NIH R01 RESEARCH PROJECT GRANT

# Targeted Epigenetic Gene Silencing of PCSK9 via CRISPR-dCas9 for Long-Term Cardiovascular Protection


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**Funding Mechanism** NIH R01 Research Project Grant (PA-25-100)
**Proposed Period** July 1, 2026 – June 30, 2031 **Direct Costs Requested:** $1,250,000


## SPECIFIC AIMS

Coronary heart disease is the leading driver of global mortality, claiming over 9 million lives annually. Elevated low-density lipoprotein cholesterol (LDL-C) is a primary causative risk factor for atherosclerotic cardiovascular disease. Standard therapeutic interventions rely on daily oral statins or monthly subcutaneous injections of PCSK9-targeting monoclonal antibodies. While effective, these therapies suffer from poor long-term compliance, high cumulative costs, and systemic access barriers. Consequently, a significant clinical gap exists for a durable, single-dose solution that provides lifetime cholesterol lowering without requiring daily patient adherence.

Gene editing via CRISPR-Cas9 offers a potential one-time cure, but nucleases introduce double-strand DNA breaks (DSBs), raising critical safety concerns regarding off-target mutagenesis, insertions/deletions (indels), chromosomal translocations, and potential oncogenic transformation. To circumvent these genotoxic risks, this proposal introduces a non-mutagenic alternative: CRISPR interference (CRISPRi). By utilizing a catalytically inactive dCas9 fused to the Krüppel-associated box (KRAB) repressor, we will induce targeted epigenetic silencing of the PCSK9 gene. This approach deposits repressive histone marks (H3K9me3) at the PCSK9 promoter, inducing chromatin compaction and durable gene silencing without breaking the DNA backbone.

**Specific Aim 1: Design, Optimize, and Screen CRISPR-dCas9-KRAB sgRNAs for PCSK9 Repression.** We will design a library of 20 sgRNAs targeting the proximal promoter and transcriptional start site (TSS) of human and murine PCSK9. Candidate sgRNAs will be screened in vitro using primary human hepatocytes and transgenic mouse hepatoma cells. Repression efficacy will be quantified by qPCR and Western blot, identifying the top two candidate sgRNAs that yield >90% PCSK9 knockdown with minimal off-target activity.

**Specific Aim 2: Evaluate Efficacy, Biodistribution, and Genomic Safety in Humanized Mouse Models.** The lead sgRNA candidates and dCas9-KRAB mRNA will be co-formulated into hepatotropic lipid nanoparticles (LNPs). We will administer these LNPs via tail-vein injection into humanized PCSK9 transgenic mice (huPCSK9-Tg). In vivo knockdown durability, liver specificity, serum lipid changes, and chromatin marks (H3K9me3) will be monitored over 24 weeks. Epigenetic off-target assessments will be performed using ChIP-seq and RNA-seq.

**Specific Aim 3: Assess Long-Term Durability, Safety, and Lipid Changes in Non-Human Primates.** To facilitate clinical translation, the optimized LNP-CRISPRi system will be evaluated in cynomolgus macaques (NHPs). Animals will receive a single systemic dose of LNP-CRISPRi. We will track serum PCSK9, total cholesterol, and LDL-C levels for 12 months. Comprehensive systemic toxicity, liver histopathology, and humoral/cellular immune responses against dCas9-KRAB will be rigorously assessed.

**Expected Impact:** Successful completion of this work will establish a novel, non-cleaving gene regulation paradigm, providing a safe, durable, and cost-effective clinical strategy for cardiovascular protection.

# 1 RESEARCH STRATEGY

## 1.1 Significance

**Clinical Burden of Hypercholesterolemia:** Atherosclerotic cardiovascular disease (ASCVD) remains the leading driver of global mortality, with low-density lipoprotein cholesterol (LDL-C) acting as its primary driver. Monoclonal antibodies targeting proprotein convertase subtilisin/kexin type 9 (PCSK9) have emerged as highly effective alternatives for patients refractory to high-intensity statins. By blocking PCSK9, these antibodies prevent the degradation of hepatic LDL receptors (LDLR), thereby enhancing blood clearance of LDL-C. However, monoclonal antibodies require lifelong, bi-weekly or monthly injections, which drastically reduces patient compliance (less than 50% at 12 months) and incurs substantial financial burdens on healthcare systems. A therapeutic approach capable of providing durable, single-dose PCSK9 inhibition is urgently needed to address this public health challenge.

**The Case for Epigenetic Silencing:** Standard CRISPR-Cas9 platforms achieve PCSK9 disruption by creating double-strand DNA breaks (DSBs) that recruit error-prone non-homologous end-joining (NHEJ) pathways. Although effective, this nuclease-mediated approach carries inherent risks of permanent, undesirable genomic alterations, including off-target insertions/deletions (indels) and large chromosomal deletions. Our proposed CRISPRi technology (dCas9-KRAB) bypasses the need for DSBs by utilizing a catalytically inactive Cas9 to recruit endogenous chromatin-modifying complexes. This leads to the deposition of repressive H3K9me3 histone marks at the target promoter, triggering local heterochromatin formation. This project is highly significant because it aims to validate a completely non-mutagenic, highly durable, and reversible method of epigenetic silencing, creating a safer class of genetic therapeutics.

## 1.2 Innovation

**Non-Cleaving Epigenetic Silencing:** Unlike current base editors or prime editors that still require transient single-strand nuclease nicking, our platform utilizes a strictly non-cleaving dCas9-KRAB mechanism. We innovate by leveraging local chromatin structure manipulation rather than nucleotide sequence modification. This avoids any permanent alteration to the primary sequence of the human genome, aligning with clinical safety priorities.

**Advanced Liver-Targeted LNP Delivery:** Traditional gene delivery systems rely on viral vectors (e.g., AAV) which cause prolonged dCas9 expression, leading to severe immunogenicity and increased off-target risks. We innovate by utilizing lipid nanoparticles (LNPs) composed of novel ionizable lipids designed for transient, liver-specific delivery of dCas9-KRAB mRNA and sgRNA. This transient expression window ensures effective epigenetic writing while minimizing immunogenicity and potential long-term toxicity.

## 1.3 Approach

**Aim 1: Design, Optimize, and Screen CRISPR-dCas9-KRAB sgRNAs for PCSK9 Repression: Rationale:** Achieving robust repression requires high-affinity targeting of the transcriptional machinery. We will target the chromatin environment flanking the PCSK9 transcriptional start site (TSS). **Research Design:** We will synthesize 20 sgRNAs targeting the region from $-150$ to $+50$ base pairs relative to the TSS of human PCSK9. sgRNAs will be cloned into expression plasmids containing the dCas9-KRAB sequence. Transfection will be performed in primary human hepatocytes. Control groups will receive non-targeting sgRNAs or dCas9-KRAB alone. We will measure PCSK9 mRNA levels via qPCR and secretable PCSK9 protein via ELISA. Chromatin alterations at the promoter will be assessed via ChIP-qPCR targeting H3K9me3. **Expected Outcomes:** We anticipate identifying at least two sgRNAs that consistently reduce PCSK9 expression by $> 90\%$ without inducing cytopathic effects. **Potential Pitfalls & Alternative Approaches:** High GC-content in the promoter region may limit guide binding or induce low transcription efficiency. If so, we will screen guides targeting the upstream enhancers or test alternative chromatin repressors like dCas9-DNMT3A-DNMT3L.

**Aim 2: Evaluate Efficacy, Biodistribution, and Genomic Safety in Humanized Mouse Models: Rationale:** In vitro screening cannot model systemic clearance, tissue-specific biodistribution, or blood-lipid kinetics. We must validate our candidate sgRNAs in a physiologically relevant animal model. **Research Design:** The lead sgRNAs and dCas9-KRAB mRNA will be encapsulated in LNPs using a microfluidic mixing system. We will utilize humanized PCSK9 transgenic mice (huPCSK9-Tg). Mice will be randomized into treatment and control groups (n = 8 per group) and injected with a single tail-vein dose ranging from 0.1 to 1.5 mg/kg. Livers will be harvested at day 7, 30, 90, and 180 post-injection. We will track serum PCSK9 and LDL-C levels weekly. Genomic safety will be verified by liver-specific RNA-seq to screen for

global transcriptional changes and ChIP-seq to verify the absence of off-target H3K9me3 marks. **Expected Outcomes:** Dose-dependent reduction of serum PCSK9 and LDL-C by at least 80%, with stable therapeutic suppression sustained over a 6-month period, without significant elevations in serum liver enzymes (ALT/AST). **Potential Pitfalls & Alternative Approaches:** Liver-specific delivery may be suboptimal. If liver uptake is low, we will optimize LNP surface properties using ApoE-targeting ligands or adjust the ionizable-to-neutral lipid ratio.

**Aim 3: Assess Long-Term Durability, Safety, and Lipid Changes in Non-Human Primates: Rationale:** Non-human primates represent the gold standard for validating lipoprotein metabolism and safety before clinical trials, owing to their close homology to human PCSK9 and lipid biochemistry. **Research Design:** Healthy cynomolgus macaques (n = 4 per group) will receive a single intravenous infusion of LNP-CRISPRi at the optimal dose determined in Aim 2. Serial blood samples will be collected to monitor serum PCSK9, total cholesterol, LDL-C, high-density lipoprotein cholesterol (HDL-C), and triglycerides. Hepatic tissue biopsies will be performed at week 4, 24, and 48 to assess tissue architecture, inflammatory cell infiltration, and dCas9-KRAB clearance. Cellular immune responses will be measured by interferon-gamma ELISpot assays using macaque PBMCs stimulated with dCas9 peptides. **Expected Outcomes:** Durable silencing of hepatic PCSK9 resulting in a stable >60% decrease in circulating LDL-C for up to 12 months, with no chronic hepatic inflammation or adverse systemic immune reactions. **Potential Pitfalls & Alternative Approaches:** Transient immune responses against the bacterial-derived dCas9 protein may occur. If neutralizing antibodies or T-cell responses are observed, we will test transient immunosuppressive regimens or utilize engineered, low-immunogenicity dCas9 variants.

### 1.4 Project Timeline & Milestones

The proposed 5-year timeline is designed to ensure systematic progress from in vitro optimization to NHP preclinical validation.

Table 1: **Project Timeline and Milestone Matrix.** Shaded cells indicate active years for each sub-task.

| Research Activities & Milestones | Year 1 | Year 2 | Year 3 | Year 4 | Year 5 |
|---|---|---|---|---|---|
| **Specific Aim 1: sgRNA Design and Screening** | | | | | |
| sgRNA Library Design & Synthesis | ■ | ■ | | | |
| In vitro screening | | ■ | ■ | | |
| Chromatin remodeling profiling (ChIP-qPCR) | | ■ | ■ | | |
| **Specific Aim 2: Mouse Model Efficacy & Safety** | | | | | |
| LNP formulation and physical characterization | | ■ | ■ | | |
| In vivo mouse trials (qPCR, ELISA, LDL-C tracking) | | | ■ | ■ | ■ |
| Off-target genomic & epigenomic sequencing | | | ■ | ■ | ■ |
| **Specific Aim 3: Preclinical NHP Validation** | | | | | |
| NHP protocol approval and baseline profiling | | | | ■ | ■ |
| In vivo NHP studies (12-month longitudinal study) | | | | ■ | ■ |
| Long-term safety, histology, and immunology assays | | | | ■ | ■ |

## 2 Literature Cited

[1] Cohen JC, et al. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. *N Engl J Med.* 2006;354:1264-1272.

[2] Thakore PI, et al. Highly specific epigenome editing by CRISPR-Cas9 repressors for targeting diverse gene promoters. *Nat Methods.* 2016;13:127-137.

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[4] Thakore PI, et al. Highly specific silencing of select genes by dCas9-KRAB in human cells. *Genome Res.* 2018;28:1163-1174.

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