Confirmation of RelB Knockout Using Western Blotting and Proliferation Study

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Abstract

Glioblastoma Multiforme (GBM) is a common brain tumor with a median survival rate of 15 months. RelB is a transcription factor that is commonly associated with aggressive forms of cancer, and a known marker of the Mesenchymal (MES) phenotype of GBM. It is currently unknown how RelB contributes to the transition of GBM to the MES phenotype. Our hypothesis is that RelB is an integral part of the mechanism that transitions glioma cells to the MES phenotype. In order to determine the role RelB has in this development, we need 2 colonies of cells: one wildtype and one knockout of RelB. We collected single cell clones from a pool of mouse glioma cells that were treated with a CRISPR target for RelB. We then performed a Western Blot to determine the amount of RelB protein in the clones 6, 9, 10, 12, 13, and 14. Our results indicated that clones 9, 10, and 14 appeared to be complete knockouts of RelB. We performed a proliferation study of wildtype, clone 9, and clone 14 to see if growth rates differentiated. We found that both RelB KO clones 9 and 14 proliferated significantly less than the wildtype at 24 and 48 hours. This finding supported our hypothesis that RelB is associated with increased aggressiveness in glioma cells. Our western blot results will be evaluated using DNA sequencing. If clones 9 and 14 are confirmed to be RelB knockouts at the genomic level, the wild type and RelB knockout glioma cells can be subsequently used for *in vitro* and *in vivo* studies.

Objective

- Use a CRISPR target to knockout RelB from a pool of glioma cells
- Perform a western blot to confirm the absence of the RelB protein
- Perform a proliferation study to identify differences in growth rates between wildtype and RelB knockouts

Methods

RelB knockout and western blot. A pool of glioma cells were treated with a CRISPR/Cas9 gene editing system that contained a target for RelB. Single cell clones were then made by diluting a single cell-suspension until there was only one cell per microliter. One microliter of cell suspension was then put into three 96 well plates with the intention of growing colonies from single cells. These clones were then put in cell culture and lysed in order to start a western blot to measure the amount of RelB protein they had. First, a variable volume of sample was used so each lane contained 5 micrograms of protein. Enough RIPA buffer was then used to total 25 microliters to dilute the LB loading buffer 1:1. An electric current was then run through the buffer to separate all the proteins by size. All of the proteins were then transferred from a gel to a nitrocellulose membrane. To ensure that the primary RelB antibody did not bind to any other proteins, a blocking solution was added first. Once the primary antibody was added, it was placed in a 4 degree room to rock over night. The next day, the excess primary antibody was washed off and the secondary antibody was added. Once the excess secondary antibody was washed off, the membrane was ready to be imaged. The results of the imaging will show the amount of RelB protein in each clone.

Proliferation Study. On the first day, 1000 cells were added to a 96-well plate. The three cell lines occupied 3 columns of 6 wells. The first column acted as the control, the second column was the 24 hour measurement, and the third column was the 48 hour measurement. 100 microliters of media were added to the columns and the cells were given 3 hours to adhere to the bottom of the plate. Then, 10 microliters of cell counting dye were added and given 1 hour to incubate before measuring. The plate was then put in a microplate reader that will give a number directly correlated to the amount of live cells. Measurements were taken at all 3 timepoints and then the growth of the cells was compared.

Results

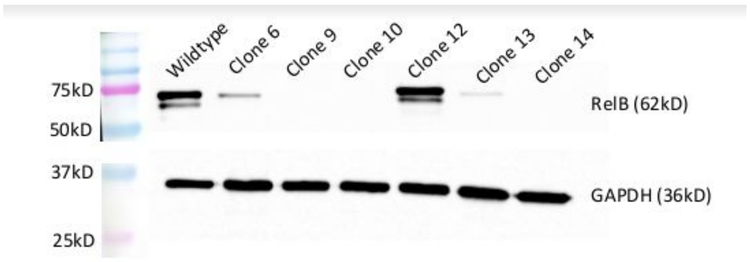


Figure 1: Image of the western blot performed to measure the amount of RelB protein in wildtype and clones. It appears that clones 9, 10, and 14 are complete RelB knockouts.

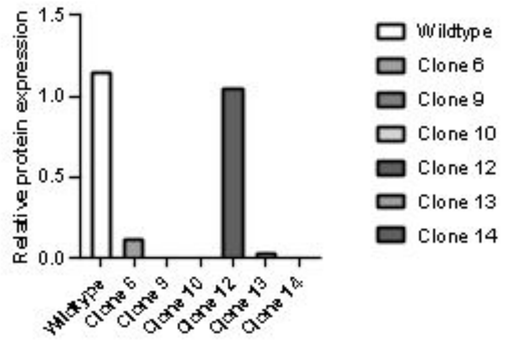
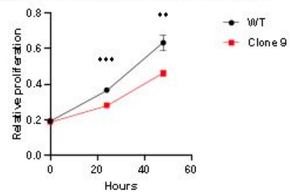


Figure 2: Quantification of bands in figure 1. Again, clones 9, 10, and 14 appear to be complete RelB knockouts.

A.



B.

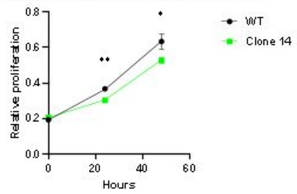


Figure 3: Results of the proliferation study. Both panels A and B show that the wildtype cells had a faster rate of growth than clones 9 and 14.

Conclusions

- Clones 9, 10, and 14 all appeared to be RelB knockouts as shown by figure 1.
- Clones 6, 12, and 13 all appeared to have some expression of RelB, therefore they will not be used in future experiments.
- The proliferation study showed that the growth rate of the wildtype was higher than both the RelB knockouts.
- Our hypothesis is that RelB is associated with more aggressive glioma cells. Our findings from the proliferation study help to support our hypothesis.

Future Directions

- The DNA of clones 9, 10, and 14 must be sent to get sequenced in order to confirm that the gene responsible for producing RelB is absent.
- Once confirmed, glioma cells will be intracranially injected into mice to produce tumors that are both wildtype and RelB knockout.
- Doing this will allow us to identify the effect RelB has on tumor development within GBM.

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