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***In vivo* genome editing of ANGPTL3: a potential therapeutic strategy for coronary atherosclerosis?**

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Standfirst (summary of the article)

Hyperlipidemia is an important risk factor for coronary heart disease. Chadwick and colleagues report significantly reduced blood lipid levels following CRISPR-based *in vivo* genome editing in mice to introduce loss-of-function mutations in ANGPTL3, a lipoprotein lipase inhibitor. The treatments were effective in both healthy and LpI^{-/-} mice and comparable to PCSK9-targeted genome editing, without causing off-target mutations.

There have been remarkable advances in understanding and treating coronary heart disease (CHD). Most notably, low-density lipoprotein (LDL) was identified to play a critical role in atherogenesis. Extensive efforts have been made to develop LDL-lowering therapies such as HMG-CoA reductase inhibitors (statins) and proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors with significant clinical success. High triglycerides (TG) have also been linked to increased risk of CHD, but initial results of treatments to block TG synthesis such as niacin and fibric acid derivatives have not yielded a clinically significant improvement in outcomes. Recently, a number of genes regulating TG-rich lipoprotein metabolism such as angiopoietin-like 3 (ANGPTL3) have been found to modulate CHD risk¹ and thereby emerged as possible therapeutic targets.

TG and other cholesterol circulate in the blood in the form of lipoprotein particles. By inhibiting lipoprotein lipase, ANGPTL3 regulates metabolism of these lipoproteins and subsequently alters blood lipid levels (Figure). In 2002, Koishi et al. first reported significant hypolipidemia in mice deficient in Angptl3.² Later, Musunuru et al. described a family whose members were affected by combined hypolipidemia due to compound heterozygous nonsense loss-of-function mutations in ANGPTL3.³ These family members exhibited marked reduction in the levels of TG and LDL, two important drivers of CHD risk, without evidence of coronary atherosclerotic plaque. These findings were further corroborated by a population-based study that demonstrated significantly decreased risk of CHD among heterozygous carriers for ANGPTL3 loss-of-function mutations. Given its distinct mechanism primarily targeting TG-rich lipoproteins, ANGPTL3 is poised to be a novel drug

target providing additional or synergistic therapeutic efficacy beyond LDL-lowering therapies.

Two ANGPTL3 inhibition therapies, an antisense oligonucleotides targeting Angptl3 messenger RNA and a human monoclonal antibody against ANGPTL3, recently advanced to phase I/II clinical trials.^{4,5} The results from the two trials are encouraging thus far. Both treatments were well tolerated, decreasing the levels of various lipid fractions such as LDL and TG. Notably, a small subset of patients with marked familial hyperlipidemia due to LDL receptor knockout (homozygous or composite heterozygous) also responded positively to the antibody treatment, demonstrating its potential to treat those who are otherwise resistant to currently available therapies. Presently, these two therapies are also being investigated in multiple cohorts of dyslipidemic patients.

Another emerging approach to inhibit ANGPTL3 is via *in vivo* gene-editing to permanently induce ANGPTL3 loss-of-function mutations. Since its first discovery in 2012, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology has garnered great excitement for its ability to introduce specific mutations at a desired location in the genome.⁶ However, potential undesired on-target mutagenesis from DNA double-strand breaks, off-target mutagenesis from insufficient specificity of guide RNA (gRNA), and inefficient on-target mutagenesis due to low rate homology-directed repair have combined to prevent effective *in vivo* genome editing.⁷ To circumvent these problems, researchers have recently developed a novel CRISPR-derived method to edit gene sequence without inducing DNA double-strand breaks.⁸ Known as a base editor, the system is composed of Cas9 nickase and gRNA to target a specific gene locus and a cytidine deaminase to convert cytosine to uracil at a particular position in the free strand. Cells then repair and replicate DNA using a uracil-containing strand as a backbone, ultimately changing the base C to T at a precise location. This ability to alter DNA sequence without cleaving its strands enables more precise and predictable gene editing by significantly lowering undesired indels at the site of double-strand breaks.

Using the third generation base editor (BE3) delivered via adenoviral vectors, Chadwick et al.⁹ now reported successful generation of ANGPTL3 loss-of-function mutations in mice. The authors first injected BE3 without gRNA (BE3-control) or BE3 with Angptl3-targeting gRNA (BE3-Angptl3) into healthy mice. After a week, deep sequencing of Angptl3 target sites in the liver tissue, the primary organ producing Angptl3, showed highly efficient genome editing with a median rate of 35% and virtually no off-target mutagenesis. In addition, blood levels of TG and total cholesterol were significantly reduced in BE3-Angptl3 group compared to the control. Similar treatment effects were also observed in *Ldlr*^{-/-} mice, consistent with prior observations. When comparing lipid-lowering efficiency of BE3-Angptl3 vs. BE3 targeting PCSK9 alone vs. BE targeting a combination of Angptl3 and PCSK9, there were comparable declines in cholesterol levels with all three therapies without evidence of combination approach superiority.

Overall, the study by Chadwick et al. successfully demonstrated the feasibility to safely and effectively introduce loss-of-function mutations in dyslipidemia-associated genes *in vivo* with significant lipid-lowering effects. There are several aspects that make ANGPTL3 an

attractive target for the BE therapy. First, ANGPTL3 is primarily synthesized in the liver. Liver is an ideal tissue type for *in vivo* genome editing because of its proliferative potential as well as tissue accessibility by intravenous treatments. Second, the complete knockout of ANGPTL3, a possible outcome of genome editing, has been shown to be safe based on both mouse and human data. Lastly, ANGPTL3 inhibition requires introduction of inactivating mutations, which is technically easier than precisely introducing a specific gene mutation.

While the study demonstrated comparable cholesterol-reducing ability between ANGPTL3-targeted therapy and PCSK9-targeted therapy, its effectiveness in preventing CHD is yet to be determined given their distinct cholesterol reducing profiles. ANGPTL3-targeted therapy is a more potent TG lowering therapy, whereas PCSK9-targeted therapy is a more potent LDL-lowering therapy. Interestingly, inhibiting both ANGPTL3 and PCSK9 did not result in any synergistic or additive effects. This may be in part due to inter-species differences in the way cholesterol is metabolized. Also, a combination of halved equal doses of BE-ANGPTL3 and BE-PCSK9 may not be the adequate dosage to create such effects. The combined therapy may also have more dynamic effects in dyslipidemic states, as reflected in possible changes in how these treatments might affect other forms of lipid fractions (e.g., LDL) and metabolic markers. While this study did not establish comparative efficacies of Angptl3 targeted therapy versus PCSK9 inhibitors or other cholesterol lowering therapies, it demonstrated great promise for lowering blood cholesterol and TG.

Will *in vivo* genome editing provide a potential curative strategy for CHD in humans? A chief benefit of genome editing lies in its ability to achieve reliable and durable therapeutic effects without requiring repeated administration of drugs by permanently modifying genes of interest. As medication non-adherence and variable therapeutic response to conventional therapies are the major hurdles in caring for patients with dyslipidemia, genome editing may one day provide an attractive lifelong solution especially for those with a severe form of familial hyperlipidemia. Prior to consideration for possible clinical translation, ethical and regulatory challenges as well as multiple hurdles must be overcome. First, continuing concerns over the risk of off-target mutagenesis and unintended on-target mutagenesis must be addressed, as these treatments cause irreversible genetic changes with possible serious adverse consequences. Second, prior or ongoing exposure to Cas9 may cause undesired immunologic side effects or treatment failure, preventing possible future use.¹⁰ Third, the use of viral vectors may cause potentially life-threatening reactions and therefore other methods of safe delivery should be explored.

In summary, the study by Chadwick et al. represents a proof-of-concept and hypothesis-generating work that demonstrate the feasibility of *in vivo* genome editing to induce inactivating mutations in Angptl3 and subsequently lower blood lipids. While further studies are needed to assess its efficacy and safety, the present work is an important addition to the field and a promising step toward potential clinical translation.

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Figure. Strategies to inhibit ANGPTL3 to lower blood lipids.

Various approaches have been developed to inhibit ANGPTL3, which include blocking antibodies, antisense oligonucleotides to block translation of ANGPTL3 messenger RNA, and finally genome editing to induce loss-of-function mutation at a gene level. When ANGPTL3 is inhibited, free lipoprotein lipase hydrolyzes TG-rich lipoproteins, subsequently lowering TG and other cholesterol levels. ANGPTL3, angioprotein-like 3; CRISPR, clustered regularly interspaced short palindromic repeats; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9