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Notch Inhibitors and the BET Inhibitor JQ-1 Decrease the Growth of Primary Tumor Cells Derived from a Novel Mouse Model of C11*orf*95-RELA Induced Brain Tumor

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I. ABSTRACT

Brain tumors are the most common childhood solid malignancy, and because of remarkable advances in treating many cancers outside of the brain, they have become the leading cause of cancer mortality in children. Ependymomas are a class of brain tumors which can be further subdivided into three groups based upon their location and genetic features. Of the three classes, supratentorial ependymomas are the only subgroup known to be marked by an oncogenic driver gene, which consists of a fusion mutation between the C11*orf*95 and RELA genes. C11*orf*95-RELA positive tumors are the most aggressive and lethal of all ependymomas. With a high mortality rate in ependymoma patients and the current lack in effective treatment methods for such brain tumors, advancement in treatment targets and mechanisms is necessary to improve patient survival. In this study, we assess the use of Notch inhibitors and JQ1 for their effectiveness in inhibiting the growth of *C11orf95*-RELA transformed cells isolated from primary brain tumors in mice. We find that both the BET bromodomain inhibitor JQ-1 and the FGFR inhibitor AZD4547 inhibit growth of C11*orf*95-RELA transformed cells and that the BET domain inhibitor JQ1 does so with the highest potency.

II. INTRODUCTION

Ependymomas are a class of brain tumors characterized by neoplasms containing a mixture of abnormal glial and endothelial cells. The third most common brain tumor children, ependymomas tumors can be divided into several subclassifications based upon their location and molecular genetic features[1]. An important subclass of these tumors are supratentorial ependymomas (ST-EPN), which develop in the third or lateral ventricles and have been found to be significantly correlated with a novel fusion involving the C11*orf*95 and RELA genes[2]. The cellular origin of ST-EPN is an incompletely defined population of neural stem cells (NSCs), possibly including radial glial cells (RGCs), intermediate progenitor cells (IPCs), or other related NSCs.[3, 4] The known source of ependymal cells and glial cells in normal development are RGCs, thus providing evidence suggesting that RGCs may be the starting cell type of ST-EPN[5].

Ependymoma, like other cancers, is a genetic disease, with multiple subtypes often having distinguishing genetic characteristics. Recently, a *C11orf95*-RELA fusion mutation has been identified as the recurrent genetic alteration driving the ST-EPN subtype of ependymoma[2, 6, 7, 8, 9, 11]. RELA encodes a well-studied protein known as p65, which plays an integral part in the NF-κB pathway, which functions in many cellular processes, including metabolism, modulation of immunity, and cell death[10,11]. The function of *C11orf95*, on the other hand, is unknown, although it is believed to encode a protein with zinc finger domains suggesting a role in transcriptional regulation[11]. The *C11orf95*-RELA fusion found in ST-EPN tumors has been shown to activate the NF-κB signaling pathway, and to be sufficient to immortalize and transform neural stem cells forced to express *C11orf95*-RELA by viral infection. Taken together, two, not mutually exclusive, hypotheses for why the *C11orf95*-RELA fusion may transform some types of neural stem cells into ependymoma have been proposed: i) inappropriate activation of the NF-κB pathway in a population of cells that usually expresses *C11orf95* but not RELA, and/or ii) altered and dysregulated NF-κB pathway activity because *C11orf95* protein domains (exons 1-2) become fused to the N-terminus of RELA[8, 10, 11].

Currently, the primary treatment for ependymoma remains to be surgical resection followed by radiotherapy. Although postoperative radiotherapy may induce stabilization and, occasionally, regression of residual disease, most incompletely resected tumors ultimately progress[7]. Seeing as no chemotherapy regimen has prolonged overall survival in children with ependymoma, development of new therapeutic options is needed to increase survival and reduce long-term sequels of current treatments.

Support for the implication of the *C11orf95*-RELA fusion in both the NF-κB and NOTCH signaling pathways as a means of tumorigenesis is well documented[8, 11, 19, 20, 21, 22]. Nuclear factor- κB (NF-κB) functions in transcriptional regulation to influence gene expression events that impact the immune system as well as cell survival, differentiation, and proliferation. The basic scheme of NF- κB signaling consists of a series of positive and negative regulatory elements. Activating stimuli trigger activation of IKK, leading to the phosphorylation, ubiquitination, and degradation of IκB proteins, which normally function to bind NF- κB and prevent its entry into the nucleus[23]. When released from IκB proteins, NF-κB may enter the nucleus and promote transcription of genes that function in the production of inflammatory mediators, inhibition of apoptosis, and promotion of cell differentiation and division. The NF-κB family of transcription factors consists of five members, one of which is RelA (p65) [23].

The NOTCH signaling pathway also plays a role in cell survival, differentiation, and proliferation. *NOTCH* genes encode single-pass transmembrane proteins that are activated upon binding of their respective ligands[22]. Upon ligand binding, Notch receptors undergo a cascade of activating cleavages mediated by a γ-secretase complex. This γ-secretase releases the Notch intracellular domain (NCID), enabling it to translocate to the nucleus and associate with transcription factors that ultimately lead to transcriptional activation of Notch target genes such as HES1, HEY1, and MYC, among many others[22, 23].

Given the inability to effectively treat ST-EPN with current standard practices, researchers have focused their efforts on evaluating genetic expression profiles and molecular pathways. While understanding of the intricate interplay between the various pathways involved in tumor initiation, development, and progression still require deeper investigation, potential therapeutic targets have been identified, providing key areas of focus for future research. Downregulation of the Notch pathway via γ-secretase inhibitors has recently been studied as a means of tumor suppression[21, 22, 24]. Puget, et. Al. demonstrated a downregulation of the Notch pathway with subsequent reduction in the size, number, and proliferation of neurospheres in a 2009 trial of ependymoma stem cell cultures[24]. Similarly, Dantas-Barbosa et. Al. found a reduction in cell density and viability of ependymoma short-term cell cultures, and tumor growth inhibition in advanced-stage glioblastoma xenografts when combined with irradiation[21].

In targeting the NF-κB pathway, the efficacy of the BET-bromodomain inhibitor JQ-1 as a therapeutic target for ST-EPN has also been studied[25]. The bromodomain and extra-terminal domain (BET) family comprises four adaptor proteins (Brd2, Brd3, Brd4, and Brdt) that regulate transcription by RNA polymerase II. These proteins function by recognizing and binding to acetylated lysine residues on histone tails, leading to increased gene expression of the targeted genes[26]. BET bromodomain inhibitors can thus serve as effective measures to reduce gene expression. Coactivation of the transcriptional activity of NF-κB has been found to be facilitated by the BET bromodomain protein Brd4 binding to acetylated RelA. Specifically, RelA, one of the subunits of NF-κB, became acetylated at lysine 310 to facilitate interactions with the

bromodomains of Brd4[27]. Treatment of the irregular upregulation of NF-κB in ST-EPN may thus be possible via the use of BET bromodomain inhibitors. The efficacy of the BET bromodomain inhibitor JQ-1 in reducing cell proliferation has been tested in both ependymoma and neural stem cell lines[25], but has not yet been tested on cells specifically transformed by the C11*orf*95-RELA oncogene.

Animal models can be used to test candidate pharmacological therapies against tumor cells transformed by defined molecular causes. Previous attempts to develop ST-EPN animal models have utilized viral infection. We recently developed an additional model of C1*1orf*95-RELA positive ST-EPN by combining *in utero* electroporation with a binary *piggyBac* transposon system (PB-IUE). In this system, a single transfection of neocortical radial glia in the fetal brain results in stable transgene expression in the neural lineage of radial glia, including neurons, astrocytes, and oligodendrocytes[28]. We have found that introduction of the C11*orf*95-RELA oncogene in this system is sufficient to induce tumors resembling ST-EPN in humans, and the aggressiveness and lethality of these tumors can be increased by mutating the tumor suppressor gene TRP53. In addition, we have isolated primary cells from these tumors which can be propagated in cell cultures over multiple passages. Both the animal model and primary cell cultures of C11*orf*95-RELA transformed cells can be used to test the effectiveness of different molecular and pharmacological therapies. Specifically, in this thesis project I've tested notch inhibitors and JQ1 for their effectiveness in inhibiting the growth of C110rf95-RELA transformed cells isolated from primary brain tumors in mice. Results indicate that both types of blockers inhibit growth of these cells and that the BET domain inhibitor JQ1 does so with the highest potency. The implications of these findings for future *in vivo* studies and with respect to the molecular signaling mechanisms that may underlie uncontrolled tumor growth in ST-EPN will be further discussed.

III.   METHODS

Plasmid Construction

Plasmids were constructed for development of an ST-EPN mouse model. To make pPBCAG-FUS1-HA and pPBCAG mCherry, the C11orf95-RELA fusion (termed FUS1) and mCherry sequences, respectively, were amplified and replaced the eGFP cassette of a previously constructed pPBCAG-eGFP construct. Replacement was achieved using the *EcoRI* and *Not1* sites of the original plasmid. For construction of pGLAST-PBase, the PBase coding sequence was directly inserted downstream of the GLAST promoter, a gift from Fuyi Chen.

Animals

CD1 mice were obtained from Charles River Laboratories, Inc. (Wilmington, MA), and maintained at the University of Connecticut vivarium. Animal gestation ages were determined and confirmed during surgery. Both male and female embryos were used for surgery, and all procedures and experimental approaches were approved by the University of Connecticut IACUC.

In Utero Electroporation

In utero electroporation was performed as previously described[28, 29]. Briefly, rats were anesthetized with a mixture of ketamine/xylazine (100/10 mg/kg i.p.). Metacam analgesic was administered daily at dosage of 1 mg/kg sub cutaneously at 24 and 48 hours following surgery. To visualize the plasmid during electroporation, plasmids were mixed with 2 mg/ml Fast Green (Sigma). In all conditions described, pPBCAG-FUS1-HA and the helper plasmid GLAST-PBase were used at the final concentration of 1.5 μg/μL, while the fluorescent proteins PBCAG-eGFP and PBCAG-mCharry were used at the final concentration of 1.0 μg/μL. Electroporation was performed at embryonic day 13 or 15 (E13 or E15). During surgery, the uterine horns were exposed and one lateral ventricle of each embryo was pressure injected with 1–2 μl of plasmid DNA. Injections were made through the uterine wall and embryonic membranes by inserting a pulled glass microelectrodes (Drummond Scientific) into the lateral ventricle and injecting by pressure pulses delivered with a Picospritzer II (General Valve). Electroporation was accomplished with a BTX 8300 pulse generator (BTX Harvard Apparatus) and BTX tweezertrodes. A voltage of 65–75V was used for electroporation. Hippocampal electroporation was performed as previously described[30].

Cell Culture

Development of ST-EPN Cell Line: A tumor sample from mouse neocortex (P21?) was obtained from a mouse model induced with ST-EPN via introduction of the C11*orf*95-RELA fusion by *in utero* electroporation. The tissue sample was dissociated using mechanical disruption followed by trypsin treatment for neural progenitor culture. Cells were grown and maintained in DMEM supplemented with 1% penicillin and streptomycin and 10% Fetal Bovine Serum. Cells continued to proliferate after 25 passages, demonstrating the development of an immortal primary ependymoma cell line.

Verification of ST-EPN: The C11orf95-RELA fusion was verified using a standard immunostaining protocol and microscopy (SP8 confocal microscope) for an HA-tag marking to the fusion mutation (PBCAG-FUS1-HA).

Immunohistochemistry

Animals were deeply anesthetized with isoflurane and perfused transcardially with 4% paraformaldehyde/PBS (4% PFA). Brain samples were post fixed for 24 hours in 4% PFA and sectioned at 65 m thickness on a vibratome (Leica VT 1000S). Sections were processed as free-floating sections. After blocking in PBS containing 5% of normal goat serum (Sigma) and 0.3% Triton X-100 (Sigma) for 2 hours at room temperature, tissue sections were incubated with primary antibodies overnight at 4 ∘C in the blocking solution. The following primary antibodies were used: rabbit anti-HA (1:1000, Invitrogen), and mouse anti-GFP (1:1000, Invitrogen). Tissue sections were washed in PBS, incubated with the appropriate secondary antibodies (1:2000 Alexa Fluor 647 Goat anti-Rabbit, Invitrogen; 1:2000 Alexa Fluor 488 Goat anti-mouse, Invitrogen) for 2 hours at room temperature and washed in PBS. In some tissues, nuclei were labeled 4-6-diaminodino-2-phenylindole (1:2000, DAPI, Invitrogen). Images were acquired on either a Leica TCS SP8 confocal system or Zeiss AXIO Zoom V.16.

Cellular Assays

5000 cells (from the primary ependymoma cell line) were plated into a 24-well plate in 500 μL DMEM (1% Pen./Strep., 10% FBS). Drug dilutions were prepared in DMSO. Aliquots from the drug-DMSO solutions were added to an appropriate volume of DMEM to generate a "Master Solution" in order to reduce pipetting errors. 500 μL of the appropriate solution was applied to its respective well 24 hours after cells were seeded. At 48 and 72 hours post-drug application, cells were imaged for qualitative analysis using a Leica DMI8 scope. At 72 hours post-drug application, a Trypan Blue cell assay was performed. The incubated solutions were aspirated and cells were washed once with 500 μL PBS applied carefully to the side of each well. After aspiration of the PBS wash, cells were incubated with 100 μL Trypsin for 2 minutes. During this time, 10 μL aliquots of Trypan Blue were spotted on a sterile petri dish. 100 μL DMEM (1% Pen/Strep., 20% FBS) was then added to each well to resuspend the cells. A 10 μL aliquot from a given resuspended well was then mixed with a given 10 μL Trypan blue aliquot. The resulting 20 μL solution was mixed by pipetted up and down. From this mixture, 10 μL were taken for quantification using an In-CYTO C-Chip disposable hemocytometer. Both the total number of cells and the number of blue cells were counted manually using a light microscope.

IV.   RESULTS

Transfection with C11*orf*95-RELA Fusion via IUE is Sufficient to induce ST-EPN in Mice Models

We investigated whether transfection via IUE with the C11*orf*95-RELA fusion was capable of generating ST-EPN neoplasms in mice models. Using an HA tag to the fusion, we see that tumors are predominantly comprised of C11*orf*95-RELA-HA positive cells (Figure 1). This demonstrates that transfection of the fusion into developing mice at E12-14 is sufficient to generate ST-EPN tumors. Furthermore, the neoplasms generated in our mice model stain positive for GFAP, validating the ST-EPN phenotype as tumors comprised of an abnormal proliferation of glial and ependymal cells (not shown).

Figure 1: Transfection with C11orf95-RELA Fusion is Sufficient to Induce ST-EPN Tumors in Mice. (a) Tumor from mouse transfected with FUS1 transgene via IUE. The neoplasm is comprised predominantly of HA-tagged FUS1 transfected cells. A small population of GFP-positive cells visible at the top of the neoplasm is enlarged in (b), where again it is evident that the tumor is composed predominantly of cells expressing the FUS1 transgene.
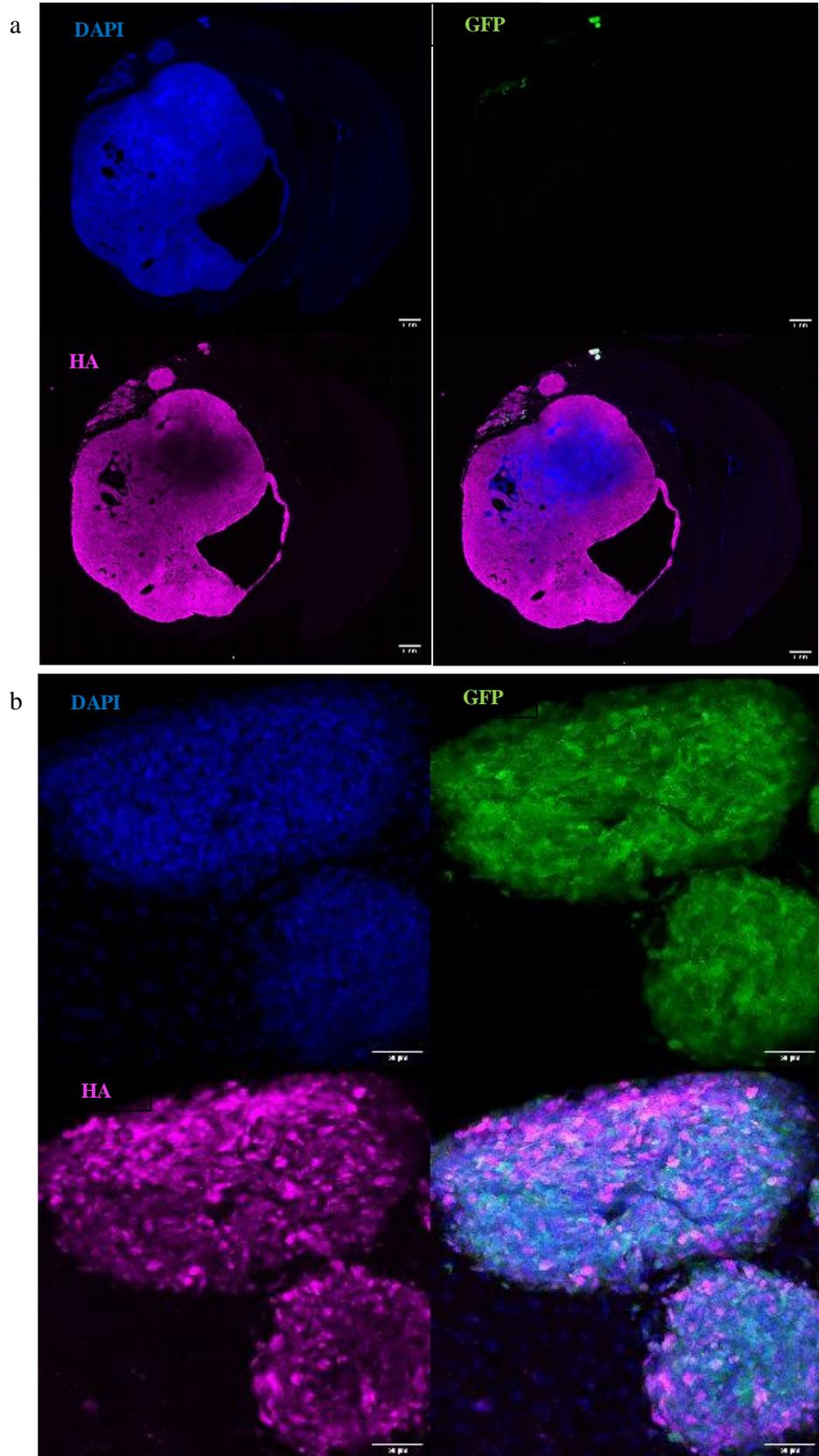
Validation of C11*orf*95-RELA Expression in Primary Cell Culture Line

Using an HA-tag to the C11*orf*95-RELA fusion, we investigated whether cells of the primary ependymoma cell line successfully expressed the fusion construct and demonstrated characteristics of ST-EPN. Imaging demonstrates successful expression of the C11*orf*95-RELA fusion, localized to cell nuclei (Figure 2).
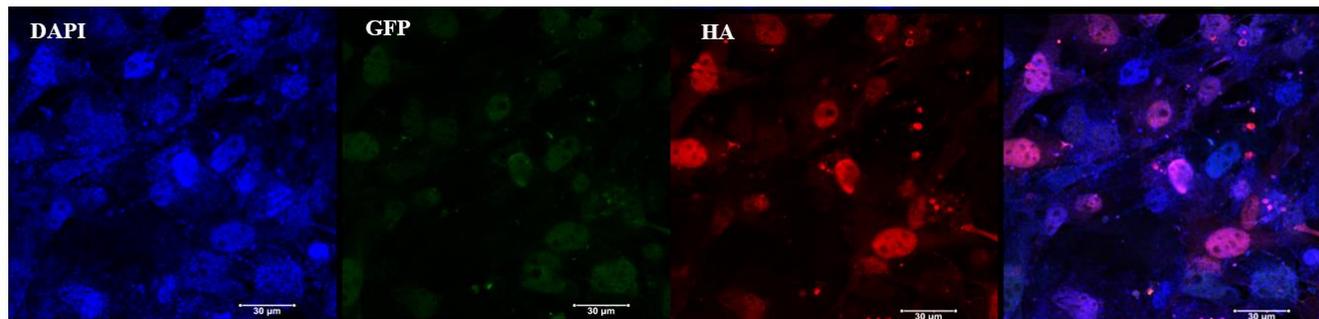

Figure 2: Primary Ependymoma Cell Line Demonstrates C11*orf*95-RELA Positive Tumor Characteristics. The HA-tagged C11*orf*95-RELA fusion is localized to cell nuclei, where it may be acting to upregulate oncogenic transcriptional targets. Some endogenous GFP is also present.

JQ-1, RO4929097, and AZD5447 Exhibit Concentration Responses at 72 hours

We investigated whether the pharmacological agents JQ-1, RO4929097, and/or AZD5447 could limit cell proliferation of the primary Ependymoma cell culture line over the course of 72 hours. Qualitatively, it can be seen that JQ-1 and AZD5447 inhibit cell division at concentrations greater than 10μM, while RO4929097 has no effect. Although both JQ-1 and AZD5447 limit cell proliferation, it appears that JQ-1 serves to limit cell division while maintaining normal cell morphology. In contrast, application of AZD5447 generates an irregular cell morphology (Figure 3).

Quantitatively, at concentrations of 30μM, all three drugs demonstrated a reduction in cell growth relative to media and DMSO controls. The IC_{50} of JQ-1 and AZD5447 however, was far lower than that of RO4929097, demonstrating a higher efficacy in the former over the latter. RO4929097 and AZD5447 both exhibited clear dosage dependence. Both JQ-1 and AZD4557 reduced cell proliferation at all concentrations tested, although JQ-1 proved the most efficacious; significantly limiting cell growth at 1μM (Figure 4).
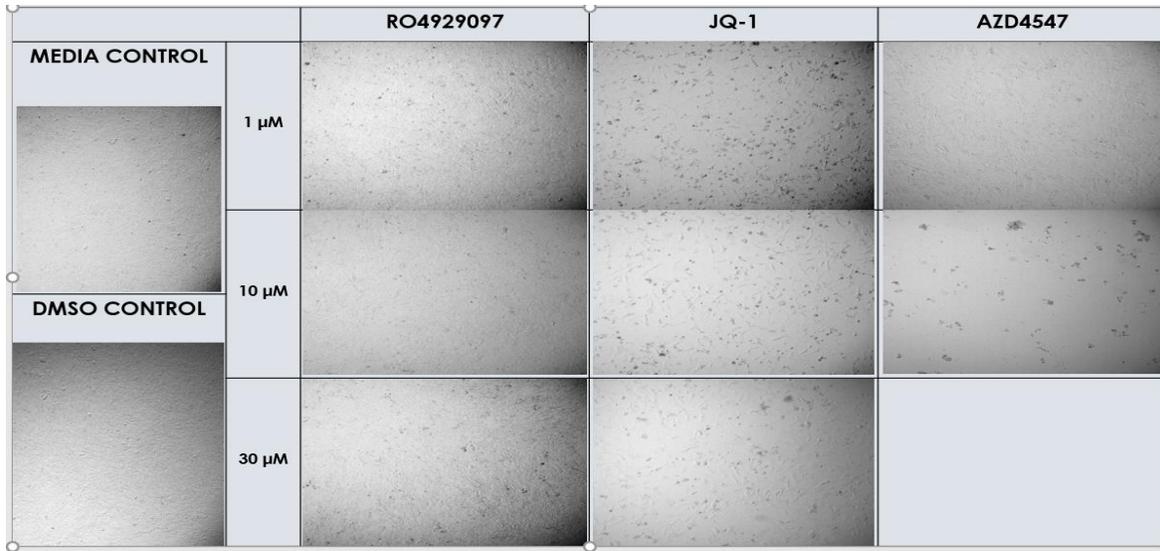
Figure 3: Qualitative analysis of cultures at 72 hours. While both JQ-1 and AZD4547 exhibited clear effects at concentrations above 10µM, JQ-1 appears to reduce cell proliferation while maintaining normal cell morphology. In contrast, AZD4547 appears to limit cell growth in addition to having effects on cell health, as seen by the irregular cell morphology and clumping exhibited at 10µM AZD4547.
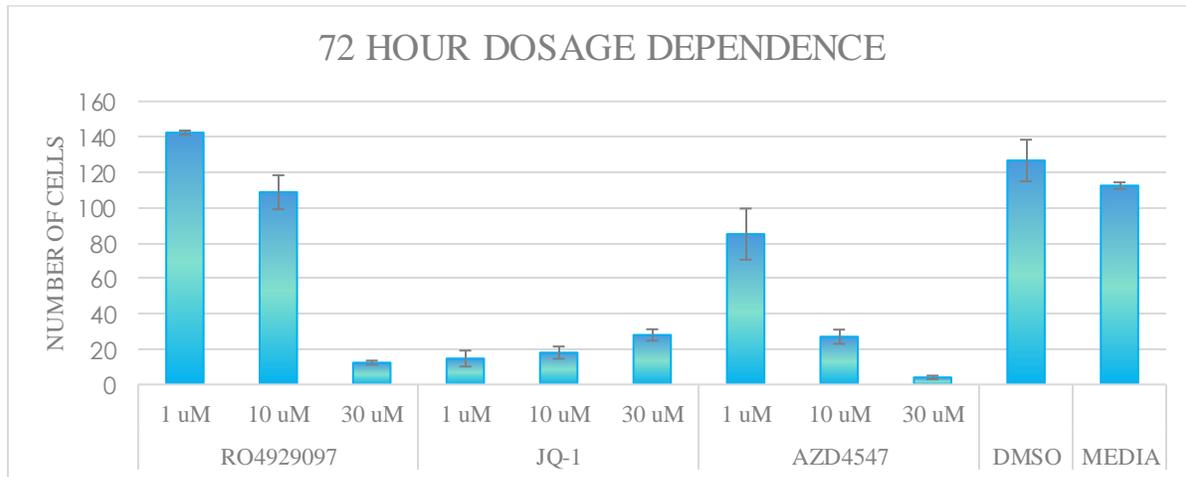

Figure 4: Quantitative analysis of cultures at 72 hours. While all three drugs demonstrated clear effects at concentrations above 30µM, JQ-1 appears to reduce cell with the most potency; significantly limiting cell growth at 1µM.

JQ-1 Exhibits Dosage Response in Primary Ependymoma Cell Line

Identifying JQ-1 as the most effective candidate for further analysis, we then performed a full dosage response curve for JQ-1 to better characterize its concentration response. Cell proliferation was analyzed at concentrations of 1, 0.3, 0.1, 0.03, and 0.01 µM JQ-1. Qualitative analysis demonstrates a clear dosage response curve, with increasing drug concentrations eliciting greater inhibition of cell growth in comparison to media and vehicle controls, as

expected (Figure 5). Concentrations of 0.1 μM or greater are sufficient to reduce cell proliferation; results that match closely with those obtained by Mack et. Al.[25].
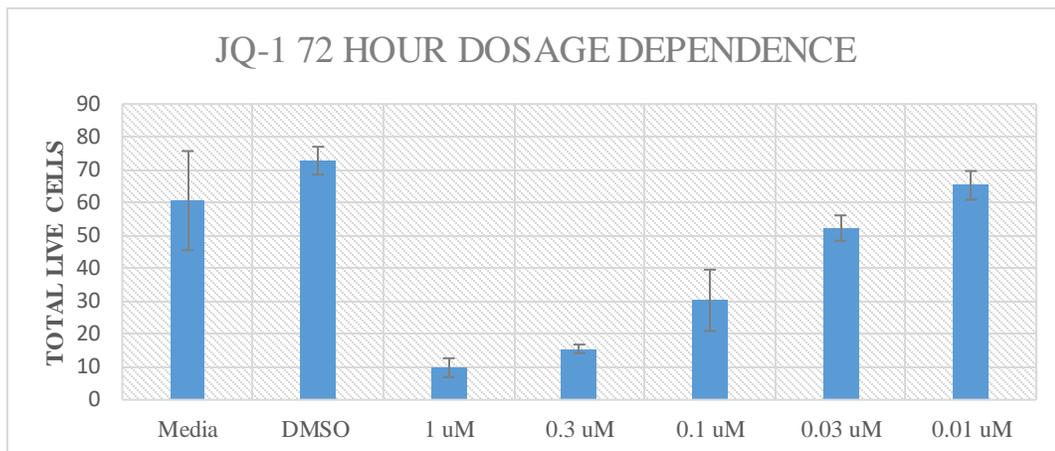

Figure 5: Quantitative analysis of JQ-1 cultures at 72 hours demonstrates clear dosage dependence for JQ-1 on primary ependymoma cell culture. JQ-1 exhibits effects on cell proliferation at concentrations above 0.1 μM, with an IC_{50} of approximately 0.1 μM.

V.   DISCUSSION

Ependymomas are associated with significant mortality. The 10-year overall survival rate is 64% in patients with ependymomas[32]. As such, development of effective and efficient treatment methods is crucial to improving patient outcomes. In this study, we assess the use of Notch inhibitors and JQ1 for their effectiveness in inhibiting the growth of *C11orf95*-RELA transformed cells isolated from primary brain tumors in mice. Major findings of the study are as follows: 1) both the BET bromodomain inhibitor JQ-1 and the FGFR inhibitor AZD4547 inhibit growth of C11*orf*95-RELA transformed cells and 2) the BET domain inhibitor JQ1 does so with the highest potency.

In 2018, Mack et. Al demonstrated that the FGFR blocker AZD4547 and the BET bromodomain inhibitor JQ-1 are effective in reducing cell growth in ependymoma cells and a neural stem cell line. They did not, however, assess the effectiveness of these drugs in C11*orf*95-RELA positive transformed cells. Our results show that both drugs can effectively limit proliferation in this cell line, and demonstrate similar IC_{50} values as to those found by Mack et. Al. These results indicate JQ-1 and AZD4547 may be effective treatment methods for limiting the growth of ST-EPN tumors.

Multiple prior studies have found that the NF-κB and Notch singlaing pathways are upregulated in ependymoma. The reduction in cell growth elicited by JQ-1 and AZD4547 application demonstrates that BET bromodomain proteins and Notch signaling may be required for EPN growth.

Seeing as BET bromodomain proteins – particularly Brd4 – play a role in upregulating transcription of the NF-κB pathway, specifically via interaction with acetylated RelA, they may be required for EPN growth and tumorigenesis. Our results support this hypothesis, as growth is

significantly reduced with application of the BET bromodomain inhibitor JQ-1. Further biochemical analysis, however, is necessary to gain insight into the mechanism by which JQ-1 acts to reduce cell proliferation in ependymoma. Additional cell-culture studies utilizing mutated RelA constructs to prevent acetylation would help elucidate the interaction of JQ-1 with the NF-κB pathway in reducing tumor growth. Future work should also involve *in vivo* analysis of JQ-1 application on tumorigenesis in our mouse models.

The composition of the NF-κB dimer is unknown. A better understanding of the makeup of the NF-κB components specifically in ST-EPN is needed to develop effective treatment methods. Biochemical immunoprecipitation assays to pull down the NF-κB dimer expressed in ST-EPN would allow for such identification and aid in the understanding of JQ-1's application for treatment. Additional work analyzing the effects of *in vivo* overexpression of the different NF-κB family members on tumor generation, and whether JQ-1 has any effects in such conditions, would also help to further highlight targets driving ST-EPN growth.

In summary, this study demonstrates the development of the first primary ST-EPN cell line that can effectively be used to study ST-EPN tumors. We find that the BET bromodomain inhibitor JQ-1 and the FGFR inhibitor AZD4547 limit cell proliferation in this cell line. JQ-1 specifically limits cell growth at concentrations potentially sufficient for further clinical assessment. With the high mortality rate of ST-EPN tumors, development of effective treatment methods is crucial to improving patient outcomes. This study highlights two drugs that may serve as useful treatment methods with which to treat ST-EPN tumors. With the current lack of a chemotherapy regimen that can generate prolonged overall survival in children with ependymoma, further development of the use of JQ-1 and/or AZD4547 in our cell line and animal models is needed to progress treatment to clinical trials and increase survival in ST-EPN patients.

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