Peer-Review

Xu, Jenny, Amanda Zou, Xiaojuan Zhang, and Xuedong Liu. 2025.
"Disrupting *PCSK9* and *ANGPTL3* Genes with CRISPR Gene Editing Technology for Hypercholesterolemia Treatment." *Journal of High School Science* 9 (3): 1–13. https://doi.org/10.64336/001c.141775.

I found the experiments to be well designed. The execution was performed well and the results were presented in accordance with generally accepted scientific rigor, clarity and erudition. I do have some concerns which I have stated below. Please discuss these in the manuscript.

1.you state "…..However, LDL cholesterol can be regulated in both LDLR-dependent and LDLR-independent ways (9), meaning the suppression of one pathway may trigger compensatory upregulation of the other through feedback mechanisms….." Do you have any references for this conjecture? I have not seen literature on this aspect of PCSK9 inhibitor resistance. If there are no resistance pathways mediated through ANGPTL3, then it may be futile to repress both genes?

2.Please present all centrifugation parameters in RCF or "times g" to enable different labs to duplicate your work, regardless of centrigure model or design.

3.You state "….suggesting the addition of one more sgRNA may reduce the transient transfection ratio of SaCas9 and/or sgRNAs….." Do you think the knockdown TIME may be affected as well ? Do you think that multiplex transfection efficiency increases may decrease the duration of effect ? Please discuss in the manuscript.

4.Is the 'weightage' of both genes the same in controlling LDL levels? Does a 22% knowckdown of PCSK9 contribute to a 22% decrease in LDL levels ? Does a 29% knockdown of ANGPTL3 contribute to a 29% decrease in LDL levels ? Disproportionate knockdown/levels may need re-calculation on how much of one gene needs knockdown relative to the other for maximum decrease in LDL levels. Some discussion along this line is necessary so that the relative efficiencies of knockdown of both genes can be addressed in terms of LDL levels.

5.Did you try 'staggering' the CRISPR delivery ? i.e. deliver to target one gene on day 1, then deliver to target another gene on day 2 (alter the kinetics of delivery/transfection)? Would this be any more successful? see this reference: https://doi.org/10.1016/j.omtn.2024.102172 , please discuss in the manuscript.

6.you state "….each sgRNA with SaCas9 plasmid was transfected into N2a cells and incubated for 3 days…." Why 3 days?

7.Can you provide a concentration (ng/uL) and a purity ratio (260/280) and (260/230) for the nanodrop spectrophotometer measurement?

8.Did you run an experiment with a negative control with no sgRNA to assess background editing levels ? Explain and describe in the manuscript.

9.Although your graphs do contain error bars and p values, please list the number of replicates for each experiment (or a general statement of how many replicates were run for each experiment) in the manuscript.

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1. you state "…..However, LDL cholesterol can be regulated in both LDLR-dependent and LDLR-independent ways (9), meaning the suppression of one pathway may trigger compensatory upregulation of the other through feedback mechanisms….." Do you have any references for this conjecture? I have not seen literature on this aspect of PCSK9 inhibitor resistance. If there are no resistance pathways mediated through ANGPTL3, then it may be futile to repress both genes?

   Response: We agree with the reviewer that drug resistance to PCSK9 inhibitors has not been reported in FH patients. However, PCSK9 inhibitors efficacy depends on LDL receptor (LDLR) activity. In HoFH patients with null/null LDLR mutations, PCSK9 inhibitors have minimal LDL-C–lowering effects. In those with partially functional LDLR, the average reduction is ~30%. While this may lower ASCVD risk, LDL-C levels often remain above recommended goals for most HoFH patients(Cuchel M, 2023). Therefore, combining PCSK9 inhibitors with LDLR-independent therapies, such as statins, may be necessary to achieve treatment goals (Cuchel M, 2023). In this paper, we are trying another option by simultaneous disruption of PCSK9 and ANGPTL3 which is supposed to downregulate the LDL-C level in LDLR dependent and independent ways.

   To make our statement more accurate, we revised our statement as follows: ***However, LDL cholesterol can be regulated in both LDLR-dependent and LDLR-independent ways (9), suggesting that the simultaneous knockout of the PCSK9 and ANGPTL3 genes could produce synergistic effects, similar to the combination of statins and PCSK9 inhibitors(10, Cuchel M, 2023).***

   Cuchel M, Raal FJ, Hegele RA, Al-Rasadi K, Arca M, Averna M, Bruckert E, Freiberger T, Gaudet D, Harada-Shiba M, Hudgins LC, Kayikcioglu M, Masana L, Parhofer KG, Roeters van Lennep JE, Santos RD, Stroes ESG, Watts GF, Wiegman A, Stock JK, Tokgözoğlu LS, Catapano AL, Ray KK. 2023 Update on European Atherosclerosis Society Consensus Statement on Homozygous Familial Hypercholesterolaemia: new treatments and clinical guidance. Eur Heart J. 2023 Jul 1;44(25):2277-2291. doi: 10.1093/eurheartj/ehad197. PMID: 37130090; PMCID: PMC10314327.

2. Please present all centrifugation parameters in RCF or "times g" to enable different labs to duplicate your work, regardless of centrifuge model or design.

Response: Thanks for pointing out this and all centrifugation parameters as shoed below were replaced with RCF in the paper.

14000 rpm = 18440 rcf

8000 rpm = 6021 rcf

12000 rpm = 13548 rcf

The rpm was converted to rcf using calculator: converter (https://www.marshallscientific.com/rpm_to_rcf_calculator_a/2235.htm?utm_medium=chat&utm_campaign=link-shared-in-chat&utm_source=livechat.com&utm_content=www.marshallscientific.com) based on the centrifuge we used (Eppendorf Centrifuge 5424) the radius of rotor of which has a 84 mm.

3. You state "….suggesting the addition of one more sgRNA may reduce the transient transfection ratio of SaCas9 and/or sgRNAs….." Do you think the knockdown TIME may be affected as well ? Do you think that multiplex transfection efficiency increases may decrease the duration of effect ? Please discuss in the manuscript.

   Response: Thanks for these nice suggestions. We added these discussions in the Discussion part: It is possible that the dual-gene knockdown takes a slightly longer duration than single gene knockout because of the competition which may exist between two different sgRNAs. In that case, a sequential knockdown of PCSK9 and ANGPTL3 could be another strategy for the dual-gene editing which may increase the therapeutic efficacy by fastening the gene editing process and avoiding sgRNAs competition.

   In addition, delivery of multiplex gene editing machinery into hepatocytes may decrease the duration of therapeutic effects due to the increase in innate response caused by more double strand DNA breaks that may lead to the elimination of the edited cells. We are going to address this issue in our future work.

4. Is the 'weightage' of both genes the same in controlling LDL levels? Does a 22% knowckdown of PCSK9 contribute to a 22% decrease in LDL levels ? Does a 29% knockdown of ANGPTL3 contribute to a 29% decrease in LDL levels ? Disproportionate knockdown/levels may need re-calculation on how much of one gene needs knockdown relative to the other for maximum decrease in LDL levels. Some discussion along this line is necessary so that the relative efficiencies of knockdown of both genes can be addressed in terms of LDL levels.

   Response: One major limitation of our research is that we didn't evaluate the weightage of disruption of PCSK9 and ANGPTL3 in downregulation of LDL-C levels in this paper. This is a critical question which needs to be addressed soon. We are planning to address this question by using hepatocytes freshly isolated from mice. Finding out the weightage of each gene knockout in LDL-C deregulation would be critical for optimizing our dual-gene knockout system before they are applied to mice. Accordingly, we discussed it in our paper.

5. Did you try 'staggering' the CRISPR delivery ? i.e. deliver to target one gene on day 1, then deliver to target another gene on day 2 (alter the kinetics of delivery/transfection)? Would this be any more successful? see this reference: https://doi.org/10.1016/j.omtn.2024.102172 , please discuss in the manuscript.

Response: Thanks for digging out this reference for us. It is a brilliant idea to test optimizing the doses of CRISPR/Cas9 editing machinery and the gene editing cohort which would improve dual-gene editing efficiency. To do that, we are going to use Gectosome, a VLP like particle developed in our lab, as a delivery vehicle to address these questions. We discussed this issue together with the response to question 3.

6. you state "….each sgRNA with SaCas9 plasmid was transfected into N2a cells and incubated for 3 days…." Why 3 days?

   Response: We followed the protocol in our previous paper(17). We found that 3 days is the least duration that allows the maximum gene editing readout.

7. Can you provide a concentration (ng/uL) and a purity ratio (260/280) and (260/230) for the nanodrop spectrophotometer measurement?

   Response: we added these data into our Result section.

   Table 1. Concentrations of DNA samples used in paper.

Genomic DNA Samples		**Concentration (ng/uL)**	**OD260/ OD280**	**OD260/ OD230**
Figure 2	N2A + SaCas9/gPCSK9	28.088	1.93	0.82
	N2A Blank	48.518	2.05	1.33
Figure 3	N2A + SaCas9/AB557	39.686	2.02	1.05
	N2A + SaCas9/AB554	50.127	1.94	1.31
	N2A + SaCas9/AB514	23.089	2.27	0.97
	N2A + SaCas9/AB510	54.270	1.94	1.51
	N2A + SaCas9/AB507	48.465	1.92	1.20
	N2A + SaCas9/AB505	22.491	2.19	0.95
	N2A + SaCas9/AB502	32.146	2.04	1.03
	N2A + SaCas9/AB501	34.335	1.98	1.14
Figure 4	N2A + SaCas9/AB514/gPCSK9	139.638	1.99	1.64
	N2A + SaCas9/gPCSK9	244.763	2.04	1.93
	N2A + SaCas9/AB514	273.172	2.04	1.76
	N2A Blank	205.172	2.00	1.68

*The 260/280, indicating DNA purity, is best as close to 2.00 as possible. The 260/230, indicating RNA purity, isn't really significant in our project.

8. Did you run an experiment with a negative control with no sgRNA to assess background editing levels ? Explain and describe in the manuscript.

   Response: When we screened for effective guides for *ANGPTL3* gene, the guides that didn't work served as negative control, as their little to none editing efficiencies show possible background editing levels when no sgRNAs are present.

9. Although your graphs do contain error bars and p values, please list the number of replicates for each experiment (or a general statement of how many replicates were run for each experiment) in the manuscript.

   Response: we included this information in the figures legends.

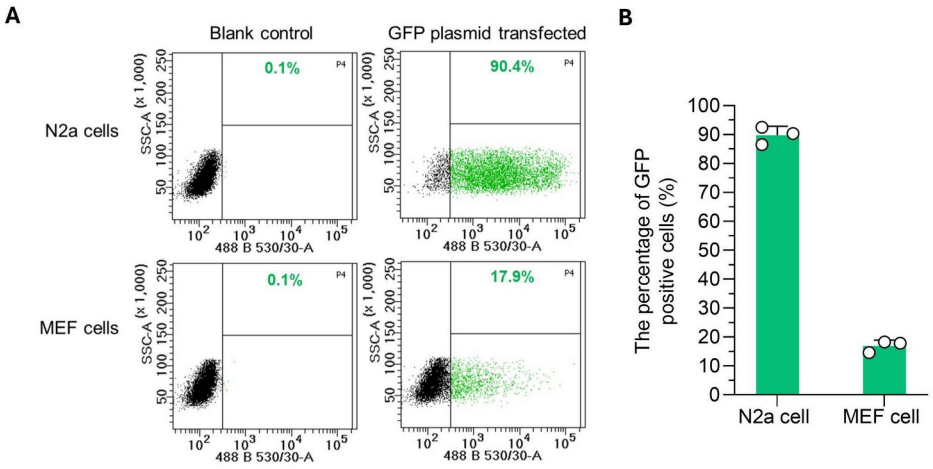
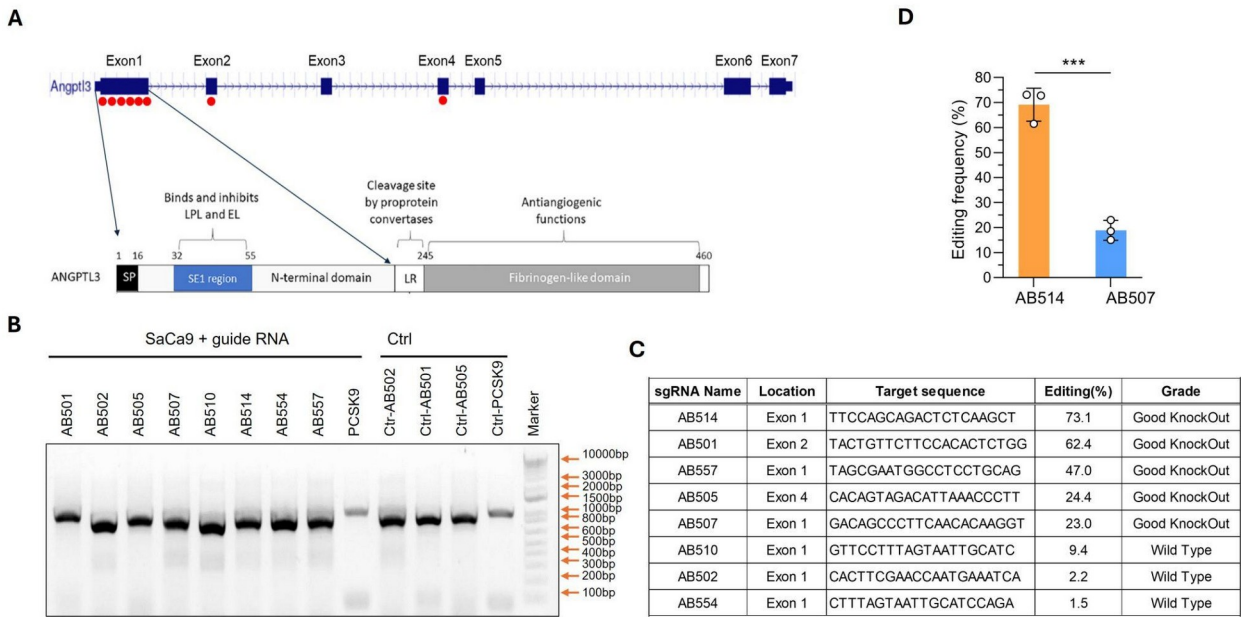


Figure 1: Detection of the efficiency of transient transfection through flow cytometry. A. 1E+5 of N2a or MEF cells were transfected with 1 microgram of pEXL-GFP reporter plasmid, and the gene transfer ratios were determined by flow cytometry analysis. **B.** The statistical result for transfection efficiency comparison of N2a and MEF cells (Student *t* test, n=3, **** p< 0.0001).

We conducted three repeats of GPF plasmid transfection with each cell type, as indicated by the number of white dots on the bar graph.



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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: D: We did three repeats for each guide (AB514 and AB507) on the N2A cells, as shown by the number of white dots on the bar graph (Student *t* test, n=3, *** p< 0.001).

Thank you for addressing my comments. Accepted. I have written a conclusion, which was missing, as well as made minor changes to content.