**CRISPR/Cas9 as a tool for creation of**

**p53 knock-outs in human glioma cells**

**I. Introduction**

As the second deadliest disease in the United States, cancer is as varied as it is difficult to treat. In the US, over 500,000 people are claimed by the various forms of cancer annually.1 However, even amongst cancers, certain types are infamous for their aggressive nature and poor prognosis – one such cancer is Glioblastoma Multiforme. This cancer is known as the most malignant and lethal form of brain cancer.2

Although mutations creating oncogenes that lead to cellular deregulation are random, certain genes are of great importance for controlled cell proliferation; among these is the p53 gene. The product of this gene is the first protein in a pathway that regulates a cell division-stimulating protein (cdk2). If the final protein in this pathway, p21, is not available, then the cell will begin to proliferate uncontrollably (**Fig. 1)**3. Cells that divide in this way become tumors.4 Research has also shown that mutated p53 genes tend to have additional oncogenic properties that are separate from the normal duties of normal p53. Mutant p53 may increase a tumor’s ability to migrate and promote angiogenesis, as well as boost a tumor’s resistance to chemotherapy.5

**Figure 1**: Basic protein pathway of normally functioning p53. Adapted from Figure 2 of Ref 3.

Mutations in the p53 gene are one of the most significant aspects of the origin of cancers. In order to reduce the potential dangers of mutated p53, artificial inhibition and stimulation of the cell’s normal DNA repair mechanisms have been considered. For example, Biddlestone-Thorpe *et al.* (2013) demonstrated that human glioma cells (strain U87, details may be found in Golding *et al.* 2009)6 with mutated/defective p53 (strain U87-281G, which can be generated from U87 by expressing mutant p53, or mp53) were more sensitive to radiotherapy than glioma cells with wild-type (wt) p53 (U87), after one of the genes that plays a role in regulating DNA damage to p53, ataxia-telangiectasia mutated (ATM), was inhibited by a kinase inhibitor (KU-60019). The result was two cell strains, U87 and U87-281G, that were otherwise isogenic, or genetically identical. However, since mp53 is dominant to wtp53, and because the expression of mp53 does not have any detectable physiological effects, Biddlestone-Thorpe *et al.* were unable to conclude whether mp53 was responsible for improved sensitivity to radiosensitization of KU-60019.7 If I were instead able to create U87 cell pairs with inactivated, or knockout (KO) p53, then I would be able to confirm the differences in ATM inhibitor radiosensitivity of wtp53 versus mp53 cells - this would have huge implications for the future of cancer treatment.

One of the methods I might use to facilitate the inactivation of p53 is the Clustered Regulatory Interspaced Short Palindromic Repeats/Cas9 system, or simply CRISPR/Cas9. CRISPR consists of a non-coding guide RNA coupled with a Cas protein. In this system, the RNA associates with a complementary strand of target DNA. Additionally, the coupling of RNA and Cas protein imparts nuclease activity upon the complex. This complex was identified to be a defense mechanism in microorganisms – this system has protected prokaryotic genomes from viral DNA by limiting horizontal gene transfer. However, CRISPR/Cas9 also has potential as a genomic engineering tool. By designing a CRISPR/Cas9 nuclease construct that has high specificity for a particular gene target, and delivering the complex to a cell’s nucleus, it is possible to alter the genome at selected sites.8

A sequence specific nuclease like the CRISPR/Cas9 complex cannot simply glue itself to the target gene after excising the unwanted sequence, however. Doench *et al.* (2014) have used single guide RNA (sgRNA) mediated CRISPR to create KO organisms.9 KO organisms are created by removing a small section of the genome of a plant or animal zygote at the germ-line (**Fig 2)**. As long as the target gene is not necessary for survival, the organism will grow up without it – similarly, all of its offspring will lack the target gene. This technique is extremely useful for studying the functions of one gene in isolation.10

CRISPR/Cas9 is not a perfect tool, however. While the results of a CRISPR mediated system are often reproducible, they do not work most of the time.11 Furthermore, experimental evidence has demonstrated that this system may generate off target effects – unintentional changes made in areas other than the target gene.8 Since exacting specificity of CRISPR/Cas9 has not yet been achieved, it is clear that this tool is still in its infancy in terms of a practical and clinical approach. However, it is conceivable that CRISPR/Cas9 might be used to knock-out the p53 gene in U87 in order to set up an experiment with three varieties of U87 glioma cells: wtp53 cells (no manipulation, just regular U87), heterozygous p53 cells (which I will call HU87), and full knockout p53 cells (KOU87). The aim of this investigation is to use the CRISPR/Cas9 system to manipulate p53 to confirm the findings of the Biddlestone-Thorpe paper – whether the enhanced response to the radiosensitizing of ATM *was caused by* a mutant/defective p53 or not!

**Figure 2**: General strategy for application of the CRISPR/Cas9 system. Adapted from Figure 3 of Ref 8.

**II. The Experiment**

There is one central goal of this investigation: to successfully create multiple variations of human glioma cell knockouts for p53 (U87, HU87, and KOU87) in order to confirm the previous findings in the Biddlestone-Thorpe paper. In addition, a set of control experiments will be carried out to determine any differences in the off-target effects of U87, HU87, KOU87, or an unanticipated double KO U87, which I tentatively call DKOU87.

**II.A.** **CRISPR/Cas9 Design & Expression**

In order to accomplish the primary objective, guide RNA (gRNA) sequences must be designed for the CRISPR system. Delivery of the CRISPR/Cas9 system to a target gene is outlined in **Figure 3**: after unique gene sequences on the target gene are identified, a gRNA strand complementary to a target sequence forms an endonuclease by associating with Cas9. This complex seeks out the DNA sequences complementary to the guide strand and facilitates a double stranded DNA break. In most instances, after the target strand is removed, DNA is repaired via non-homologous end joining (NHEJ), which is efficient, but frequently introduces insertions or deletions (InDels).12 For this experiment, the practical aim will be to remove a portion of the p53 gene with this method to render the gene a KO. I will consider that, depending on how well the endonuclease does its job that the cell might be KOed heterozygously or homozygously. The gRNA design process will be carried out via BLAST search of the human genome; by using BLAST to identify potential binding points, as many unique sets of oligonucleotides as are available on p53 will be identified. This technique was used by Zhen *et.al* (2004)13 in order to design oligonucleotide gRNA primers targeting genes associated with human papillomavirus.

**Figure 3:** Basic outline of Cas9 endonuclease formation and functionality. Adapted from Figure 1 of Ref 12.

Luckily, it is possible to computationally identify, with a high degree of accuracy, potential off-target sites given a gRNA nuclease. Drost *et al.* used software to determine an ‘off-target score’ based on how similar a particular gRNA sequence was to its target sequence. In cases where the sequences perfectly match, the software assigns a score of 1.0; as compared sequences become more different, this value decreases, to a minimum of 0.14 For this experiment, each candidate gRNA strand will be assessed in this way with the Cas9 Online Designer (COD), a free online resource that can estimate off-target score.15 gRNAs with an off-target score greater than 0.30 will be considered for p53 KO trials. At least three trials may be necessary in order to see varying levels of p53 KO. Obviously, each of these trials will depend on the delivery of a gRNA/Cas9 nuclease to its target sequence. To this end, the gRNA will be synthesized using human codon-optimized Cas9 expression plasmid, obtained from Addgene (# 41815) as a template.16

This experiment will utilize the lipofection transfection protocol as first described in Drost *et al.* to deliver the Cas9 endonucleases. First, the original U87 glioma cell cluster will be dissociated from its petri dish using trypsin at 37 °C for 10 minutes. Then, the cells will be equally divided into separate plates. The total number of plates will be equal to (3 \* candidate gRNAs) because each gRNA will be featured in 3 trials. A growth medium will be added to prevent the cells from reattaching to the dish. Lipofectamine, a transfection agent, or compound that encourages the opening of pores in cell membranes, will be mixed with varying amounts of the gRNA/Cas9 endonuclease, and added to the growth medium solution containing the glioma cells.14 The first trial will include 0µg of the gRNA/Cas9 endonuclease (control group). The second cell line will be transfected with 1µg the gRNA/Cas9 endonuclease. The third trial will include 3µg of the gRNA/Cas9 endonuclease. It is predicted that higher levels of the gRNA/Cas9 endonuclease will result in a more complete KO of p53. However, it is unknown how differing degrees of KO might affect the radiosensitivity of ATM. The purpose of this triple-trial method is determine which, if any, degree of p53 KO is capable of radiosensitizing ATM. If more than one gRNA/Cas9 endonuclease is able to accomplish this goal, then the endonuclease that generates the fewest off-target effects as predicted by COD will be considered the ‘winner.’ All resulting cell pairs featuring a KO will be examined using COD and a Western Blot, to determine both genotypically and phenotypically, whether a more aggressive KO of p53 results in more severe off-target effects.

 I predict that the 1µg trial might generate HU87 or KOU87 cells, while the 3µg trial might generate KOU87 or DKOU87 cells. In the event that only KOU87 and DKOU87 cells are created, another set of trials will be carried out with 0.5µg of the Cas9 and gRNA expression vectors, in an effort to attain HU87 cells.

**II.B. Determining Knockdown & Off-Target Effects:**

After all lipofection transfection trials are completed, each glioma cell line will be incubated until it has cloned itself several times. It will be necessary to acquire the genotype of the p53 gene for all of my trials. In order to do this, I will adopt a genotyping method used by Drost *et al.* known as Viagen Direct PCR, which is a form of the polymerase chain reaction that can amplify specific genes14. The primer sequence for the PCR amplification of the target, p53, will be:

5’ – CCCATCTACAGTCCCCCTTG – 3’14

From here, the products of the PCR chain reaction will be cloned into a pGEM-T easy vector system, and sequenced by a T7 sequencing primer. Easy vector systems consist of a vector, insert DNA, ligation buffer, and DNA ligase, and are tools that are used to clone PCR products.17 The T7 sequencing primer has the sequence:

5’ – TAATACGACTCACTATAGGG – 3’18

To conduct the Western Blot, samples from each cell line will first be lysed. Then, protein concentrations will be determined via Bradford assay.14 Equivalent amounts of protein will also be taken for SDS-PAGE analysis – determining whether proteins in different KO cells have different electrophoretic mobility will reveal phenotypic off-target effects.19

**III. Discussion**

If all goes well, I will end up with at least one HU87/KOU87/DKOU87 cell line with minimal off-target effects – ideally there will be no observable phenotypic effects in the colony compared with the original U87 glioma cells. From this position I would hope to conclude that I had successfully optimized a CRISPR mediated p53 knockout procedure for human glioma cells, and that such a feat had laid the groundwork for a future experiment that focused on knocking-in wtp53 to prevent and reverse dysregulation of glioma cell. This translates to a major step in curing cancers in which p53 is defective. However, this experiment does little to investigate whether a p53 knock-in procedure would create any InDels and/or off-target effects that I am hoping to avoid! Based on my Western Blot and derived genotypes of any KO cell lines I would also hope to conclude definitively that mp53 is not necessary for the radiosensitization of ATM.

A potential concern are any potential InDels that the NHEJ process may induce within the target sequence itself. In general, gRNA oligonucleotides are around 20 bases in length, but this length is significantly smaller than most genes.12 Therefore, although a deletion of 20 or so nucleotides in p53 may succeed in creating a p53 KO, the unpredictable nature of mutation may induce changes in functionality of genes that lay elsewhere on chromosome 17 entirely, via frameshift4. Depending on where the mutation occurs, the change may be phenotypically undetectable if any related proteins are not immediately affected. Despite these potential setbacks, optimizing a p53 KO is a preliminary step in utilizing CRISPR/Cas9 to treat dangerous cancers.

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