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Disrupting NFkB/BRD4 function in glioblastoma as a means to block tumor cell communication

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Disrupting NFkB/BRD4 function in glioblastoma as a means to block tumor cell communication

A Thesis Submitted in Partial Satisfaction of the Requirements for the Degree

Master of Science

in

Biology

by

Alfonso Alejandro Izurieta Muñoz

Committee in Charge:

    Professor Frank Furnari, Chair

    Professor James Kadonaga, Co-Chair

    Professor Amy Kiger

The thesis of Alfonso Alejandro Izurieta Muñoz is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2020

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DEDICATION

I dedicate this work to my family who has been extremely supportive of everything in my life. I have grown up to truly appreciate and admire all that they do, and I love them with all my heart. May we share many more years being as close together as we have become.

Secondly, I dedicate this work to all my teachers throughout the years. From my teachers at The Edron Academy in Mexico City to my teachers at the University of California San Diego. In these trying times the importance of education has really been made evident to me, I hope to continue to foment education and keep learning anywhere I go as I would not be here if it were not for the guidance that I have had from all of you along the way.

Special thanks to ML, CJ, EL, BH, MM, NC, SB, PC, AS, NB, GS, OF for their friendship, you all are my family away from home.

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PUBLICATIONS

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ABSTRACT OF THE THESIS

Disrupting NFkB/BRD4 function in glioblastoma as a means to block tumor cell
communication

by

Alfonso Alejandro Izurieta Munoz

Master of Science in Biology

University of California San Diego, 2020

Professor Frank Furnari, Chair
Professor James Kadonaga, Co-Chair

Glioblastoma multiforme (GBM) is an aggressive type of cancer, whose therapeutic resistance stems from its heterogeneity which refers to diversity in mutations and cell types as well as diversity in the epigenome of cancer cells (Inda et al. 2014). A common scenario of heterogeneity in GBM is the overexpression and mutation of the Epidermal Growth Factor Receptor (EGFR) referred to as WTEGFR and EGFRvIII respectively (Gay et al. 2010).

Tumor heterogeneity contributes to the recalcitrant nature of GBM, making pathway-specific and mutation-specific therapy less effective. (Neftel et al. 2019, Verhaak et al. 2010). It was found that WTEGFR and EGFRvIII-expressing GBM cancer cells both converge to signal through an NFkB and BRD4 mediated mechanism to remodel their enhancer landscapes (Zanca et al. 2017). This results in increased expression of survivin, which is known to prevent tumor death, as well as IL-6 expression, which is known to promote tumor proliferation and survival (Wheatley et al. 2019).

With this information we sought to explore the molecular and epigenetic modifications that the NFkB/BRD4 interaction causes. To do so a mutation that inhibits the interaction between NFkB and BRD4 was CRISPR engineered into iPS cells which have GBM driver mutations. So far, the iPS cells were successfully engineered to express this NFkB/BRD4-disrupting mutation and differentiated into NPCs for subsequent lentiviral transduction to express WTEGFR and EGFRvIII as well as engraftment in mice for tumor development. The engineering and differentiation of these cells provides a platform for a novel approach of targeting both WTEGFR and EGFRvIII through a shared epigenome landscape facilitated by NFkB/BRD4 activity.

INTRODUCTION

Glioblastoma (GBM), with over 10,000 new patients per year (Cloughesy et al. 2014, Ostrom et al. 2016), is the most lethal form of brain cancer with a medium survival of 12-15 months after diagnosis. In general, treatment for cancer is centered around surgical debulking, cytotoxic therapy and/or radiotherapy. However, glioblastoma (GBM) poses a significant challenge due to its localization to the brain and aggressiveness. In many cases, surgery damages the surrounding brain tissues. Cytotoxic and radiotherapies also have significant side effects in areas surrounding the brain tumor, meaning that treatment for GBM carries with it major risks for decreased quality of life (Moiyadi and Shetty. 2012, Davis et al. 2016).

One of the main issues for ineffective treatment of GBM is the heterogeneous nature of the tumor (Inda et al. 2014). Heterogeneity presents itself as multiple cell subtypes within the same tumor that have distinct phenotypes, structures and differences in gene expression. It is a common occurrence found in many cancer types, including tumors in brain, kidney, lung and colon (Gay et al. 2016). GBM tumors have been shown to harbor cancerous cells similar to neural progenitors, oligodendrocyte progenitors, and astrocytes. Because of these different cellular states, pathway-specific and mutation-specific therapy is much less effective for GBM patients (Neftel et al. 2019, Verhaak et al. 2010).

Epidermal Growth Factor Receptor [EGFR] gene overexpression is a common mutation in GBM that is found in 60% of cases (Brennan et al. 2013). The prevalence of EGFR overexpression in GBM provides a target for therapy. The

EGFR gene encodes a transmembrane glycoprotein receptor which is a member of the ERBB receptor Tyrosine Kinase family (Hynes et al. 2005). EGFR overexpression has been shown to correlate with faster relapse and lower survival in cancer patients (Hurt et al. 1992, Schlegel et al. 1994). This overexpression of EGFR, known as Wild Type EGFR (WTEGFR), and its activation is dependent on EGF ligand presence. 30% of GBM patients also present a truncated mutant version of EGFR named EGFRvIII which is constitutively active and does not require the binding of a ligand (Johnson et al. 2012). The EGFRvIII mutant is activated by auto-phosphorylation, which leads to activation of the Shc-Grb2-Ras and PI3-K (AKT) signaling pathways (Narita et al. 2002). The activation of these pathways results in enhanced tumorigenicity, reduced apoptosis (Nagane et al. 2001), and increased cell proliferation (Hesselager et al. 2003).

EGFR targeted therapies have been developed for different types of cancer such as non-small cell lung carcinoma (Reardon et al. 2014). EGFR-directed therapies, such as drugs which act as tyrosine kinase inhibitors, block EGFR signaling, and thus decrease survival and proliferation of tumorigenic cells. Unfortunately, these inhibitors to date have not shown efficacy in GBM clinical trials (Rich et al. 2004). These disappointing results have been hypothesized to be in part due to tumor heterogeneity where the heterotypic tumor cells interact with each other to compensate for the inhibition of an important growth factor receptor such as EGFR (Inda et al. 2010). Thus far, no approach has tried to target both WTEGFR and EGFRVIII at the same time, a commonly occurring expression scenario in glioblastoma heterogeneity (Gay et al. 2016).

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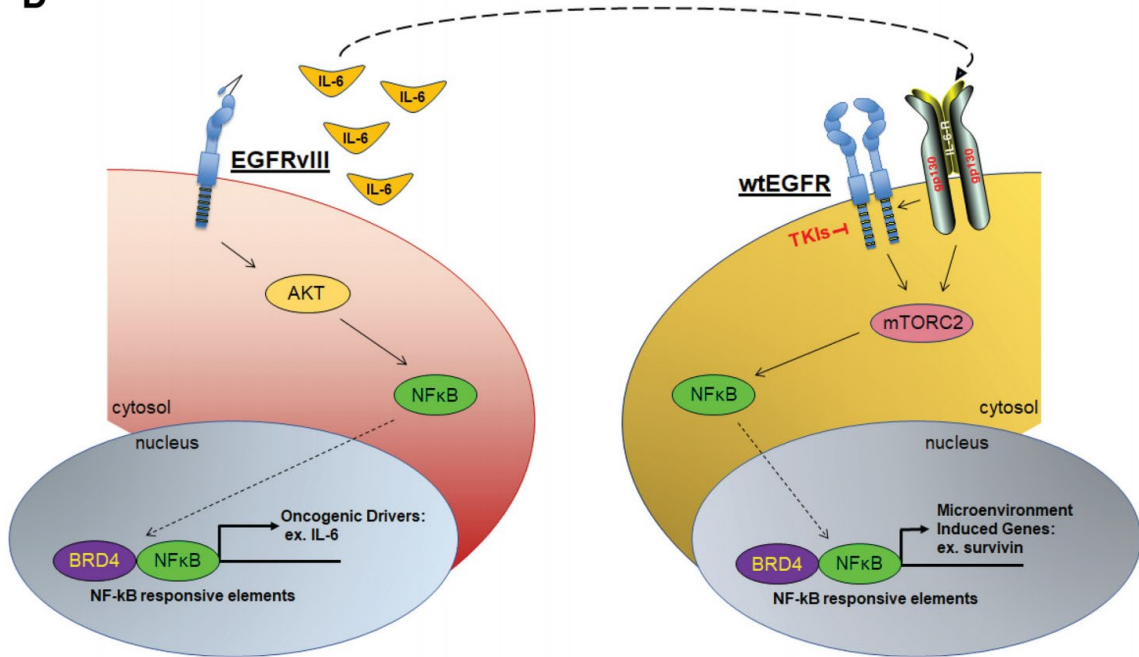
Figure 1. Illustration depicting intracellular pathway activation moderated by EGFRvIII and extracellular activation of receptor gp130 converging on activation of BRD4 and NFkB. If WTEGFR is inhibited IL-6 can activate receptor gp130 promoting cell survival through mTORC2/NF-kB signalling. Image from Zanca et al. 2017

Tumor heterogeneity is not only seen as differences in cell types but also in the epigenome of different tumor cells. Alterations in the epigenome have been characterized in cancer to be linked to genetic mutations and affect cancer phenotypes and gene expression (Mazor et al. 2016). Previous studies in tumor heterogeneity have shown that GBM cellular cross-talk, mediated by exchange of signaling cues between WTEGFR and EGFRvIII cells, converges on a shared NFkB/BRD4 signaling axis to reduce EGFR sensitivity to targeted therapy (Fig. 1, Zanca et al. 2017). Zanca and colleagues showed that a small population of

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EGFRvIII cells in a heterogeneous tumor potentiates the fitness of WTEGFR cells through IL-6-mediated cellular cross-talk.

Because of this cellular cross-talk, WTEGFR cells are able to survive receptor-targeted therapy by upregulating the survivin [BIRC5] gene (Zanca et al. 2017). Survivin expression in WTEGFR cells is usually dependent on EGF-mediated receptor activation and subsequent downstream mTORC2/NFkB pathway activation. Phosphorylation of NFkB enables it to enter the cell nucleus where it can interact with BRD4 to remodel enhancers which in turn upregulates expression of the survivin gene (Fig. 1, Zanca et al 2017). The survivin protein is an inhibitor of apoptosis which allows these WTEGFR-expressing cells to survive EGFR-directed therapy (Wheatley et al. 2019).

In contrast, in EGFRvIII cells, the receptor is constitutively active, which leads to activation of the PI3-Kinase pathway with AKT as one of its main effectors (Narita et al. 2002). The PI3K pathway through activation of AKT leads to NFkB phosphorylation and its associate with BRD4 to remodel the chromatin landscape, similar to WTEGFR activation. By virtue of the EGFRvIII/PI3K/AKT/BRD4 pathway, IL-6 is expressed which in a paracrine fashion activates the IL-6 receptor [GP130] expressed on WTEGFR cells leading to mTORC2 pathway activation similar to EGF-mediated WTEGFR activation described above, and subsequent survivin gene expression (Fig. 1, Zanca et al 2017). The activation of the mTORC2 pathway via the IL-6 receptor shows that even when WTEGFR is inhibited, the IL-6 receptor provides a backup mechanism for survivin expression.

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NFkB and BRD4 have been shown to establish super enhancers downstream of proinflammatory stimulation (Brown et al. 2014). These scientific findings leave questions open to how NFkB and BRD4 are remodeling the enhancer landscape in WTEGFR and EGFRvIII GBM cancer cells. This project will focus on exploring and identifying the mechanisms by which tumor heterogeneity in GBM, specifically with a WTEGFR/EGFRvIII heterotypic population, promotes enhancer remodeling through NFkB/BRD4 signaling, thus leading to upregulation of survivin and IL-6.

To effectively survey the importance and differences in the NFkB/BRD4 complex during enhancer remodeling, patient derived cell lines TS576 (glioma cells), GSC11 (GBM stem cell) and induced pluripotent stem (iPS) cells with GBM driver mutations, will be utilized. The iPS cells to be used are Cyclin Dependent Kinase Inhibitor 2A/2B (CDKN2A/2B) deleted, PTEN null, and TERT promoter mutated (conveying expression). These mutations occur in 61%, 42% and 55-83% of GBM tumors respectively, thus effectively recapitulating the most aggressive form of this cancer (Brennan et al. 2013, Sturm et al. 2014).

The CDKN2A/2B gene codes for proteins p16 and p14 which function as inhibitors of CDK4 kinase, which is required for G1 to S-Phase transition (Leseur et al. 2008). The PTEN gene encodes for the PTEN enzyme which acts as a tumor suppressor helping to regulate cell division through downregulation of PI3K/AKT activation (Shi et al. 2012). Finally, TERT promoter activation through its mutation is responsible for maintaining and extending telomeric DNA (Liu et al. 2016), thereby preventing cellular senescence. With these driver mutations GBM is accurately

modelled in iPS cells. These iPS cells will be differentiated to Neural Progenitor Cells (NPC) which are capable of forming brain tumors in mice (Koga et al. 2020).

These engineered iPS cells will be further altered by CRISPR editing to contain amino acid substitution lysine 310 to arginine (K310R) in the p65 subunit of NFkB. The objective here is to inhibit acetylation of lysine 310, which has been shown to mediate interaction with BRD4 (Huang et al. 2009), thus allowing for interrogation of NFkB/BRD4-specifically bound enhancers separate from BRD4 bound enhancers.

The above CRISPR edited cell lines will then be probed to show that NFkB and BRD4 no longer physically interact with each other through an immunoprecipitation analysis. The p65 subunit of NFkB will also be probed through immunoblot to ensure that the p65 protein is stable and the K310R mutation is not causing destabilization of the protein. The disruption of the interaction of BRD4 and NFkB will allow us to probe levels of IL-6 production in edited vs unedited cells to show that the K310R mutation is working correctly.

MATERIALS AND METHODS

Cell Culture: All experiments utilizing human pluripotent stem cells were done following the regulations of UCSD Human Research Protections Program, project number 151330ZX. All culture of IPS cells were done in plates coated with matrigel hESC-Qualified Matrix (Corning) in mTESR1 plus media (Stemcell Technologies). NPCs were also cultured on matrigel-coated plates in NPC maintenance media which contains DMEM/F12 with GlutaMAX (Thermo Fisher Scientific), 1 X N-2

Supplement (Thermo Fisher Scientific), 50mM ascorbic acid (Tocris), 3μM CHIR99021 (Tocris) and 0.5μM purmorphamine (Tocris). TS576 and GSC11 cells were cultured in suspension in DMEM/F12 with 1X B-27 supplement, 20ng/ml EGF (Stemcell Technologies) and 20ng/ml bFGF (Stemcell Technologies).

Generation of genetically engineered hiPSC clones: Human iPSCs were cultured in mTeSR1 Plus media (Stemcell Technologies) in a matrigel hESC-Qualified Matrix (Corning). The iPS cells were allowed to grow to 80% confluency before using Lipofectamine 3000 (Thermo Fisher Scientific) containing 4ug total of the N2-1 plasmid and 1ug of single strand oligonucleotide DNA. The lipofectamine was left with the cells for 48 hours. After this period GFP positive cells were sorted by flow cytometry using SONY SH800 flow cytometer. $1-2x10^4$ sorted cells were then plated on a 10cm matrigel-coated plate in mTeSR1 with 10μM Y-27632 RHO/ROCK pathway inhibitor for 24 hours before switching to mTeSR1 Plus media. Isolated colonies were manually picked and plated in duplicated matrigel-coated 96-well plates.

hiPSCs clones on one of the duplicated 96-well plates were lysed using 50μl QuickExtract DNA Extraction Solution (Epicenter). Genotyping PCR was performed using the QuickExtract DNA using a Platinum Taq DNA Polymerase (Thermo Fisher Scientific) in 10μl reaction volume containing 0.2μM of each primer with the following conditions 94°C for 2 min, 35 cycles of 94°C for 30 s, 55°C for 30s, and 72°C for 1 min. The PCR products were then visualized in 15% agarose gels. Primers used for the genotyping PCR are as follows:

RelA-K310R-F: 5'-GGACATATGAGACTTCCGC-3'

RelA-R: 5'-AGGGCTAGGTCAGTTGTTCTCAG-3'

Differentiation of hiPSC's to neural progenitor cells (NPC): The method for differentiation of iPS cells to the NPC state was adapted from a previous study (Reinhard et al. 2013.). Human iPSCs were grown until 70-80% confluency, they were then dissociated using accutase (Innovative Cell Technologies) and resuspended at $1x10^6$cells/ml in N2B27 medium (DMEM/F12 with GlutaMAX (Thermo Fisher Scientific), 1 x N-2 supplement (Thermo Fisher Scientific), 1 x B-27 supplement (Thermo Fisher Scientific), 150mM ascorbic acid (Tocris), and 1% Penicillin/Streptomycin). This was also supplemented with 1µM Dorsomorphin (Tocris), 10µM SB43152 (Tocris), 3µM CHIR99021, 0.5µM Purmophamine and 5mM Y-26732 (Stemcell Technologies). Three million cells were transferred into one well of an uncoated six well tissue culture plate and incubated at 37°C, 5% CO_2 on a shaker at 90 rpm. Small Embryoid Bodies were observed within 24 hours and increased in size over time. After 48 hours, a full media change was done with N2B27 medium supplemented with Dorsomorphin, SB431542, CHIR99021, and Purmorphamine. On days 3–5, half media change was performed with fresh N2B27 media supplemented with Dorsomorphin, SB431542, CHIR99021, and Purmorphamine. On day 6, Dorsomorphin and SB431542 were withdrawn and a full media change with smNPC media (N2B27 media supplemented with 3 µM CHIR99021 and 0.5 µM Purmorphamine) was performed. On day 8, EBs were triturated by pipetting 10-15 times with a P1000 pipette and plated onto matrigel

coated 10-cm plates. After 3-4 days the attached EB fragments were dissociated to single cells with accutase (Innovative Cell Technologies) and split at a 1:6-1:8 ratio onto matrigel coated plates. After the first passage, cells were passaged at a 1:10-1:15 ratio every 3-6 days. In the first passages, large flat non-smNPCs could be observed between smNPC colonies, but progressively disappeared no later than 3-6 passages.

RT-qPCR: Total RNA was extracted from cells using the RNeasy Plus Mini Kit (Qiagen) and was reverse transcribed using RNA to cDNA EcoDry Premix (Clonetech) according to the manufacturer's instruction. Triplicate RT-qPCR reactions containing cDNA obtained from 10ng equivalent RNA were run on a CFX96 Real Time system (Bio-Rad) to confirm designated targeting of the genes with the following reaction conditions to check for IL-6 and EGFRvIII expression: 95°C for 3 minutes, 40 cycles of 95°C for 10s, 58°C for 30s. The data was normalized to GAPDH and the relative transcript levels were calculated using the 2-ΔCt formula. Primers used for RT-qPCR are as follows:

GAPDH-RT-f: 5′-AATTTGGCTACAGCAACAGGGTGG-3′

GAPDH-RT-r: 5′-TTGATGGTACATGACAAGGTGCGG-3′

IL-6-qPCR-F: 5'-AGCCACTGACCTCTTCAGAACGAA-3'

IL-6-qPCR-R: 5'-AGTGCCTCTTTGCTGCTTTGACAC-3'

EGFRvIII-qPCR-F: 5'-GGGCTCTGGAGGAAAAGAAAGGT-3'

EGFRvIII-qPCR-R: 5'-CTTCTTACACTTGCGGACGC-3'

For NPC cells the reaction conditions for RT-qPCR were 95 °C for 5 min, 40 cycles of 95 °C for 15 s, 56 °C for 30 s. The primers for these cells are as follows:

Nanog-RT-f: 5′-GAAATACCTCAGCCTCCAGC-3′,

Nanog-RT-r: 5′-GCGTCACACCATTGCTATTC-3′,

Oct4-RT-f: 5′-AGAACATGTGTAAGCTGCGG-3′,

Oct4-RT-r: 5′-GTTGCCTCTCACTCGGTTC-3′,

Pax6-RT-f: 5′-GCCCTCACAAACACCTACAG-3′,

Pax6-RT-r: 5′-TCATAACTCCGCCCATTCAC-3′,

Lentivirus Production and Infection: pLV puromycin lentivirus expressing EGFRWT or EGFRvIII, were generated by co-transfection with VSVg and Δ8.9 packaging plasmids in 293T cells using Lipofectamine 2000 (Life Technologies). Supernatants were collected at 48 and 72 hours after infection and virus was concentrated through ultra-centrifugation. The virus pellets were resuspended in HBSS and kept in -80°C until use. TS576 cells were infected with lentivirus for 24 hours and then subjected to 200µg/ml puromycin selection. Cells were then sorted to enrich expression of WTEGFR and EGFRvIII with U87 WTEGFR and EGFRvIII as baselines respectively.

Western Blotting: Cultured cells were lysed with RIPA lysis buffer (50mM Tris-HCl, 150mM NaCl, 0.5% Sodium Deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitor cocktail (Bimake). Protein concentration of each sample was determined by BCA assay. Cell lysates were analyzed by electrophoresis on a

10% SDS-PAGE gel. PVDF membrane (Millipore) was used for gel transfer and the membrane was probed with primary antibodies for 24 hours, followed by secondary antibodies conjugated with HRP for 1 hour. The signal was detected with Super Signal West Pico/Femto Chemiluminescent Substrate (Thermo Scientific).

Statistical Analysis: All statistical analysis was done using GraphPad Prism 8 software. Data are representative of results obtained in at least three independent experiments. The specific test used for each experiment to determine significance is indicated in the text of the results and/or figure legends.

Results

It has been reported previously that in glioblastoma, heterogeneous expression of WTEGFR and EGFRvIII cells limits sensitivity to EGFR-targeting therapeutics through an NFkB/BRD4 signaling axis. This is done through the upregulation of cytokine IL-6 in EGFRvIII cells which is able to activate gp130 receptors in WTEGFR cells leading to BRD4 dependent expression of the protein survivin which allows cells previously sensitive to EGFR-targeting therapeutics to survive (Zanca et al. 2017). In order to determine the molecular dependencies of this mechanism, the interaction between NFkB and BRD4 must be disrupted to determine promoters and enhances that are dependent on the NFKB/BRD4 complex as opposed to genes that require NFkB or BRD4 only for their activity.

BRD4 and NFkB are known to interact with each other through an acetylated lysine in position 310 of the RelA subunit of NFkB specifically (Huang et al. 2009). The initial approach to disrupting the interaction between NFkB and BRD4 was

through the generation of cell mutants that substituted the lysine of position 310 to an alanine (K310A). This substitution should prevent the acetylation at position 310 thus inhibiting the interaction between NFkB and BRD4. The TS576 and GSC11 cell mutants containing a K310A mutation were generated by Dr. Ciro Zanca, a former postdoctoral fellow in the lab. These cells were then transduced with lentivirus expressing WTEGFR or the constitutively active mutant form, EGFRvIII (Fig 2a) and selected in puromycin. Following selection, these cells were probed for IL-6 expression to determine if the K310A mutation was effectively inhibiting the BRD4/NFkB interaction. RT-qPCR analysis of IL-6 showed that the TS576-P65K310A mutants had a significant reduction in IL-6 expression (Fig 2b). It was also observed that when EGFRvIII was expressed in TS576 parental cells there was a significant upregulation of IL-6 expression but in the TS576-P65K310A clones, IL-6 expression remained at reduced levels even when EGFRvIII was expressed (Fig 2b). EGFRvIII levels were also probed to ensure that the levels of the receptor were not a factor influencing IL-6 expression. The RT-qPCR data showed that EGFRvIII levels were similar and had no significant differences between the TS576 parental cells and TS576-P65K310A cells. Next, RelA (p65) protein levels were assessed to determine if the mutation had any effect on the protein stability. A western blot analysis was performed which showed that the p65 levels in TS576 cells, when compared to TS576-K310A cells, were higher (Fig 2d). This data suggests that the K310A mutation has a negative effect in the stability or integrity of the protein.

To address the observed instability of p65 when lysine at position 310 was mutated to an alanine a new approach was taken. Instead of mutating this lysine to

an alanine it would instead be changed to arginine as this amino acid has the same polar side chain as lysine and the same charge but cannot be acetylated (Lanzilotta et al. 2010). The change from alanine to arginine required redesigning the sgRNA in order to successfully generate the mutation (Fig 3a). This Lysine to Arginine (K310R) mutation in the RelA (p65) subunit of NFkB was engineered in TS576 and GSC11 cells by Dr. Raghavendra Vadla, a postdoctoral fellow in the lab. The mutation was also generated in iPS cells which have GBM driver alterations; CDKN2A/B and PTEN deletion and heterozygous for TERT promoter mutation (Fig 3b). The K310R mutation was validated through Topo TA sequencing in order to ensure both alleles had been successfully edited. Analysis of the sequences showed that one allele presented the desired substitution while the second allele had a truncating in/del mutation (Fig 3b). This same K310R mutation was also observed and validated in TS576 and GSC11 cells.

To assess the stability of p65 K310R in TS576, GSC11 and iPS cells, western blots were performed which showed p65 K310R was more stable than the K310A mutant (Fig 4 a-c). These cells were then probed for IL-6 expression levels and showed that there is a significant decrease in the expression levels of IL-6 in cells with the K310R mutation (Fig 4d). TS576 and GSC11 RT-qPCR quantification of IL-6 was obtained by Dr. Raghavendra Vadla and correlates with iPS cell model results. This RT-qPCR functional validation of the K310R mutation suggests that this mutation is disrupting the interaction between NFkB and BRD4.

Now that the iPS mutants had been confirmed through sequencing and exhibited lower levels of IL-6 the next step was to differentiate them into NPC for engraftment into mice brains to form tumors. The differentiation protocol that was followed was adapted from Reinhard et al.2013 as described in Methods (Fig 5a). To confirm that the cells had indeed been differentiated fully into NPCs, RT-qPCR analysis was conducted to quantify key factors that discriminate iPS from NPC state. Oct4 and Nanog are pluripotency factors which are upregulated in the iPS state (Takahashi & Yamanaka 2006) while Pax6 is a marker for cells in the NPC state (Gerrard et al. 2006). It was found that after completion of the differentiation protocol the iPS cells had downregulated Oct4 and Nanog expression and upregulated Pax6 expression, demonstrating that successful differentiation into NPC had occurred (Fig 5c). The stability of p65 was also surveyed through western blot analysis in the newly generated iNPC cells and it was found that p65 continued to be more stable in the K310R mutants when differentiated to the NPC state than cells with a K310A mutation (Fig 5b).

Discussion and Future Directions

In an effort to uncouple EGFRvIII to WTEGFR tumor cell communication, the p65 subunit of NFkB was edited to disrupt its interaction with BRD4 in GSC11, TS576 and human iPS cells. NFkB and BRD4 mediated upregulation of IL-6 in EGFRvIII cells and survivin in WTEGFR cells allow for increased tumor survival and attenuation of therapy (Zanca et al. 2017). This NFkB and BRD4 mechanism is prevalent in both heterogenous populations of the tumor making it a particularly

interesting target as identification of sensitivities in both cell types should result in an effective treatment for GBM patients.

The initial approach to this project was through the use of previously engineered TS576 and GSC11 cells that had a Lysine to Alanine mutation in position 310 (K310A) of the p65 subunit of NFkB. Further analysis into the K310A mutants revealed that while levels of IL-6 transcription were reduced (Fig 2b), the level of p65 protein was unexpectedly reduced (Fig 2d). This observed decrease in protein level prompted reconsideration of the K310A mutants as a viable approach for this project. In order to address this concern an alternative approach was taken to substitute an Arginine instead of an Alanine in the same 310 position (K310R).

The K310R mutation showed similar results to the K310A mutation regarding transcriptional levels of IL-6 (Figure 4d). Western blot analysis revealed that the K310R mutation had higher levels of p65 than the K310A mutation. This indicated that the K310R mutation was a more viable option to continue the project and perform further experiments. While these results point towards disruption of NFkB and BRD4 as leading to the decrease in target gene (IL-6) expression, a co-immunoprecipitation analysis is required to show that NFkB and BRD4 no longer physically interacting with each other. Preliminary data (data not shown) showed that the NFkB and BRD4 interaction was too weak to be detected in parental cells. This can be explained by low levels of p65 acetylation in the cells. To remedy this, cells will be transfected to over-express the acetylase, P300, to elevate levels of p65 acetylation thus promoting detectable interaction between NFkB and BRD4

(Darcy et al. 2015). It is expected this experiment will show interaction in parental cells but not in mutant K310R cells.

The success at generating the K310R mutation in the p65 subunit of NFkB in iPS cells with a GBM driver mutation background, and differentiating these cells to the NPC state along with similarly engineered glioma sphere lines, TS576 and GSC11 (by Dr Raghavendra Vadla), provides a foundation to begin an interrogation of NFkB/BRD4 bound enhancers separate from BRD4 bound enhancers. The generation of these cell lines is crucial to answering questions regarding the interaction of NFkB and BRD4 in WTEGFR and EGFRvIII heterogeneous tumor populations. It is important to use multiple cell lines as they will give an insight into any variations in the mechanism that BRD4 and NFkB utilize to remodel enhancers resulting in increased IL-6 expression in EGFRvIII cells and survivin expression in WTEGFR cells. Although there are limitations as to what information cell lines can provide due to their genetic variations (Borrel et al. 2010), it is expected that using two patient derived tumor cell lines GSC11 and TS576 as well as an iPS derived cell model, will paint a more accurate picture that can be validated with GBM tissues from patients that exhibit EGFRvIII and WTEGFR tumor heterogeneity.

The K310R edited cells will be transduced with lentiviruses expressing WTEGFR or EGFRvIII. These cells will allow us to assess levels of IL-6 production in EGFRvIII-expressing edited vs unedited cells. It is expected that p65 K310R-edited cells will produce lower levels of IL-6 as this gene requires the BRD4/NFkB complex to activate the IL-6 enhancer. This inhibition of BRD4/NFkB-mediated
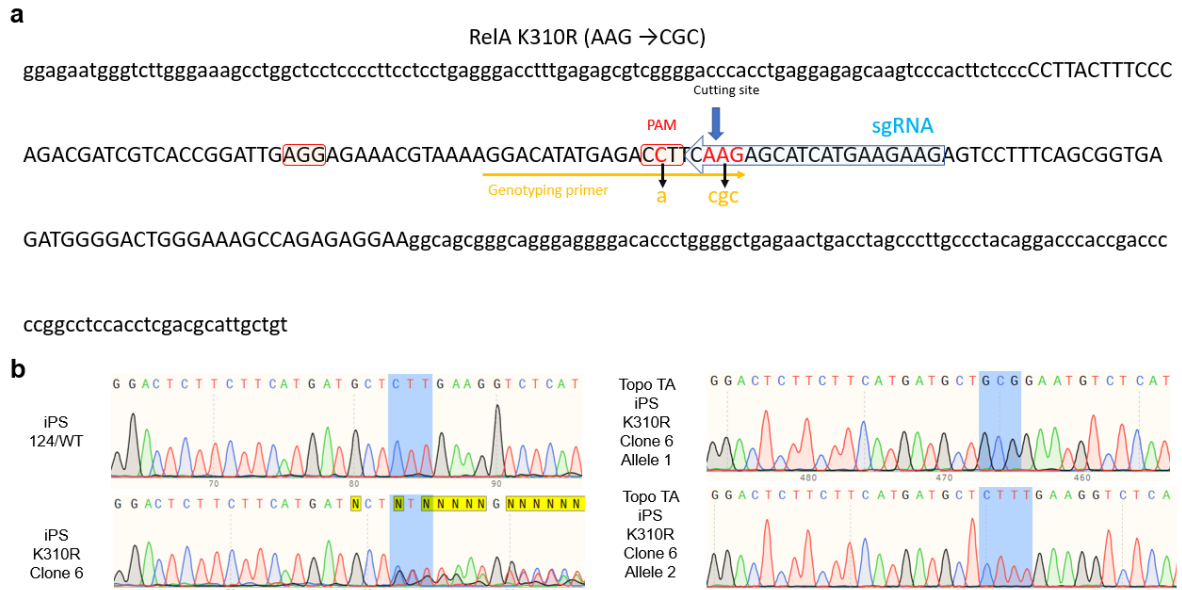
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enhancer remodeling also allows us to quantify survivin expression levels in these cells, which should be similarly affected.

The generation and validation of these cells establishes the groundwork for future experiments. All engineered cell lines will be orthotopically engrafted into mice brains to generate tumors that reflect the characteristics of human GBM. Tumor-derived cell lines will then be probed for IL-6 and survivin expression. To analyze alterations in their enhancer landscapes, ChIP-seq will be performed to identify areas of active promoters (H3K27ac) and sites with active transcription (RNA Pol II), as well as p65 and BRD4 localization to segregate NFkB from BRD4/NFkB bound enhancers. This information will then be compared to control cells lacking the K310R mutation and any changes in gene expression patterns should provide valuable insight on how EGFRvIII-expression globally alters the enhancer landscape in WTEGFR/EGFRvIII heterotypic tumors. There is optimism that these observed changes in the enhancer landscape mediated by the NFkB/BRD4 complex might reveal novel therapeutic targets for treating GBM patients. Dr. Raghavendra Vadla from the Furnari Lab is continuing to actively investigate this project to bring it closer to completion.
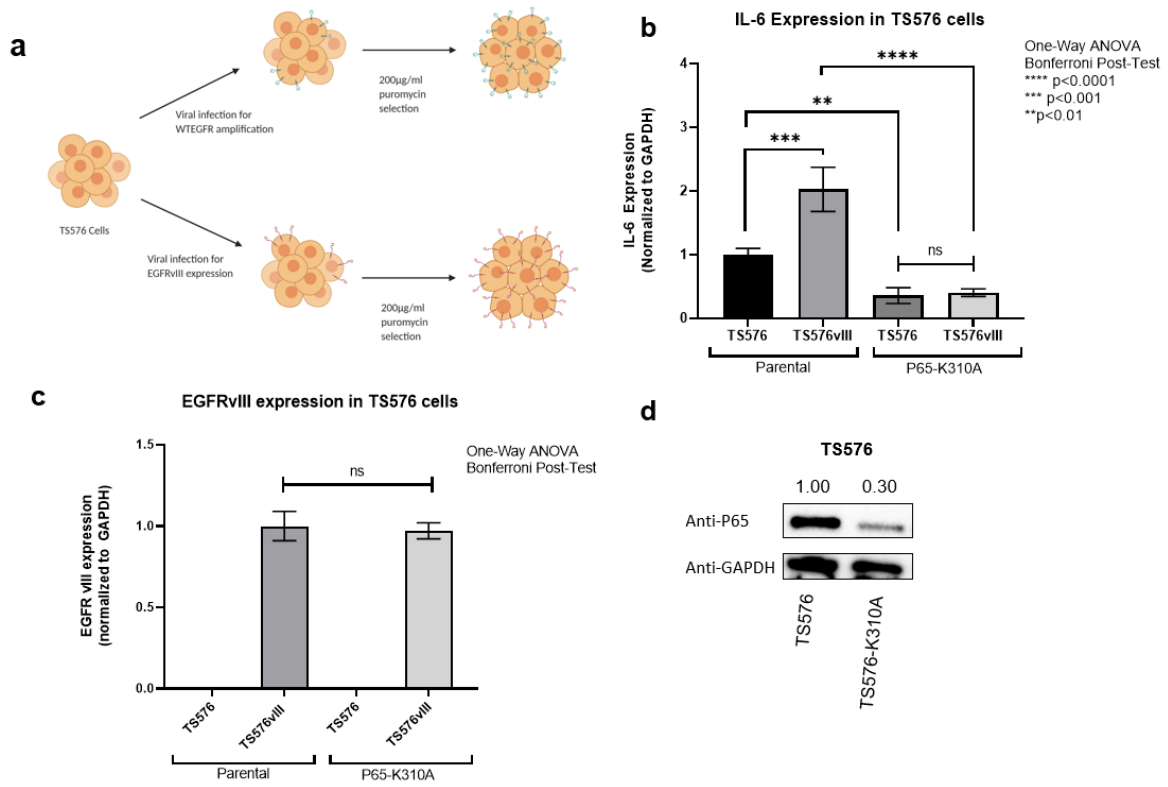
Figure 2. TS576-K310A mutants show decreased IL-6 expression and exhibit reduced p65 protein levels. **a** Schematic representation of EGFRvIII and EGFRWT lentiviral infection on TS576 cells. **b** RT-qPCR quantification of IL-6 expression in parental and EGFRvIII overexpressing TS576 cells. Data are representative of three replicates n=3. Data are shown as mean ±SD. **c** RT-qPCR quantification of EGFRvIII expression levels in parental and EGFRvIII overexpressing TS576 cells. Data are representative of three replicates n=3. Data are shown as mean ±SD. **d** Western blot of anti-P65 and GAPDH in TS576 cells. Quantification of protein levels was done utilizing image J software.

a

RelA K310R (AAG →CGC)

ggagaatgggtcttgggaaagcctggctcctcccctt cctcctgagggacctttgagagcgtcggggacccacctgaggagagcaagtcccacttctcccCCTTACTTTCCC

Cutting site

PAM   sgRNA

AGACGATCGTCACCGGATTGAGGAGAAACGTAAAAGGACATATGAGACCCTCAAGAGCATCATGAAGAAGAGTCCTTTCAGCGGTGA

Genotyping primer   a   cgc

GATGGGGACTGGGAAAGCCAGAGAGGAAggcagcgggcagggaggggacaccctggggctgagaactgacctagcccttgccctacaggacccaccgaccc

ccggcctccacctcgacgcattgctgt

b

iPS 124/WT

iPS K310R Clone 6

Topo TA iPS K310R Clone 6 Allele 1

Topo TA iPS K310R Clone 6 Allele 2

Figure 3. **Generation of RelA K310R by CRISPR/Cas9 editing in iPS Cells. a** Experimental design of sgRNA to generate a Lysine to Arginine mutation in RelA at position 310. **b** Representative sequencing results showing mutation of RelA K310R in iPS cells.

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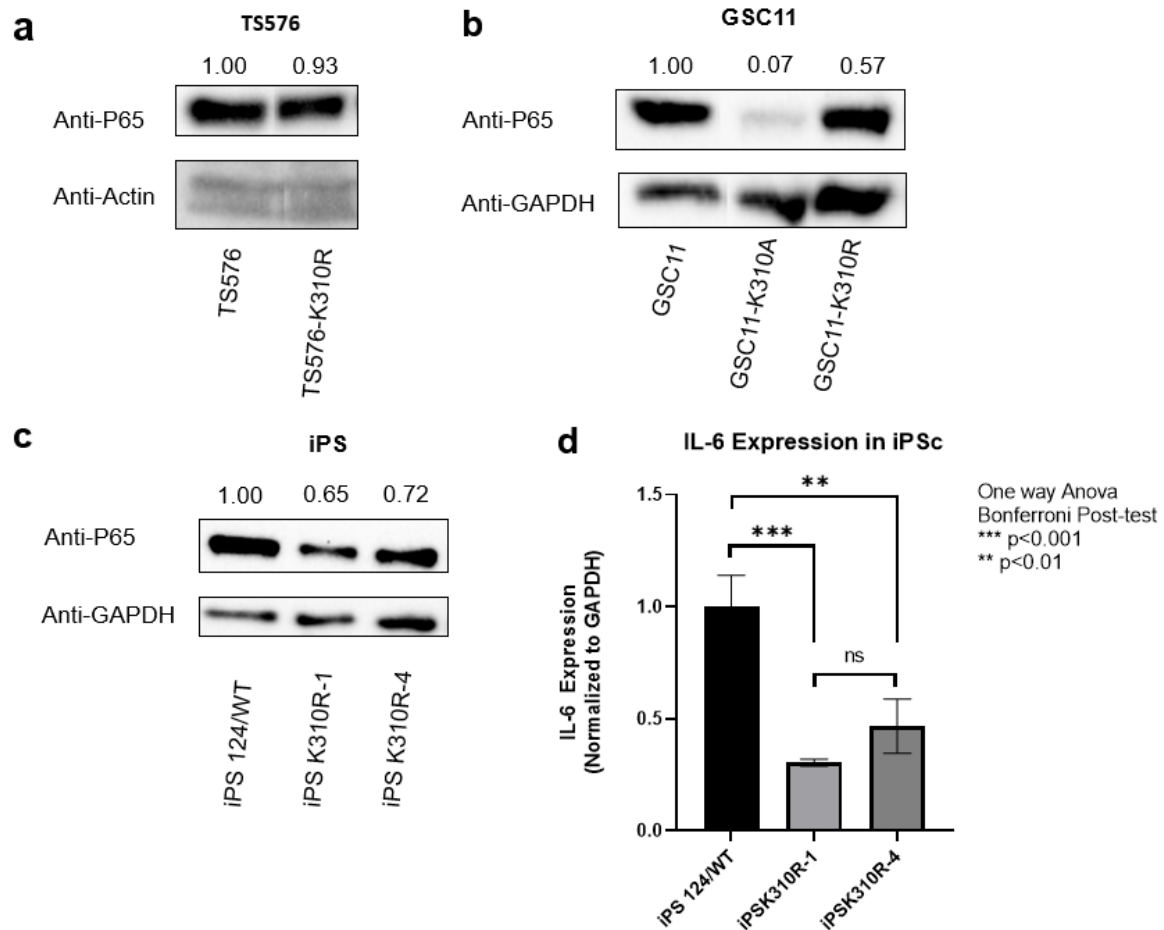
Figure 4. Mutation of Lysine 310 to Arginine shows stability of p65 and decreased IL-6 expression. a,b,c Representative western blots of anti-p65 in parental cells and mutant cells. Quantification of protein levels was done utilizing image J software. **d** RT-qPCR quantification of IL-6 expression in parental and RelA K310R iPS cells. Data are representative of three replicates n=3. Data are shown as mean ±SD
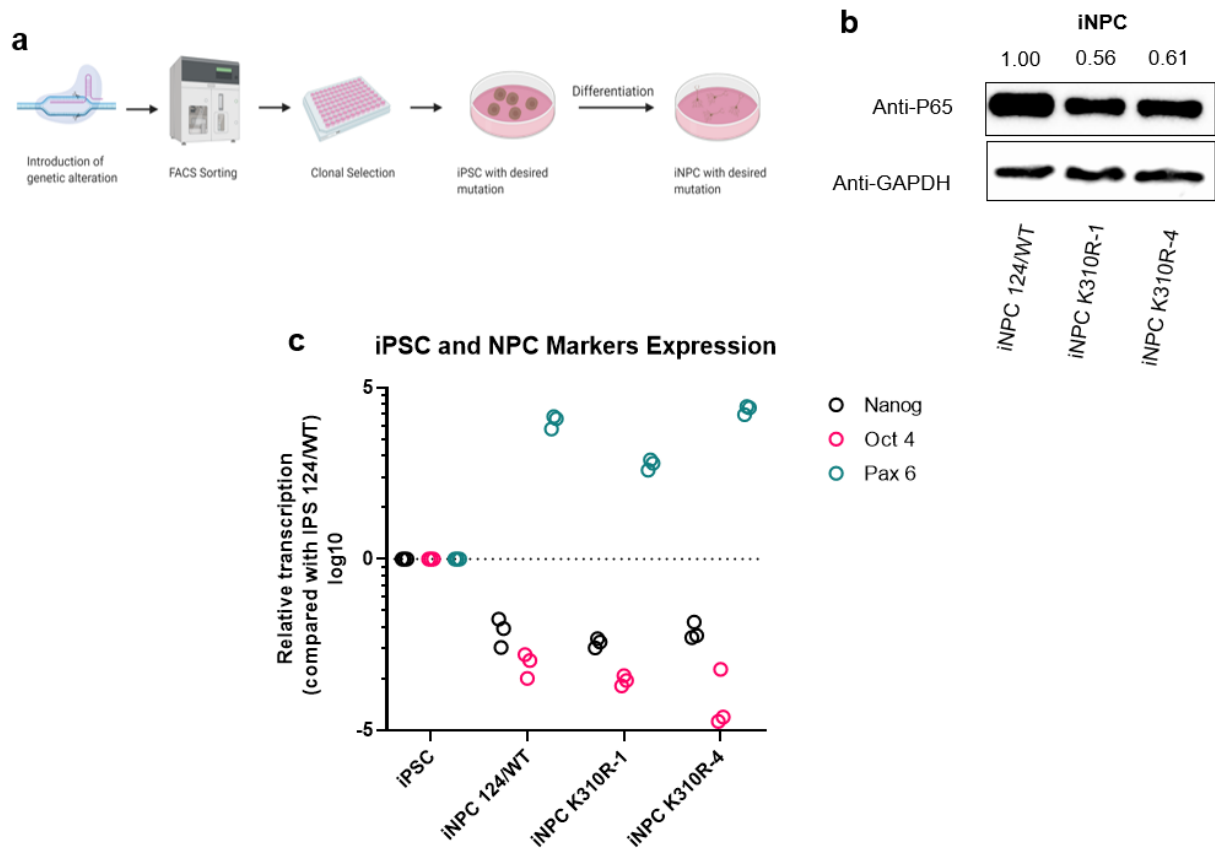
Fig 5. Successful differentiation of iPSC's to iNPC's. a Schematic representation of differentiation from gene editing in iPSC to full differentiation to iNPC. **b** Western Blot of anti-P65 and GAPDH in iNPC cells. Quantification of protein levels was done utilizing image J software. **c** RT-qPCR quantification of markers for iPSC and NPCs. Data are representative of three replicates.

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