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c-Myc Knockout as a Model for Gene Editing for Training Healthcare Professional Students

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Abstract

Correction of genetic errors, commonly known as gene editing, holds promise to treat diseases with unmet medical needs. However, gene therapy trials do encounter unwanted outcomes, because of an incomplete understanding of the disease states, and gene therapy processes, among others. This situation encourages a concept that healthcare professionals receiving laboratory research training will not only identify inadequacies in basic biomedical knowledge of gene therapies but also provide tangible refinements. To this end, we have undertaken the PharmD student training in gene editing in a basic research laboratory setting. As a model, *MYC* gene was chosen for knockout using CRISPR-Cas9 method in HT29 and OVCAR8 cells. Students were involved in the design of *MYC*-specific gRNAs, subcloning into Cas9-carrying plasmid, and selection of knockout clones from the transfected cells. Subsequently, genomic DNA isolation and sequencing, analysis of clonal DNA sequences using online bioinformatics tools, western blotting, cell proliferation and cell division cycle experiments, were performed to characterize the *MYC* knockout clones. Results presented in this communication suggest that healthcare professionals who received laboratory training gain a better understanding of the disease states and mechanisms, gene therapy protocols, limitations of gene therapies, ability to critically evaluate the literature and confidence in the oversight of gene therapies in the clinic.

Keywords

CRISPR-Cas, *MYC*, Gene Disruption, Healthcare Professional, Laboratory Training, Genome Editing

1. Introduction

The Human Genome Project elicited enormous influence on the development of newer and more efficient cost-effective technologies for DNA sequencing, bioin-

formatics, molecular diagnostics, genetic manipulations, and disease treatments. Bioinformatics, for instance, has uncovered various abnormalities in the human genome, including single nucleotide polymorphisms, indels, gene copy number variation, and alternative splicing, which are being annotated in genotype-phenotype databases to gain insights into the genetic basis for various human diseases and disorders [1] [2]. Importantly, these developments have accelerated the development of newer tools and methodologies to manipulate genetic errors in human diseases with precision, in a process known as gene therapy [3]. Although earlier methods of genome editing employed extracellular DNA fragments in altering the genome, several tools such as zinc finger nucleases, transcription activator-like effector nucleases, meganucleases and clustered regularly interspersed short palindromic repeats (CRISPR)-associated system (CRISPR-Cas) are efficient in modifying the genome at desired locus [4] [5]. Because of its precision, ease and adaptability to the needs, the CRISPR-Cas system is now widely adapted not only for the analysis of the gene function but also for therapeutic intervention of human diseases and disorders. Understandably, gene therapy has elicited keen interest in clinicians, researchers, and patients, which is evident from the large number of clinical trials that are currently undertaken [6] [7]. Progress is evident from the approval of many therapeutic interventions by the Federal Drug Administration (www.fda.gov), for diseases and disorders that were hereto considered intractable [8].

While gene therapies are welcoming developments, inadequate oversight by the personnel involved may have contributed to the previously unanticipated health deterioration and death among the study participants [9]. To predict and effectively address such unforeseen consequences, all stakeholders, including clinical professionals, pharmacists and nurse practitioners, require foundational knowledge in genome editing procedures, critical evaluation of literature, and comprehension of the associated ethical issues. In fact, the Food and Drug Administration requires that the healthcare personnel involved in prescribing, dispensing, and administrating gene therapies have training in all facets of this therapy.

Undoubtedly, hands-on laboratory research training undoubtedly reinforces the knowledge gained in the classroom [10]. Extending this view, healthcare professionals receiving training in genome editing are better equipped with knowledge and insights into the complex issues associated with this method, enabling them to take part in the clinic effectively. Toward this end, we have recently introduced a didactic Elective Course on therapeutic genome editing in our College PharmD curriculum. A few students who completed this Elective have enrolled in the laboratory research rotation as a part of our Advanced Pharmacy Practice Education (APPE) curriculum. In this laboratory research training, *MYC* was selected as a target for gene disruption in two different well-established cancer cell lines. Details of the planning and execution of the experiments, unbiased interpretations of the data, and critical analysis of the published works are presented in this report. We expect the knowledge and

problem-solving skills gained in this training to be useful and promote the active participation of these students in gene therapy trials and treatments. It is expected that this training program may serve as a model for preparing medical and other healthcare professionals in gene therapy technologies as well.

2. Materials and Methods

Research Ethics Committee approval. This research study and the purchase of cell lines used in this study were reviewed by the Institutional Biosafety Committee and the Institutional Research Review Board of the Appalachian College of Pharmacy and approved with Exempt Status as this study does not involve small laboratory animals.

Cancer Cell Lines and Culturing Conditions. The human colorectal carcinoma cell line, HT-29 and the high-grade human ovarian serous adenocarcinoma cell line, OVCAR8 were purchased from the National Cancer Institute cancer cell line repository and stored in liquid nitrogen. Authentication of cell lines was done by the supplier by the DNA-short tandem repeat profiling. Cells were cultured, aliquoted and stored in liquid nitrogen for long-term storage or −80˚C freezer for short-term use. All the experiments were carried out within two years from the time the cells were purchased. Cells were cultured in the Supplier-recommended growth media containing 10% (v/v) fetal bovine serum (Cytiva, Marlborough, MA) in a 37˚C incubator containing 5% CO_2, as reported [11].

Selection of knockout gene. The *MYC*, a well-characterized oncogene, was chosen as a target for knockout using CRISPR-Cas9 method.

Synthesis of *MYC*-specific gRNA. Although many online bioinformatics platforms are available (https://zlab.bio/guide-design-resources), the Synthego-based bioinformatics tool (design.synthego.com) was used in designing small guide RNA (gRNA), setting the following parameters: Genome: Homo sapiens (Genome Reference Consortium Human Build 38.p10); Gene *MYC* (Ensembl link: ENST00000621592); Nuclease: SpCas9 (Streptococcus pyogenes CRISPR-as-socated-9). These parameters have identified four gRNA sequences in exon 2 of the *MYC,* which are: gRNA1 (ACGUUGAGGGGCAUCGUCGC), gRNA2 (AACGUUGAGGGGCAUCGUCG), gRNA3 (UUGGUGAAGCUAACGUUGAG) and gRNA4 (UUGAGGGGCAUCGUCGCGGG). Among these gRNA1 was used in these studies. The corresponding DNA sequence ACGTTGAGGGGCATCGTCGC to which a CACCG, was added to its 5'-end, resulting in 5'-CACCGACGTTGAGGGGCATCGTCGC-3'. The reverse complementary oligo to this was designed with an additional AAAC sequence to the 5'-end, and a C at the 3'-end. These bases were added to enable subcloning into the pX459 plasmid (see below). Thus, the reverse complementary sequence is 5'-AAACGCGACGATGCCCCTCAACGTC-3'. The PAM sequence was omitted as required by the gRNA design guidelines [12], which is however present at the 5'- of this gRNA sequence in the reverse strand of exon 2 (3'-GGGCGCTGCTACGGGGAGTTGCA-5', wherein PAM sequence is underscored). These oligos were synthesized at IDT DNA Technologies, Coralville, Iowa.

Subcloning of gRNA duplexes in pX459 plasmid. The forward and the corresponding reverse-complementary DNA oligoes were mixed, annealed and their 5'-ends were phosphorylated using polynucleotide kinase by following these steps: incubation at 37˚C for 30 min, followed by 95˚C for 5 min, and then cooling down slowly (5˚C/min) to 25˚C. This oligo duplex was cloned into the pSpCas9(BB)-2A-Puro plasmid (pX459) (Addgene, Addgene.org) that was previously digested with Bbs I (New England Biolabs, Ipswich, MA), dephosphorylated with calf intestine alkaline phosphatase and then ligated with T4 DNA ligase. This reaction mixture was then transformed into *E.coli*, by following the standard molecular biological procedures. Plasmid DNAs were isolated from ampicillin-resistant *E.coli* colonies and incorporating MYC gRNA sequences was confirmed by DNA sequencing.

Cell Transfection, Genomic DNA isolation, and Sequencing. Cells growing in 6-well plates were transfected with pX459/MYC-gRNA plasmids (2.5 μg each) using Lipofectamine 2000 reagent (Invitrogen, CA). As controls, cells were transfected with pX459 plasmid vector. The transfected cells were grown for 48 h in the complete growth medium, which was then replaced with puromycin (EMD Millipore, Billerica, MA) (0.3 μg/ml) supplemented growth medium and incubated for an additional 10 days. Single-cell colonies observed in the plates were marked and then carefully collected using a sterile pipette tip and transferred to 12-well plates. Clones were propagated in regular growth media without puromycin and used in further analysis.

Genomic DNA was extracted from several clones obtained from transfection with the vector-, and pX459/MYC plasmids using PureLink Genomic DNA kit (Invitrogen), according to the manufacturer's instructions. Using the genomic DNA as a template, regions surrounding the gRNA target sites in *MYC* gene were amplified using specific primer sets (forward primer: 5'-CTTTAGGGGATAGCTCTGCAA-3', reverse primer: 5'-TTCTTCCAGATATCCTCGCTG-3') by polymerase chain reaction (PCR) (Fisher Scientific, Waltham, MA). The PCR amplification conditions were: 95˚C for 2 min, followed by 30 cycles of 95˚C for 20 sec, 55˚C for 20 sec, and 72˚C for 20 sec, and 3'-extension at 72˚C for 3 min.

The PCR products were separated on agarose gels (1% w/v) containing ethidium bromide (10 mg/ml) and the expected size DNA fragments were excised and purified using PureLink Gel Extraction Kit (Invitrogen, CA). The PCR-amplified DNA fragments were sequenced using the forward primers used in the PCR, at the Genomic Sequencing Center, Biocomplexity Institute, Virginia Tech, Blacksburg, VA on a pay-for-service basis.

Cell proliferation. The effect of gene knockout on cell proliferation was evaluated by the 3-(4,5-dimethylthazol-2-yl)-2,5diphenyltetrazolium bromide) MTT assay, as described by others [13]. Briefly, cells were seeded in 96-well plates in octuplicates (10,000 cells per well) and allowed to attach to the plates in a phenol red-free RPMI culture medium overnight at 37˚C/5% CO_2 in a cell culture incubator. To establish the 0-time cell content, MTT assay was carried out 5 h after cells were attached to the plates. At regular time intervals, cell growth was quan-

tified.

Cell cycle analysis: Cells (100,000 /well) were plated in 12-well plates and grown for 48 h. Cells were then collected after trypsinization, fixed in ice-cold 70% (v/v) ethanol and stored overnight at 4˚C. The cells were then washed in PBS and incubated in Guava Cell Cycle Reagent (Lumenix, Austin, TX) in the dark for 30 min, and then analyzed by flow cytometry using Guava Flow Cytometer [14]. Data were collected using Incyte data acquisition software (Millipore Guava Instruments) and the cell cycle phases were quantified by Modfit 5.0 software (Verity Software House, Topsham, ME).

Western blotting. Preparation of whole cell lysates and protein estimation was carried out using BioRad DC protein assay kit using bovine serum albumin as standard, as described previously [15]. Proteins (10 μg) in cell lysates were separated in mini-acrylamide gels (BioRad Laboratories) and transferred onto PVDF membranes. The western blots were developed using specific antibodies mentioned in the Results Section (Cell Signaling Technology, Danvers, MA) and WesternSure PREMIUM reagents (Li-COR, Lincoln, NE). The blots were scanned using Li-COR c-DIGIT equipment.

Materials and reagents. All materials and reagents used in this study were obtained as reported [11] [14] [16].

Statistical analysis. Each cell proliferation experiment was carried out in octuplicates and repeated a minimum of three times. The data points presented in **Figure 1D** and **Figure 2C** represent mean ± s.e.m. Similarly, each of the cell cycle analysis was carried out in triplicates and repeated a minimum of three times. Data presented in **Figure 1E** and **Figure 2D** represent the mean of three measurements ± s.e.m.

3. Results

Selection of gRNA for *MYC* disruption.

Using the online bioinformatics tools, four gRNA sequences that could disrupt the human *MYC* were identified (see Materials and Methods), all of which bind to exon 2 with 100% homology. These gRNAs could also bind to other loci only when there are over 3 mismatches, and such mismatch binding regions were however uncharacterized so far (synthego.com). Although all four gRNAs are appropriate for disrupting *MYC*, we employed gRNA1 in the following studies.

Disruption of *MYC* in HT29 cells.

Bioinformatics tools, mentioned under Materials and Methods, have identified four overlapping guide RNA (gRNA) sequences at the 5'-end of the exon 2 in *MYC* (**Figure 1A**). We selected gRNA1 (**Figure 1A**) for this study. The gRNA1 was subcloned into pX459 plasmid, transfected into human colorectal cancer HT29 cells and several puromycin-resistant clones were isolated for further characterization, according to Ran *et al.* [12]. The c-Myc expression and β-actin levels in these clones were determined by western blotting using equal amounts of total cellular protein lysates from these samples and the results are shown in **Figure 1B**. The data shows these clones contained variable amounts of

c-Myc protein. Clone 4 contained similar amounts of c-Myc as in the vector control, suggesting that *MYC* in clone 4 was not disrupted. Importantly, the c-Myc protein was nearly absent in clones 5 and 7, suggesting that all *MYC* copies in these clones were likely disrupted. Finally, clones 1, 2, 3, 6 and 8 contained lower amounts of c-Myc when compared to control, suggesting that at least one copy of the *MYC* was intact.

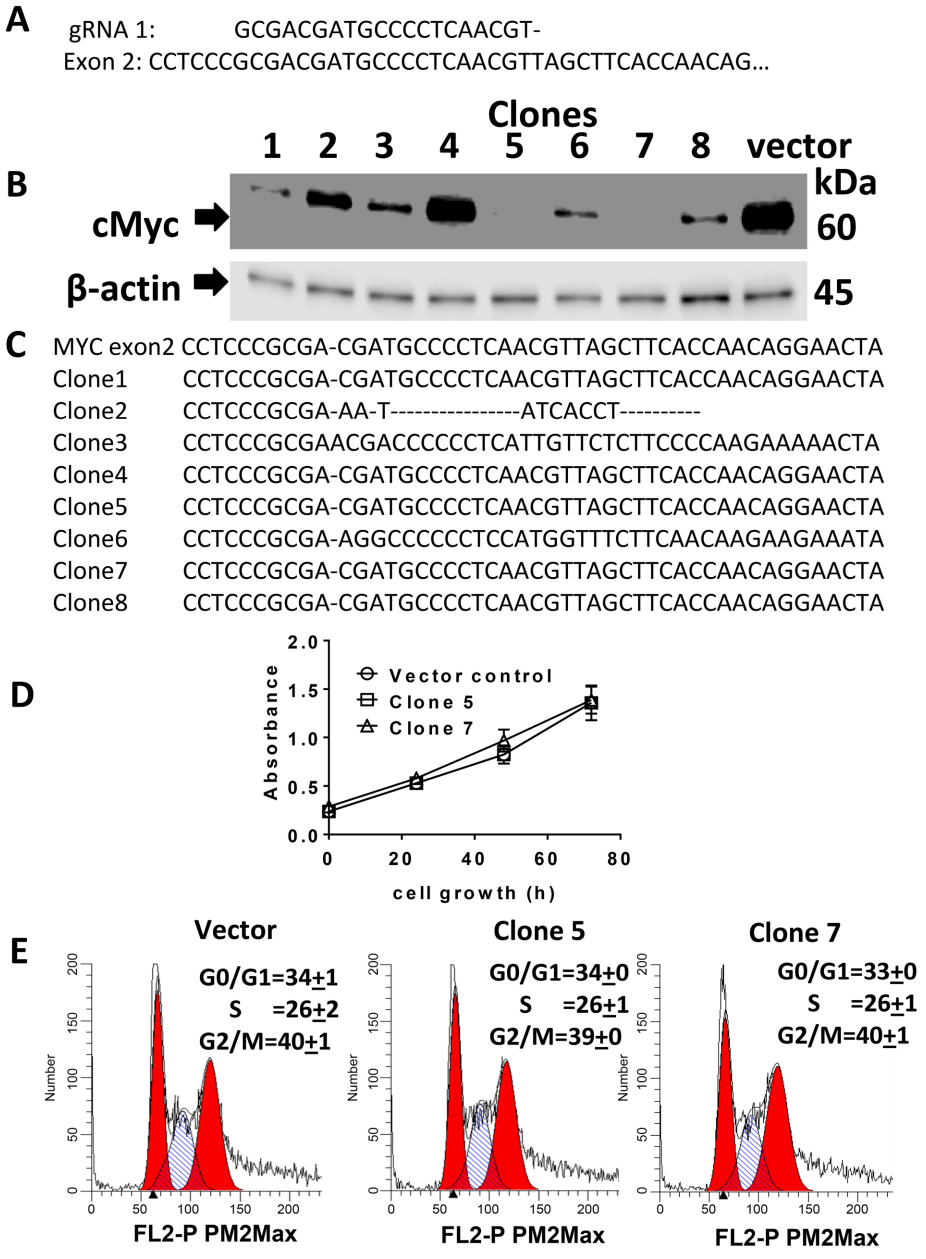

Figure 1. Disruption of *MYC* in HT29 cells. (A) The gRNA sequence and its alignment with the exon 2 of MYC. (B) Analysis of c-Myc expression in clones by western blotting. (C) Analysis of *β*-actin levels in clones by western blotting. (D) Alignment of clonal DNA sequences with the MYC exon 2. Gaps in clonal DNA indicate deletions and, gaps in the MYC exon 2 indicate insertions in the clonal DNAs. (E) Cell proliferation analysis of vector control, clones 5 and 7. F. Cell division cycle analysis on vector control, clones 5 and 7. See text for details.

To confirm knockout, genomic *MYC* DNA encompassing the gRNA1 region was amplified by PCR from all clones and subjected to sequencing. The DNA sequences thus obtained were aligned with *MYC* exon 2 using bioinformatics tools mentioned in the Materials and Methods. **Figure 1C** shows that the DNA sequences from clones 1, 4, 5, 7 and 8 aligned perfectly with the wild-type *MYC* exon 2 sequence, although c-Myc levels in these clones were lower than in the control. The exon 2 sequence from clones 2, 3, and 6 was disrupted with indels, transitions and transversions, which was evident from the gaps and mismatches in the sequence alignments (**Figure 1C**). Comparison of the peptide sequences predicted (web.expasy.org/translate) from these DNA sequences was shown below:

Vector control: …PPATMPLNVSFTNRNYDLDYD…

Clone 1: …PPATMPLNVSFTNRNYDLDYD…

Clone 2: …PPAKYHLPYDSLHP…(premature stop)…

Clone 3: …PPANDPPHCSLPQEKL…(premature stop)…

Clone 4: …PPATMPLNVSFTNRNYDLDYD…

Clone 5: …PPATMPLNVSFTNRNYDLDYD…

Clone 6: …PPAKAPLHGFFNKKKYHLHHHAV…(premature stop)…

Clone 7: …PPATMPLNVSFTNRNYDLDYD…

Clone 8: …PPATMPLNVSFTNRNYDLDYD…

The above protein sequences clearly show that clones 2, 3, and 6 are completely altered because of frameshift mutations in the DNAs, when compared to the normal c-Myc amino acid sequence. This analysis provided evidence for the reduced levels of c-Myc detection in these clones by the western blotting analysis. However, the loss of c-Myc in clones 5 and 7 could not be explained by the DNA sequence analysis, which showed that *MYC* was intact in these clones. Interestingly, it is known that colorectal cancers contain multiple pseudogenes of *MYC* [17], and one of which was likely sequenced, yielding wild-type sequence in our analysis.

To determine the functional consequences of MYC knockout in clones 5 and 7, cell proliferation assays were carried out as described under Materials and Methods. **Figure 1D** shows that the cell proliferation rates of these clones were nearly identical to that of the control, suggesting that the cell proliferation was unaffected with *MYC* disruption. To further analyze the MYC knockout, cell division cycle analysis was also carried out on these clones as described under Materials and Methods. **Figure 1E** shows that the distribution of cell population in G0/G1, S and G2/M phases in both clones was identical to that in the control. These results suggested that *MYC* is not involved in the regulation of cell division cycle processes in this cancer cell line. These results together suggest that c-Myc may not be the only cellular protein regulating these cellular processes in HT29 cells.

Disruption of *MYC* in OVCAR8 cell line.

In the absence of any functional alterations due to *MYC* disruption in HT29 cells, we performed these experiments on the human ovarian carcinoma OVCAR8

cell line. The OVCAR8 cells were transfected with the gRNA1 carrying pX459 plasmid and 24 puromycin-resistant clones were collected, as described under Materials and Methods. However, many of these clones could not be propagated due to extremely slow proliferation. The c-Myc expression and β-actin levels in 7 of these clones were determined by western blotting as mentioned in the Materials and Methods. **Figure 2A** shows the results. Among these clones, c-Myc expression was minimal in clones 9 and 21 when compared to other clones and vectors, suggesting that *MYC* was likely disrupted in these clones. To establish *MYC* disruption, the *MYC* exon 2 segment encompassing the gRNA1 region from clones 9, 21 and the vector control, was PCR-amplified, sequenced and aligned with the exon 2 sequence, as described under Materials and Methods. **Figure 2B** shows that the DNA from clones 9 and 21 contained widespread mismatches with indels. Comparison of the peptide sequences predicted (https://web.expasy.org/translate) from these DNA sequences is shown below:

Vector control: … PPATMPLNVSFTNRNYDLDYD…

Clone 9: … PPAICLSMWFSHINYNTVPPSLCSYISCD (premature stop)

Clone 21: … PPALCAPLQHAPAN (premature stop)

These data showed that c-Myc protein in clones 9 and 21 was prematurely truncated with stop codons introduced by indels and other alterations in exon 2, when compared to control, suggesting that *MYC* was disrupted. These data provided evidence for the decreased expression of c-Myc in these clones.

To further characterize the effect of MYC disruption, cell proliferation was carried out as described under Materials and Methods. **Figure 2C** shows that clones 9 and 21 exhibited decreased rates of cell proliferation when compared to control. Although not shown, clones 1, 2, 4 and 8 exhibited normal cell proliferation, similar to controls. Together, these data suggested that c-Myc is an important protein, playing a key role in OVCAR8 cell proliferation, corroborating the well-established role of *MYC* in this cellular process [18]. As performed on HT29 cells, the cell division cycle analysis was carried out on clones 9 and 21, and the results were shown in **Figure 2D**. These data show that clones 9 and 21 contained a significantly increased percentage of cells in G0/G1 phase, with a concomitant decrease in cells in G2/M phase, when compared to controls. These data indicated that *MYC* disruption leads to slow progression of cells from G0/G1 to S phase, suggesting that MYC regulates the cell division cycle in OVCAR8 cells.

To determine the mechanism of *MYC*-regulated cell division cycle, we analyzed the expression of phospho-cdc2 and p21 proteins in clones 9 and 21. **Figure 3** shows that the phospho-cdc2 level was nearly similar in clones 9 and 21, to that in control cells. Interestingly, there was a significant increase in p21 levels in these clones, when compared to control. These data extend strong support for the well-established observations that MYC represses the p21 expression [19]. Together, these data suggested that *MYC* is an important gene in OVCAR8 cells regulating the cell proliferation and cell division cycle, and its disruption leads to downregulation of these processes.

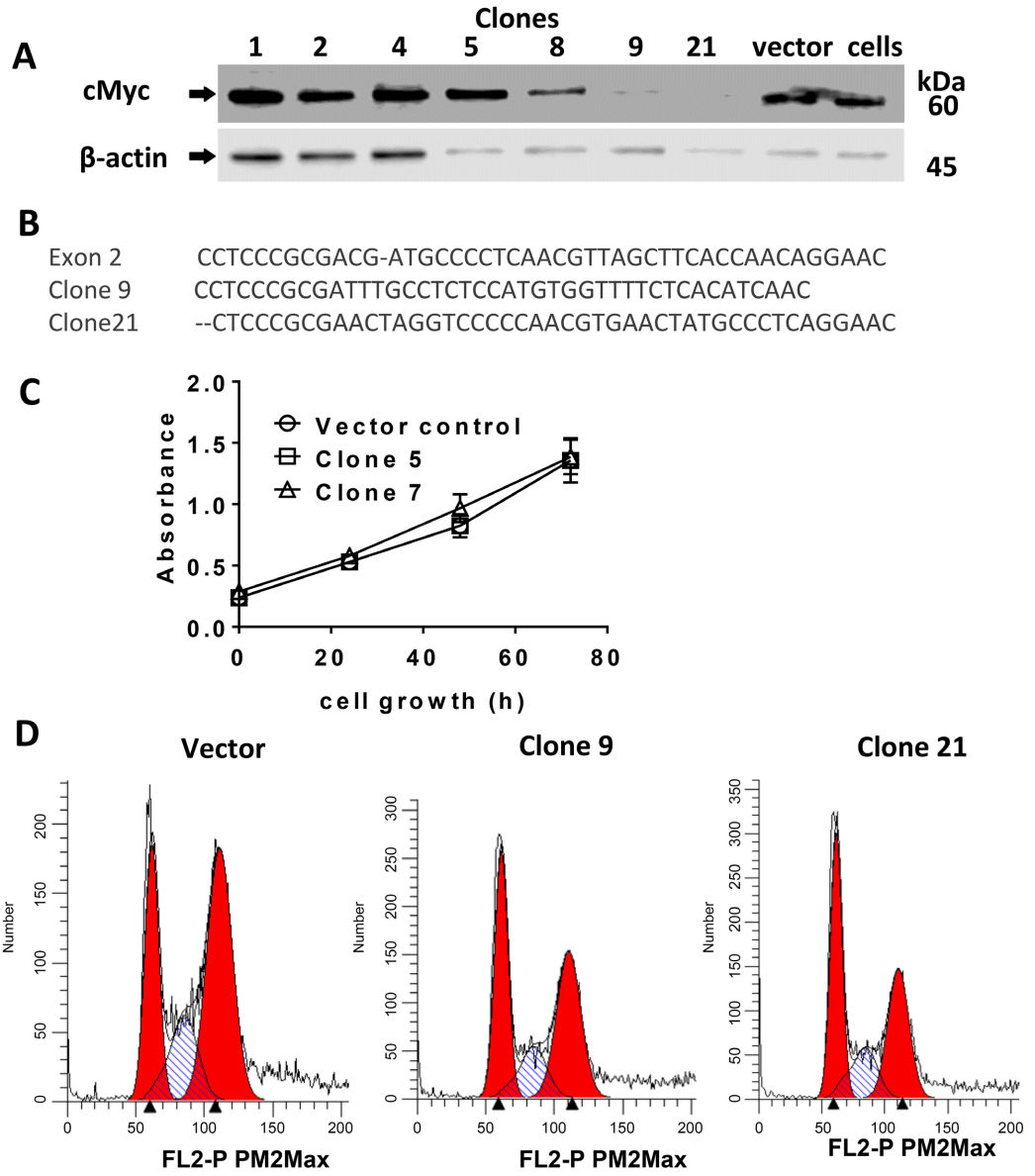

Figure 2. Disruption of *MYC* in OVCAR8 cells. (A) Analysis of c-Myc and β-actin expression in clones by western blotting; (B) Alignment of clonal DNA sequences with the MYC exon 2. Gaps in clonal DNA indicate deletions and, gaps in the MYC exon 2 indicate insertions in the clonal DNAs; (C) Cell proliferation analysis of vector control, and clones 5 and 7; (D) Cell division cycle analysis on vector control, clones 5 and 7. See Text for details.

4. Discussion

Several clinical gene therapy trials conducted over the past four decades to treat various genetic defects met with limited success. The initial optimism at each gene therapy trial about restoring gene function was plagued by unforeseen safety issues due to vector-triggered immune responses, off-target gene editing, insertional mutagenesis, patient selection criteria and others [20]-[25]. These failures, although have raised doubts about the clinical efficacy of gene therapy, could be prevented with better understanding of diseases, clinical trial designs,

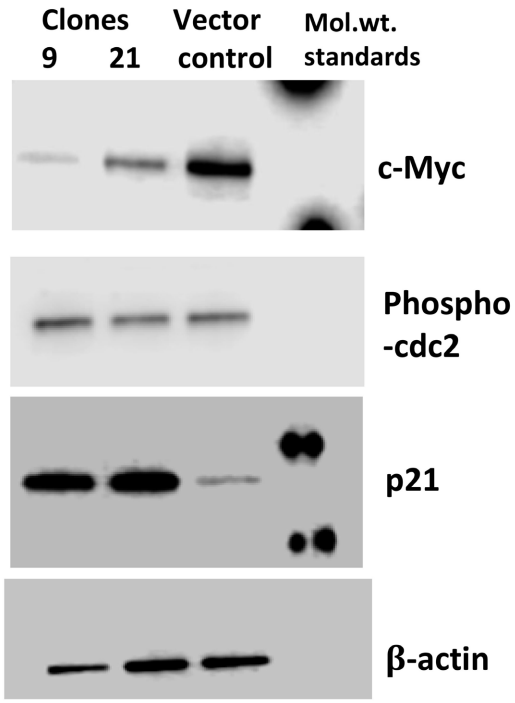

Figure 3. Western Blot analysis of c-Myc, phospho-cdc2 and p21 expression in MYC knockout OVCAR8 cells. See Text for details.

and patient selection. Pharmacists, who receive education in all fields of basic sciences, are suitably qualified for training in genome editing research. Such training prepares these healthcare professionals to take part in developing policies and protocols for gene replacement, identifying risk levels, post-therapy care and resolving ethical issues. Thus, we have undertaken training of PharmD students in gene editing in our research laboratory and the results are presented here.

Our laboratory has been training PharmD students in cancer biomarker validation [26] [27], and pharmacogenetics [28]. This is our first attempt to train students in genome editing research. We chose *MYC*, hereto a non-druggable oncogene highly expressed in nearly 50 percent of all cancers, as a target for gene disruption. To compare the c-Myc expression levels among the clones and controls, we used both the estimates of total cellular protein and β-actin staining methods. Since β-actin levels are known to change in response to various biochemical stimuli, such as growth arrest and apoptosis [29], we relied on total protein loading estimates to interpret the western blotting. As noted in the Results section, *MYC* was disrupted in several clones isolated from both cell lines. However, the amounts of c-Myc were variable in the isolated clones, suggesting that these cell lines carry more than two copies of *MYC*. Students were thus challenged to review the literature and then interpret the western blotting data in deriving the number of *MYC* copies in these cell lines. The general assumption is that the amount of c-Myc in controls is due to the transcriptional activity of two

MYC copies and the clones would express either half or nil amounts of c-Myc. Contrary to this expectation, the clones in **Figure 1B** and **Figure 2A** contained more than or less than half the amount of c-Myc. The students thus recognized that cancers may harbor additional genetic alterations leading to the existence of additional copies of *MYC*.

The results presented here support the view that these cell lines contained greater than two copies of *MYC*. To validate this interpretation, the DNA fragments covering the CRISPR-Cas9 site from all these clones were sequenced. The data revealed indels, transitions and transversions in exon2 at the gRNA1 binding site, indicating that the predictive algorithms accurately identify gRNA sequence targets for Cas9 cleavage. In addition, indels and other alterations at the CRISPR-Cas9 site suggested that cells, *a priori,* adapt to non-homologous end-joining mechanism of repair, in the absence of homology-directed repair sequences. Closer examination of these DNA sequences revealed that *MYC* gene in HT29 clone 4 was intact, supporting the normal levels of c-Myc expression (**Figure 1B**). Importantly, HT29 clones 5 and 7 although do not express c-Myc (**Figure 1B**), the *MYC* DNA was intact. This observation suggested that HT29 cells might contain transcriptionally inactive *MYC* pseudogenes. To determine this, we carried out karyotyping on HT29 cells which indicated that this cell line contains three copies of chromosome 8 (data not shown) on which *MYC* was localized at 8q24.21, supporting the previous studies [30]. Importantly, earlier studies have shown that the *MYC* locus on chromosome 8 in the HT29 cells is enriched as a homogeneously staining region (HSR) with many *MYC* pseudogenes [17]. Together, these previous studies suggested that HT29 cells contain a minimum of 3 transcriptionally active copies of *MYC*.

Previous studies have shown that OVCAR8 cell line carries four copies of chromosome 8 [31], suggesting that this cell line carries 4 copies of *MYC*. Since clones 9 and 21 obtained from OVCAR8 cell line contained indels (**Figure 2B**) leading to premature truncations of c-Myc, it is likely that many of these *MYC* copies were disrupted. On the other hand, clones 1, 2, 4, 5 and 8 express variable levels of c-Myc, suggesting one or more copies of *MYC* are transcriptionally active. An important outcome from these analyses is that gene therapy clinical trials must ensure that all gene copies in the target cells are edited to achieve optimum results in the clinic. These observations and interpretations are relevant to student training in gene therapies as these issues demand special attention in clinical trials.

Since gene knockout must lead to the loss of function in order for clinical benefit, we analyzed cell proliferation and cell division cycle analyses to determine the role of *MYC* in HT29 and OVCAR8 cell lines. Our data showed that these cellular processes were affected with *MYC* disruption in OVCAR8 cells, but not HT29 cells, which pointed out that gene function is cell type-dependent. This observation is important because all the cells in an organism, though contain the same gene, its function is likely restricted to specific tissues. Thus, the

students learned that gene delivery procedures must be directed to the desired tissue in which the gene is active to achieve maximum benefits of gene therapies. Analysis of cell proliferation and cell division cycle studies emphasized the gene function.

Finally, the students were directed to recognize the possible off-target genes in CRISPR editing using bioinformatics tools [32] [33] [34] [35] [36]. If any of these off-target genes are functionally important, students were advised to design alternative gRNAs to minimize disruption of such off-target important genes in their clinical gene therapies. In conclusion, students trained in laboratory research gained in-depth working knowledge and skills useful in forecasting and addressing the unforeseen setbacks continually encountered in gene therapy trials.

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Author Contribution

PSR and USR conceived and designed the study. PSR performed the experiments. PSR and USR analyzed the data and prepared the figures. USR wrote the manuscript and PSR reviewed the manuscript.

Data Availability Statement

The authors declare that all data supporting the findings of this study are available within the paper. The DNA sequence obtained from each clone mentioned in this paper was deposited at https://ncbi.nlm.nih.gov/nuccore/ with identities OQ865414 to OQ865425.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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