Int Cardiovasc Res J.2022;**16**(4):129-134.icrj.131422

International Cardiovascular Research Journal
www.ircrj.com

LIMA1 Gene Knockout by CRISPR/Cas9 System Using Lentiviruses as an in Vitro Model for Reducing Cholesterol Absorption

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ARTICLE INFO

Article Type:
Research Article

Article History:
Received: 2 Sep 2022
Revised: 15 Dec 2022
Accepted: 25 Dec 2022

Keywords:
Cardiovascular Diseases
Cholesterol
CRISPR/Cas9
EPLIN
LIMA1

ABSTRACT

Background: Cardiovascular diseases, with an estimated 18.6 million deaths per year, are the leading cause of death worldwide. One of the major risk factors is elevated blood low-density lipoprotein cholesterol (LDL-C) secondary to multiple environmental and genetic factors. Genes involved in LDL-C metabolism are the targets of the most common treatment options. Advanced molecular techniques could pave the way for identifying novel targets in dyslipidemia therapies. The LIM domain and actin-binding 1 (*LIMA1*) gene binds to the NPC1L1 protein and facilitates its more efficient recycling to the plasma membrane. Inhibition of LIMA1 could disrupt cellular cholesterol hemostasis with a probable decrease in blood LDL-C levels.

Objectives: The present study was designed to knock out exon 2 of the *LIMA1* gene using lentiviruses as an in vitro model for reducing cholesterol absorption.

Methods: A CRISPR/Cas9 system with dual guide RNAs (gRNAs) was designed to completely excise exon 2 of *LIMA1*. Two gRNAs (gRNA1 and gRNA2) were cloned in the LentiCRISPR v2 vector. LentiCRISPR viruses were produced in the HEK293T cell line to encode the CRISPR/Cas9 complex structure. HepG2 cell lines were transduced with two different LentiCRISPR viruses simultaneously.

Results: Exon 2 deletion was detected by PCR, gel electrophoresis, and subsequent Sanger sequencing of the PCR product. Exon 2 deletion caused a frameshift mutation, and the subsequent production of nonfunctional transcripts led to gene knockout. The dual gRNA CRISPR/Cas9 system could be used in gene editing setups.

Conclusions: The in vitro knockout model of *LIMA1* could be considered as preliminary work to study the role and mechanism of action of the LIMA1 protein, along with its potential as a target for hypercholesterolemia therapy.

1. Background

Cardiovascular diseases (CVDs), with an estimated prevalence of 523 million in 2019 and correlated with 18.6 million deaths, have remained the most common cause of death for over a decade worldwide (1). Major CVD risk factors include high blood cholesterol, high blood pressure, high blood glucose (diabetes), and metabolic syndrome. Another major contributing component is genetic factors. Any form of hyperlipidemia with an increased level of blood low-density lipoprotein cholesterol (LDL-C), increased triglycerides level, and low blood high-density lipoprotein cholesterol (HDL-C) could have underlying genetic factors (2, 3).

In 2018, during the Cardiovascular Risk Survey in western China, a Chinese Kazakh family with inherited low levels of LDL-C was identified (4). Whole-exome sequencing of the affected family members detected a heterozygous frameshift deletion in exon 7 of the "LIM domain and actin-binding 1" (*LIMA1*) (OMIM 608364) gene (5).

The *LIMA1* human gene is located on chromosome 12q13.12 and was previously known as Epithelial Protein Lost in Neoplasm (EPLIN). It contains two actin-binding

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regions on each side of the LIM domain and single domains to interact with Myosin Vb and NPC1L1 (Figure 1). LIMA1 interactions with these molecules lead to the maintenance of the cellular cytoskeleton, cell-cell adhesive contacts, and an intact cytokinesis process during cell division (6, 7).

Further investigations demonstrated LIMA1 as the missing structural connector between NPC1L1 and Myosin Vb in the NPC1L1 recycling route to the plasma membrane. NPC1L1 conserved amino acids of Q1277KR residues and the C164LG residues of LIMA1 proteins interact with each other, while the amino acid 21 - 40 region of Myosin Vb and the amino acid 491 - 511 region of LIMA1 facilitate Myosin Vb–LIMA1 interaction (5).

Mutations in *LIMA1* reduce NPC1L1 cellular recycling rate, leading to lower dietary cholesterol uptake, increased fecal cholesterol excretion, and ultimately lower plasma LDL-C levels (8, 9).

Since the discovery of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated 9 (Cas9) system, it has been widely used as a popular genome-editing technique due to its simplicity, robustness, and its considerably higher efficiency in comparison with other genome editing tools (8, 9). The CRISPR/Cas9 ribonucleoprotein structure comprises two units, Cas9 endonuclease and guide RNA (gRNA). Each gRNA is composed of a 17 - 20 nucleotide sequence complementary to the target sequence and guides the Cas9 protein to cleave the DNA. Host target DNA sequence is followed by Protospacer Adjacent Motif (PAM) sequences, and Cas9 usually makes a double-strand break 3-4 nucleotides upstream of the PAM sequence. DNA breakage induces a cellular non-homologous end joining (NHEJ) pathway to repair the scission. However, the NHEJ pathway is an error-prone mechanism that could result in small random insertions or deletions (indels) at the cleavage site, resulting in a frameshift mutation and the subsequent production of nonfunctional transcripts leading to a gene knockout. The CRISPR/Cas9 gene regulation potential could be a promising approach to treating CVDs (10).

2. Objectives

We aimed to knock out exon 2 of the *LIMA1* gene in HepG2 cell lines via two gRNAs using LentiCRISPR viruses as an in-vitro model for reducing cholesterol absorption.

3. Methods

3.1. CRISPR/Cas9 Complex Construction

3.1.1. gRNAs design

A dual system of gRNA 1 and gRNA 2 was employed to excise exon 2 entirely. The gRNA oligos were annealed and ligated to the digested plasmid (Table 1).

3.1.2. LentiCRISPR v2 Digestion

LentiCRISPR v2 (Addgene plasmid #52961) (11) was digested by the Esp3I (BsmBI) enzyme using 0.5 µL of LentiCRISPR v2 with 0.5 µL of Esp3I enzyme, 1 µL of Tango buffer, 0.5 µL of 100 mM dithiothreitol (DTT), and 10 µL of distilled water.

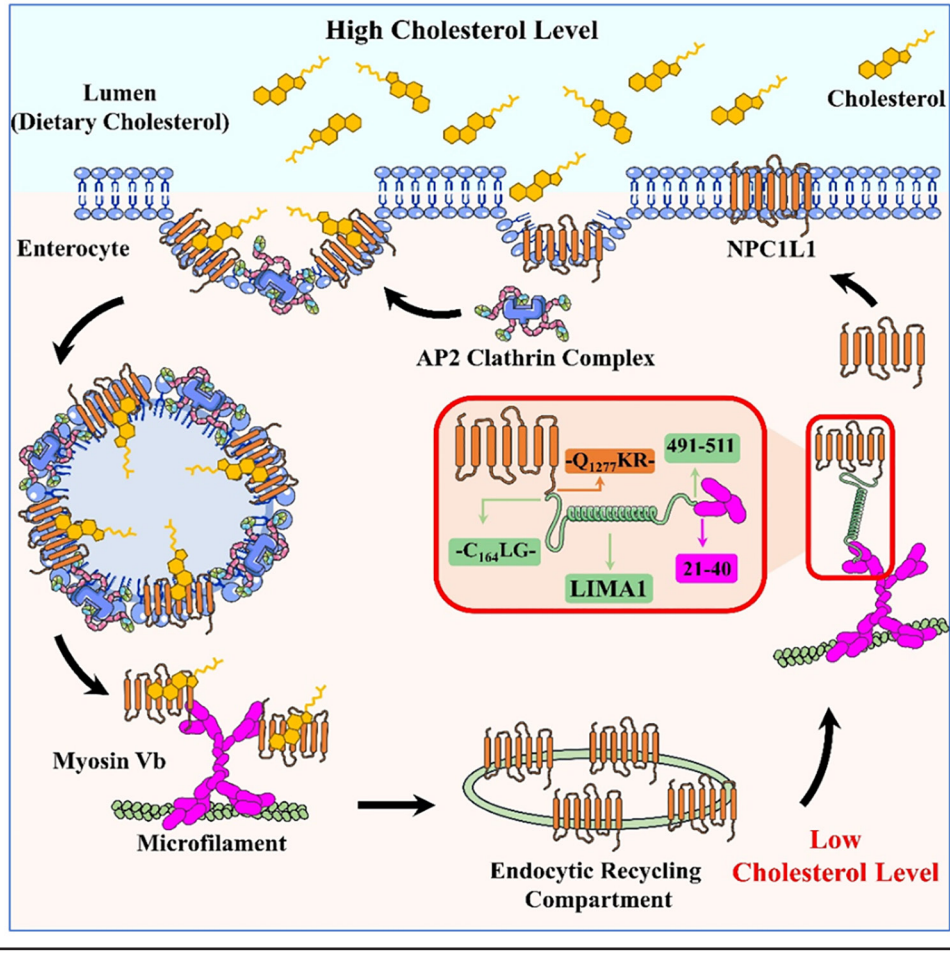

Figure 1. An Overview of NPC1L1, Myosin Vb, and LIMA1 Roles in Dietary Cholesterol Absorption

Table 1. The Guide RNAs (gRNAs) Targeting Exon 2 of the *LIMA1* Gene

gRNA	Guide Oligos
gRNA 1	5' CACC G TCATTAGAACACATCCGGTC 3' 3' C AGTAATCTTGTGTAGGCCAG CAAA 5'
gRNA 2	5' CACC G CTATCCGTAATTAATGGTAT 3' 3' C GATAGGCATTAATTACCATA CAAA 5'

Table 2. The Primer Sequences. Two Knockout Primers Were Used to Detect the Deletion of the Entire Exon 2 of *LIMA*1

Primer Name	Primer Sequence	Product Length in Undeleted DNA	Product Length in Deleted DNA
2-KO	F: 5′- GACGGCAATGGACCTCACTA -3´ R: 5′- CCGTCCTTGATGTGGGGATA -3´	26,522 bp	1,550 bp

3.2. Ligation

1 µL of digested LentiCRISPR v2 was ligated to 1 µL of ligated oligos, 0.5 µL of T4 DNA ligase, 1 µL of T4 DNA ligase buffer, and 6.5 µL of distilled water. The reaction mix was incubated at room temperature for 30 minutes and later at 4 °C overnight.

3.3. Cloning

The LentiCRISPR v2 constructs were transformed into *DH5α* competent *Escherichia coli* cells. Subsequently, selected colonies were cultured in Luria-Bertani (LB) liquid medium. Plasmid DNA was extracted using a Miniprep kit (QIAprep Spin Miniprep Kit, USA), then PCR amplification was performed. Each PCR product was later validated by Sanger sequencing.

3.4. LentiCRISPR v2 Virus Production

A second-generation packaging system was used to produce recombinant lentiviruses. The LentiCRISPR v2 transfer plasmid together with psPAX2 packaging plasmid (Addgene plasmid# 12260) and pMD2.G (Addgene plasmid# 12259) envelope plasmid were transfected into HEK293T cells using the calcium phosphate method.

A T-75 flask was seeded with HEK293T cells. Cell cultures with ~60% confluency were ideal for transfection, and their medium was replaced with 15 ml of antibiotic-free culture media. For each flask, 440 µL of TE 0.1X buffer, 233 µL of deionized distilled water, and 75.3 µL of calcium chloride 2.5 M solution were prepared in 50 ml falcon tubes.

In the next step, the plasmid DNA transfection mix with 3:2:1 (wt/wt/wt) transfer/packaging/envelope plasmid ratio was added. A total amount of 30 µg of DNA, including 15 µg of LentiCRISPR v2 transfer plasmid, 10 µg of psPAX2 packaging plasmid, and 5 µg of pMD2.G envelope plasmid were used. Ultimately, under agitation by vortexing, 760 µL of 2X HEPES buffered saline (HBS2X) solution was added to the mixed solution. After 20 min, the produced viruses were harvested by collecting the cell culture medium.

The collected virus supernatant was precipitated with polyethylene glycol-8000 (PEG-8000) solution in a 1:3 (v/v) ratio of PEG to viral supernatant. All the above processes were performed separately for gRNA1 and gRNA 2. The concentrated virus was titrated with an ELISA kit by measuring the amount of HIV p24 Gag antigen.

3.5. Lentiviral Transduction of HepG2 Cells

For the transduction, HepG2 cells were seeded in a six-well plate. Next, a volume of 15 µL of each of the synthesized recombinant lentiviruses, 1 µL of 10 µg/mL Polybrene, 0.5 µg/mL of Puromycin, and 1,969 µL of DMEM culture media supplemented with 10% FBS were added to each well. After 72 hours of incubation, only transduced cells would survive and were later maintained by transferring to two T-75 flasks.

3.6. PCR Analysis of Transduced Cells

Transduced cells were lysed, and their genomic DNA was extracted. Identification of deleted DNA sequences was achieved by amplifying correlated sequences using designed primers (Table 2). The PCR products were visualized by Safe dye and were analyzed using agarose gel electrophoresis. Related PCR products were extracted from the gel and were further studied by Sanger sequencing.

4. Results

In the present study, *LIMA1* exon 2 was selected as a suitable knockout target. Based on our study design, two target sites were chosen in the upstream and downstream introns of exon 2. It was hypothesized that the complete deletion of exon 2 would lead to a frameshift mutation, and since the mutation is in early exons, it would inhibit the synthesis of the functional protein (Figure 2a) (5). Two guide RNAs (gRNA1 and gRNA2) were cloned in the LentiCRISPR v2 vector. Eighteen hours after the cloned guides were transformed into separate *DH5α* competent *E. coli* cells, white colonies appeared in the LB agar plates supplemented with ampicillin.

Recombinant colonies carrying gRNAs were detected by colony PCR and observation of the 320 bp PCR products on gel electrophoresis (Figure 2b). Successful cloning of gRNA1 and gRNA2 to the LentiCRISPR v2 vector was further validated by Sanger sequencing (Figure 2c).

HepG2 cells were transduced with two LentiCRISPR v2 viruses simultaneously. Only a small fraction of cells would receive both gRNA lentiviruses simultaneously and would have exon 2 completely excised out. Recombinant HepG2 cells would be Puromycin-resistant and would therefore continue growing in a cell culture medium.

After DNA extraction and PCR amplification with the intended primers, the PCR products were analyzed by agarose gel electrophoresis (Table 2). DNA samples with knocked-out *LIMA1* demonstrated a 1,550 bp product band (Figure 3).

The negative control cells that were not transduced by any

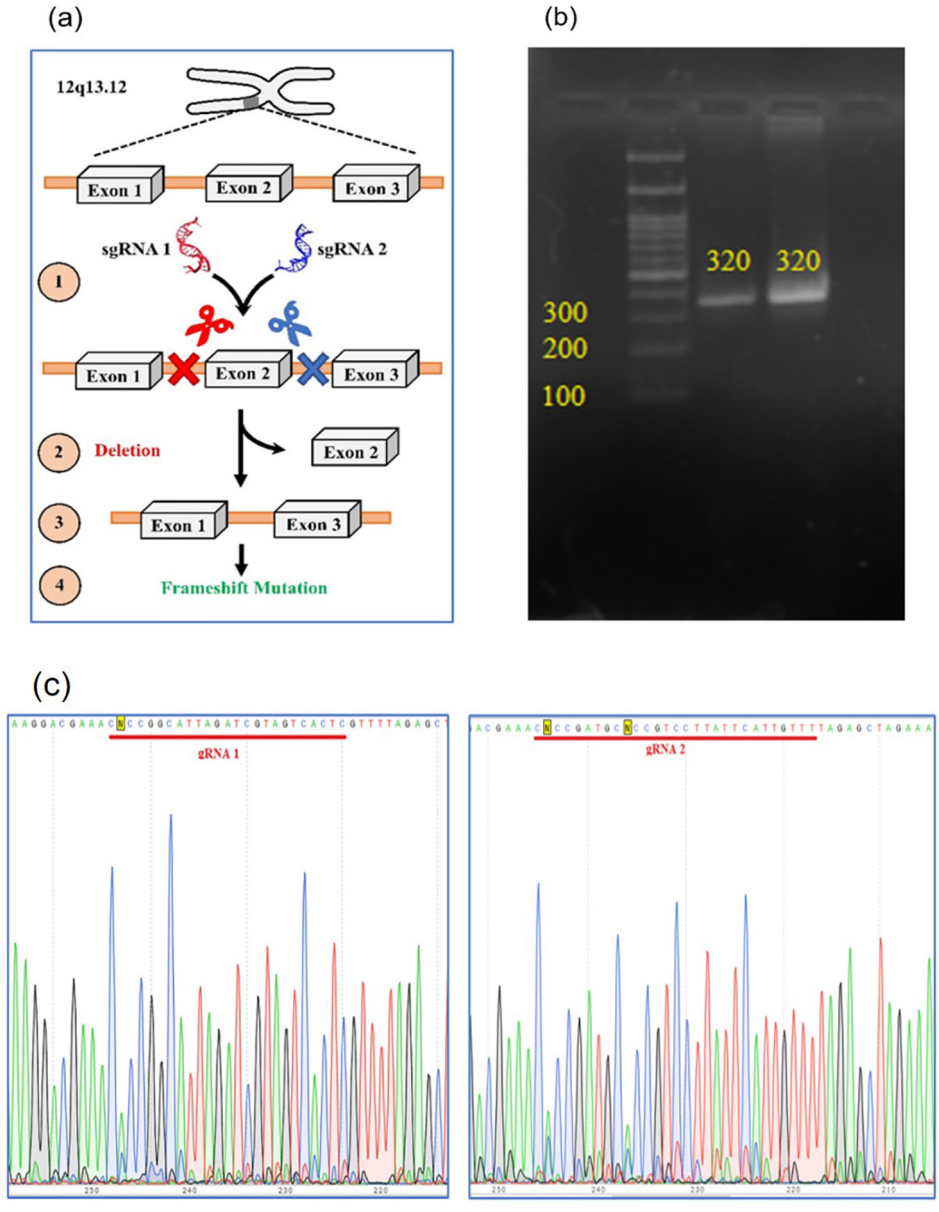

Figure 2. (**a**) Schematic Diagram of the Designed gRNAs' Locations and Deletion of the *LIMA1* Exon 2; (**b**) 320 bp PCR Products on Agarose Gel Electrophoresis Indicating Correct Guide Cloning; (**c**) Sequencing Results of Cloned LentiCRISPR v2 by gRNA1 and gRNA2.

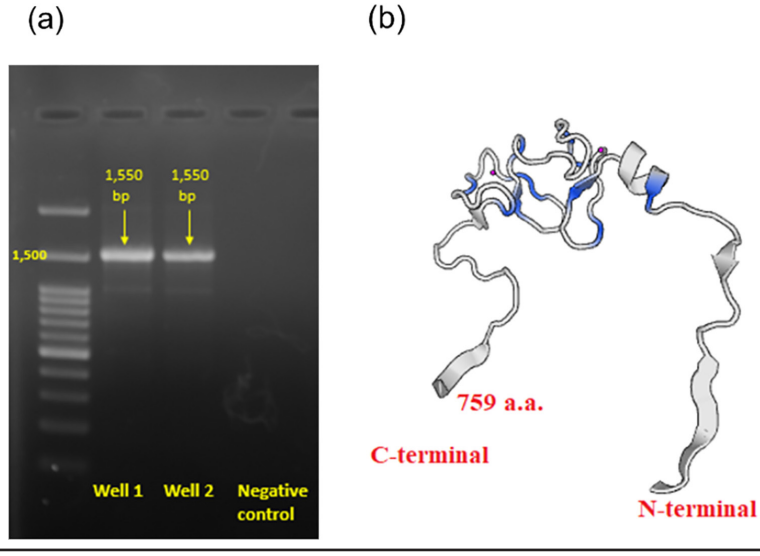

Figure 3. (**a**) PCR Products of 1,550 bp on Agarose Gel Electrophoresis Indicate Successful *LIMA1* Exon 2 Knockout; (**b**) Schematic Structure of Wild-Type LIMA1 Protein.

LentiCRISPR viruses demonstrated a homozygote state with no PCR product band. To ensure the correct knocked-out state of cells, 1,550bp PCR products were extracted from the gel and were further confirmed by Sanger sequencing (Figure 3a).

According to the analysis of the *LIMA1* gene, deletion of exon 2 would result in a frameshift mutation. As a result, around 720 amino acids in the protein structure would not be synthesized (Figure 3b).

5. Discussion

The latest guidelines for managing dyslipidemia have shifted to the paradigm that a lower LDL-C is better, and maximal reduction of blood LDL-C is the primary target in preventing CVDs (12-16). Currently, various treatment options are designed based on the different molecules involved in cholesterol metabolism (16-18).

One of the most common non-statin cholesterol-lowering drugs is ezetimibe, which inhibits NPC1L1 action in the intestine and reduces dietary cholesterol absorption. As a result of decreased dietary LDL-C absorption, the liver overexpresses the LDL receptor (LDLR) gene, leading to further cholesterol reduction in the blood (19, 20). A new category of drugs includes proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors. Upon binding to LDLRs, PCSK9 increases the lysosomal degradation of cellular LDLRs, leading to increased circulating LDL-C (21, 22).

Despite clinically implementing these newly discovered drugs and higher-intensity statin therapies, most patients can not reach the ideal blood LDL-C, and CVDs are still the main cause of death worldwide. This indicates the need for studies to identify novel therapeutic targets (23).

Our previous works demonstrated that multiple exon deletions induced by CRISPR-directed gene editing cause exon skipping in the *dystrophin* gene and could restore dystrophin expression in a human skeletal muscle cell line, representing a probable therapeutic approach to muscular dystrophies (24, 25). In the present study, we expanded our work to cardiovascular diseases. We knocked out exon 2 of the human *LIMA1* gene via the CRISPR/Cas9 genome editing system using lentiviruses as an in-vitro model for reducing cholesterol absorption. LIMA1 is a scaffold protein involved in cellular cholesterol homeostasis through the regulation of NPC1L1 recycling to the plasma membrane. Theoretically, since NPC1L1 is a dietary cholesterol receptor, *LIMA1* knockout could result in lower dietary cholesterol absorption and lead to lower blood LDL-C levels (5). Considering that HepG2 expresses both LIMA1 and NPCILI, it can be a suitable in-vitro model for such novel hypercholesterolemia therapies. However, our work should be considered as a preliminary step in proving the role of *LIMA1,* and in vivo studies are essential to demonstrate its comprehensive mechanism of function.

5.1. Ethical Approval and Consent to Participate

This study was ethically approved by the Ethics Committee of Shiraz University of Medical Sciences under the ethical approval code IR.SUMS.REC.1398.122.

(https://ethics.research.ac.ir/EthicsProposalView.php?&code=IR.SUMS.REC.1398.122).

5.2. Availability of Data and Material

All data generated or analyzed during this study are included in the final published article.

Acknowledgements

The results presented in this paper are part of the Ph.D. thesis of Mahdieh Talebzadeh, supported by a grant (No. 95-01-74-15989) from Shiraz University of Medical Sciences, Shiraz, Iran.

Authors' Contribution

This study was designed by TM and MD. The study procedures were carried out by TM, MD, HJK, MR, and FT. The final manuscript was written and edited by MT and MN. All contributing authors approved the final manuscript.

Funding/Support

The results presented in this paper are part of the Ph.D. thesis of Ms. Mahdieh Talebzadeh, supported by a grant (No. 95-01-74-15989) from Shiraz University of Medical Sciences, Shiraz, Iran.

Financial Disclosure

The authors declare that they have no competing interests.

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