RESEARCH HIGHLIGHT

Persistent in vivo epigenetic silencing of *Pcsk9*

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The quest for safer therapeutic alternatives to genome editing heralds epigenome editing as a promising approach. In a recent study published in *Nature*, Cappelluti et al. demonstrated robust, long-term *Pcsk9* silencing in vivo with a single administration of epigenetic effectors delivered by lipid nanoparticles, marking a significant stride towards the clinic for epigenetic editing therapies.

The epigenome consists of various chemical modifications to DNA and chromatin that modulate gene expression without altering DNA sequence.[1] Epigenome editing, which manipulates these modifications, has emerged as a safer approach than traditional genome editing for controlling gene expression. Several studies have accomplished targeted, durable silencing, in vivo, albeit using persistently expressed epigenetic effectors; however, long-term gene repression via transient epigenetic effector delivery has remained elusive until now.[2] Previously, the combination of three epigenetic effectors, Krüppel-associated box (KRAB), the catalytic domain of DNA methyltransferase 3A (cdDNMT3A), and DNA methyltransferase 3L (DNMT3L), has been shown to trigger persistent gene silencing in vitro.[3] KRAB, a transcriptional repressor, establishes repressive histone marks (H3K9me3 and H3K27me3) while DNMT3A and DNMT3L install DNA methylation at CpG dinucleotides.[4,5] Thus, fusion of these effectors to DNA-binding domains (DBDs) enables targeted, robust, long-term gene repression. In a recent study published in *Nature*, the authors showed that lipid nanoparticle (LNP)-mediated delivery of these engineered transcription repressors (henceforth referred to as ETRs) enables long-term silencing of *Pcsk9* in mouse livers for almost one year.[6]

Pcsk9 expression is tightly linked to low-density lipoprotein (LDL) cholesterol levels: repression of *Pcsk9* ameliorates hypercholesterolemia. Thus, persistent epigenetic silencing of *Pcsk9* holds promise as a long-term therapeutic solution that does not require DNA sequence alteration.[7] Although there exist several Food and Drug Administration approved strategies for *Pcsk9* inhibition, including monoclonal antibodies and siRNA-based therapeutics,[8] treatment of hypercholesterolemia by silencing *Pcsk9* serves as a benchmark for new therapies, such as epigenetic editing, due to the established biological and pathological mechanisms and the capacity for efficient LNP-mediated mRNA delivery into the liver.

Prior to evaluating the efficacy of ETRs in vivo, the authors performed multiple rounds of in vitro optimization in a Hepa1-6 cell line to identify the optimal system to target *Pcsk9*. Of the DBDs tested, which included zinc-finger proteins (ZFPs), nuclease-deactivated CRISPR-associated protein (dCas9), and transcription activator-like effectors (TALEs), ZFP produced the most robust *Pcsk9* silencing effect. Upon LNP-mediated delivery of the three separate ZFP-ETRs in vivo, the authors observed a ~60% reduction in PCSK9 levels for 330 days, accompanied by a ~35% decrease in LDL cholesterol levels. Comparable *Pcsk9* silencing was observed in a subset of mice subjected to partial hepatectomies, indicating that ETR-mediated *Pcsk9* repression could withstand cell division. Repression was likely induced by an increase in CpG methylation at the *Pcsk9* promoter, which remained stable over time in ZFP-ETR-treated mice, regardless of partial hepatectomy status (Fig. 1).

Combinatorial delivery of ZFP-KRAB, ZFP-cdDNMT3A, and ZFP-DNMT3L enabled durable *Pcsk9* silencing (Fig. 1), but a three-component system makes therapeutic delivery challenging. As such, the authors engineered EvoETR-8, a single, tripartite molecule consisting of a ZFP fused to cdDNMT3A and DNMT3L at its C-terminus and KRAB at its N-terminus. In vivo, EvoETR-8 also effectively repressed *Pcsk9* expression, reducing serum levels by 75% and LDL cholesterol levels by 26%, over 43 days. However, the duration of long-term silencing past day 43 remains to be evaluated, as measurements were not taken past this time point. As with the three ZFP-ETRs, EvoETR-8 repressed *Pcsk9* expression via deposition of CpG methylation at its promoter region (Fig. 1).

Although other examples of epigenetic silencing have been reported, this study represents the first instance of durable gene silencing in vivo following transient delivery of epigenetic effectors.[2] As such, this work not only has the potential to revolutionize the treatment of hypercholesterolemia but also paves the way for other epigenetic therapies by serving as a platform for modulation of gene expression in the liver without altering DNA sequence. While promising, significant work is needed to translate this breakthrough into the clinic.

One major benchmark for safety of genetic and epigenetic therapeutics is specificity. In vitro analysis of the specificity of EvoETR-8 in Hepa1-6 cells revealed three off-target differentially expressed genes (DEGs) and four off-target differentially methylated regions (DMRs). Overall, the genome-wide methylation levels were comparable between treated and untreated cells, and only one DMR corresponded to a DEG. Although the genome-wide off-target analysis was not repeated in vivo, quantification of methylation levels at the four DMRs identified in the in vitro analysis indicated that three of the four were also differentially methylated in vivo. While this data suggests a correlation between in vitro and in vivo off-target sites, a comprehensive evaluation of the specificity, and therefore safety, of EvoETR-8 requires further analysis at both transcriptional and methylation levels.

The application of EvoETR-8 in a therapeutic setting requires a thorough toxicity assessment. After LNP delivery of EvoETR-8 in vivo, transient increases were observed in the levels of several

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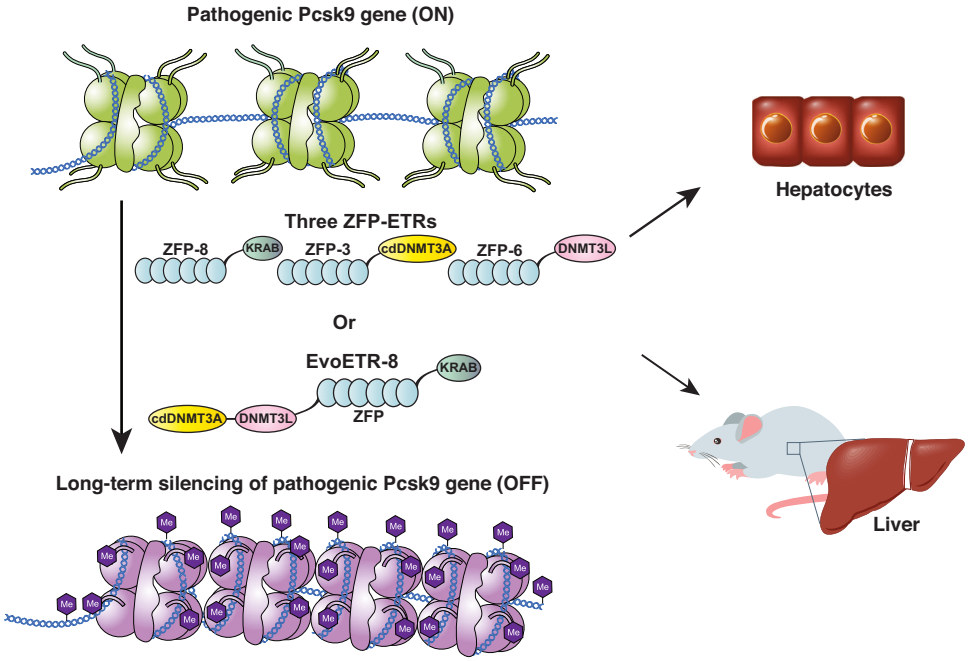

Fig. 1 ETRs and EvoETR-8 cause durable repression of *Pcsk9* in hepatocytes and in vivo. ETRs or EvoETR-8 install targeted histone and DNA methylation at *Pcsk9*, resulting in its long-term silencing in hepatocytes and mice. ETR engineered transcriptional repressor, EvoETR evolved engineered transcriptional repressor, ZFP zinc finger protein, KRAB Krüppel-associated box, cdDNMT3A DNA methyltransferase 3A catalytic domain, DNMT3L DNA methyltransferase 3L, Me methyl group.

liver enzymes, but they returned to baseline six days later. Notably, comparable elevation in liver enzyme levels was observed in mice treated with empty LNPs, suggesting that the vehicle itself triggered the toxicity response, not EvoETR-8. As such, further LNP optimization may mitigate toxicity.

Hypercholesterolemia is a promising therapeutic target for proof-of-concept demonstration of durable epigenetic silencing therapies, and several treatment options are currently available. Nevertheless, this study opens doors for the treatment of other liver diseases that could benefit from this technology, but several questions remain to be addressed. First, since the ZFP is driver of the EvoETR-8's silencing duration, efficacy, and specificity, EvoETR-8 must be re-optimized for each new target gene. Further, LNP-mediated delivery of EvoETR-8 limits the tissues to which it can be targeted. LNPs have natural liver tropism and can be engineered to target the spleen and lungs, but not yet other tissues.[9] Thus, safe epigentic editing of other organs awaits advancements in LNP technology, specifically in detargeting the liver. To apply EvoETR-8 to a wider spectrum of diseases, alternative delivery approaches must also be explored. Finally, despite the impressive demonstration of the durability of epigenetic silencing post hepatectomy, concerns persist regarding the potential long-term effects and stability of epigenetic modifications in a dynamically changing in vivo environment.

Overall, the authors demonstrated durable, robust epigenetic silencing of *Pcsk9* in vivo with a one-time administration of LNP. This study not only advances our understanding of epigenome editing as a viable strategy for durable gene silencing in vivo but also represents a promising step towards the development of an epigenetic therapeutic for hypercholesterolemia. Further, this work suggests a general approach for treating a wide range of diseases through durable epigenetic silencing. The continued exploration of specificity and long-term stability will be crucial in translating these innovations from bench to bedside.

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COMPETING INTERESTS

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ADDITIONAL INFORMATION

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