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Expression of Single mRNA Constructs Encoding Both CRISPR-CAS9 Protein and Guide RNAs for Future Gene Therapy Applications

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ABSTRACT: The basis of many life-threatening diseases is disruption in key genes. In many cases, repairing these disruptions can prevent or reverse disease. The development of CRISPR-Cas9 technology, which consists of Cas9 nuclease directed to specific genomic locations by guide RNA (gRNA), has significantly progressed in the past decade and has shown signs of promise for treating diseases such as Alzheimer's and cystic fibrosis. One integral issue of gene editing therapy is the method and effectiveness of delivery. Current approaches such as lentiviral and adeno-associated virus vectors suffer from either stable, constant expression of CRISPR components that causes unintended gene editing or an inability to efficiently carry large cargoes such as two independent genes: Cas9 and guide RNA. To begin to bypass these cargo limitations, we created a CRISPR-Cas9 mRNA structure that encompasses all of the necessary components for gene editing on a single RNA. These constructs consist of a promoter, followed by a Cas9 open reading frame, a triplex region from *MALAT1* that protects the Cas9 open reading frame, and then either 1, 2, or 4 gRNAs that target specific reporters, with each gRNA between two self-cleaving ribozyme sequences. These constructs successfully drove Cas9 editing of two distinct reporters in human cells and thus open the door for many more experiments such as incorporation into various delivery constructs to further develop this technology for gene editing therapy.

INTRODUCTION

CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-CRISPR associated protein 9) gene editing technology has been shown to be a promising solution to genetic diseases. Cas9 proteins are naturally found in *Streptococcus thermophilus* as a defense system against invading viruses, but now scientists use this system to perform gene editing in many organisms.[1] In the CRISPR system, guide RNAs bind to a specific region of DNA as well as Cas9 nuclease, guiding Cas9 to the targeted DNA region to cause double-stranded breaks in an organism's genome. The cell's own non-homologous end joining DNA repair system is used to repair the break made by Cas9 which induces changes to targeted sequences in the genome (Figure 1).[2] This system has been extensively researched and expanded upon for the past decade, and more systems have been developed that use Cas9 as well such as base and prime editors. In these systems, various proteins have been fused to nuclease-deficient Cas9 to modify the genome in several different ways.

The advent of base editing technology grants the ability to perform specific types of point mutations to nucleotides. The first effective iteration of this technology, a cytosine base editor, arose in 2016 where Komor et al. was able to fuse rat derived deaminase protein to the amino terminal end of an inactive Cas9 protein.[3] Instead of causing double stranded breaks, this editor is able to deaminate cytosine to uracil. Since DNA replication machinery does not recognize uracil, the replication results in a C-G to T-A mutation.[4] Through further research, this editing system was able to become more specific and effective. An adenine base editor was also developed using a similar method to the cytosine base editor.[4] Like Cas9, cytosine and adenine base editors can cause targeted mutations, however these base editors are more specific due to the ability to specify the point mutation. In a gene therapy context, adenine base editors are being used to mutate premature stop codons, which are thought to cause faulty gene expression in up to a third of genetic diseases.[5]

Despite the incredible capabilities of base editors, they are unable to perform more than specific point mutations. However, another Cas9 related system, prime editors, can perform any type of mutations that can be encoded in a gRNA.[4] Simply speaking, these systems are created by fusing a reverse transcriptase to an inactive Cas9 protein. A prime editing guide RNA then directs targeted transition and transversion mutations.[4] Although there is great potential for this system, it still requires much more research to improve its efficacy and specificity. Other applications include gRNA targeted modulation of transcription or epigenetic marks by fusing transcriptional or epigenetic regulators to inactive Cas9.[6] These methods can be used to up or downregulate genes like those related to cancer, therefore showing a lot of promise for therapeutic uses.[4]

In several mouse models, researchers have been able to reduce the severity of

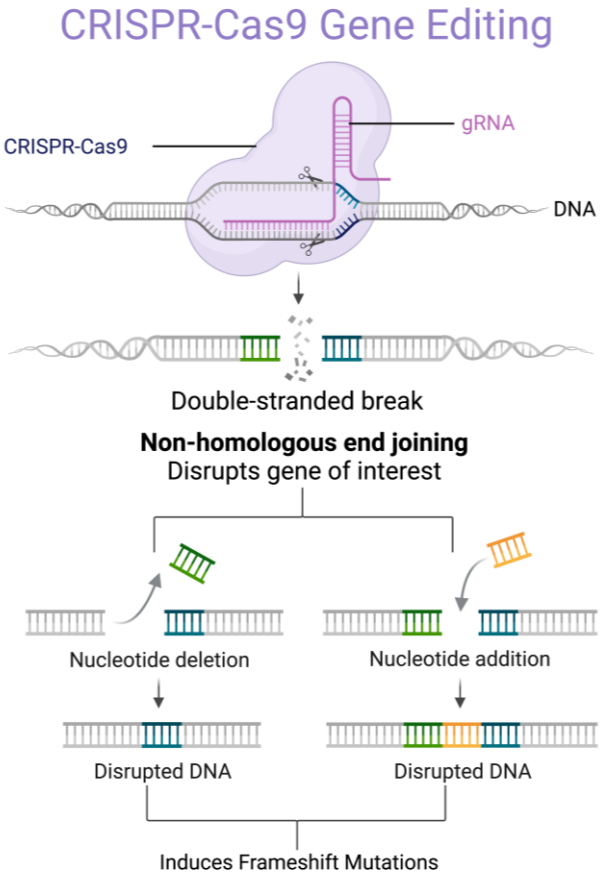

Figure 1: Illustration of how CRISPR-Cas9 performs gene editing.

neurodegenerative diseases by editing genes with CRISPR.[7] Recent studies have demonstrated Cas9 to be promising in decreasing the effects of Alzheimer's and Parkinson's disease through the mutation of the *APP* and *LRRK2* gene respectively.[7] A study from Yang et al. has shown that CRISPR technology can be used to ameliorate the symptoms of Huntington's disease in mouse models through mutations in the *HTT* gene.[8] In addition, clinical trials for CRISPR related therapies have already begun to treat cystic fibrosis by mutating the *CFTR* gene.[9] As a result, CRISPR, while still being heavily researched and developed, shows extremely promising capabilities for mitigating genetic diseases.

CRISPR-Cas9 Delivery

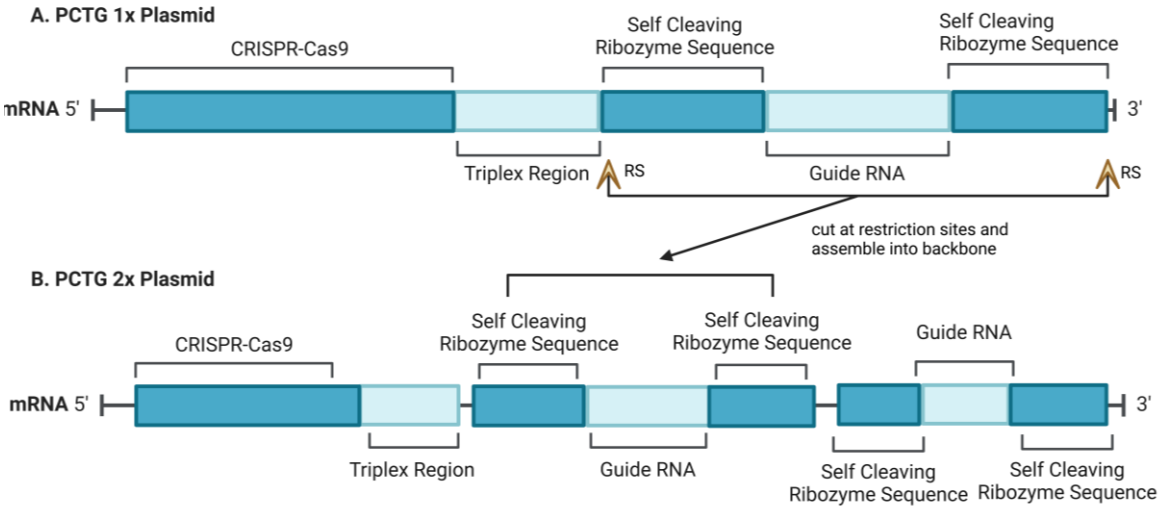

Figure 2: Sequence of PCTG plasmids involved with Cas9 gene editing. 1x refers to one copy of the gRNA between two self-cleaving ribozyme sequences. The PCTG 2x plasmids have two ribozyme + gRNA + ribozyme sequences subsequently and the PCTG 4x (not shown) plasmid has four.

One major hurdle to fully realizing the potential of Cas9-based approaches is delivery to tissues and cells. There are two primary ways currently used to deliver Cas9 and gRNA: viral and non-viral delivery. With viral delivery, there are methods that use adeno-associated viruses (AAVs) or lentiviral vectors to encapsulate both Cas9 and gRNA genetic information and deliver it to cells through a mechanism similar to viral infection. On the other hand, non-viral methods utilize other macromolecules like lipid nanoparticles (LNPs), protein polymers, and Au nanoparticles to deliver gRNA and Cas9 mRNA, protein, or DNA.[10] Delivering the protein form of CRISPR-Cas9 along with gRNA is efficient and doesn't result in much off-target editing, however it is very expensive and there is risk of endotoxin contamination.[10] Cas9 mRNA + gRNA delivery has similar

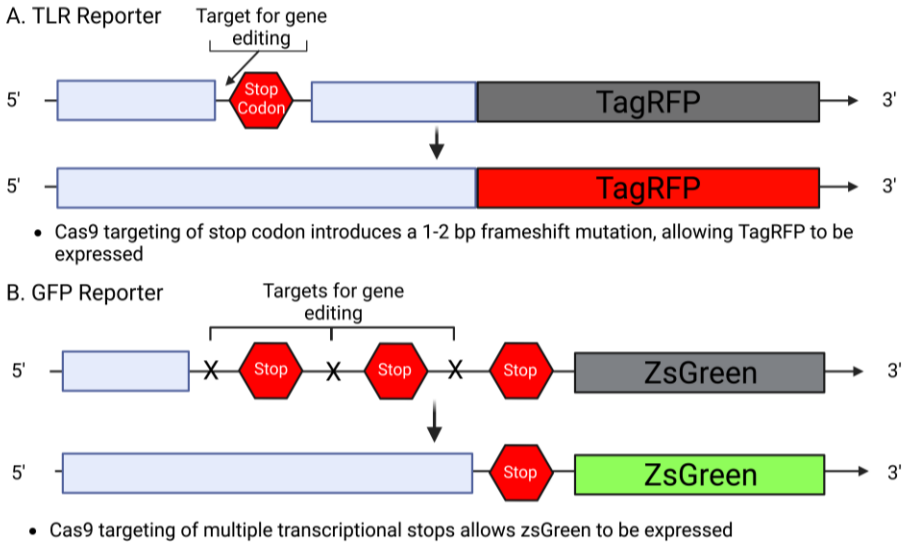

Figure 3: Schematic of RFP and GFP Reporters Used. Gene editing of TLR reporter causes a frameshift which makes RFP. GFP reporter is activated through editing multiple sites to delete transcriptional stops.

benefits to the protein form; however, it requires two components to get into particles and cells.[10]

Alternatively, Cas9 delivery can also be performed through complexing and conjugating with proteins. Ramakrishna et al. were able to design a cell-penetrating protein (CPP) that was conjugated to Cas9 and combined with a CPP complexed to a gRNA, resulting in efficient endogenous gene editing.[11] This technology has been proven effective in HEK293T cells, embryonic stem cells, embryonic carcinoma cells, and dermal fibroblasts.[11] Additionally, this system was able to produce less off-target editing events, however it is not perfect and it is difficult *in vivo* without a protective lipid nanoparticle.[11]

While there have been studies showing effective delivery of CRISPR-Cas9 into tissues using lentiviral and adeno-associated virus vectors, these methods of long-term expression can be problematic if used as a therapeutic[12,13]. For example, lentiviruses involve integration into the genome and potentially permanent, constant expression of Cas9 along with a gRNA[14]. Despite the specificity of Cas9, the technology is not guaranteed to always edit targeted gene sequences, and this can potentially lead to malignant or deleterious mutations.

In many of the previous methods, the size of cargo is also a significant barrier for the efficient delivery of gene editing technology. For instance, AAV packaging limits require separate viruses to be made for Cas9 and gRNA. Lentiviral packaging limitations lead to reduced efficacy when carrying large Cas9 and gRNA genes together. Several non-viral approaches depend on two different components (Cas9 and gRNA) to get into the same particles and cells. Therefore, to increase the efficiency of delivery, we engineered both the gRNA and Cas9 segments to be expressed from a single piece of RNA. With this construct design, cargo size would be significantly reduced and in the case of RNA delivery methods, two molecules reduced to one. This configuration has the potential to open the door for more efficient and a greater variety of delivery methods.

METHODS

Plasmids

pCMV-VSV-G was a gift from Bob Weinberg (Addgene plasmid # 8454 ; http://n2t.net/addgene:8454 ; RRID:Addgene_8454).[15] lentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid # 52961 ; http://n2t.net/addgene:52961 ; RRID:Addgene_52961).[16] pAAVS1-TLR targeting vector was a gift from Ralf Kuehn (Addgene plasmid # 64215 ; http://n2t.net/addgene:64215 ; RRID:Addgene_64215).[17] pCAG-loxP-STOPloxP-ZsGreen was a gift from Pawel Pelczar (Addgene plasmid # 51269 ; http://n2t.net/addgene:51269 ; RRID:Addgene_51269).[18]

Cloning of constructs encoding Cas9 and gRNA on a single RNA

A pCMV-Cas9 plasmid was cloned by replacing the VSV-G open reading frame in pCMV-VSV-G with Cas9 open reading frame from lentiCRISPR v2 using PCR followed by HiFi assembly (New England Biolabs). To clone in gRNAs, pCMV-Cas9 was linearized downstream of the Cas9 stop codon using StuI, then *MALAT1* Triplex (from Campa et al.)[19] + hammerhead (HH) ribozyme DNA and guide RNA sequence + hepatitis delta virus (HDV) ribozyme DNA were ordered from Integrated DNA Technologies as gBlocks with 20 bp overhangs between all three DNA fragments for HiFi assembly. XmaI, NheI, and HindIII sites as well as homologous regions for HiFi assembly were created in these DNAs in order to allow for expansion of the gRNA cassette according to the approach of Spakman et al. (Figure 2).[20]

The result of these steps was pCMV-Cas9-*MALAT1* Triplex-HH ribozyme-gRNA-HDV ribozyme, or PCTG-1x, where the 1x

denotes a single gRNA. In the text, gRNA targeting is added to the name: PCTG-TLR-1x encodes gRNA targeting traffic light reporter (GGTAGCGGGCGAAGCACTGC) and PCTG-GFP-1x encodes gRNA targeting GFP reporter (sgTOM from Wei et al.).[20,21]

To create 2x and 4x gRNA constructs, we used the approach of Spakman et al. 2020 but with different restriction enzymes. Briefly, 1x constructs were linearized with NheI and separately the entire HH ribozyme-gRNA-HDV ribozyme region was cut out with XmaI and HindIII then gel purified. This creates overhangs that allow the entire HH ribozyme-gRNA-HDV ribozyme region to be cloned upstream of the other copy of the gRNA cassette with HiFi assembly to create 2x gRNA constructs. The same expansion was repeated starting with the 2x construct to get the 4X construct encoding 4 identical gRNAs. Plasmid sequences were confirmed by Sanger sequencing.

Transfection of PCTG-TLR and PCTG-GFP plasmids

The day before transfection, HEK293T cells were seeded in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum at roughly 25% confluency in 6-well plates and incubated overnight at 37°C and 5% CO_2. The next day, PCTG-TLR 1x, 2x, 4x, or PCTG-GFP 1x and 2x were transfected into HEK293T cells with either pAAVS1-TLR or pCAG-loxP-STOPloxP-ZsGreen using JetOptimus transfection reagent. Plates were then left to incubate for 48 hours at 37°C and 5% CO_2.

Imaging of all constructed plasmids for gene editing activity

All samples were imaged after 48 hours incubation using the Lionheart FX automated microscope with the 4X objective. The plates transfected with pAAVS1-TLR reporter were imaged for RFP and the plates that received pCAG-loxPSTOPloxP-ZsGreen reporter were imaged for GFP. Gen5

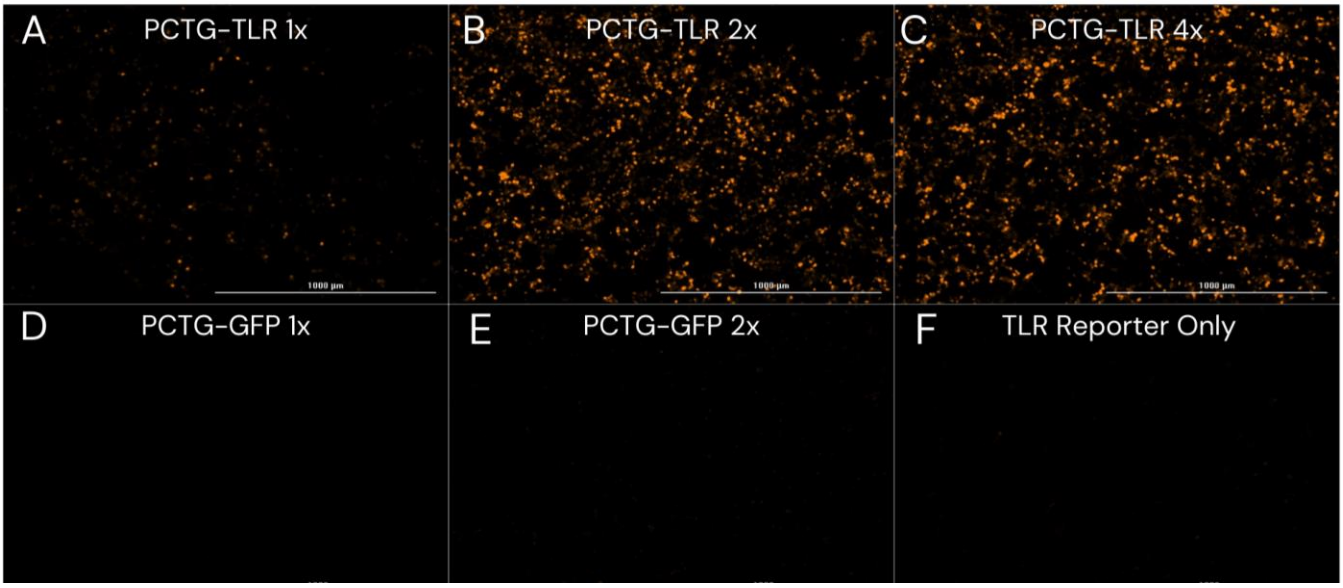

Figure 4: PCTG-TLR constructs efficiently edit TLR reporter. HEK293T cells were transfected with indicated Cas9-gRNA plasmid and traffic light reporter plasmid, then imaged for TagRFP after 48 hours. Fluorescence imaging was done with the Lionheart FX automated microscope using the 4X objective. Each red particle shown represents one cell expressing TLR. Each panel represents 1 of 16 fields imaged. Brightfield images were taken of all wells and showed similar cellular confluency (data not shown).

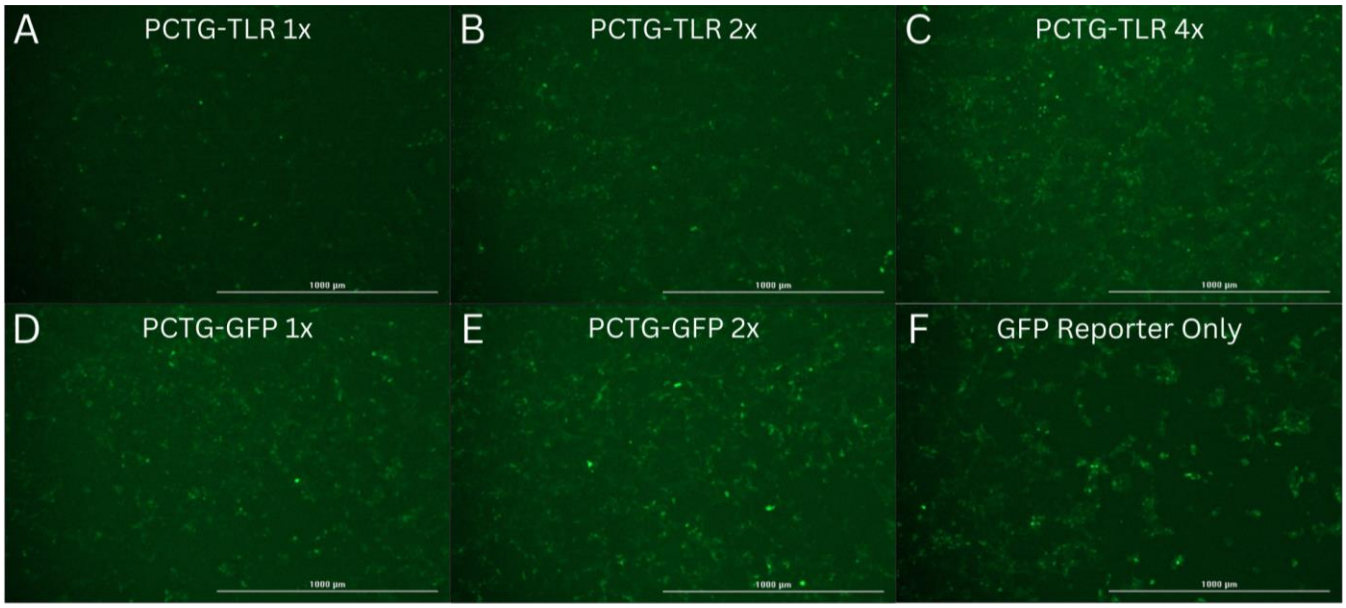

Figure 5: PCTG-GFP-2X construct edits GFP reporter. HEK293T cells were transfected with indicated Cas9-gRNA plasmid and green fluorescent protein reporter plasmid, then imaged for GFP after 48 hours. Fluorescence imaging was done with the Lionheart FX automated microscope using the 4X objective. Each bright green particle shown represents one cell expressing GFP. Each panel represents 1 of 16 fields imaged. Brightfield images were taken of all wells and showed similar cellular confluency (data not shown).

software was used to count RFP+ or GFP+ cells as well as calculate intensity of expression per cell. After imaging, a two-tailed, two sample equal variance, statistical t-test was performed to determine significance of RFP or GFP expression (n=2). Figures 1, 2, and 3 were made with BioRender software.

RESULTS

In order to test Cas9 and gRNA single RNA configurations, we created constructs that contain Cas9 open reading frame followed by an RNA region that forms a protective triplex.[22] This sequence is then followed by gRNA sequence sandwiched between self-cleaving ribozymes (Figure 2). In the cell, these self-cleaving ribozymes cleave out gRNA so that it can bind to Cas9 and the targeted DNA. The triplex is needed because these ribozyme cleavages would expose the 3' end of the Cas9 open reading frame to nucleases in the absence of the triplex.[22] We created versions expressing 1, 2, or 4 gRNAs that target traffic light reporter (TLR), or 1 or 2 gRNAs targeting the STOP region of pCAG-loxPSTOPloxP-ZsGreen (Figure 3). These reporters were targeted because both are well characterized and are also integrated into mouse lines, and thus are useful gRNAs for future *in vivo* experiments.[23]

To test targeted editing of TLR, we transfected each of these constructs into HEK293T cells along with traffic light reporter (TLR). Traffic light reporter encodes an upstream stop codon as well as an out-of-frame TagRFP. Upon Cas9 editing upstream of the early stop codon, a portion of editing events will cause the upstream stop codon to go out-of-frame and bring TagRFP in frame, resulting in RFP expression (Figure 3). Cells expressing TLR gRNAs exhibit RFP expression, which indicates successful gene editing by Cas9 that targeted the RFP reporter (Figures 4A-C). The quantification of RFP+ cells also demonstrate a significant number of cells underwent gene editing (Figure 6A). When comparing the difference between 1x, 2x, and 4x TLR gRNAs, there is a significant increase in

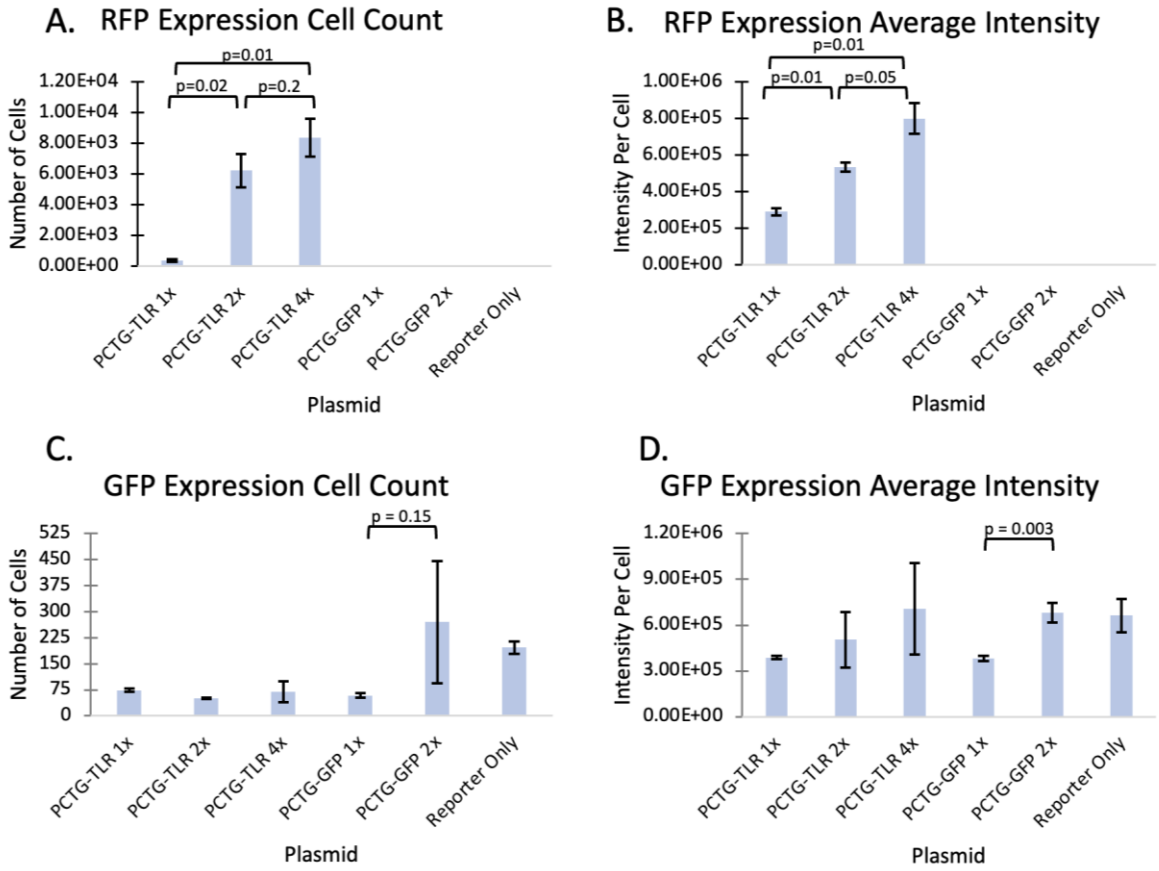

Figure 6: Quantitative analysis of imaging from figures 4 and 5. Corresponds with Figure 4: TagRFP+ cell counts (A) obtained over 16 images for each sample were obtained using Gen5 software. Average RFP intensity per cell (B) was also obtained using Gen5 software. Corresponds with Figure 5: GFP+ cell counts (C) obtained over 16 images for each sample were obtained using Gen5 software. Average GFP intensity per cell (D) was also obtained using Gen5 software. These data are the result of two independent experiments (n=2).

the number of cells that displayed gene editing (Figure 6A). Additionally, the magnitude of RFP expression per cell increased corresponding to the number of guide RNA sequences, meaning that 1x TLR gRNA showed the lowest magnitude, followed by 2x, and finally 4x demonstrated the largest magnitude of fluorescence (Figures 4A-C and 6B). Importantly, cells not expressing gRNA (Figure 4F) or expressing constructs encoding GFP targeting gRNA (Figure 4D-E) do not result in RFP expression, indicating that editing of TLR is selective.

Similarly, constructs expressing Cas9 and gRNAs targeted to the STOP region of pCAG-loxPSTOPloxP-ZsGreen were tested and demonstrated selective gene editing as well. pCAG-loxPSTOPloxP-ZsGreen encodes a ZsGreen reporter downstream of 3 successive transcription termination sites. Normally ZsGreen expression would be low but targeting Cas9 to regions between transcription termination sites with gRNA results in removal of termination sites and ZsGreen expression (Figure 3). Cells expressing Cas9 and TLR gRNAs along with reporter exhibited some GFP (Figures 5A–C), but co-expression of Cas9 and 2 GFP gRNAs resulted in increased GFP expression (Figures 5E and 6C-D).

When quantified, there was no statistical significance between the GFP reporter only negative control (Figure 5F) and the PCTG-GFP 2x version of the plasmid with

regards to both number of cells that express GFP and the magnitude of expression/gene editing (Figure 6C-D). This reporter has a higher background signal in the absence of editing as well as a higher threshold for signal (multiple Cas9 have to hit the same reporter DNA). In addition, the reporter only control is a transfection with only one plasmid and neither Cas9 nor gRNA, meaning that the reporter only control is not the best direct comparison. However, there was a significant increase in both number of GFP+ cells, as well as GFP intensity per cell when comparing PCTG-GFP 2x to PCTG-GFP 1x or any of the PCTG-TLR constructs (Figure 6C-D) indicating some level of selective gene editing. For future research, it will be valuable to create a PCTG-GFP 4x plasmid and assess whether or not it demonstrates an increase in gene editing activity similar to the PCTG-TLR plasmids.

DISCUSSION

The single RNA encoding Cas9 + guide RNA constructs were successful in performing gene editing on two different reporters. The plasmids containing TLR guide RNA were able to produce significant gene editing only on TLR and the duplication of guide RNA sequences was shown to improve not only the number of cells that had gene editing but also increased the magnitude of gene editing per cell as well.

With regards to the GFP guide RNA containing plasmid, there was a significant increase in the number of cells with gene editing between the 2x version of PCTG-GFP when compared to the 1x version or any of the PCTG-TLR versions, demonstrating the increase in number of gRNAs is also able to increase the level of selective gene editing in cells. While this result was clearly weaker in magnitude than the TLR system, there are some limitations to using this GFP reporter that likely account for this. First, there are nonzero amounts of background green fluorescence for negative controls (Figure 5). This higher background is likely worse in the reporter only control in which only one plasmid is being transfected as opposed to all other samples which get two plasmids. In addition, the GFP system requires multiple editing events per cell to get GFP (Figure 3), making the signal weaker than the single editing event required for the TLR system.

An article written by McCarty et al. reviews construct designs somewhat similar to ours, including one encoding Cas12a.[19,24] We selected Cas9 for our configuration due to the wealth of knowledge about this system and Cas9's high activity relative to other Cas proteins. In addition, the selected self-cleaving ribozymes that process gRNAs are well-characterized and regulated cleavage can be engineered.[25,26] Since we found that this kind of construct design works, we can potentially also test other designs, such as one where different gRNA sequences are encoded on one RNA strand which would allow for simultaneous gene editing at multiple gene loci.[24] This kind of structure could be promising for gene therapy in the future and is worth considering.

CONCLUSION

These results are significant because after demonstrating that these Cas9/gRNA single RNA constructs function in HEK293T cells, future experiments can now be designed to test whether this configuration improves delivery of Cas9/gRNA using systems that have been shown to be limited by cargo size like AAVs, lentiviruses, and mRNA delivered by nano-lipid particles. AAVs are known to have limited cargo capacity despite being very effective for delivery[27]. Combining our configuration with smaller promoters, more compact Cas enzymes, and regulatable ribozymes may even allow a single AAV or more efficient lentiviruses to be produced.[28,29]

With regards to nonviral delivery, lipid nanoparticles are already known to produce efficient delivery of cargo, as well as target

specific cells. While nonviral delivery systems are generally able to hold larger cargos, it is still valuable to test this single RNA construct's effectiveness in lipid nanoparticles and whether it demonstrates improvement in gene editing frequency and magnitude since current methods often rely on getting two separate molecules, gRNA and Cas9, into particles.

In the future, these delivery approaches could be directly used in animal models too as the guide RNAs we have used in this study target commercially available TLR and Ai9 mice. Beyond this, testing for effective modification of disease associated genes using other gRNAs would provide valuable insight for the prospect of using our single RNA structure for gene therapy applications. In short, this technology has the potential to improve both existing and emerging Cas9/gRNA delivery methods.

AUTHOR INFORMATION

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Author Contributions

Elvis Lang conceived experiments, performed the cloning, and performed data analysis. He also wrote the manuscript and generated all the figures used. Dr. John Tilton provided direction and guidance during this project. Dr. Thomas Sweet also provided direction and including with the manuscript, conceiving experiments, and also did some of the plasmid design, cell culture work, and imaging for this project.

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ABBREVIATIONS

CRISPR: clustered regularly interspaced short palindromic repeats
Cas9: CRISPR associated protein 9
gRNA: guide RNA
AAV: adeno associated virus
RNP: ribonucleoprotein complex
LNP: lipid nanoparticles
CPP: cell penetrating protein
HEK293T: human embryonic kidney 293T
TLR: traffic light reporter
RFP: red fluoresce ent protein
GFP: green fluorescent protein
PCTG: pCMV-triplex-gRNA
CMV: cytomegalovirus

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