexa Fluor 488 antibi-
rabbit secondary antibody as per the manufacturer’s protocol. The TUNEL assay was performed per Roche’s instructions.

**Quantitative analysis.** All immunohistochemical slides were scanned. One observer (O.J.B.) performed the quantifi-
cation of TUNEL, phospho-H3, and Ki-67 using the program MetaMorph, where a threshold was set for TUNEL, phos-
pho-H3, and Ki-67 and applied to all sections analyzed. At least five high-magnification 20× representative areas were
analyzed per tumor. For quantification of phospho-H3, positive cells were at least 5 pixels of positive area. For quantification of TUNEL-positive nuclei, we excluded pseudopalisades.

**Magnetic resonance imaging.** Magnetic resonance imaging (MRI) of murine gliomas has been previously described (26).

**Results**

**PDGFRα is overexpressed in pediatric high-grade BSGs.** To specifically investigate the PDGFRα expression in pediatric high-grade BSGs biopsy and autopsy specimens, we immunostained paraffin sections of high-grade BSGs for PDGFRα and noted that 12 of 18 (67%) of high-grade BSGs contained tumor cells that express PDGFRα (Table 1). Interestingly, we noted that 7 of 8 surgical biopsies of BSGs had PDGFRα overexpression (87.5%), whereas only 5 of 10 autopsies expressed PDGFRα (50%). The difference in expression of PDGFRα between surgical biopsies and autopsies was not statistically significant ($P = 0.15$, two-tailed Fisher’s exact test).

**Generation of PDGF-induced BSGs in mice.** PDGFRα overexpression is a common event in pediatric high-grade BSGs, and so, we were interested to determine if overexpression of PDGF in the posterior fossa of neonatal Ntv-a mice can induce BSGs. We infected RCAS-PDGF into the posterior fossa of Ntv-a neonatal mice within 72 hours of birth ($\sim 2 \text{ mm posterior and midline to the bregma}$) and observed the mice for signs and symptoms of brain tumor formation. PDGF overexpression in Ntv-a mice resulted in low-grade BSGs (grade 2; Fig. 1B). The incidence of tumor formation was 80% (12 of 15), and most of the mice were asymptomatic until euthanasia at 12 weeks (Fig. 1A). Of note, no embryonal tumors (such as medulloblastomas) were observed, and the cerebellar external granule layer was not altered.

The tumor cells of these low-grade gliomas were proliferating at a low rate with a Mib1 index of 0.83 ± 0.16%. There was minimal baseline apoptosis as observed with cleaved caspase-3 immunohistochemistry (data not shown). The low-grade gliomas were not purely astrocytic as most of the cells in these lesions immunostained for Olig-2. These lesions were not simply reactive gliosis, as they were quite large at 12 weeks with several tumors occupying $\sim 50\%$ of the pons. These lesions were easily visible on MRI (Fig. 1B). Mice injected with vector alone did not show any lesions. The single mouse that died developed obstructive hydrocephalus due to tumor. Characteristic immunostains of these lesions are illustrated in Fig. 2C (Ki-67, Olig-2, and PDGFR) and can be contrasted with immunostains of normal brainstem (Fig. 2D). In addition there is a GFAP immunostain of a low-grade BSG in Supplementary Fig. S1A.

High-grade BSGs in humans show *Ink4a-ARF* loss, and therefore, we added this genetic alteration to our model. Ntv-a mice with deletion of *Ink4a-ARF* infected with RCAS-PDGF developed symptoms of brain tumor formation such as macrocephaly, lethargy, and hemiparesis between 4 and 8 weeks. We sacrificed these symptomatic animals and extracted their brains. All of the mice had grade 4 BSGs with pathologic elements of microvascular proliferation and pseudopalisading necrosis (Fig. 1C). The incidence and latency for

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<th>Table 1. Human high-grade BSGs overexpress PDGFRα</th>
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Abbreviation: IHC, immunohistochemistry.
*Negative = no staining; 1+ = weak staining of tumor cells; 2+ = strong immunostaining of tumor cells.
PDGF overexpression in Ink4a-ARF-null mice was 96% (68 of 71), with a median survival of 31.5 days (Fig. 1A). The survival of mice with high-grade BSGs induced in Ink4a-ARF−/− background was significantly shorter than that of mice with low-grade BSGs (P < 0.0001, log-rank test). We obtained a MRI of a subset of symptomatic mice before sacrificing the animals and could easily visualize the brainstem tumors in all cases (Fig. 1C). We examined the brainstems of at least 50 un.injected Ntv-a mice with deletion of Ink4a-ARF and did not observe any preneoplastic lesions.

The anatomic extent of the tumors at 4 weeks was that 80% of the tumors localized completely within the brainstem (based on T2-weighted MRI images). The natural course of these tumors based on serial MRIs was that the tumors would go on to invade into the cerebellum posteriorly and invade anteriorly and superiorly into the thalamus and top of the brainstem, respectively. Eventually, all the mice developed obstructive hydrocephalus that likely resulted in obstruction of the cerebrospinal fluid flow at the level of the fourth ventricle and aqueduct and its associated symptoms. Mice were euthanized at that point. The tumor volume estimate at 4 weeks (measurements were done on T2-weighted MRI images of at least 10 high-grade BSGs) was 11.9 ± 4.7 mm³. Histologic examination revealed that 70% of the tumors occupied at least 50% of the pons.

The histologic appearance of our murine BSGs was similar to human pediatric BSGs, particularly with regard to the diffusely infiltrating arrangement of the tumor cells (Fig. 2A and B). We immunostained our murine BSGs for Ki-67, Olig-2, GFAP, and PDGFRα and noted further similarities between these tumors and pediatric BSGs. Pediatric BSGs stained positive for Olig-2 but with large variability in the percent of positive cells in different tumors ranging from 10% to ∼100%, whereas >90% of the tumor cells of our PDGF-induced BSGs in Ink4a-ARF−/− mice stained strongly for PDGFRα and Olig-2 (Fig. 2A and B; Supplementary Fig. S2). GFAP immunostaining was positive in both low- and high-grade murine BSGs, with similarity to pediatric BSGs (Supplementary Figs. S1 and S2). Of note, Mib1 labeling was 10.2 ± 5.6% for PDGF-induced BSGs in Ink4a-ARF−/− background.

RCAS infections target cells on the ventricular surface of the fourth ventricle. The RCAS/tv-a system allows for lineage tracing of tv-a–expressing cells infected with RCAS. As we were using the Ntv-a mouse, we were infecting only nestin-expressing cells. Nestin expression in the ependymal layer of the fourth ventricle has been previously described before in humans (27). In addition, it has been hypothesized that cells lining the floor of the fourth ventricle may serve as cells of origin for BSGs (28). We therefore decided to investigate which cells we were targeting with our infections (29, 30).
We injected DF1 cells producing RCAS-RFP into the posterior fossa of neonatal Ntv-a mice at postnatal day 2 ($n = 2$). Twenty-four hours later, we euthanized both neonatal mice, extracted their brains, sectioned, and looked for RFP-labeled cells. Both mice had RFP-labeled cells floating in the cerebrospinal fluid and were localized around the fourth ventricle and aqueduct (Supplementary Fig. S3). In parallel, we injected DF1 cells producing RCAS-GFP in a similar fashion at postnatal day 2 ($n = 6$). We monitored the mice for 4 weeks (DF1 cells have been previously reported to survive up to 3 weeks after injection), euthanized the mice, extracted their brains, and looked for GFP-labeled cells. The cells expressing GFP were located mostly in the outer layer of the fourth ventricle (Fig. 3A) or aqueduct. Of note, the distribution was not random (Fig. 3B). We found that $>90\%$ of the GFP-labeled cells were located symmetrically at specific locations on the ventricular surface of the floor of the fourth ventricle, whereas only 5% were from the roof of the fourth ventricle (266 GFP-labeled cells counted). In addition, we immunostained sections of neonatal pups at postnatal days 1 to 3 and noted strong nestin expression in the cells lining the fourth ventricle during this early postnatal age (both roof and floor of fourth ventricle as seen in Fig. 3C).

To confirm that the nestin-expressing cells of the fourth ventricle and aqueduct are the cells of origin for our murine BSG model, we infected RCAS-PDGF as described above, observed the mice for 3 weeks, euthanized them, and looked for neoplastic lesions. We noted that all of the mice had small precursor lesions and 80% of the precursor lesions (12 of 15) were arising from the floor of the fourth ventricle or aqueduct (Fig. 3D).

In summary, we have developed a genetically engineered BSG model that has histologic similarities to human BSGs and has comparable immunostaining characteristics. Our BSG model arises from the cells of the fourth ventricle or aqueduct. Next, we were interested to use this BSG model in preclinical trials.

**Preclinical trials on the mouse BSG model.** RT is the only known therapeutic modality with antitumor activity for children with BSGs. Therefore, we investigated this treatment in...
our mouse model. We irradiated PDGF-induced high-grade BSG-bearing mice with single-dose RT at 2 Gy (approximately the dose used in the clinic), 6 Gy, and 10 Gy, and sacrificed the mice at three time points: 6, 24, and 72 hours (minimum of five mice per group). We analyzed the response of these tumors to irradiation using TUNEL and evaluated the proliferation rate using phospho-H3. Doses of 10 and 6 Gy induced a near-complete cell cycle arrest 6 hours after treatment as evaluated by phospho-H3, but 2 Gy did not significantly affect phospho-H3 (Fig. 4A and B). We also found significantly increased TUNEL-positive nuclei relative to unirradiated BSGs at 24 hours after RT for all doses of RT analyzed (Fig. 4C and D). Time course (10 Gy) showed that the peak inhibition of proliferation was 6 hours after RT and that the highest percent of TUNEL-positive nuclei was 24 hours after RT (Supplementary Fig. S4A and B). To determine if RT prolongs survival in this BSG model, we treated BSG-bearing mice with a single dose of 10 Gy (by only irradiating the head). Treatment was begun after pretreatment MRIs at 4 weeks so that similarly sized tumors were assigned to each treatment group (including untreated group). A single dose of 10 Gy prolonged survival by 12 days (57 days versus 45 days; \( P = 0.0002 \); Fig. 5D). A single dose of 2 Gy did not prolong survival significantly (\( P = 0.14 \)). In summary, high-dose RT is effective in prolonging survival in this BSG model.

**Combination of perifosine and RT in this high-grade BSG model.** We recently noted that perifosine, an inhibitor of AKT signaling, cooperates with radiation in medulloblastomas (31). In addition, the AKT pathway is active in radioresistant glioma cells, and we have previously shown that perifosine cooperates with temozolomide in gliomas (25, 32). Therefore, we determined whether perifosine has a therapeutic effect in BSGs as a single agent and in combination with 10 Gy RT, the most effective single radiation dose. We treated a group of PDGF-induced high-grade BSGs with perifosine alone for 3 to 5 days, as well as a second group with 3 days of perifosine followed by a single dose of 10 Gy. Perifosine treatment did not affect proliferation significantly as a single agent, and there was no statistically significant difference in proliferation with the addition of perifosine to 10 Gy.
RT at 6, 24, and 72 hours as measured with phospho-H3 (P = 0.7, P = 0.28, and P = 0.06, Mann-Whitney, respectively; Fig. 5A). The percent of TUNEL-positive nuclei increased significantly with treatment with perifosine (P = 0.005, Mann-Whitney), but the combination of perifosine + 10 Gy did not significantly affect the percent of TUNEL-positive nuclei relative to 10 Gy alone at 6, 24, and 72 hours after RT (P = 0.46, P = 0.15, and P = 0.09, Mann-Whitney, respectively; Fig. 5B). To investigate the long-term effect of these treatments, survival analysis was carried out to determine if perifosine will prolong survival as monotherapy or in combination with RT (using head irradiation) in this high-grade BSG model. Single-agent daily perifosine treatment for 1 week did not prolong survival (43 days versus 45 days; P = 0.92), and pretreatment with perifosine before 10 Gy RT did not result in a significantly increased survival relative to 10 Gy RT alone (64 days versus 57 days; P = 0.23; Fig. 5C and D). Our results suggest that perifosine, when used a single agent or with a single dose of 10 Gy, does not have sufficient antitumor activity to prolong survival in this model.

**Discussion**

In this study, we applied RCAS/tv-a technology to generate BSGs that can be used for preclinical trials. There are numerous advantages to this preclinical model. First, it is consistent with the genetic alterations of a subset of the human disease according to the published literature and our analysis.
of human specimens. Second, it is histologically similar to the human disease. Third, the tumors arise in immunocompetent mice. Fourth, the model has high penetrance and short latency and forms in the appropriate microenvironment—the brainstem. Fifth, this model responds to, but is not cured with, RT like the clinical experience of treating children with DIPGs with focal RT. Sixth, the pattern of invasion of the PDGF-induced high-grade BSGs is similar to the pattern of invasion of human high-grade BSGs, as a subset of the latter has been described to contiguously involve the cerebellar peduncles, cerebellum, and/or thalamus (33). It thus may serve as a preclinical model for screening of novel agents. There are only a few hundred new cases of pediatric BSGs in the world each year, and as there are now an increasingly large number of novel agents and exponential combinations of these agents, a genetic mouse model may be useful to screen for the most active combinations and schedules.

The cell of origin for supratentorial NF-1-deleted gliomas has been proposed to be neural stem cells from the subventricular zone (34). However, other modeling systems indicate that nestin-expressing cells in other areas of the brain are also able to serve as the cell of origin (26). Here, we present evidence that the cells of origin for this murine BSG model are mostly localized to the surface of the fourth ventricle and aqueduct. Other cells in the brainstem may also serve as the cells of origin for the development of murine BSGs, but as we used the Ntv-a mouse, we restricted our investigation to nestin-expressing cells in the brainstem in the neonatal period, which are the cells lining the fourth ventricle and aqueduct.

We evaluated external beam irradiation in this BSG model and noted that, similar to the clinical experience of treating children with DIPGs with focal RT, RT is active in this model. It is important to note that the results of our preclinical testing with single doses of irradiation do not suggest that higher...
doses of irradiation will be more beneficial to treat children with DIPGs. A single dose of 10 Gy is a potentially toxic dose to the normal cells in the brainstem, and higher total doses of radiation have been tested in clinical trials for DIPGs using hyperfractionated radiation and did not show increased efficacy (35). As BSGs are heterogeneous tumors, observations with this mouse model may only apply to BSGs that overexpress PDGFR and have lost Ink4a-ARF. If this was the case, pretreatment tissue to determine genetic status would be needed, and obtaining biopsies for DIPGs has recently been reported to have minimal complications (36). However, it is important to note that biopsy DIPGs is a controversial topic, its success is highly dependent on who performs it, and it is hard to obtain approval for it in clinical trials (37).

We extended the preclinical testing by evaluating perifosine, an inhibitor of AKT signaling, alone and in combination with RT using this model. Our analysis showed that although perifosine monotherapy has modest activity in inducing cell death in short-term analysis in this model, it does not have enough antitumor activity to increase survival. In addition, perifosine did not provide additional survival benefit to high-dose RT. This is perhaps not surprising, as there is no drug that has been shown to increase survival of children with high-grade BSGs in numerous clinical trials even in combination with RT and may be due to the poor drug delivery into the brainstem due to the blood-brain barrier. This observation reinforces our recommendation that novel combinations should be tested in a genetically and histologically accurate preclinical model first before their translation to the clinic.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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