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ARTICLE

Systemically administered AAV9-sTRAIL combats invasive glioblastoma in a patient-derived orthotopic xenograft model

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Adeno-associated virus (AAV) vectors expressing tumoricidal genes injected directly into brain tumors have shown some promise, however, invasive tumor cells are relatively unaffected. Systemic injection of AAV9 vectors provides widespread delivery to the brain and potentially the tumor/microenvironment. Here we assessed AAV9 for potential glioblastoma therapy using two different promoters driving the expression of the secreted anti-cancer agent sTRAIL as a transgene model; the ubiquitously active chicken β-actin (CBA) promoter and the neuron-specific enolase (NSE) promoter to restrict expression in brain. Intravenous injection of AAV9 vectors encoding a bioluminescent reporter showed similar distribution patterns, although the NSE promoter yielded 100-fold lower expression in the abdomen (liver), with the brain-to-liver expression ratio remaining the same. The main cell types targeted by the CBA promoter were astrocytes, neurons and endothelial cells, while expression by NSE promoter mostly occurred in neurons. Intravenous administration of either AAV9-CBA-sTRAIL or AAV9-NSE-sTRAIL vectors to mice bearing intracranial patient-derived glioblastoma xenografts led to a slower tumor growth and significantly increased survival, with the CBA promoter having higher efficacy. To our knowledge, this is the first report showing the potential of systemic injection of AAV9 vector encoding a therapeutic gene for the treatment of brain tumors.

INTRODUCTION

In recent years, adeno-associated virus (AAV) vectors have gained an increasing attention as a gene therapy vector for several diseases, some of which have made it to clinical trials.1 The first approved AAV-based gene therapy in the Western world is alipogene tiparvovec for the treatment of lipoprotein deficiency, which shows that this approach can be successfully and safely applied to monogenic diseases.2 Additionally, AAV vectors for the treatment of more complex diseases such as heart failure have seen some success in clinical trials,3,4 and many advances are made using these vectors as cancer therapeutics.5

Glioblastoma (GBM) is the most common and highest-grade malignant primary brain tumor in adults. Despite aggressive therapies, median survival is generally just over one year following diagnosis.6 This underscores the need for novel treatments to be developed. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is considered as a potent anti-cancer agent, capable of inducing cell death in a variety of tumor cells, including GBM.7–10 Direct intracranial injection of different AAV vectors into the primary tumor mass have been used for the treatment of GBM (and other brain tumors) with some success, however, due to the invasive nature of this type of cancer, tumor recurrence is typically observed showing that a vector with widespread gene delivery in the brain is required for efficient therapy.11–13

AAV9 serotype is very efficient in transducing cells in vivo, and has been shown to cross the blood–brain barrier (BBB) upon intravenous injection, making it an ideal candidate for whole brain transduction and potential gene delivery to brain tumors.14–19 However, AAV9 shows high tropism towards the liver, which may cause off-target toxicity and limit the potential therapeutic effect.18,20,21 In order to restrict AAV-mediated expression to the brain/tumor environment, a brain cell-specific promoter could be used, such as the neuron-specific enolase (NSE) promoter, which is predominantly active in neurons, thus limiting expression in other cell types/tissues.22,23 The aim of the current study is to evaluate systemic injection of AAV9 vector expressing a therapeutic gene (secreted soluble TRAIL as a model) under the control of either a constitutively active promoter or neuron-specific promoter for potential therapy of tumors in the brain.

RESULTS

AAV9 transgene expression driven by different promoters has a similar kinetics profile.

We cloned the Firefly luciferase bioluminescent reporter under the control of either a constitutively active CBA promoter or neuron-specific NSE promoter and packaged them into AAV9 vector generating AAV9-CBA-Fluc and AAV9-NSE-Fluc (Figure 1a). Athymic nude...
mice (n = 4 per group) were then injected i.v. via the tail vein with 1.5 × 10¹² g.c./kg of each vector and bioluminescence imaging (BLI) was performed at three and twelve days post-injection to quantify the distribution of transgene expression. Widespread delivery of both vectors was observed throughout the animal’s body at both time points (Figure 1b). Analysis of the signal from the head and the abdomen of mice at the two time points showed that, at twelve days post administration, AAV9-NSE-Fluc vector yielded an average of 100-fold lower BLI signal in the abdomen (P = 0.0171) as well as the brain (P = 0.0193) of mice compared to the AAV9-CBA-Fluc vector, indicating that the NSE promoter could be used to decrease transgene expression and potential cytotoxicity in the liver and other tissues (Figure 1b,c). Despite the difference in transgene expression, both vectors yielded a similar expression kinetics profile without significant differences in absolute brain-to-liver expression ratios (Figure 1d). To confirm these results ex vivo, mice were sacrificed 20 days postinjection of either vector, and the brain and liver were harvested, homogenized, and analyzed for Fluc expression (normalized to total amount of protein). In line with the expression ratio results obtained by BLI (Figure 1d), an average of 10-fold lower signal in both liver (P = 0.004) and the brain (n.s.) was observed with the NSE promoter compared to the CBA promoter (Figure 1e), with no significant differences in the brain-to-liver ratio between both vectors (Figure 1f).

NSE promoter in AAV9 vector transduces mainly neurons in the brain upon i.v. injection

The observed decreased overall expression of the AAV9-NSE-Fluc vector could be due to several factors, including a lower number of cells transduced, expression level per cell, or both. To confirm cell-type selectivity of the NSE promoter, we generated AAV9 vectors expressing GFP under either promoter (AAV9-CBA-GFP and AAV9-NSE-GFP; Figure 2a). These vectors were injected i.v. into nude mice (n = 3 per group) at a dose of 5 × 10¹³ g.c./kg. Two weeks post-vector injection, mice were sacrificed, and the brain and liver were collected, sectioned, and stained for GFP expression. In mice injected with AAV9-CBA-GFP, expression of GFP was detected in endothelial cells, neurons and astrocytes in the brain (Figure 2b). On the other hand, and as expected, the most common cell type with GFP expression in the brain of mice injected with AAV9-NSE-GFP were neurons (Figure 2c). In these mice, some dim GFP expression was also observed in endothelial cells while no GFP expression correlated with astrocytes. Furthermore, GFP staining in AAV9-NSE-GFP mice brains coincided mostly with NeuN staining and not with GFAP staining (Supplementary Figure S1), demonstrating that indeed neurons were transduced. Liver sections showed GFP staining, although it was more widespread and intense in sections from mice injected with AAV9-CBA-GFP compared to those injected with AAV9-NSE-GFP vector (Figure 2c). We also quantified the number of GFP+ cells in three sections and averaged the total number of GFP+ cells per mm² showing no significant differences in the number of GFP+ neurons, however significantly more astrocytes (P < 0.01) and liver cells (P < 0.05) expressed GFP in mice injected with AAV9-CBA-GFP as compared to AAV9-NSE-GFP (Figure 2d).

Intravascular administration of AAV9-sTRAIL vectors inhibits GBM growth in a patient-derived orthotopic xenograft mouse model

In order to assess the potential use of AAV9 vector for systemic delivery of therapeutics to brain tumors, we used the secreted soluble variant of TRAIL as a transgene model and cloned it under the control of NSE or CBA promoter (AAV-CBA-sTRAIL or AAV-NSE-sTRAIL; Figure 3a). Initially, to assess that sTRAIL is secreted in an active form using both constructs, we packaged them into an AAV2 vector and then transduced 293T cells with either vectors or AAV2-NSE-Fluc as a control. Conditioned medium from these cells were collected and used to treat primary GBM neurospheres in triplicate. The AAV-NSE-Fluc control medium had no effect on cell viability. On the other hand, a significantly lower number of viable cells were observed in wells treated with AAV2-CBA-sTRAIL medium, killing 60% of GBM neurospheres as compared to the control group (P = 0.0008) as well as in GBM neurospheres treated with AAV2-NSE-sTRAIL medium (P = 0.0006; Figure 3b).

We then assessed whether systemic delivery of AAV9-sTRAIL could target the brain tumor environment, while evaluating both CBA and NSE promoters to drive sTRAIL transgene expression, to determine if a restricted promoter also has a similar therapeutic effect in vivo. For evaluation of these vectors, we used an invasive patient-derived xenograft orthotopic mouse model. Mice were intracranially injected in the left striatum with 2 × 10⁶ of primary GBM stem-like cells culture as neurospheres and expressing Firefly luciferase (GBM-Fluc). Twenty-eight days post-tumor cells implantation, mice were pseudo-randomized into treatment groups so that each group had a similar average Fluc signal (P > 0.9999; Figure 4 and Supplementary Figure S2) and each group received i.v. injection of 2 × 10¹⁵ g.c./kg of either AAV9-CBA-Fluc (control, n = 5), AAV9-CBA-sTRAIL (n = 10) or AAV9-NSE-sTRAIL (n = 10). Tumor volume was monitored weekly by Fluc bioluminescence imaging. After the first week post-vector injection, the Fluc signal in the control group had increased significantly, compared to the AAV9-NSE-sTRAIL group (P = 0.0419) and compared to the AAV9-CBA-sTRAIL group (P = 0.0356), but there was no significant difference between both treatment groups (Figure 4a,b). Between 2 and 3 weeks post-vector injection, all mice in the control group had reached their maximum Fluc tumor signal, after which they were sacrificed due to predetermined humane endpoints. Imaging showed that tumors in both the AAV9-NSE-sTRAIL group and AAV9-CBA-sTRAIL group continued to slowly grow, with the former group showing slightly more growth. However, no significant differences were found at any time point in the Fluc signal between AAV9-NSE-sTRAIL and AAV9-CBA-sTRAIL groups.

Survival analysis showed that there was an overall significant difference between all groups (P < 0.0001). Post-hoc analysis showed that the survival of control mice was significantly shorter than that of mice treated with AAV9-NSE-sTRAIL (P = 0.0002) and those treated with AAV9-CBA-sTRAIL (P < 0.0001). This shows that sTRAIL treatment using i.v. administered AAV9 vectors under either promoter provided a therapeutic benefit. Although there was a trend for longer survival in mice treated with AAV9-CBA-sTRAIL as compared to AAV9-NSE-sTRAIL, there was no significant difference between the two vector-treated groups (P = 0.0998), despite the fact that Fluc imaging showed a larger tumor size in mice treated with AAV9-NSE-sTRAIL, and that these mice subsequently died sooner. Median survival for the control group was 38 days, while mice treated with AAV9-NSE-sTRAIL or AAV9-CBA-sTRAIL had a median survival of 47 and 54.5 days, respectively (Figure 4c). Furthermore, a small subset of mice (n = 3) with AAV9-CBA-sTRAIL survived out to the predetermined end of the study (135 days; Figure 4c). This indicates a trend for longer survival in mice treated with the vector using the CBA promoter over the NSE promoter. To our knowledge, this is the first report showing systemic injection of an AAV vector to treat tumors in the brain.
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Figure 1  Expression profile and quantitation of adeno-associated virus (AAV9)-mediated bioluminescence expression using chicken β-actin (CBA) or neuron-specific enolase (NSE) promoters. Plasmids were designed for the production of AAV9-CBA-Fluc or AAV9-NSE-Fluc (a). Female nude mice \( n = 4 \) per group were injected with \( 1.5 \times 10^{12} \) g.c./kg of each vector and images were taken from the dorsal side and ventral side at three and twelve days after administration. A representative mouse at each time point is shown for AAV9-CBA-Fluc (b, left panel) and AAV9-NSE-Fluc (b, right panel). At 3 and 12 days post vector administration, quantification of the average radiance showed that there is a 100-fold higher signal from the head and abdomen (c) in mice injected with AAV9-CBA-Fluc compared to AAV9-NSE-Fluc. No differences in head-to-abdomen ROI ratios were found (d). Tissue homogenates showed 10-fold higher relative light units (RLU)/mg in the brain and liver of mice injected with AAV9-CBA-Fluc compared to AAV9-NSE-Fluc (e), with no significant differences in the brain-to-liver tissue homogenate ratios (f). Note the scale difference between left and right axis in c and e \( (**P < 0.05; ***P < 0.005)\).
Next, we performed ex vivo histological analysis using H&E staining and Ki-67 staining for cell proliferation on brains of mice sacrificed at 6 weeks post-tumor inoculation. In addition, as TRAIL might induce hepatotoxicity and AAV9 is known to efficiently transduce cells in the liver, we also performed H&E staining on liver sections to establish whether this therapy caused any histopathological changes. Gross histological analysis did not reveal any visible differences in liver sections across the different groups (Supplementary Figure S3). Large tumors were found at the injection site in the brains of mice from the control group. In contrast, much fewer tumor cells were found in the brains of mice injected with AAV9-sTRAIL. This indicates that AAV9-sTRAIL is an effective therapy for glioblastoma.
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Figure 3 Cells transduced with AAV-CBA-sTRAIL or AAV-NSE-sTRAIL secrete TRAIL in an active form. 293T cells were transduced with either AAV2-CBA-sTRAIL, AAV2-NSE-sTRAIL (a) or AAV2-NSE-Fluc control vector. Three days later, conditioned medium was transferred to wells containing GBM neurospheres, and the next day cell viability was measured (b). GBM cell viability decreased significantly in wells treated with AAV2-CBA-sTRAIL or AAV2-NSE-sTRAIL compared to control wells (**P < 0.001).

Discussion

The use of AAV as a preclinical gene therapy delivery vector has resulted in significant successes.1 The ability of AAV9 to efficiently pass the BBB after intravascular (i.v.) administration makes this serotype uniquely suited for gene therapy of the CNS, particularly the brain.16,18 GBM is an aggressive form of primary brain cancer that is notoriously resistant to therapies. The presence of death signaling receptors on the membrane of GBM cells makes their ligand, TRAIL, a potential therapy. Unfortunately, the short half-life and its inability to cross the blood-brain barrier are a major problem when using recombinant TRAIL protein for therapy in the brain.25,26 AAV vectors encoding TRAIL have been utilized for the treatment of many forms of cancer, although most studies use intratumoral or near-tumor administration27–29 rather than systemic administration.30,31 In case of the brain, systemic delivery is hampered due to limited BBB penetration and subsequent poor brain distribution of many AAV vectors and TRAIL protein.11,21,22 Here we used i.v.-administered AAV9 vectors for delivery of sTRAIL (as a transgene model) to the primary tumor mass and infiltrating tumor cells in the brain. Although TRAIL's proapoptotic effects are confined to tumor cells,12–14 high levels of TRAIL or other transgene expression in the liver21,23 become a concern leading to toxicity.26,37 To overcome potential liver toxicity, we used the neuron-specific enolase (NSE) promoter, predominantly active in neurons,32 and compared it to the ubiquitously active chicken beta-actin (CBA) promoter. Our data show that the NSE promoter reduced transgene expression in the brain and the liver by at least 10-fold compared to the CBA promoter, although the brain-to-liver ratio remained the same. This is likely due to the fact that in adult mice, AAV9 primarily transduces astrocytes.17 By restricting expression to mostly neurons in the brain, we likely lowered the overall transgene expression levels in the brain. Indeed, we observed that while the CBA promoter leads to expression in most cell types in the brain, most notably neurons, astrocytes and endothelial cells, the NSE promoter leads to a more confined expression in neurons with low expression in endothelial cells and much less expression in the liver. Further, the NSE promoter may also be weaker on a per neuron basis than the CBA promoter, in line with other studies.38,39 In the future, we may be able to maintain high expression of transgene in the brain and lower expression in the liver by employing miR122 target sites into the 3’UTR of the transgene cassette as previously described.40

We have previously shown that intracranially administered AAVrh.8-sTRAIL was capable of combating primary GBM tumor mass in a nude mouse model, however, single vector injection was not able to combat invasive cells, which managed to escape the zone of resistance and form a new tumor elsewhere in the mouse brain leading to tumor recurrence.11 In addition to intracranial injections being a laborious procedure, i.v. administration of a therapeutic vector with widespread CNS transduction would be advantageous since it could target both the primary tumor mass as well as infiltrative tumor cells in the brain. Indeed, a single i.v. injection of either AAV9-CBA-sTRAIL or AAV9-NSE-sTRAIL vectors lead to a significantly slower tumor growth...
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and increase in survival rate as compared to mice treated with AAV9-CBA-GFP control vector. Although there was a general trend in slower tumor growth and increase in survival of mice treated with AAV9-CBA-sTRAIL as compared to AAV9-sTRAIL vector, there were no significant differences between both groups showing the efficacy of neuron-specific promoter with lower liver transduction in combating aggressive GBM cells. To our knowledge, this is the first report showing that systemic injection of an AAV vector could treat tumors in the brain using a brain-specific promoter with lower liver expression.

Massive invasion of the brain is a major problem in controlling GBM growth. Unfortunately, this therapy did not sufficiently inhibit the spread of GBM cells to other parts of the brain, away from the primary tumor site and eventually all mice died. Additionally, resistance to therapy (including TRAIL) is a major problem for the treatment of GBM.41–43 We initially tried a relatively low dose of AAV9-sTRAIL (2 × 10^13 g.c./kg). A higher dose of AAV9-sTRAIL or multiple rounds of therapy, combined with TRAIL-sensitizing compounds,44–47 may achieve a greater benefit for inhibiting the invasive growth of GBM and prevent intrinsic or acquired TRAIL resistance. TRAIL did not appear to have affected the liver, as there was no indication of liver toxicity in both AAV-sTRAIL-treated groups as observed in the H&E stained sections. Therefore, promoter restriction might not be necessary for limiting TRAIL expression in non-target tissues such as the liver (at least in mice). However, restricting transgene expression of TRAIL from liver could still be a concern due to cell-mediated immunity against AAV capsid or the transgene,35,48 especially in patients with underlying liver pathologies.49

In conclusion, systemically-delivered AAV9 encoding a therapeutic protein is a promising approach to GBM therapy. Future experiments using higher vector doses, different transgene expression cassettes, combined with TRAIL-sensitizing compounds should lead to a more pronounced survival benefit.

MATERIALS AND METHODS

Cloning

The pAAV-NSE-Fluc vector was constructed by digesting pAAV-NSE-GFP50 and pAAV-CBA-Fluc51 with NheI and SacI (New England Biolabs, Ipswich, MA), replacing GFP with Fluc. The pAAV-CBA-sTRAIL vector has been previously described.52,53 From this vector, sTRAIL was amplified by polymerase chain reaction (PCR) using the following primers: AgeI forward 5′-GACACGGGTGACGCCGCCACCATGACAG-3′ and SpeI reverse 5′-GGACTAGTACCTAGAATTCAACTAAAAAGCCGAA-3′. After digesting sTRAIL with AgeI and SpeI, respective inserts were ligated into a similarly digested pAAV-NSE-GFP, generating pAAV-NSE-sTRAIL.

Cell culture and viral vector production

293T cells, for AAV production, obtained from American Type Culture Collection (ATCC, Manassas, VA) were cultured in high-glucose Dulbecco’s modified eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO), 100 U of penicillin/0.1 mg/ml streptomycin complex (Pen/Strep; Invitrogen, Carlsbad, CA). Primary stem-like GBM8 glioblastoma cells44 were cultured as neurospheres in serum-free NeuroCult NS-A Basal Medium with Proliferation Supplement (StemCell Technologies, Vancouver, BC), supplemented with 20 ng/ml recombinant epidermal growth factor (EGF; R&D Systems, Minneapolis, MN), 10 ng/ml basic fibroblast growth factor (bFGF; Peprotech, Rocky Hill, NJ), and 10% dextran-coated charcoal-treated FBS.
and 2 μg/ml heparin (Sigma). All cells were cultured at 37 °C under 5% CO₂ humidified atmosphere.

AAV vectors were produced as previously described. Briefly, triple transfection of 293T cells using calcium phosphate precipitation was used with AAV2 ITR-containing single-stranded transgene plasmid, mini-adenoivirus helper plasmid pMD6, and pH22 (AAV2 capsid; for in vitro use) or pA99 (AAV9 capsid; for in vivo use) rep/cap plasmid. Three days later, the cells were harvested, lysed by freeze/thaw, and AAV was purified using iodixanol density gradient ultracentrifugation, resulting in typical yields of 10⁹ genome copies per ml (gc/ml).

Cell viability assay
For production of STRAL protein, 4 × 10⁴ 293T cells per well were plated in a 12-well plate. The next day, cells were transduced with 1 × 10⁴ gc/ml cell AAV2-NSE-Fluc (control), AAV2-NSE-STRAL, or AAV2-CA-STRAL. We have previously shown that the NSE promoter is active in 293T cells. The day after 293T cell transduction, 5 × 10⁴ GBM8 cells were plated in each well of a 96-well plate. Two days later, conditioned medium from 293T producer cells was harvested and added to the GBM8 cells for all conditions in triplicate. Twenty-four hours after treatment, quantification of cell viability and ATP detection in metabolically active cells was performed using the commercially available CellTiter-Glo assay as per manufacturer’s protocol (Promega, Madison, WI). Readout of relative light units (RLUs) was performed using a luminometer (Dynex Technologies, Chantilly, VA) and control samples were used to assess background signal in the medium and cell viability in each treatment group. After subtraction of background, the mean RLU per group was plotted as a percentage, with the control group set at 100%.

GBM animal models and bioluminescence imaging
All animal experiments were approved by the Massachusetts General Hospital (MGH) subcommittee on Research Animal Care and performed according to their guidelines and regulations. Female athymic nude mice of 6–8 weeks old, weighing 20–25 g, were obtained from MGH research animal services, and housed at their animal facility. For tail vein injections, each mouse was placed in a restrainer (Braintree Scientific, Braintree, MA); the tail was warmed up in a beaker with water at 40 °C and swabbed with alcohol. Mice were injected i.v. with a 200 μl mixture of PBS containing 1.5 × 10⁵ gc/ml of either AAV9-CBA-Fluc or AAV9-NSE-Fluc vector, or 5 × 10⁴ gc/ml/kg of AAV9-CBA-GFP or AAV9-NSE-GFP vector, or 2 × 10⁵ gc/ml/kg of AAV9-CBA-STRAL or AAV9-NSE-STRAL vector.

To generate GBM models, mice were anesthetized with a mixture of 100 mg/kg ketamine and 5 mg/kg xylazine in 0.9% sterile saline. Mice were placed in a stereotaxic frame and intracranially injected with 2 × 10⁴ GBM8-Fluc cells using a Micro 4 Microsyringe Pump Controller (World Precision Instruments, Sarasota, FL) attached to a Hamilton syringe with a 33-gauge needle (Hamilton, Reno, NV), at the following coordinates in mm from Bregma: -0.5 antero-posterior, +2.0 medio-lateral, -2.5 dorso-ventral.

To assay the transduction efficiency of AAV9-Fluc vectors or GBM8-Fluc tumor growth, Fluc expression in mice was imaged noninvasively using a Xenogen IVIS Spectrum charge-coupled device (CCD) camera (Caliper Life Sciences, Hopkinton, MA), equipped with an XGl-8 gas anesthesia system. Mice were anesthetized with 2.5% isoflurane in oxygen and the Fluc signal was acquired 10 minutes after i.p. injection of 200 μg/kg Fluc. Mice were anesthetized and sacrificed by i.p. injection with an overdose mixture of ketamine (150 mg/kg) and xylazine (15 mg/kg) in sterile saline. Liver and brain were harvested, snap frozen on liquid nitrogen and stored at −80 °C. Tissues were embedded in Neg-50 freezing medium (Fisher Scientific, Thornwood, NY) with an automated stage for capturing images using the Zen software package (Carl Zeiss Microscopy, Thornwood, NY). Cell type was determined based on morphology. The number of GFP+ cells for each cell type was counted per section and then averaged over the total area in mm².

Statistics
Statistical analysis was performed using GraphPad Prism (v6.01; LaJolla, CA). A P value ≤ 0.05 was considered statistically significant. To compare patient differences between tissues or between days, a two-way analysis of variance was used followed by a Sidak Multiple Comparison test to compare individual groups. Otherwise, comparisons between two samples were analyzed using an unpaired two-tailed t-test. For analysis between multiple groups, a one-way analysis of variance was performed, again followed by a Sidak Multiple Comparison Test. Survival was analyzed using Kaplan-Meier curves and log-rank (Mantel-Cox) tests to compare all or individual groups.

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