ORIGINAL ARTICLE

Genome Editing: A Comparative Study on the Efficiency of CRISPR/Cas9 Nuclease Versus Nickase Using HIV as a Model System

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ABSTRACT

Introduction: CRISPR/Cas9 nuclease has gained popularity as a genome editing tool due to its straight-forward mechanism. However, there are concerns that CRISPR nuclease would cause off-target and toxicity. The CRISPR/Cas9 D10A nickase was designed to enhance genome editing. Nevertheless, this raised the question of whether the efficiency of nickase is compromised compared to CRISPR/Cas9 nuclease. Targeting HIV genes, we investigated if CRISPR nuclease performed better than the nickase in efficacy and safety. **Methods:** CRISPR nucleases and nickases were designed to target Gag, Pol, Rev, Vif, Tat and LTR. HIV latently infected cell line, ACH-2, was transfected with the nucleases and nickases. Changes to viral load after CRISPR treatment was measured using p24 ELISA. Safety of nuclease and nickase was monitored using GFP expression with fluorescence microscopy and flow cytometry. Targeting two sites within the same gene, and targeting multiple genes concurrently were also studied to determine efficacy of CRISPR in reducing viral load. **Results:** A 44.9 to 68.1% and a 34.4 to 49.7% decrease in viral load was seen in CRISPR nuclease and nickase respectively. Microscopy and flow cytometry results showed that the nickase system was slightly toxic with a 0.31 to 0.7-fold cell death. There was a 34% decrease in viral load when two sites were targeted within a gene, and the largest decrease was seen when all the nucleases were combined, giving a 75.4% decrease in viral load at day 5. **Conclusion:** The knowledge gained from this study will be employed to improve genome editing in other disease models.

Keywords: CRISPR/Cas9, Gene editing, Genome engineering, HIV

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INTRODUCTION

Genome engineering refers to the act of DNA manipulation, including inserting, deleting or modifying DNA sequences. The earlier generations of the genome editing tools include zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALENS). More recently, the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system has gained popularity due to the simplicity of the tool, and now, most genome editing work is primarily conducted with CRISPR (1).

ZFN and TALEN both rely on protein-DNA as the DNA recognition, while CRISPR has an RNA-DNA system. ZFN and TALENs use an obligate heterodimer FokI, as the DNA cleavage, while CRISPR utilizes Cas9, which has less off-targets from non-specific binding. Two sets of proteins need to be cloned for each ZFN and TALEN target, whereas CRISPR just requires swapping in a 20 base pair oligonucleotide. There are no preferential target sites with CRISPR and it is small in size, increasing its flexibility in targeting any region (1-3). Lastly, CRISPR has a multiplex ability, enabling several genes to be targeted simultaneously. Despite the differences, ZFNs, TALENs and CRISPR can all be used for gene activation, targeted DNA cleavage, targeted integration, gene repression, chromatin modification and high-throughput screening (4).

However, as mentioned above, the ease of using CRISPR has made it the preferred genome editing tool amongst researchers. In just the span of a few years, CRISPR has been used to target various genes in many cell types across a huge range of organisms, including

mammalian cells, plants and microorganisms. In its natural settings, CRISPR is part of the adaptive immune system of bacteria that serves as a protection mechanism in the event of a phage and virus attack. CRISPR is made out of three core components – CRISPR RNA (crRNA), trans-activating crRNA (tracrRNA) and Cas9. In bacteria, pre-crRNA, which is interspersed with phage or viral sequence, combines with tracrRNA to form an RNA duplex. This duplex is then cleaved by RNA III, forming a crRNA/tracrRNA hybrid, which acts as a signal for Cas9 cleavage (5, 6).

The CRISPR that is used in laboratories today has been modified into a simplified system. crRNA and tracrRNA have been combined to form a single guide RNA (sgRNA). The single guide RNA contains the sequence of the targeted gene of interest, and endonuclease enzyme Cas9 with two active sites RuvC and HNH, functions like a molecular scissors. When sgRNA binds to a target site, Cas9 induces a site directed double-stranded break (DSB). The cell would repair the break using non-homologous end joining (NHEJ) which causes insertions and deletions (indels) at the cleavage site. These indels typically cause a frame shift, which disrupts the function of the gene (4, 7-9).

The modest mechanism of CRISPR popularized its usage within different research groups. However, with the first generation of CRISPR/Cas9, some researchers worried that the simple mode of 'binding and cutting' would cause CRISPR to be promiscuous and bind to several genomic locations, causing off-target and toxicity. For a genome editing tool to be used in a clinical setting, it is crucial for the tool to have a high efficacy, while remaining safe to the patients. Bearing that in mind, scientists mutated one of the active sites, so that the CRISPR/Cas9 D10A nickase was capable of only a single strand break (SSB) and would require a pair of CRISPR to create a DSB. However, the question of whether a paired nickase system functions as efficiently as the wild type CRISPR/Cas9 nuclease remains speculative (10-14). Improving the safety of nickase may compromise its efficiency in comparison to the nuclease system. It is also crucial to investigate other aspects of improving the efficiency of the CRISPR/Cas9 systems such as knocking down two sites within the same gene, and targeting two genes simultaneously. These questions were investigated by performing a comparative study on the efficiency and safety of nuclease versus nickase using HIV as the target organism. HIV was chosen as the model system for this study due to the virulence and complexity of this virus, as well as its suitability to be compared to other pathogens.

The knowledge gained from this study would enable us to understand the balance between safety and efficacy of genome editing, especially in bringing this tool forward for translational research. While there are other types of CRISPR systems, this study focuses on CRISPR/Cas9 nuclease and nickase as most researchers typically use CRISPR to knockdown or knockout DNA in their genes of interest. The results from this study can be used to improve the techniques for other disease models.

MATERIALS AND METHODS

Design and construction of CRISPR/Cas9 against HIV-1

The sequences of Gag, Pol, Rev, Vif and Tat were retrieved from the HIV Sequence Database (https://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html) and the guide RNAs (gRNA) were selected from an in silico program, Target Finder (https://www.crispr.mit.edu). The gRNAs were screened for sequence homology against the human genome (GRCh37.72) and targets with high similarities with the human genome were removed. The complementary 20-nucleotide sequences with BbsI enzyme overhangs were synthesized as oligonucleotides (IDT) for CRISPR cloning. A CRISPR/Cas9 targeting LTR in the HIV genome was included as a positive control in this study (15). The sequences for the guides are as listed in Table I.

Cloning of CRISPR/Cas9 nuclease in expression vector px330-U6-Chimeric-BB-CBh-hSpCas9 (nuclease, nuc) and px335-U6-Chimeric-BB-CBh-hSpCas9 (nickase, nic)

pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene plasmid # 42230; http://n2t.net/addgene:42230; RRID: Addgene_42230) and pX335-U6-Chimeric_BB-CBh-hSpCas9n(D10A) were gifts from Feng Zhang (Addgene plasmid # 42335; http://n2t.net/addgene:42335; RRID: Addgene_42335). First, 5 mM forward and reverse oligonucleotides were annealed in 0.5X T4 ligation buffer (NEB) and 0.025X T4 PNK (NEB) in the following thermocyclic conditions – 37°C for 30 min, followed by 95°C for 5 min, and ramped down to 25°C at 5°C/min. Next, the annealed oligonucleotides were cloned into pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene: 42230) and pX335-U6-Chimeric_BB-CBh-hSpCas9n(D10A) (Addgene: 42335) respectively following manufacturers' protocol. Briefly, 200-fold diluted annealed oligonucleotides, 50 ng vector digested with FastDigest BbsI, 1X Quick Ligase and 0.1X Quick Ligation buffer were mixed and incubated at room temperature for 10 min. The ligation reaction was then treated with 1X RecBCD and incubated at 37°C for 30 min. The products were then transformed into DH5-alpha competent cells and confirmed with Sanger sequencing. The CRISPR/Cas9 plasmids will be referred to as nuclease (nuc) and nickase (nic) from here onwards.

Measurement of viral load in CRISPR/Cas9 treated ACH-2 cells to determine efficacy of nuclease versus nickase

ACH-2, the T lymphocyte and model cell line for latent HIV infection (16, 17), was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: ACH-2 Cells from Dr. Thomas Folks (Cat#

Table I: Oligonucleotide sequences for gRNA targeting HIV

Oligonucleotide	Name	Oligo sequence	gRNA sequence
HIV Gag F	Gag_Nuc	CACCCCCGGCCATAAGGCAAAGAGTTT	CCCGGCCATAAGGCAAAGAGTTT
HIV Gag R		AAACTCTTGCCTTATGGCCGGG	
HIV Pol F	Pol_Nuc	CACCGCAGAAACCTTCTATGTAGA	GCAGAAACCTTCTATGTAGA
HIV Pol R		AAACTCTACATAGAAAGGTTTCTGC	
HIV Rev F	Rev_Nuc	CACCCTTCAGCTACCACCGCTTGA	CTTCAGCTACCACCGCTTGA
HIV Rev R		AAACTCAAGCGGTGGTAGCTGAAG	
HIV Vif F	Vif_Nuc	CACCGTCAGGGAGTCTCCATAGAA	GTCAGGGAGTCTCCATAGAA
HIV Vif R		AAACCCATTCTATGGAGACTCCCTGAC	
HIV Tat F	Tat_Nuc	CACCAAGCCTTAGGCATCTCCTAT	AAGCCTTAGGCATCTCCTAT
HIV Tat R		AAAC CCATAGGAGATGCCTAAGGCTT	
HIV Tat 1 F	Tat_Nuc_1	CACCGCTTAGGAATCTCCTATGGC	GCTTAGGAATCTCCTATGGC
HIV Tat 1 R		AAACGCCATAGGAGATTCCTAAGC	
HIV Gag P1 F	Gag_Nic	CACCGAGAGACAGGCTAATTTTTT	GAGAGACAGGCTAATTTTTT
HIV Gag P1 R		AAACAAAAAATTAGCCTGTCTCTCGGTG	
HIV Gag P2 F		CACCCCCGGCCATAAGGCAAAGAGTTT	CCCGGCCATAAGGCAAAGAGTTT
HIV Gag P2 R		AAACTCTTGCCTTATGGCCGGG	
HIV Rev P1 F	Rev_Nic	CACCCTTCAGCTACCACCGCTTG	CTTCAGCTACCACCGCTTG
HIV Rev P1 R		AAACTCAAGCGGTGGTAGCTGAAAA	
HIV Rev P2 F		CACCACTTCATCTTGATTGCAGCG	ACTTCATCTTGATTGCAGCG
HIV Rev P2 R		AAACCGTTACAATCAAGAGTAAGT	
HIV LTR-D F	Positive control	CACCGTTAGACCAGATCTGAGCCT	GTTAGACCAGATCTGAGCCT
HIV LTR-D R		AGGCTCAGATCTGGTCTAACCAAA	

349). 6.25 x 105 cells were seeded in a 6-well plate containing Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS, 1% penicillin/ streptomycin and incubated at 37°C and 5% CO2. After 12 hours of incubation, cells were treated with 100 unit (U) of tumor necrosis factor alpha (TNFα) to activate viral production. After 24 hours of incubation, ACH-2 cells with TNFα stimulation were transfected with 2.5 µg CRISPR/Cas9, 7 µL Lipofectamine 3000 and 5 µL P3000 reagent (Invitrogen). Untransfected cells with TNFα stimulation were included as a negative control, and cells treated with CRISPR/Cas9 targeting LTR were included as a positive control. Viral particles were harvested from the supernatant at days 1, 3 and 5 post-transfection, and p24 expression in cells was measured using ELISA p24 quantification kit (Cellbiolabs, Inc) according to the manufacturer's protocol.

Cytotoxicity of CRISPR/Cas9 nuclease and nickase in HEK 293 cells

Human embryonic kidney 293 (HEK 293) was purchased from ATCC. 6.25 x 105 cells were seeded in a 6-well plate containing Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FBS, 1% penicillin/ streptomycin, and incubated at 37°C and 5% CO2. After 24 hours, cells were transfected with 1.5 µg CRISPR/Cas9, 0.5 µg pQBI-eGFP (plasmid that constitutively expresses GFP), 7 µl Lipofectamine 3000 and 5 µL P3000 (Invitrogen). CRISPR/Cas9 targeting LTR and pQBI-eGFP transfected cells without CRISPR were included as positive and negative controls respectively. GFP expression in the cells was monitored with fluorescence microscope (Olympus) at 100x and fluorescence activated cell sorter (FACS) at days 1, 2 and 3 post-transfection.

RESULTS

Efficiency of nuclease versus nickase

To investigate the efficiency of CRISPR/Cas9 in latently infected cells, ACH-2 cells were treated with CRISPR nucleases and nickases targeting Gag and Rev. Viral load was determined by measuring the expression of p24 on days 1, 3 and 5 post-treatment using ELISA. Viral load on day 1 for treated and untreated cells was normalized to 100%.

In CRISPR nuclease and nickase treated cells on day 3 (Fig.1), there was a significant 2.2-fold reduction (54.4% decrease) in viral load in the positive control compared to the untreated cells. Gag_Nuc and Rev_Nuc had a 1.39-fold reduction (28% decrease) and a 1.74-fold reduction (42.4% decrease) respectively, compared to untreated cells. Gag_Nic and Rev_Nic had a 1.21-fold reduction (17.31% decrease) and 1.40-fold reduction (31.61% fold decrease) respectively, compared to untreated cells.

On day 5, all treated cells showed a significant decrease in viral load. There was a 3.94-fold reduction (74.64% decrease) in positive control, 1.82-fold reduction (44.93% decrease) in Gag_Nuc, 3.14-fold reduction

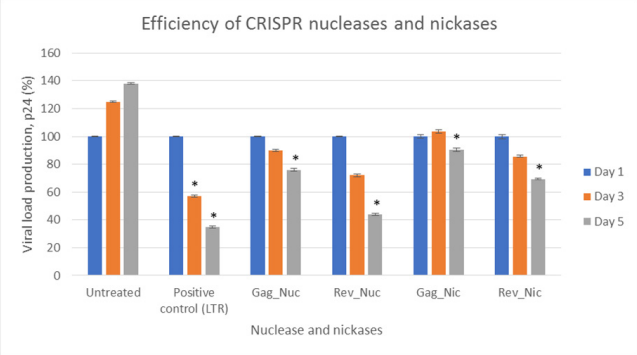

Figure 1: The percentage of viral load production in latently infected cells treated with CRISPR/Cas9 nuclease and nickase on days 1, 3 and 5. There was an increase in viral load in untreated cells, and there was a significant decrease in viral load on day 5 in all CRISPR nuclease and nickase treated cells (* = $p < 0.05$).

(68.12% decrease) in Rev_Nuc, 1.52-fold reduction (34.38% decrease) in Gag_Nic and 1.99-fold reduction (49.72% decrease) in Rev_Nic, compared to untreated cells. Other than positive control, the biggest decrease seen on days 3 and 5 was Rev_Nuc. There was a constant increase in viral load in untreated cells on days 1, 3 and 5.

The nuclease and nickase systems impaired HIV replication between 1.21 to 3.14-fold in comparison to the untreated cells. There was no significant difference between the efficiency of nuclease versus nickase CRISPR systems for viral load reduction.

Cytotoxicity of nuclease vs nickase

In addition to efficacy, the safety of both systems was investigated. The cellular cytotoxicity of CRISPR/Cas9 system in non-specific mammalian cells and HEK 293 was done in triplicates and assessed by monitoring the expression of GFP-retained cells over time via fluorescence microscope (Fig.2). The number of GFP-retained untreated cells was used as a reference of no cytotoxicity. While there may be variation in transfection efficiency on day 1, the increase in GFP cells over time would indicate no cytotoxicity, while a decrease in GFP cells would indicate cell death and cytotoxicity. GFP expression was quantified using ImageJ.

There was an increase in GFP-positive cells for all CRISPR treated cells from day 1 to day 3, except in Gag_Nic and Rev_Nic (Fig.2). In Gag_Nic and Rev_Nic, a decrease in GFP positive cells were observed in days 2 and 3 compared to day 1 and compared to untreated. This suggests the possibility of Gag_Nic and Rev_Nic potentially being toxic, while the CRISPR nucleases did not seem to exhibit any toxicity.

To further verify the fluorescence microscopy results, the cytotoxicity of CRISPR/Cas9 was also determined using flow cytometry on days 1, 2 and 3. For flow cytometry analysis, which was also conducted in triplicates, the number of cells on day 1 was normalized to 1, and the

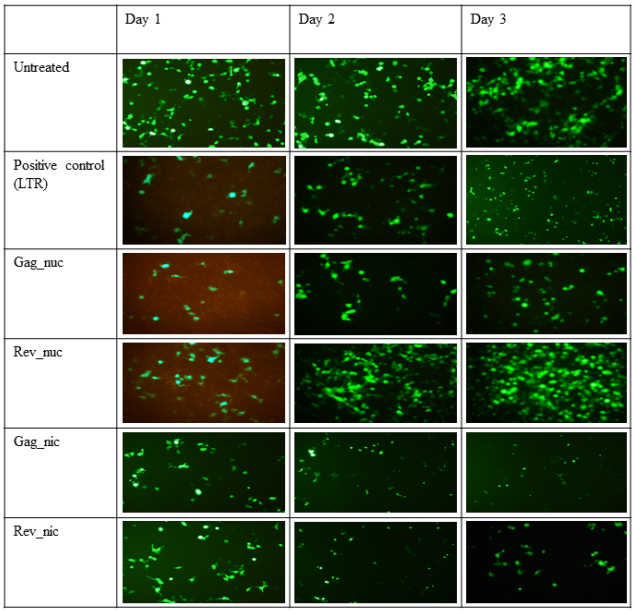

Figure 2: The changes in GFP expression from Day 1 to Day 3 for all CRISPR treated cells associated with cell proliferation. There was an increase in GFP expression in all treated cells except Gag_Nic and Rev_Nic, implying toxicity with the nickase system. Cells were further quantified with FACS to determine cell proliferation.

fold difference of the number of cells on days 2 and 3 were determined. The fold difference on day 3 was then divided by day 2 (Fig.3). If the value was less than 1, that indicated that CRISPR potentially had off-targets and caused cell death.

Similar to the results for fluorescence microscopy, the fold change in cell growth treated with positive control (LTR), Gag_Nuc and Rev_Nuc was equal to, or more than 1, implying that the CRISPR nucleases were not toxic. However, the fold change in cell growth treated with Gag_Nic and Rev_Nic was 0.3 and 0.69 respectively, implying severe toxicity in cells treated with CRISPR nickase (Fig.3). These results were in agreement with that seen in fluorescence microscopy.

Although the nickases caused a decrease in viral load, both Gag_Nic and Rev_Nic were found to be toxic. From both sets tested, the fluorescence microscopy and flow cytometry data seemed to suggest that the nickase system caused more toxicity in comparison to the nuclease system. As the nickases seemed to be toxic, further tests on improving CRISPR efficacy were conducted using only nucleases.

One versus two sites in a gene

We further explored whether targeting one or two sites within a gene would show a significant knockdown. CRISPR Tat_Nuc_1 was less efficient in comparison to the other nucleases as it did not manage to reduce the viral load on day 4. In comparison, positive control (LTR) and Tat_Nuc were efficient at reducing viral load by 50% and 1.6% respectively (Fig.4). For cells treated with

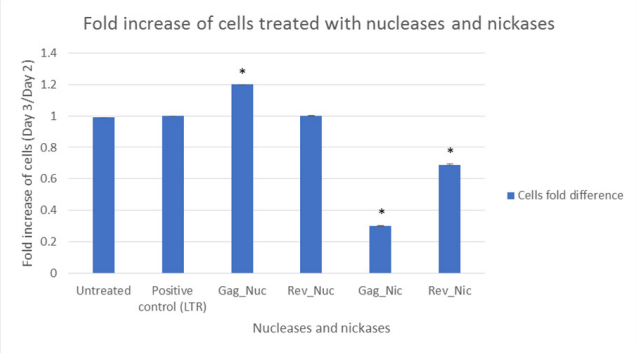

Figure 3: The fold increase of GFP positive cells on day 3 compared to day 2 after treated with CRISPR/Cas9 nuclease and nickase. A fold increase of less than 1 implies that the CRISPR may have off-targets, and may be causing cell death, as seen in Gag_Nic and Rev_Nic. The cells treated with nuclease had a fold increase of 1 or more than 1, implying that they were not-toxic (* = $p < 0.05$).

both Tat_Nuc and Tat_Nuc_1 simultaneously, there was a 35.2% reduction in viral load (Fig.4) on day 4, while there was an increase in viral load in untreated cells. The results of this experiment seem to suggest that there was a synergistic effect causing a huge knockdown in viral load when two sites within a same gene are targeted simultaneously. Knockdown efficiency was increased by almost 10.0-fold (Fig.4) when two sites were targeted concurrently.

From this experiment, we discovered that targeting two different sites within a gene gave optimal knockdown compared to one site targeting. A cytotoxicity analysis for this portion of the study was not conducted, so we were unable to determine if there was an increase in toxicity when two sites were targeted as opposed to one.

One versus many genes

After establishing that targeting two sites within one gene gave a better knockdown, the next step was to prove if targeting two or more genes gave a bigger knockdown compared to one target. CRISPR nuclease was used to target five genes simultaneously; Gag, Rev, Tat, Pol, and Vif of HIV, and p24 expression was measured on days 1, 3 and 5 post-treatment using ELISA (Fig.5).

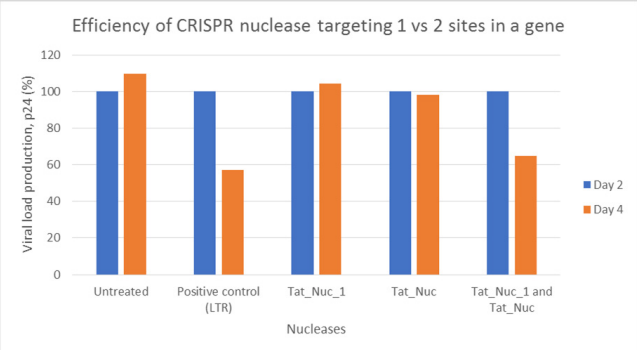

Figure 4: The percentage of viral production in latently infected cells treated with CRISPR/Cas9 nuclease on days 2 and 4. There was a decrease in viral load on day 4 in the positive control (LTR) and Tat_Nuc treated cells. There was a huge decrease when targeting two sites (Tat_Nuc_1 and Tat_Nuc) concurrently in a gene implying a synergistic effect.

Results showed a major viral production decrease when all the CRISPR nucleases were combined. On day 3, there was a 2.02-fold reduction (50.4% decrease) in viral load production and on day 5, there was a 4.06-fold reduction (75.36% decrease) in viral production when treated with the combined CRISPR nucleases. The decrease in viral production on day 5 seen with the combination of all CRISPR nucleases was comparable only to positive control (LTR) and Pol_Nuc at 3.94-fold reduction (74.64% decrease) and 3.73-fold reduction (73.19% decrease) respectively (Fig.5).

CRISPR nickase was also used to target two genes concurrently, Gag and Rev. For the combined CRISPR nickases, there was a 1.68-fold reduction (40.40% decrease) and a 2.01-fold reduction (50.13% decrease), which was similar to the efficiency of Rev_Nic on days 3 and 5. There was a constant increase in viral load in untreated cells for days 1, 3 and 5.

Based on the results of this research, it is clear that a combination of multiple CRISPR nucleases caused a synergistic effect as early as day 3, with the knockdown being about 1.45-fold more effective compared to just using a single nuclease. The significance of these results was more obvious on day 5 with a 2.29-fold increased efficacy seen in the combination nucleases.

DISCUSSION

CRISPR offers stable genetic manipulation attributes that have been successfully applied in different cell types. However, the simplicity of CRISPR/Cas9 led to speculations about its 'binding and cutting' mechanism specificity, as well as the safety of these genome editing tool. Using HIV as a model system, we explored three aspects related to the issue: a) The efficacy and safety of nickase compared to nuclease, b) The changes in

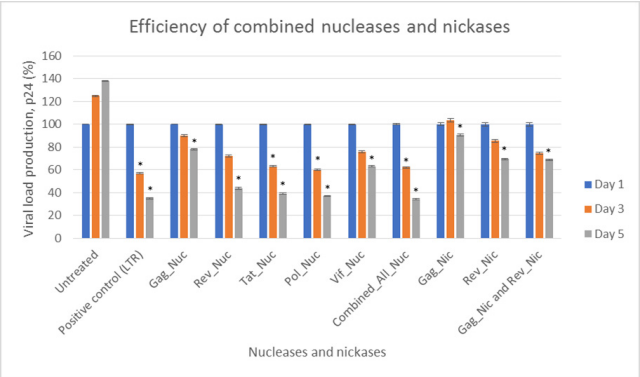

Figure 5: The percentage of viral production in latently infected cells treated with CRISPR/Cas9 nuclease and nickase on days 1, 3 and 5. There was an increase in viral load in untreated cells, and there was a significant decrease in viral load on day 5 in all CRISPR nuclease and nickase treated cells. The largest decrease in viral load was seen on day 5 when cells were treated with combined CRISPR nucleases, which targeted Gag, Rev, Tat, Pol and Vif (* = $p < 0.05$).

efficacy when targeting two sites within the same gene as opposed to one, and c) Obtaining optimal knockdown efficiency by targeting two genes simultaneously in comparison to single gene targeting.

From our studies, we discovered that there was no difference in the efficiency of single gene targeting using the nuclease and the nickase system, with both systems causing a significant decrease in viral production on day 5. However, the nickase system was more toxic to the cells with a significant decrease in cell numbers over time. Even though the nickase system is predicted to be safer due to nicking the DNA strand as opposed to creating a double stranded break, two CRISPR nickases need to be introduced to the cells each time for the gene of interest to be edited. This means that the chances of off-target may be multiplied by two, hence why there may have been cell death seen in the cells treated with CRISPR nickases, Gag_Nic and Rev_Nic. Despite its efficacy, since both nickases were toxic to the cells, we only proceeded with nucleases for further experimentation, as the nucleases were efficient and specific.

We also wanted to determine if there was a way to increase knockdown efficiency. We investigated if targeting two sites within the same gene gave a greater decrease in viral production compared to targeting one site. In the experiment, even though there was an increase in viral production on day 4 when just treated with Tat_Nuc_1, treating the cells with Tat_Nuc_1 and Tat_Nuc caused a huge decrease in viral load production, implying a synergistic and a more efficacious approach in knocking down genes when targeting two sites within a gene. There may be challenges when targeting one site as perhaps the targeted region may be heterochromatin, a part of the chromosome that is firmly packed and genetically inactive, making it not easily accessible to CRISPR (18). Therefore, targeting two sites within the same gene ensures that at least one of the CRISPR nucleases bind and cleave the region of interest.

Lastly, we examined if targeting multiple genes concurrently would provide the ideal knockdown efficiency, in comparison to targeting a single gene. Our findings showed that targeting five genes of HIV, namely Gag, Rev, Tat, Pol and Vif significantly improved knockdown efficacy. This suggests that when dealing when pathogenic organisms such as HIV, a multi-pronged approach such as knocking down the structural genes, the regulatory genes, and the accessory genes ensures that the virus is left helpless to fight back and unable to replicate. It is however interesting to note that the efficiency of the combinatorial knockdown was similar to that of our positive control (LTR) on both days 3 and 5.

This raises an interesting point that perhaps targeting the promoter of the gene of interest, in this case, LTR, is what gives the largest effect of knockdown. Due to the homologous sequences on both the 5′ and 3′ end of the HIV-1 genome, the CRISPR targeting LTR is capable of targeting two locations. This also means that rather than just introducing indels at the cleavage site, it might also be possible to cause a large deletion in the virus if the CRISPR acts at the 5′ and 3′ end concurrently, removing almost the entire genomic region, severely impairing the virus. We did not however perform sequencing for the entire genomic region of HIV, so we are unable to verify the specific molecular changes to the genomic sequence. In any case, modifications to the promoter do cause a huge change to the transcription level of the gene, so the largest decrease with a single target seen when targeting LTR, the promoter, is not surprising (15, 19, 20).

The knowledge gleaned from this last aspect of our experiment should be evaluated in a case-by-case basis. Targeting single genes of a pathogen might be risky if the targeting efficiency is not perfect, as the indels caused by CRISPR may prevent it from further recognizing the same target site after a few rounds of replication (21). Likewise, if the pathogen has a high mutation rate, or has a high error-prone replication mechanism (22), its fitness level may not be affected by the indels caused by a single CRISPR nuclease. In this case, a safer method of ensuring a successful knockdown would be to target multiple genes at various sites that may be important to the replication of the organism (19, 23).

While our CRISPR was designed based on a thorough in silico analysis, we plan to conduct a more thorough cytotoxicity assay in the future to determine the presence of off-targets, if any, when targeting two sites within a gene, and targeting multiple genes simultaneously. The entire HIV genome will be sequenced to determine the specific insertions and deletions that occurred. Somewhat similar human genome sequences to the CRISPR targets will also be sequenced to ensure safety of this tool. This is crucial knowledge prior to bringing this tool forward in the future.

Expanding from this study, when targeting an essential gene in humans, and not a pathogenic gene, knocking down one gene might be a better approach to control the downstream effects, and to ensure no toxicity (24, 25). Alternatively, CRISPR/Cas mediated base-editing can also be done, which enables bases to be edited without inducing a DNA break. In these cases, caution has to be taken to ensure that the mutation introduced is conservative, and does not impair the function of the essential gene. Bioinformatics analysis can be conducted to identify enhancer regions, or other interacting proteins to be targeted to not affect the essential gene.

CONCLUSION

From these experiments, we can conclude that the

CRISPR nuclease is a more suitable system for gene knock down by either targeting multiple sites within the same gene, or multiple genes simultaneously, as we saw a synergistic effect in both experiments. These encouraging results give us a glimpse of efficient methods that could be used in pathogens, as these results are not only applicable to HIV but to other organisms as well. In the future, we plan to investigate other CRISPR knockdown approaches for essential genes in humans, where there are reported cases of aberrant expression which has led to illnesses.

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