Journal of High School Science

Disrupting *PCSK9* and *ANGPTL3* genes with CRISPR gene editing technology for Hypercholesterolemia treatment

Xu J[1,5], Zou A[2,5], Zhang X[3], Liu X[4]

Submitted: May 31, 2025, Revised: version 1, June 30, 2025
Accepted: July 2, 2025

Abstract
Homozygous familial hypercholesterolemia (HoFH) is a rare and severe condition resulting from genetic mutations and characterized by extremely high levels of low-density lipoprotein (LDL) cholesterol, thus increasing the risk of heart disease. Gene therapy provides the potential for lifelong therapeutic benefits with a single treatment. CRISPR/Cas9 systems based on *Streptococcus pyogenes* Cas9 (SpCas9) have been employed to disrupt the *PCSK9* or *ANGPTL3* genes, reducing circulating LDL cholesterol through LDL receptor (LDLR)-dependent and -independent mechanisms, respectively. However, the use of SpCas9 for multiplex gene editing remains largely limited in preclinical research due to suboptimal editing efficiency and delivery constraints. Recent studies suggest that *Staphylococcus aureus* Cas9 (SaCas9), a smaller ortholog with higher editing efficiency, may overcome these limitations. To explore a dual-pathway gene therapy strategy for HoFH, we hypothesized that CRISPR/SaCas9 could simultaneously disrupt *ANGPTL3* and *PCSK9* genes in mammalian cells. Using mouse N2a cells as a model, we first identified a highly efficient novel *ANGPTL3* guide RNA; AB514. By delivering AB514 and a validated PCSK9 guide RNA with SaCas9 into N2a cells, we found that *ANGPTL3* and *PCSK9* genes could be edited simultaneously. Our findings demonstrated that SaCas9 mediated multiple gene editing in mammalian cells is feasible and the dual gene editing approach targeting *PCSK9* and *ANGPTL3* genes may represent a promising strategy for future HoFH therapy.

Keywords
Hypercholesterolemia, LDL cholesterol, CRISPR, *PCSK9*, *ANGPTL3*, Gene therapy, Dual-gene editing, Cas9, Multiplex gene editing

[1]Jenny Xu, Fairview High School, 1515 Greenbriar Blvd, Boulder, CO 80305, USA. jzxu01@bvsd.org
[2]Amanda Zou, Peak to Peak Charter School, 800 Merlin Dr, Lafayette, CO 80026, USA. amanda.r.zou@gmail.com
[3]Corresponding author: Xiaojuan Zhang, Department of Biochemistry, University of Colorado, 3415 Colorado Avenue C318, Boulder, CO 80303, USA. xiaojuan.zhang@colorado.edu
[4]Corresponding author: Xuedong Liu, Department of Biochemistry, University of Colorado, 596 UCB, Boulder, CO 80309, USA. xuedong.liu@colorado.edu
[5]These authors contributed equally to this work.

https://doi.org/10.64336/001c.141775

Introduction

Homozygous familial hypercholesterolemia (HoFH) is a rare, severe condition resulting from genetic mutations characterized by extremely high levels of low-density lipoprotein (LDL) cholesterol (LDL-C, a form of cholesterol transport protein that can cause plaque to build up in arteries and can cause heart disease and strokes) and early-onset atherosclerotic cardiovascular disease (ASCVD) (1). The condition affects ~ 1 in 250,000 individuals globally (2). Without treatment, HoFH can cause serious heart problems such as coronary artery disease and early death (2). On average, people with untreated HoFH usually live to ~ 18 years old (3). Most cases of HoFH are caused by mutations in both alleles of the gene encoding the LDL receptor (LDLR) (3). In addition, pathogenic variants in other genes, such as apolipoprotein B100 (APOB), proprotein convertase subtilisin/kexin 9 (PCSK9), and LDLR adapter protein 1 (LDLRAP1), which lead to autosomal recessive hypercholesterolemia, can also produce the HoFH phenotype (3,4). The LDL-C level can be modulated by LDLR-dependent and -independent pathways (5). Proprotein convertase subtilisin/kexin type 9 (PCSK9) is an LDLR negative regulator that can bind and direct LDLR to lysosomes for degradation; angiopoietin-like-3 protein (ANGPTL3) is a well-studied key player of the LDLR-independent pathway that can prevent disassembly of LDL cholesterol by inhibiting the activity of key lipases (6-8).

Early diagnosis and treatment are crucial to avoid medical emergencies, with genetic testing aiding in confirmation. The main LDL-C lowering treatments for early diagnosed HoFH patients include lifestyle changes and lipid-lowering therapies such as ezetimibe, statins, and PCSK9 inhibitors (9). However, due to the absence or impaired LDL-receptor activity, most individuals with HoFH show limited response to conventional LDL-C lowering agents that act by upregulating LDL-receptors to increase LDL-C clearance (10). Even for patients who partially respond to LDL-C treatments, lifelong medication remains a significant challenge. Cholesterol-lowering therapies that do not depend on LDL receptor expression are urgently needed for HoFH patients.

Gene editing technologies have rapidly advanced in the last decade. Casgevy® is the first FDA-approved CRISPR/Cas9-based gene therapy for sickle cell disease, highlighting the potential of this technology for treating other genetic disorders (11). Recent studies demonstrate the potential of CRISPR-based gene editing for treating hypercholesterolemia and related conditions, particularly HoFH. For instance, lipid nanoparticles containing CRISPR base editor targeted to *PCSK9* were injected into cynomolgus monkeys, resulting in significant reductions of LDL-C (60%) and PCSK9 (90%), with near-complete clearance of PCSK9 from the liver, sustained for eight months after a single dose (6). CRISPR/Cas9 was also used to knock down *ANGPTL3* in mice, and a single dose treatment was found to cause dose-dependent reductions in ANGPTL3, LDL-cholesterol, and triglycerides for more than 100 days (12). Despite these promising results, these studies targeted only one gene, even though LDL cholesterol levels are regulated in both LDLR-dependent and

independent pathways. Gene editing efficiency is a major challenge that hinders the development of multiplex gene editing. SpCas9 from *Streptococcus pyogenes*, as the earliest developed gene editing tool, has been applied extensively, including gene editing of *PCSK9* and *ANGPTL3* mentioned above. Recent studies show that SaCas9 from *Staphylococcus aureus* outperforms SpCas9 in both editing efficiency and specificity (13,14), highlighting the feasibility of the application of CRISPR/SaCas9 to target multiple genes for genetic conditions, such as HoFH.

The discovery that CRISPR systems in bacteria and archaea can target multiple phage genes has prompted several studies exploring the potential of multiplex gene editing in animals (15,16). For instance, Wang et al. demonstrated that CRISPR/Cas-mediated editing could efficiently disrupt five genes simultaneously in mouse embryonic stem cells (15). Similarly, another study in zebrafish successfully targeted five distinct genomic loci in a single experiment, resulting in multiple loss-of-function phenotypes in the same injected fish (16). Based on these studies, we postulate that multiple gene editing by CRISPR/SaCas9 is feasible in mammalian cells, since it works in bacteria.

In this study, we first performed sgRNA screening for SaCas9-mediated *ANGPTL3* gene editing and identified five sgRNAs that could potentially edit the mouse *ANGPTL3* gene. To achieve higher efficiency in lowering LDL cholesterol in future experiments, we developed a dual-gene strategy that could disrupt *PCSK9* and *ANGPTL3* genes simultaneously. Our results demonstrated that dual-gene editing was feasible in mouse N2a cells and dual-gene editing of *PCSK9* and *ANGPTL3* could be developed as a potential treatment strategy for HoFH.

Methods

Cell culture

Mouse neuroblast N2a cells (CCL-131™) and Mouse Embryonic Fibroblast MEF cells (SCRC-1008™) were purchased from American Type Culture Collection (ATCC). Both cell lines were cultured in DMEM complete media supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 mg/L streptomycin. Cells were maintained at 37°C in a humidified incubator with 5% CO_2.

Transient transfection

Transient transfection of plasmids was performed using the Lipofectamine™ 3000 Transfection Reagent (Cat# L3000015, Thermo Fisher Scientific) according to the manufacturer's instructions. To determine the optimal cell lines for efficient transfection, $1x10^5$ cells in 2 ml of DMEM complete media were seeded in a 6-well plate overnight. Then, 1 μg of reporter plasmid pEXL-GFP was diluted in 125 μL of Opti-MEM™ Reduced Serum Medium in a 1.5 mL microcentrifuge tube, followed by the addition of 4 μL of P3000™ reagent. In a separate tube, 4 μL of Lipofectamine™ 3000 reagent was diluted in 125 μL of Opti-MEM™. The plasmid DNA–lipid complexes were then prepared by adding the diluted DNA solution to the diluted Lipofectamine™ 3000 reagent. The mixture was gently mixed by tapping the bottom of the tube and incubated at room temperature for 10 minutes before being added to the cells.

For gene editing experiments, 1 µg of SaCas9 plasmid (pBbsr-SaCas9) and 1 µg of sgRNA plasmid pBbsr-gRNA were diluted in 125 µL of Opti-MEM™ and processed as described above. For dual-gene editing, 1 µg of pBbsr-SaCas9, plus 1 µg of *ANGPTL3* sgRNA and 1 µg of gPCSK9 plasmid, were diluted in 125 µL of Opti-MEM™. The subsequent steps, including lipofectamine™ 3000 preparation, were conducted in a similar way as described earlier. Transfected cells were cultured at 37°C for 72 hours before harvesting for genomic DNA extraction.

DNA extraction

Genomic DNA was extracted from treated N2a or MEF cells using the QIAGEN DNeasy Blood and Tissue Kit (Cat# 69504, QIAGEN) following the manufacturer's instructions. Cells from the well of a 6-well plate were resuspended in 200 µL PBS using a sterile scraper. Cell suspensions were then transferred to 1.5 mL microcentrifuge tubes. 20 µL Proteinase K and 200 µL Buffer AL were added to the cell suspension, followed by vortexing and incubation at 56°C for 10 minutes. After incubation, 200 µL of 100% ethanol was added, and the mixture was vortexed. Lysates were loaded onto DNeasy Mini spin columns in 2 mL collection tubes and centrifuged at 6021×g for 1 minute. After discarding the flow-through, columns were washed with 500 µL Buffer AW1 (6021×g, 1 minute), then 500 µL Buffer AW2 (18440×g, 3 minutes). Finally, DNA was eluted by adding 200 µL Buffer AE to the columns, incubating for 1 minute at room temperature, and centrifuging at 6021×g for 1 minute. The concentration of eluted DNA was measured using a NanoDrop Spectrophotometer (DeNovix DS11) before being applied for subsequent agarose gel electrophoresis and PCR amplification. The OD 260/280 ratio was > 1.9 for all the samples, generally considered indicative of pure samples.

PCR amplification and product purification

Polymerase chain reactions (PCR) were performed using GoTaq® Master Mix (Cat# M7122, Promega), which contains all components except the DNA template and primers. Each 50 µL reaction includes 20 µL distilled water, 1 µL of 100 ng genomic DNA, 4 µL primer mix (10 µM forward and 10 µM reverse primers), and 25 µL 2×GoTaq® Master Mix. PCR amplification was carried out in a thermocycler (Bio-Rad, T100) under the following conditions: initial denaturation at 95°C for 3 minutes; 34 cycles of denaturation at 95°C for 30 seconds, annealing at 59°C for 30 seconds, and extension at 72°C for 1 minute; followed by a final extension at 72°C for 5 minutes. Samples were held at 12°C until further processing.

The resulting PCR products were then purified using the QIAquick PCR Purification Kit (Cat# 28104, QIAGEN) following the manufacturer's protocol. Briefly, 45 µL of PCR product was transferred to a microcentrifuge tube, mixed with 135 µL Buffer QG and 45 µL isopropanol, and vortexed thoroughly. The mixture was applied to a QIAquick spin column placed in a 2 mL collection tube and centrifuged at 13,548×g for 1 minute to bind DNA. The flow-through was discarded, and the column was returned to the same collection tube. The column was washed twice with Buffer PE: first with 750 µL and then with 500 µL, centrifuging each time at 13,548×g for 1

minute. After discarding the flow-through, the column was centrifuged again at 13,548×g for 1 minute to remove residual wash buffer. The spin column was transferred to a clean 1.5 mL microcentrifuge tube, and DNA was eluted by adding 50 µL Buffer EB, incubating at room temperature for 5 minutes, and centrifuging at 13,548×g for 1 minute. The purified PCR product was submitted for Sanger sequencing.

DNA sequencing and data analysis

Sanger Sequencing analysis was used to check if CRISPR gene editing was successful. Purified PCR products were sent to a company serving Sanger Sequencing (Quintara Biosciences) and sequencing results were submitted to SeqScreener Gene Edit Confirmation Analysis (online tool, Thermo Fisher Scientific) to analyze editing efficiency. Statistical analyses of data was performed with GraphPad Prism 9.5.

Results

N2a is a suitable cell line for the gene editing analysis of mouse PCSK9 and ANGPTL3 genes

The application of gene editing analysis in mouse cell lines allowed to validate the gene editing efficacy and directly evaluate the LDL-lowering effect in mice.

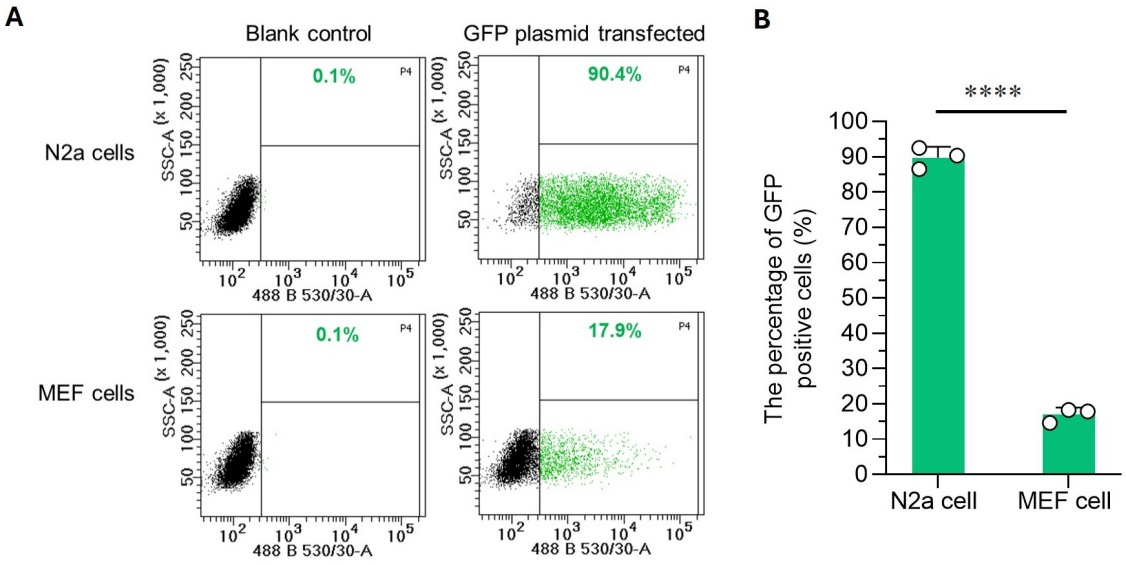


Figure 1. Detection of the efficiency of transient transfection by flow cytometry. **A.** 1x10^5 of N2a or MEF cells were transfected with 1 µg of pEXL-GFP reporter plasmid, and the gene transfer ratios were determined by flow cytometry. **B.** Transfection efficiency comparison of N2a and MEF cells (Student t-test, n=3, **** $p < 0.0001$).

To find a suitable mouse cell line for gene editing analysis that could be efficiently transfected by SaCas9 and sgRNA plasmids, we tested two mouse cell lines, Neuroblastoma-2a (N2a) cells and Mouse Embryonic Fibroblasts (MEF) cells, which were available in our lab. First, we performed a transient transfection using Green Fluorescent Protein

(GFP) reporter plasmids in both cell lines. 1×10^5 of N2a or MEF cells in a well of a 6-well plate were transfected with 1μg of pEXL-GFP reporter plasmid, and the gene transfer ratios were determined by flow cytometry analysis. As shown in Figure 1A and 1B, the percentage of GFP-positive cells was found to be 90.4% for N2a cells and 17.9% for MEF cells, with there being a significant difference between these two cell lines (Student *t* test, ****, p< 0.0001). Therefore, N2a was selected as the cell line for gene editing analysis in the subsequent experiments.

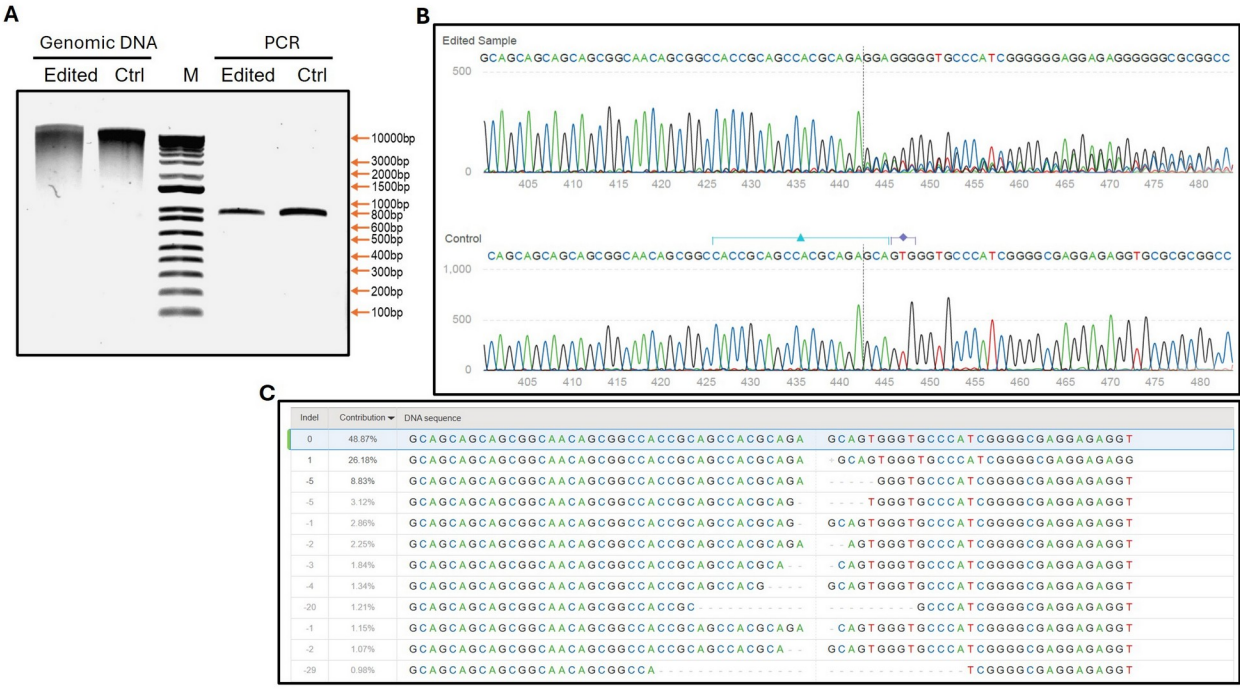


Figure 2. Detection of the editing efficiency of SaCas9/sgPCSK9 transfection in N2a cells. **A.** N2a cells were transfected with SaCas9 and sgPCSK9 plasmids. Three days later, genomic DNAs were extracted and PCRs were run with PCSK9 primers. A fragment size marker (M) was used for size reference. **B.** Sanger sequencing traces of *PCSK9* gene fragment showing the comparison of the edited and control samples in N2a cells. **C.** The indels generated by SaCas9/sgPCSK9 and their contribution to editing efficiency. The panel was created by an online software (SeqScreener Gene Edit Confirmation). The *PCSK9* gene in N2a cells can be edited by SaCas9/sgPCSK9 transfection, with upto 51% editing efficiency.

To further verify whether the cell line was suitable for gene editing analysis, we performed co-transfection in N2a cells with SaCas9 plasmid as well as gPCSK9 plasmid, the latter of which encodes a well-validated sgRNA for mouse *PCSK9* gene (17). After 3 days of transient transfection, the genomic DNA of the transfected cells was extracted, and *PCSK9* gene fragments containing the edited region were amplified using PCR amplification (Figure 2A). PCR products were purified and then sent out for Sanger sequencing. As shown

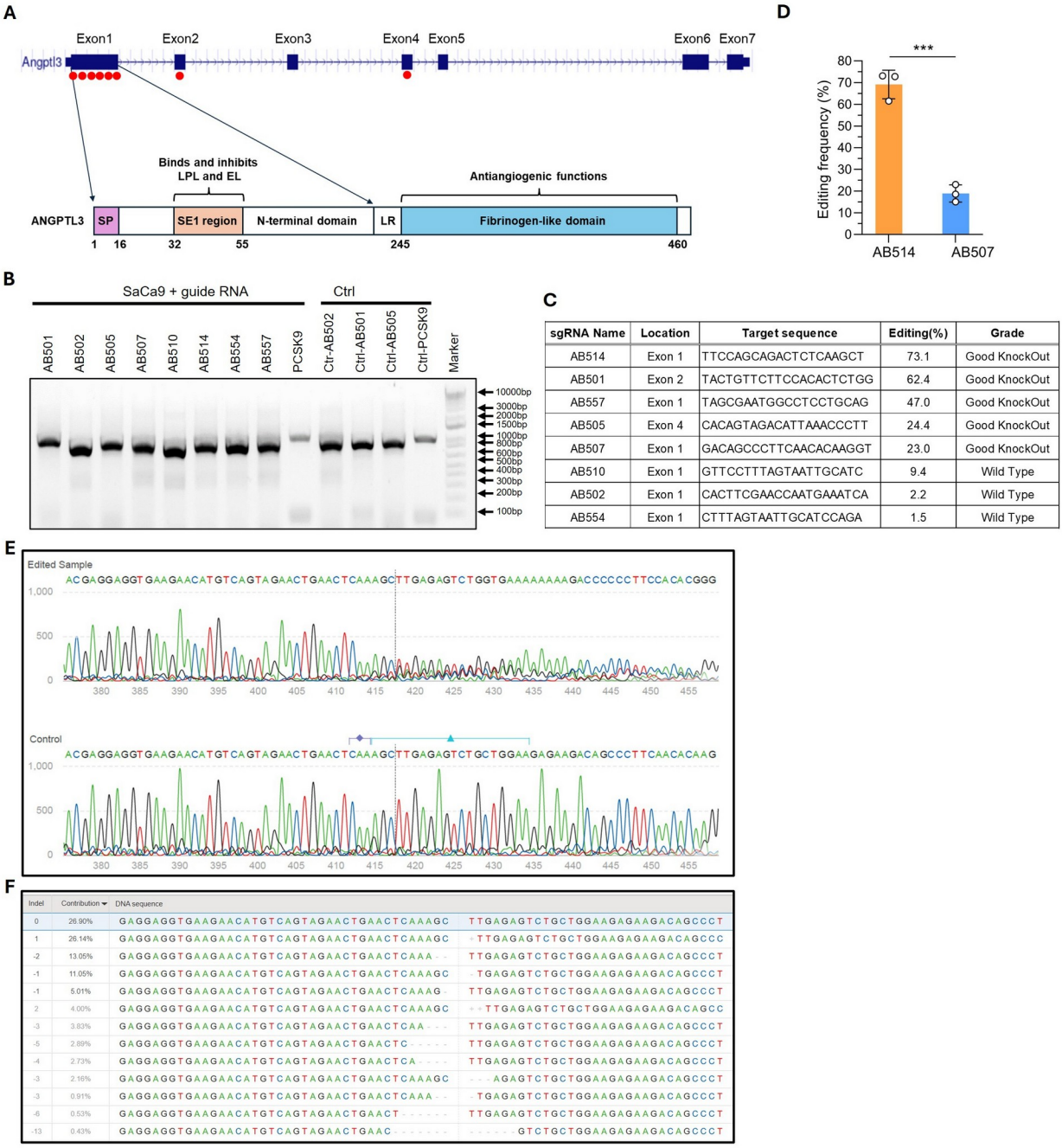
in Figure 2B, each nucleotide position shows a sharp peak in the control sample while in the edited sample, there are multiple nucleotides in each nucleotide position after the Cas9 cutting site, as demonstrated by disordered peaks (Figure 2B), a classic pattern of successful gene editing. SeqScreener Gene Edit Confirmation app analysis showed that most mutations in *PCSK9* were frameshift mutations caused by one nucleotide deletion, with the total editing efficiency being 51% (Figure 2C). Collectively, these data demonstrated that N2a cells could be transfected and edited by the SaCas9 editing machinery.

The design and screening of sgRNAs for mouse ANGPTL3 gene

To screen for a potent sgRNA, we designed a set of sgRNAs using online sgRNA design software Benchling (https://benchling.com) and (https://portals.broadinstitute.org/gppx/crispick/public) termed CRISPick. We selected eight sgRNAs based on ANGPTL3 protein domains that these sgRNAs targeted (Figure 3A, 3B). Using the same protocol established in Figure 1, each sgRNA with SaCas9 plasmid was transfected into N2a cells and incubated for 3 days. The sgRNA targeted regions of *ANGPTL3* were amplified by PCR and then subjected to PCR product purification and Sanger sequencing. Notably, the sgRNA AB507, which works for SpCas9 in the previous report, also functions for SaCas9 in our study (13, Figure 3C). Complexed with SaCas9, AB507 can lead to *ANGPTL3* gene editing in 23% of N2a cells. In the eight sgRNAs, there are three sgRNAs (AB502, AB510, AB554) showing no editing effect on the target gene, while the rest, including AB514, AB501, AB557, and AB505, all show superior editing efficiency compared to AB507. AB514 ranks at the top of the list with an editing efficiency of 73.1%, which is significantly higher than that of AB507 (Figure 3C, 3D). The most common mutation generated by AB514 is a one-nucleotide insertion that causes a frameshift mutation (Figure 3E, 3F). Collectively, we identified a highly effective novel sgRNA AB514 for SaCas9-mediated *ANGPTL3* gene editing.

Simultaneous disruption of PCSK9 and ANGPTL3 genes

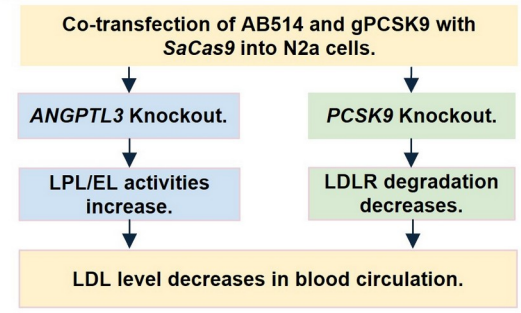
Most CRISPR/Cas9-based gene therapy targets one gene, including sickle cell disease drug Casgevy, which targets *BCL11A* gene (12, 13). However, LDL cholesterol can be regulated in both LDLR-dependent and LDLR-independent ways (9), suggesting that the simultaneous knockout of *PCSK9* and *ANGPTL3* genes could produce synergistic effects, similar to the combination of statins and PCSK9 inhibitors (18). To test this hypothesis, we explored if both *PCSK9* and *ANGPTL3* genes could be edited simultaneously in mouse N2a cells (Figure 4A). We transfected both gPCSK9 and AB514 with SaCas9 plasmids into N2a cells. Using the same procedure as shown in Figure 2, we performed DNA extraction, PCR amplification, and Sanger sequencing. In accordance with our previous postulation, we found that both *ANGPTL3* and *PCSK9* have been edited at a ratio of 28.6% and 21.7%, respectively (Figure 4B). From the result, we concluded that *PCSK9* and *ANGPTL3* genes were edited simultaneously in mouse N2a, which mimics the multiple gene editing behavior of CRISPR/SaCas9 in bacteria.



sgRNA Name	Location	Target sequence	Editing(%)	Grade
AB514	Exon 1	TTCCAGCAGACTCTCAAGCT	73.1	Good KnockOut
AB501	Exon 2	TACTGTTCTTCCACACTCTGG	62.4	Good KnockOut
AB557	Exon 1	TAGCGAATGGCCTCCTGCAG	47.0	Good KnockOut
AB505	Exon 4	CACAGTAGACATTAAACCCTT	24.4	Good KnockOut
AB507	Exon 1	GACAGCCCTTCAACACAAGGT	23.0	Good KnockOut
AB510	Exon 1	GTTCCTTTAGTAATTGCATC	9.4	Wild Type
AB502	Exon 1	CACTTCGAACCAATGAAATCA	2.2	Wild Type
AB554	Exon 1	CTTTAGTAATTGCATCCAGA	1.5	Wild Type

Figure 3. Guide RNA screening for *ANGPTL3* gene in N2a cells. A. CRISPick and Benchling tools were used to design and select sgRNAs for editing the *ANGPTL3* gene. Red dots show the genomic sites of sgRNAs. The lower panel shows the corresponding sites of sgRNAs in ANGPTL3 protein. B. N2a cells were transfected with SaCas9 and sgRNA plasmids. Three days later, genomic DNAs were extracted, and PCRs were run with ANGPTL3 primers for sgRNA-targeted gene fragments. A fragment size marker (M) was used for size reference. C. The ranking of the Gene editing ratios of screened guide RNAs. D. Gene editing efficiency comparison of AB514 and AB507 in N2a cells (Student t-test, n=3, *** $p < 0.001$). E. Sanger sequencing traces show the result of the edited *ANGPTL3* genes. F. The indels were induced by AB514 sgRNA.

A



B

Sample No.	Guide used for transfection	Editing efficiency	
		ANGPTL3 gene	*PCSK9* gene
1	AB514+gPCSK9	28.6%(±2.4%)[K]	21.7% (±3.5%)[K]
2	gPCSK9 only	-	47.0%(±5.3%)[K]
3	AB514 only	66.3%(±5.9%)[K]	-

Note: [K] Good Knockout.

Figure 4. *PCSK9* and *ANGPTL3* genes can be edited simultaneously in N2a cells. **A.** Schematic diagram of Dual-Gene editing strategy. **B.** Gene editing efficiency of *ANGPTL3* and *PCSK9* genes. Plasmids, as shown with SaCas9, were introduced into N2a cells. Three days later, the DNAs were extracted and analyzed by PCR, Sanger sequencing and SeqScreener Gene Edit Confirmation. The results show that simultaneous gene editing performed by AB514 and sgPCSK9 was successful.

Discussion

In this study, we tested multiple new guide RNAs for SaCas9-mediated *ANGPTL3* gene editing in mouse N2a cells, including AB501, AB505, AB514, and AB557. We also determined that AB514 and gPCSK9 sgRNAs could be used to achieve gene editing simultaneously. We found that co-transfecting AB514 and gPCSK9 with SaCas9 plasmids to edit *ANGPTL3* and *PCSK9* genes simultaneously decreased the gene expression of these two genes. Ultimately, we proposed that this dual gene editing strategy would lead to a more comprehensive approach to hypercholesterolemia gene therapy for both homozygous familial hypercholesterolemia and the more common heterozygous familial hypercholesterolemia by significantly lowering LDL cholesterol levels.

A dual gene editing strategy should provide a more comprehensive effect of lowering LDL cholesterol. However, the feasibility of multiple gene editing in mammalian cells is not well demonstrated despite prior literature (15,16). Our data suggested that multiple gene editing could be applicable in general in mammalian cells, though several critical aspects require optimization. For example, in dual gene editing experiments, the gene editing efficiency of both *ANGPTL3* and *PCSK9* decreased, which may impair their effectiveness. Efforts are ongoing in our lab to improve dual-gene editing efficacy to a level comparable to single-gene editing by optimizing the dual-gene editing system. Interestingly, dual-gene editing efficacy shows ~ 50% reduction compared to single-gene editing, suggesting that the addition of one more sgRNA may reduce the transient transfection ratio of SaCas9 and/or sgRNAs. Addressing the low efficiency of multi-gene editing may require further investigation into the optimal timing for editing each gene (editing kinetics) and the appropriate balance of dual gene editing effects. For instance, it is possible that the dual-gene knockdown requires a longer duration than single gene knockout because of the competition which may exist between two different sgRNAs. In that case, a

staggered sequential knockdown of *PCSK9* and *ANGPTL3* could be another strategy for dual gene editing which may increase the efficiency of the gene editing process by avoiding sgRNAs competition. Additionally, in this paper, we did not evaluate the weightage of disruption of *PCSK9* and *ANGPTL3* in the downregulation of LDL-C levels. Finding the weightage of each gene knockout in LDL-C deregulation would be critical for optimizing the dual gene knockout system before their application in *in-vivo* murine models. In addition, adjustment of SaCas9 activity could be another way of improving dual gene editing efficiency because using inducible rather than constitutive expression of Cas9 can significantly promote gene knockout efficiency (19).

In addition to the gene editing efficacy challenge, dual pathway gene disruption also has its own set of particular obstacles. For example, *PCSK9* and *ANGPTL3* could be edited in the same cell or in different cells, although it may have no difference when measuring LDL cholesterol in circulation. To solve this problem, we plan to clone the gPCSK9 and gANGPTL3 in one plasmid and analyze the gene editing efficiency using single cell sequencing technology. Off-target effects are another crucial parameter in developing CRISPR/Cas9-based gene therapy, which is in large part dependent on the specificity of sgRNA (20). Current sgRNA design programs can automatically screen the potential off-target sites in the genome. However, dual gene editing techniques may double the risk of off-target effects. We plan to address this issue by using NGS sequencing in our future experiments.

This paper demonstrated that the N2a cell line is a suitable cell line for sgRNA screening and gene editing due to its higher transfection efficiency. However, N2a cells are not the target cells of LDL cholesterol metabolism, which do not express ANGPTL3 and PCSK9 proteins, and thus prevent further validation of the phenotype induced by gene editing. Mouse hepatocytes are difficult to conduct transient transfections on, and were therefore excluded from our experiment. To fully address the gene editing efficiency in hepatocyte cells, we plan to deliver SaCas9 with gPCSK9 and *ANGPTL3* sgRNA (AB514) to mouse liver using our newly developed delivery platform, Gectosomes (17). In the mouse model, the protein levels of ANGPTL3 and PCSK9 in hepatocytes, and LDL cholesterol levels in circulation will be measured accordingly.

Conclusion

CRISPR/ *Staphylococcus aureus* Cas9 (SaCas9) was used to simultaneously edit *PCSK9* and *ANGPTL3* genes in mouse N2a cells with knockdown efficiencies of 21.7% and 28.6% respectively. A validated PCSK9 sgRNA along with a newly identified sgRNA (AB514) for targeting the *ANGPTL3* gene were used to affect the simultaneous knockdown of the two genes. The findings demonstrated that SaCas9 mediated multiple gene editing in mammalian cells is feasible and the dual gene editing approach targeting *PCSK9* and *ANGPTL3* genes may represent a promising strategy for future Homozygous familial hypercholesterolemia gene therapy.

Acknowledgements

We would like to thank Dr. Paul Strode and Mr. Bayley Zubler for their support and

discussion throughout this process. This project was supported by grants from the National Institutes of Health (R01GM144749).

Abbreviations

PCSK9: proprotein convertase subtilisin/kexin type 9, *ANGPTL3*: Angiopoietin-like 3, CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats, LDL: low density lipoprotein, N2a: also called Neuroblastoma-87902a, is a mouse neuroblastoma cell line, RNA: ribonucleic acid, Cas9: CRISPR-Associated protein 9, PAM: protospacer adjacent motif, LDLR: LDL receptor gene, PCR: Polymerase Chain Reaction, sgRNA: small guide RNA, LPL: lipoprotein lipase, FFA: free fatty acid, VLDL: very low-density lipoprotein, GFP: green fluorescent protein, MEF: Mouse embryonic fibroblasts, DNA: deoxyribonucleic acid, SP: Signaling Peptide, SE1 Region: Specific Epitope 1, ER: endoplasmic reticulum, Indel: insertion-deletion, LR: linker region, EL: endothelial lipase, LPL: Lipoprotein lipase, SaCas9: *Staphylococcus aureus* CRISPR-associated protein 9, SpCas9: *Streptococcus pyogenes* Cas9, NGS: next-generation sequencing, APOB: apolipoprotein B protein, LDLRAP1: LDLR adapter protein 1, DMEM: Dulbecco's Modified Eagle Medium, NHEJ: non-homologous end joining, ddNTP: dideoxynucleoside triphosphate, BCL11A: B-cell lymphoma/leukemia 11A, Opti-MEM: Minimal Essential Medium, pEXL: the plasmid for proteins, specifically GFP

References

1. Defesche, JC., Gidding, SS., Harada-Shiba, M., Hegele, RA., Santos, RD., Wierzbicki, AS. "Familial hypercholesterolemia" Nature reviews Disease primers, 3,17093, (2017). https://doi.org/10.1038/nrdp.2017.93

2. Homozygous Familial Hypercholesterolemia. National Organization for Rare Diseases, 22 July 2024, https://rarediseases.org/rare-diseases/familial-hypercholesterolemia/

3. Cuchel, M., Lee, PC., Hudgins, LC., Duell, PB., Ahmad, Z., Baum, SJ., et al. "Contemporary homozygous familial hypercholesterolemia in the United States: insights from the CASCADE FH registry" Journal of the American Heart Association, 12(9), e029175, (2023). https://doi.org/10.1161/jaha.122.029175

4. Cohen, JC., Boerwinkle, E., Mosley, TJ., Hobbs, HH. "Sequence variations in PCSK9, low LDL, and protection against coronary heart disease" New England Journal of Medicine, 354(12), 1264-1272, (2006). https://www.nejm.org/doi/full/10.1056/NEJMoa054013

5. Srivastava, RAK. "A review of progress on targeting LDL receptor-dependent and-independent pathways for the treatment of hypercholesterolemia, a major risk factor of ASCVD" Cells, 12(12), 1648, (2023). https://doi.org/10.3390/cells12121648

6. Musunuru, K., Chadwick, AC., Mizoguchi, T., Garcia, SP., DeNizio, JE., Reiss, CW., et al. "In vivo CRISPR base editing of PCSK9 durably lowers cholesterol in primates" Nature, 593(7859), 429-434, (2021). https://doi.org/10.1038/s41586-021-03534-y

7. Dewey, FE., Gusarova, V., Dunbar, RL., O'Dushlaine, C., Schurmann, C., Gottesmann, O., et al. "Genetic and pharmacologic inactivation of ANGPTL3 and cardiovascular disease" New England Journal of Medicine, 377(3), 211-221, (2017). https://www.nejm.org/doi/full/10.1056/NEJMoa1612790

8. Stitziel, NO., Khera, AV., Wang, X., Bierhals, AJ., Vourakis, AC., Sperry, AE., et al. "ANGPTL3 deficiency and protection against coronary artery disease" Journal of the American College of Cardiology, 69(16), 2054-2063, (2017). https://doi.org/10.1016/j.jacc.2017.02.030

9. Feingold, KR., Ahmed, SF., Anawalt, B., Blackman, MR., Boyce, A., Chrousos, G., et al. "Cholesterol lowering drugs" Endotext [Internet] South Dartmouth (MA): MDText.com, Inc.; 2000. 2024. https://pubmed.ncbi.nlm.nih.gov/27809434/
.
10. Kayikcioglu, M., Tokgozoglu, L. "Current treatment options in homozygous familial hypercholesterolemia" Pharmaceuticals 16(1), 64, (2022). https://doi.org/10.3390/ph16010064

11. Kerwash, E., Sajic, M., Rantell, KR., McBlane, JW., Johnston, JD., Niewiarowska, A., et al. "Regulatory Assessment of Casgevy for the Treatment of Transfusion-Dependent β-Thalassemia and Sickle Cell Disease with Recurrent Vaso-Occlusive Crises" Current Issues in Molecular Biology, 46(8), 8209-8225, (2024). https://doi.org/10.3390/cimb46080485

12. Qiu, M., Glass, Z., Chen, J., Haas, M., Jin, X., Zhao, X. "Lipid nanoparticle-mediated codelivery of Cas9 mRNA and single-guide RNA achieves liver-specific in vivo genome editing of Angptl3" Proceedings of the National Academy of Sciences, 118(10), e2020401118, (2021). https://doi.org/10.1073/pnas.2020401118

13. Raitskin, O., Schudoma, C., West, A., Patron, NJ. "Comparison of efficiency and specificity of CRISPR-associated (Cas) nucleases in plants: An expanded toolkit for precision genome engineering" PloS one, 14(2), e0211598, (2019). https://doi.org/10.1371/journal.pone.0211598

14. Yang, Z-X., Fu, Y-W., Zhao, J-J., Zhang, F., Li, S-A., Zhao, M., et al. "Superior fidelity and distinct editing outcomes of SaCas9 compared with SpCas9 in genome editing" Genomics, Proteomics and Bioinformatics, 21(6), 1206-1220, (2023). https://doi.org/10.1016/j.gpb.2022.12.003

15. Wang, H., Yang, H., Shivalila, CS., Dawlaty, MM., Cheng, AW., et al. "One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering" cell, 153(4), 910-918, 2013. https://doi.org/10.1016/j.cell.2013.04.025

16. Jao, L-E., Wente, SR., Chen, W. “Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system” Proceedings of the National Academy of Sciences of the United States of America, 110(34), 13904-9, (2013). https://doi.org/10.1073/pnas.1308335110

17. Zhang, X., Xu, Q., Zi, Z., Liu, Z., Wan, C., Crisman, L., et al. "Programmable extracellular vesicles for macromolecule delivery and genome modifications" Developmental cell, 55(6), 784-801, (2020). https://doi.org/10.1016/j.devcel.2020.11.007

18. Cuchel, M,. Raal, FJ., Hegele, RA., Al-Rasadi, K., Arca, M., Averna, M., et al. “Update on European Atherosclerosis Society Consensus Statement on Homozygous Familial Hypercholesterolemia: new treatments and clinical guidance” Eur. Heart J., 44(25), 2277-2291, (2023). https://doi.org/10.1093/eurheartj/ehad197

19. Weber, JA., Lang, JF., Carrell, EM., Alameh, M-G., Davidson, BL. “Temporal restriction of Cas9 expression improves CRISPR-mediated deletion efficacy and fidelity” Molecular Therapy Nucleic Acids, 35(2), 102172, (2024). https://doi.org/10.1016/j.omtn.2024.102172

20. Janik, E., Niemcewicz, M., Ceremuga, M., Krzowski, L., Saluk-Bijak, J., Bijak, M. "Various aspects of a gene editing system—crispr–cas9" International journal of molecular sciences, 21(24), 9604, (2020). https://doi.org/10.3390/ijms21249604