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Expression and targeting of transcription factor ATF5 in dog gliomas

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Abstract

Background—Activating transcription factor 5 (ATF5) is a transcription factor that is highly expressed in undifferentiated neural progenitor/stem cells as well as a variety of human cancers including gliomas.

Aims—In this study, we examined the expression and localization of ATF5 protein in canine gliomas, and targeting of ATF5 function in canine glioma cell lines.

Materials and Methods—Paraffin-embedded canine brain glioma tissue sections and western blots of tumours and glioma cells were immunoassayed with anti-ATF5 antibody. Viability of glioma cells was tested with a synthetic cell-penetrating ATF5 peptide (CP-d/n ATF5) ATF5 antagonist.

Results—ATF5 protein expression was in the nucleus and cytoplasm and was present in normal adult brain and tumour samples, with significantly higher expression in tumours as shown by western immunoblotting. CP-d/n ATF5 was found to decrease cell viability in canine glioma cell lines *in vitro* in a dose-dependent manner.

Conclusion—Similarities in expression of ATF5 in rodent, dog and human tumours, and cross species efficacy of the CP-d/n ATF5 peptide support the development of this ATF5-targeting approach as a novel and translational therapy in dog gliomas.

Keywords

astrocytoma; brain tumor; canine glioma cell lines; cell penetrating peptide; d/n-ATF5; oligodendroglioma

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Conflict of interest.

Columbia University, on behalf of inventors Dr. Angelastro awarded United States patents US 07888326 and US 08158420. Columbia University/University of California, Davis; Provisional patent application was filed, February 22, 2013 with U.S. Provisional Application Serial No. 61/768,390. Dr. Angelastro is a consultant/advisory board member for Sapience Therapeutics who hold technology licenses for ATF5 therapeutic targeting (patents held by Columbia University). Drs. DY, CDS, PJD, and NLC declare no potential conflict of interest.

Introduction

Intracranial gliomas represent approximately 30–35% of primary brain tumors in dogs with an increased incidence reported in specific breeds including Boxers, Bulldogs, Boston Terriers, Mastiffs and Jack Russell Terriers.[1, 2] Data relating to treatment outcomes in dogs are limited, however, similar to human tumor counterparts, prognosis following standard therapy (surgery, radiation therapy and chemotherapy) is poor.[3, 4] Major advances in molecular genetic phenotyping of gliomas have been made in human neurooncology allowing a more rational approach to development of novel tumor marker and pathway based targeted therapies.[5, 6] Similar approaches are ongoing for brain tumors in dogs and may provide a translational platform for therapeutic development beneficial to both species.[7–14]

The CREB family member, activating transcription factor 5 (ATF5) is an important regulator of normal central nervous system (CNS) development, and appears to be important for gliomagenesis and tumor cell survival across a range of tumor grades in humans and rodent models. Consistent with these roles, ATF5 has been shown to be expressed in rodent neural and glial progenitor cells[15–17] and human glioma associated stem cells,[18, 19] and is also highly expressed in mouse, rat, and human gliomas.[18, 20–24] Based on these data, abrogation of ATF5 function in ATF5-expressing neoplastic cells has been proposed as an interventional strategy to treat gliomas either as a monotherapy or adjunctively in synergism with standard therapeutic modalities.

Although ATF5 expression in the mature CNS is reportedly limited to reactive astrocytes[16, 20] and progenitor cells,[15–17] preferential targeting of neoplastic cells, even within this limited population of ATF5-expressing normal cells, is important to ensure a high therapeutic index for any ATF5 targeted therapies in the clinical setting. *In vitro*, targeting of ATF5 using siRNA-*ATF5* or dominant negative ATF5 (d/n-ATF5) constructs results in normal differentiation of neural progenitors in rodents,[15–17] and has no apparent deleterious effects on non-neoplastic, activated astrocytes.[20] In contrast, human and rat glioma cells *in vitro* undergo apoptosis when subjected to ATF5 expression knock down or functional abrogation.[20, 22, 25] Similarly, *in vivo* investigations utilizing different methods of ATF5 interference in a variety of rodent glioma models have demonstrated selective regression or eradication of tumors via promotion of massive apoptosis, while maintaining integrity of surrounding normal brain.[21, 22, 25]

Truncated, but fully active dominant negative ATF5 peptides have been developed that are fused to a cell penetrating domain permitting passage through the blood-brain barrier into intact cells. These peptides can be delivered systemically and provide a potential means to utilize an ATF5 targeted approach in the clinical setting.[22, 25] To validate the use of similar ATF5 targeted strategies in dogs with spontaneous gliomas, ATF5 protein expression in canine spontaneous glioma tumor samples was assessed using western blotting and immunohistochemistry, and sensitivity of canine glioma cell lines to an ATF5 dominant negative peptide was assessed *in vitro*. We report that ATF5 is overexpressed in canine gliomas, and that similar to human tumor cell lines, *in vitro* treatment with a synthetic cell penetrating dominant negative ATF5 peptide results in decreased canine glioma cell viability.

Materials and Methods

Sample Collection

Tumor tissue was obtained at necropsy or from surgical or computerized tomography (CT)-guided biopsy of clinical cases presented to the University of California, Davis Veterinary Medical Teaching Hospital. Samples were snap frozen in liquid nitrogen within 1 hour of collection. Tumors consisted of 8 high grade (III) oligodendrogliomas, 4 low grade (II) astrocytomas, 1 anaplastic astrocytoma (III), 6 high grade (IV) astrocytomas (glioblastoma), and 4 mixed oligoastrocytomas (2 grade II, 2 grade III). All tumors were histologically classified by a board-certified pathologist according to the WHO classification of human tumors of the central nervous system.[26]. Protein samples from neurologically normal adult and fetal canine cerebrum (superficial frontal/parietal) containing both white and grey matter were similarly collected at necropsy.

Cell culture

Three canine glioma cell lines (J3TBg, SDT3G, and G06A) were assayed by western blotting and the J3TBg and SDT3G cell lines were used for cell viability assays. The J3Tbg cell line was derived from a grade III astrocytoma.[27] SDT3G and G06A cell lines were derived from glioblastoma samples G3 and G6, respectively. All cell lines were grown in high glucose DMEM with 1.5% or 10% fetal bovine serum (FBS) as previously described.[7]

Western Immunoblot

Proteins were extracted from archived frozen tissue and cell lines using RIPA buffer (ThermoFisher Scientific, Waltham, MA) and quantified using a Pierce BCA Protein Assay (ThermoFisher Scientific, Waltham, MA). Western blotting was performed as previously described and 20ug of total protein were loaded for each sample.[7] Rabbit polyclonal anti-ATF5 primary antibody (1:1000, #4500895, Sigma-Aldrich, St Louis, MO) was used for quantification of ATF5 expression in tumor samples specifically on western blots. Rabbit monoclonal anti-ATF5 (1:2000, #ab184923, Abcam Cambridge, MA) was used for immunohistochemical studies and was initially validated on western blots by showing a predominant 37 kDa band using canine tissues cell lines that include fetal canine brain (40–50 weeks gestational age), dog grade III oligodendroglioma, J3TBg cell line, and G06A cell line (Supplementary Figure 1). Rabbit anti-GAPDH (1:3000, #25778, Santa Cruz Biothechnology Inc., Santa Cruz, CA) was used as a loading control. The secondary antibody used was horseradish peroxidase (HRP)-conjugated goat anti-rabbit (1:5,000 #12-348, EMD Millipore, Billerica, MA) and blots were visualized with SuperSignal West Femto solution (Thermo Fisher Scientific, Waltham, MA). Images were captured and exposure was optimized using Protein Simple FluorChemE (Bio-Techne, San Jose, CA). ATF5 bands were quantified densitometrically and normalized to GAPDH using Image J software (v1.48, National Institutes of Health, Bethesda MD). Normalized mean values for the tumour types and normal brain were statistically evaluated for significant difference among the groups by 1-way analysis of variance (ANOVA) with posthoc pairwise comparisons using Tukey's honest significant difference(HSD) procedure. Significance was defined as $P < .05$. Comparison of all tumor samples to normal brain was done using Welch's unequal variances t-test.

Immunohistochemistry

Antigen retrieval and immunofluorescence staining to define cellular localization of ATF5 expression was done using 4 formalin fixed, paraffin embedded dog tumor samples (2 anaplastic oligodendrogliomas, 2 glioblastomas) also containing areas of normal brain, and methodology as previously described[19] with minor changes. Antigen retrieval was achieved by incubating the tissue-mounted slides in a steamer (Black & Decker HS 1600, Towson, MD) in 10 mM Citrate buffer, pH 6.0 for 40 min at 100°C. Slides were kept overnight at 4°C to maximize the antigen retrieval. Tissue sections were blocked in 10% normal goat serum with 0.1% Triton-X100 for 2 hours. Rabbit anti-ATF5 monoclonal antibody (1:500, ab184923/Abcam Cambridge, MA) or rabbit irrelevant non-specific immunoglobulin (1:200) in 10% normal goat serum with 0.1% Triton-X100 were applied concurrently overnight at ambient temperature. Sections were rinsed three times for 10 minutes in Phosphate Buffered Saline (PBS prior to secondary labeling with goat anti-rabbit antibody Alexa Fluor 488 IgG (H+L) (1:1,000, #A-11034, Thermo Fisher Scientific, Waltham, MA) in 10% normal goat serum with 0.1% Triton-X100 for 2 hours at ambient temperature. Sections were rinsed three times for 10 minutes in PBS. The sections were labelled with 4,6-Diamidino-2-phenylindole, dihydrochloride (DAPI) (#D9542, 1 mg/mL, 1:1000, Sigma-Aldrich) in PBS buffer for 5 minutes with a final 10-minute rinse with PBS. Slides were mounted using Prolong diamond (#P36961, Thermo Fisher Scientific).

Immunofluorescence was subjectively assessed for localization of ATF5 as nuclear, cytoplasmic or membranous.

Cell viability assay

3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) was used to measure cell viability. Two dog glioma cell lines (J3TBg, SDT3G) were seeded in 96 well plates at a density of 2000–5000 cells depending on cell lines, for 24 hours prior to drug exposure. Cells were cultured in standard 10% FBS or 1.5% FBS to simulate a low nutrient environment. Cell penetrating-dominant negative-ATF5 peptide (CP-d/n ATF5, CS Bio Co., Menlo Park, CA)[21, 22] diluted with water (Type I) was added to cells for 5 days to achieve concentrations between 0–336 μM CP-d/n-ATF5. After 5 days, the medium was removed and cell viability was assayed using a standard MTT assay. Control groups consisted of cells with 1.5% or 10% FBS and equivalent volumes of diluent, and all experiments were done in triplicate. Formazan concentration was determined by optical density using a test wavelength of 570nm using an automated plate reader (SpectraMax M3, VWR, Radnor, PA). Results were expressed as a percentage of absorbance in treated or control wells relative to untreated wells. Significant differences in cell viability at differing drug concentrations was determined by original one-way ANOVA Fisher's least significant difference (LSD), and half maximal effective concentration (EC_{50}) values were calculated using non-linear regression (GraphPad Prism, GraphPad Software Inc., La Jolla, CA).

Results

Western blotting

Specificity of the ATF5 antibodies in canine tissues was assessed by visualization of an appropriate molecular weight-sized band on western blotting between 30–35kDa (Fig 1A, Suppl Fig 1). Protein lysates from normal adult brain, glioma cell lines and dog glioma samples produced a band of approximately 35kDa (Fig 1A). Normalization of ATF5 to GAPDH showed a general pattern of higher expression in tumor samples compared to normal brain with highest expression seen in the high grade glioma samples (Fig 1B). Expression of ATF5 was significantly different among the sample groups when comparing normal brain, astrocytomas, glioblastomas, oligodendrogliomas and mixed oligoastrocytomas ($p=0.0083$). Pairwise assessment showed a significant difference in ATF5 expression in glioblastoma samples compared to normal brain ($p=0.0037$). ATF5 expression was also significantly different comparing all tumor samples to normal controls ($p<0.0001$) (Fig 1C,D).

Immunohistochemistry

Positive immunostaining for ATF5 was seen in all tumor samples consistent with western blotting data, and was localized in both nuclear and cytoplasmic compartments. Membranous expression was not observed. (Fig 2). Immunostaining in peritumoral normal brain was minimal compared to tumor tissue (Fig 3).

Cell viability assays

Exposure of canine glioma cell lines J3TBg and SDT3G to the CP-d/n ATF5 peptide for 5 days resulted in a concentration dependent decrease in cell viability at micromolar concentrations. Similar biological effects were seen using low serum media conditions (1.5% FBS, Fig 4A,C) and standard serum conditions (10% FBS, Fig 4B,D) with no significant differences in viability curves between the different conditions ($p=0.91$). Relative to vehicle control, statistically significant decreases in cell viability were observed at CP-d/n ATF5 concentrations ≥168μM for both J3Tbg and SDT-3G cell lines, regardless of serum conditions. Apparent EC_{50} values for J3Tbg and SDT3g in 1.5% serum conditions were 192.9 μM and 218.9 μM respectively, and in 10% serum conditions, 169.8 μM and 220.1 μM respectively.

Discussion

The current study suggests that overexpression of ATF5 previously reported in human and rodent gliomas is recapitulated in spontaneous dog gliomas, and that investigated ATF5 antibody and therapeutic peptide previously described for use in rodents and humans have cross species reactivity and activity in dogs.

The pattern of ATF5 immunostaining seen in dog gliomas in this study (Fig 2) resembles that seen in human gliomas and other human tumors where ATF5 protein is increased, and is consistent with ATF5's role as a nuclear targeted transcription factor.[20, 24, 28] While ATF5 is consistently expressed in the nucleus, cytoplasmic localization is also reported,[15–17, 29, 30]

and ATF5 has been reported to be an essential pericentriolar material protein also localized in the cytoplasm.[31] Previously reported low level immunohistochemical expression of ATF5 in peritumoral non-neoplastic brain[18, 20, 24] was also seen in the dog samples, although extensive anatomical assessment of ATF5 expression beyond cellular localization was not a specific aim of the current study. Specific assessment of expression and effects of ATF5 abrogation in normal brain derived and tumor derived progenitor/stem-like cells will be necessary to determine if similarities between dogs and humans also extend to this developmental level. Overexpression of ATF5 has been associated with increased tumor grade in human rectal cancer,[32] and ATF5 levels correlate negatively with survival in human glioblastomas.[18, 23, 33] Controlled survival data for dog glioma patients are not available, however the highest expression of ATF5 on western blotting was seen in the highest grade tumors, (glioblastoma, Fig 1) consistent with the human data. Further assessment of ATF5 expression across a larger group of variably graded tumors may be indicated to define potential ATF5 targeted therapeutic trial candidates and the use of ATF5 expression as a potential biomarker for survival in dogs.

In vitro activity of the CP-d/n ATF5 peptide in cell viability assays with canine glioma cell lines was similar to that reported for human cancer cell lines, including gliomas, with EC_{50} values in the 10^2 uM concentration range.[25] Extrapolation of *in vivo* dosing approaches assessed in preclinical rodent models based on these human cell line data may therefore be a reasonable starting point for pilot studies investigating the CP-d/n ATF5 peptide in dog spontaneous tumors. Abrogation of ATF5 activity by this and other mechanisms has repeatedly been shown to have no observable effects on non-neoplastic cells, even when ATF5 expression is present.[20–22, 24, 25, 28] Systemic delivery of between 4–150 mg/kg of CP-d/n ATF5 peptide has been shown to have profound antitumor effects in rodents, with no observable effects on normal brain or other biochemical or histological parameters.[22, 25] Pilot toxicity and pharmacokinetic data in dogs following subcutaneous delivery of CP-d/n ATF5 has revealed no adverse effects systemically or histopathologically with doses of 30mg/kg (estimated mouse equivalent dose[34] = 200mg/kg) (unpublished data).

The mechanism by which CP-d/n-ATF5 achieves cytotoxicity in canine gliomas has not been investigated, however we expect the cancer cell pro-survival function of ATF5 to be analogous to that in human and mouse cells. ATF5 promotes the function of the anti-apoptotic proteins BCL2,[35] MCL1,[18] BAG3,[25] and Egr-1 protein[36] in neoplastic cells by a combination of target gene upregulation as well as decreased proteolysis through upregulation of USP9X deubiquitinase.[25] In addition to the proven efficacy of CP-d/n-ATF5 as a monotherapy in preclinical models, combinatorial therapeutic approaches may be beneficial as the peptide has been shown to be synergistic with the antineoplastic ligand TRAIL and the BH3-mimetic ABT263.[25] ATF5 also promotes radioresistance and malignancy in cancer cells following irradiation,[33] making combination anti-ATF5 therapy with radiation a logical therapeutic approach.

Dog gliomas share similarities to human tumors and preclinical rodent models in terms of ATF5 expression and functional response to available therapeutic peptides. The apparently high therapeutic index of the CP-d/n ATF5 peptide as well as the cross species reactivity

support its further evaluation in glioma and other cancers in dogs for primary therapy and translational development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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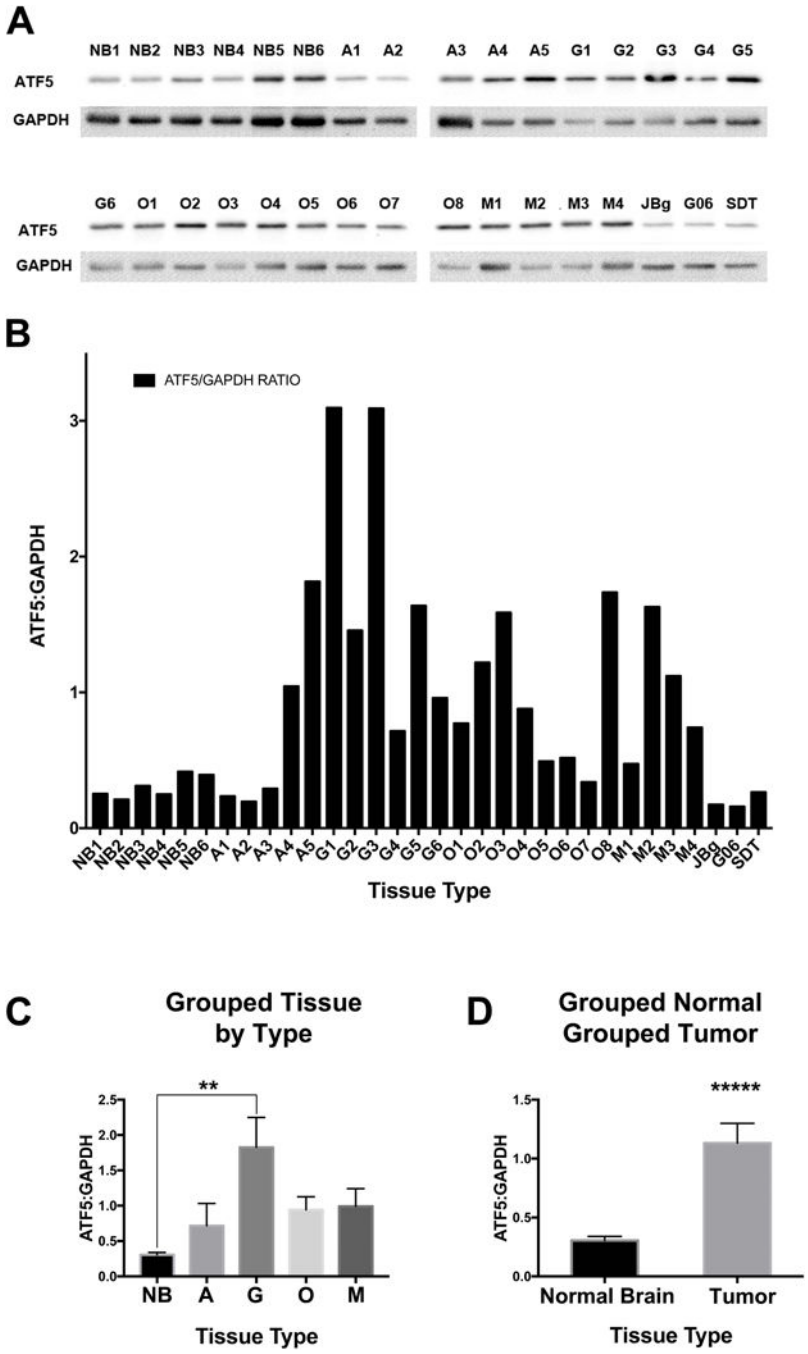

Fig 1.

Western immuno blot of ATF5 protein in canine normal brain, gliomas, and glioma cell lines J3TBg (JBg), SDT3G (SDT), and G06A (G06). Expression is seen in all samples (A), with higher expression seen generally in higher grade tumors compared to normal brain (B,C,D). NB= normal brain; A1,2,3,5=Grade II astrocytoma, A4=Grade III astrocytoma; G=glioblastoma/grade IV astrocytoma; O=Grade III oligodendroglioma, M1,4=Grade II oligoastrocytoma, M2,3=Grade III oligoastrocytoma. (** p=0.0037) (**** p<0.0001).

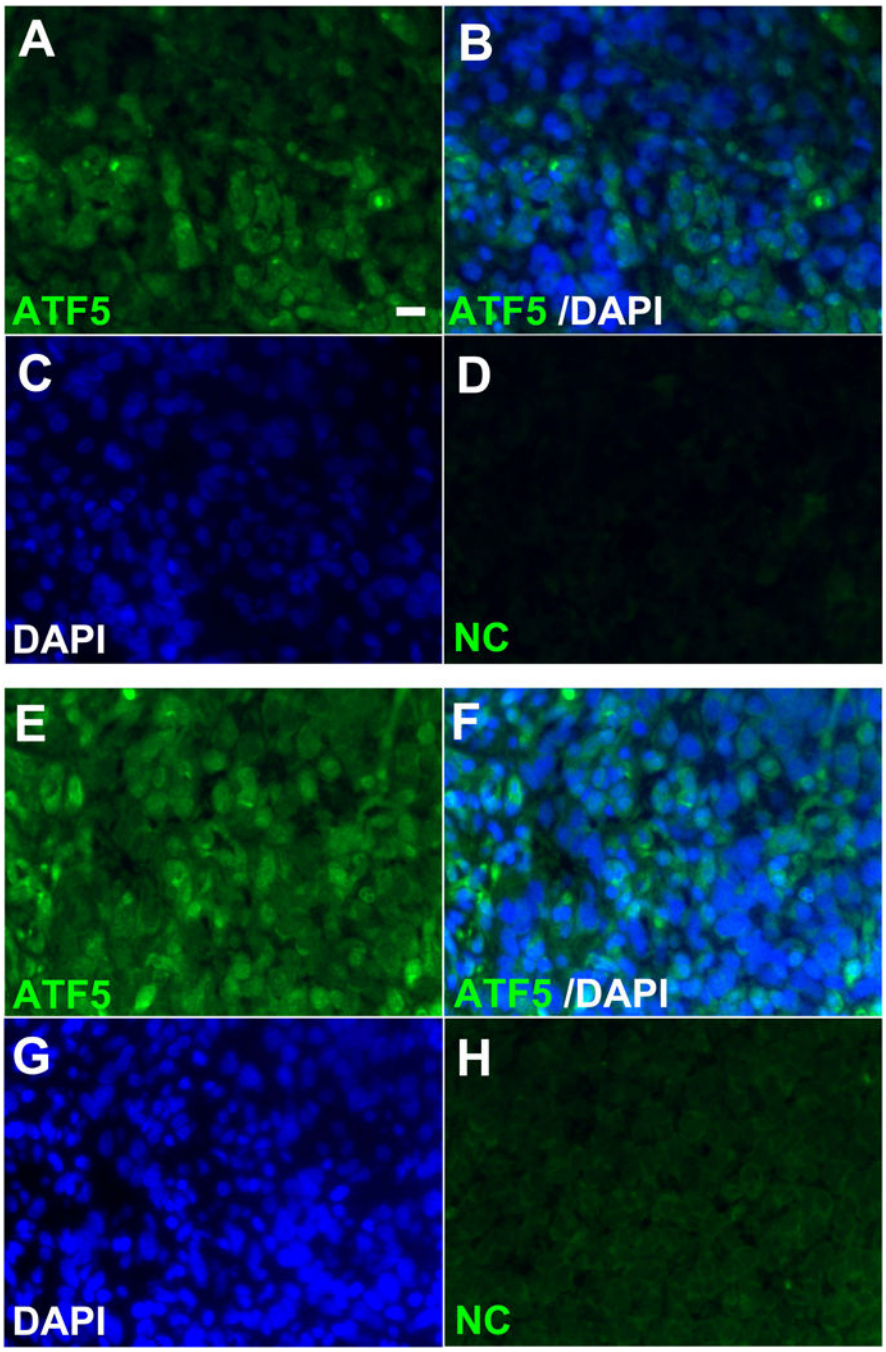

Fig 2.

ATF5 fluorescent immunostaining of two canine glioblastoma samples (A–D and E–H). (A,E) ATF5 immunostaining, (C,G) DAPI nuclear staining, (B,F) ATF5/DAPI overlay, (D,H) ATF5 negative control (NC) consisting of non-relevant rabbit immunoglobulin. Scale bar = 20μm

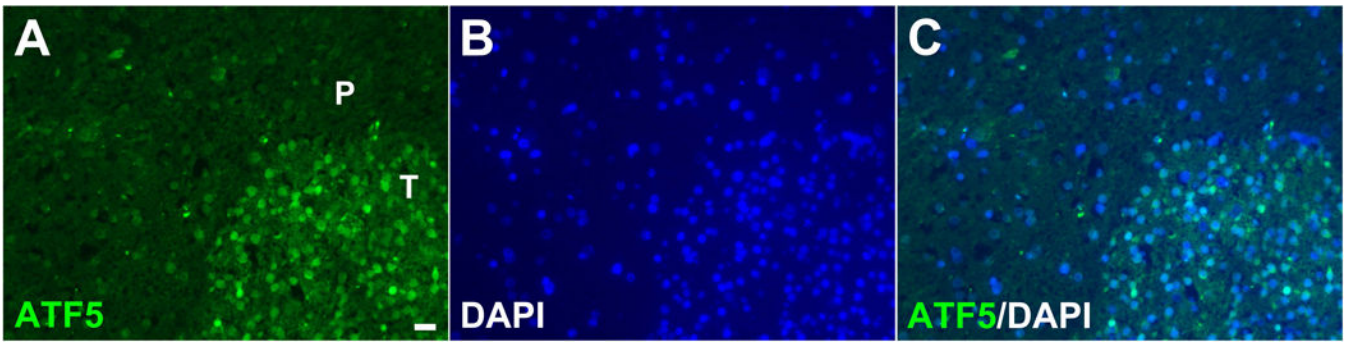

Fig 3.

ATF5 fluorescent immunostaining of peritumoral region from Fig 2A. (A) ATF5 immunostaining, (B) DAPI nuclear staining, (C) ATF5/DAPI overlay. Decreased cellularity and ATF5 immunostaining is apparent in the peritumoral tissue (P) compared to the tumor (T). Scale bar = 40μm

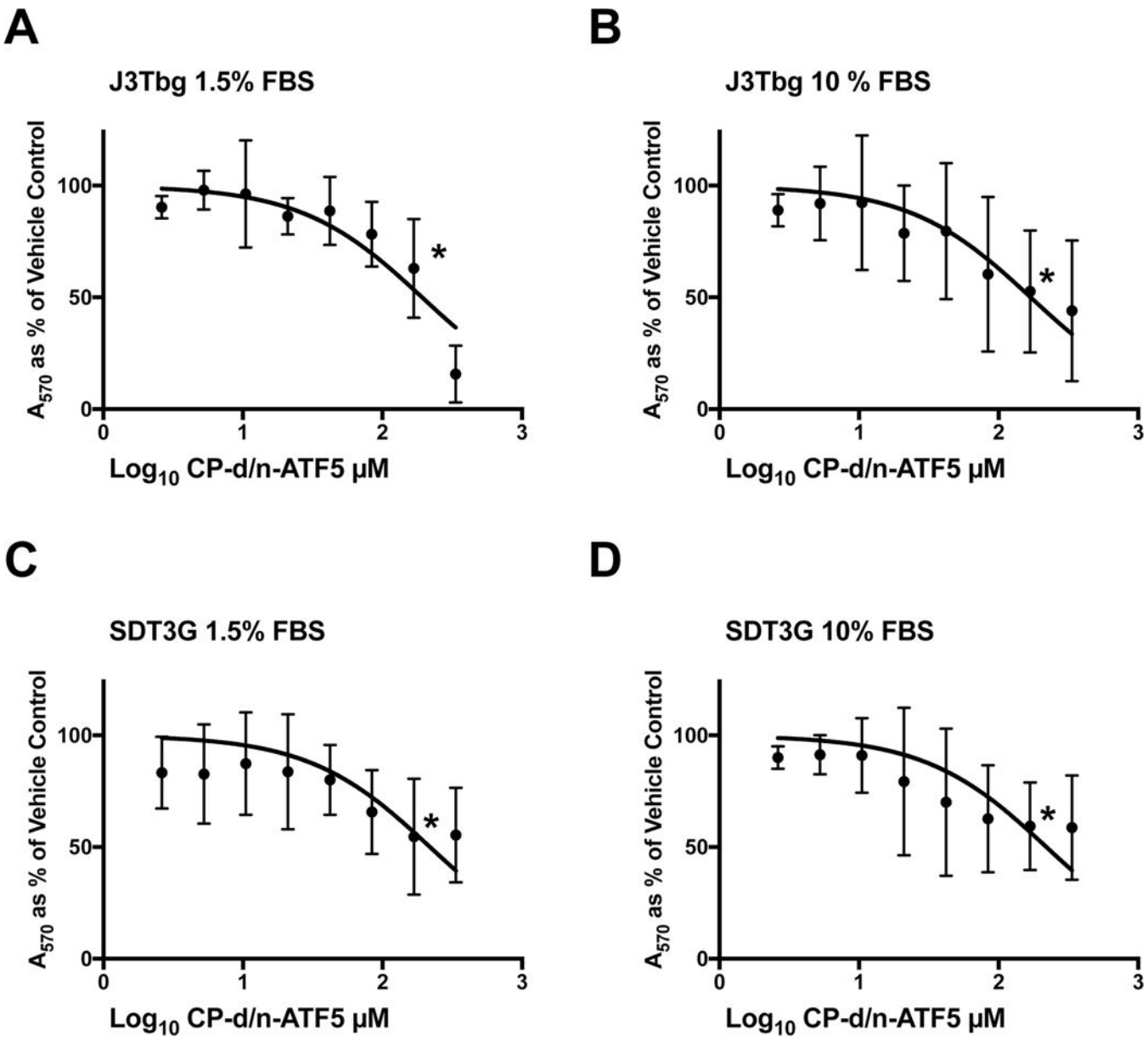

Fig 4.

MTT cell viability dose response curves for canine glioma cell lines following exposure to CP-d/n-ATF5 peptide. Peptide effects were assessed in low serum (A,C) and standard serum (B,D) conditions. Values are means +/− standard deviations. (* significantly different from vehicle control, $p<0.05$)