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A Peek into a Plausible Cool Future: Genome Editing to Delete PCSK9 and Control Hypercholesterolemia in a Single Shot

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Cholesterol management is the centerpiece of cardiovascular (CV) risk reduction. The most commonly used tool for cholesterol control is a daily statin, and the recent AHA/ACC guidelines have sanctified this approach by endorsing widespread use of statins in large groups of patients, including those with CV disease (CVD), hypercholesterolemia, diabetes, and elevated CVD risk [1]. Challenges to the notion of a daily medication for cholesterol control have been introduced with the discovery of proprotein convertase subtilisin/kexin 9 (PCSK9)[2], a secreted serine protease that binds the low-density lipoprotein (LDL) receptor (LDLR) and targets it for lysosomal destruction. Thus, a hyperactive (gain-of-function mutant) PCSK9 causes hypercholesterolemia whereas a dysfunctional (loss-of-function mutant) PCSK9 causes lifelong low LDL-cholesterol (LDL-C) levels and protection against CVD[3]. The basic mechanism of PCSK9 action[4] and its crystal structure[5] made it quite clear very early on that it would be difficult to develop a small molecule inhibitor for this protein. However, since PCSK9 is a secreted protein, it can be targeted with inhibitory antibodies, a modality that would change the paradigm of cholesterol treatment from oral to injectable and from daily to once or twice monthly dosing. A large number of clinical studies with the two leading antibodies have shown great efficacy and no safety signals, with LDL-C reductions in the range of 55-70% (even without concomitant use of statin) and no reports of myalgia (even in subjects with prior history of statin intolerance due to myalgia), transaminase elevations, or alterations in glucose metabolism[6]. In addition to neutralizing circulating PCSK9 via antibodies[6] or adnectins[7], current drug development strategies also include genetic modalities, such as antisense RNA[8] or RNA interference[9] to block PCSK9 synthesis. Given the likelihood that PCSK9 inhibitors will gain regulatory approval in the near future, it is appropriate to look into a plausible, more distant future of cholesterol management where the intervention is not bi-weekly or monthly, but a rather it is a one-time injection that permanently and selectively modifies the genome to inactivate a target gene whose function is undesirable. PCSK9 is a dream target for such strategy, as humans without circulating PCSK9 have been identified and shown to be healthy, fertile, and

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enjoying ultra low LDL-C levels and absence of CVD[3]. The paper by Ding*et al*.[10] in this issue of the *Journal* gives us a sense of where the technology stands at this point for genome editing in vivo in an adult mouse model, and of how feasible and effective such approach may be for human subjects affected by hypercholesterolemia as a way to permanently correct the metabolic problem.

Genome engineering technologies based on the RNA-guided Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR associated protein 9 (Cas9) system allow for the permanent alteration of the mammalian genome in a living organism[11]. The CRISPR/Cas9 system has been used for silencing, enhancing or changing specific genes in mice[12] and primates[13]. The CRISPR system is an exploitation of an immune mechanism used by some bacteria to defend themselves against viruses and based on the production of small RNAs matching portions of the invading viral DNA and of a nuclease (Cas) that will cleave the foreign genome at targeted sites. The technology is pretty handy and only relies on two short pieces of RNA, one that matches the targeted DNA region and one that binds to Cas9, which can be combined into a single guide RNA molecule that both targets a specific DNA sequence and recruits Cas9 nuclease. The therapeutic potential of this technology has two major applications: (1) targeted genome editing to correct genetic disorders by single-base repair[12] or promoter activation and (2) genome disruption or gene repression. Again, and at the cost of beating a dead horse, this technology can be applied to introduce permanent changes in the genome of adult animals of any species, including humans. It is apparent that this technological platform may be exploited to provide a cure for several monogenic and complex diseases, from anemia to Alzheimer's to autism.

Ding *et al.* used the adenoviral CRISPR/Cas9 system in a small, proof-of-concept study to disrupt the PCSK9 gene in adult mice via introduction of insertion and deletion mutations specifically in the liver via adenoviral infection[10]. The adenovirus targets both Cas9 and the CRISPR guide RNA to the liver, which is the main site of both PCSK9 expression and function. Using the CRISPR/Cas9 system the authors were able to mutagenize the PCSK9 gene in the liver, but the exceptionally high reduction in plasma PCSK9 levels (∼90%) cannot be explained solely by the mutagenesis rate (just above 50%). It is be kept in mind that hepatic LDLR is not only the main target of PCSK9 action but also the main regulator of circulating PCSK9 levels, because PCSK9 uses LDLR for its own plasma clearance[14]. In this case, reduced production of PCSK9 leads to high hepatic LDLR levels, which in turn further removes preexisting PCSK9 from the circulation, leading to extremely low PCSK9 levels, above what would be expected simply from the mutagenesis rate. In this scenario, the altered balance between LDLR and PCSK9 (increased LDLR levels due to reduced PCSK9 production) creates a cycle of events which leads to a new homeostatic balance between PCSK9 and LDLR (reduced PCSK9 levels due to increased LDLR-dependent clearance), caused by the well characterized reciprocal regulation between these two proteins[14,15]. The biology of PCSK9-LDLR interaction outside of the liver is much less defined, as there are tissues, such as the adrenal glands, where PCSK9 knockout or overexpression do not have an effect on LDLR levels [3]. Since PCSK9's effect on cholesterol metabolism is through its extracellular interaction with membrane LDLR, serum PCSK9 levels may be used as marker for PCSK9 activity. Indeed, PCSK9 and LDL-C levels are highly correlated not only

because they are both cleared by the LDLR, but also because up to 40% of the active form of PCSK9 (uncleaved and unaggregated) is physically associated with the LDL particle[16,17]. It is important to mention that the ELISA assays currently used to evaluate serum PCSK9 levels, including the one employed by the authors of this study, do not distinguish between active and inactive forms, and thus do not capture the specific information that adds insight on PCSK9 activity.

The CRISPR/Cas9 system has been used in adult mice to correct the Fumaryl-Acetoacetase Hydrolase (FAH) mutation in a model of the human disease Type I Tyrosinemia, a rare and severe autosomal recessive condition[12]. In this case the objective was to express functional FAH protein. Ding *et al.* used a different strategy, exploiting the unwanted consequences of PCSK9 action on cholesterol metabolism, thus targeting the gene for deletion. Using the CRISPR/Cas9 system the authors were able to induce a mutagenesis of the PCSK9 gene in the liver, followed by impressive increases in hepatic LDLR levels, decreases in total cholesterol levels from 35-40% (due by decreases in both LDL-C and HDL-C levels), and no changes in control analytes such as plasma triglyceride and transaminase levels[10]. Earlier studies showed similar effects from the removal of endogenous PCSK9 in the mouse system via standard gene deletion approaches[18].

It is important to note that there are differences between the mouse and human system in how PCSK9 affects serum lipids. These include: (1) modulation of HDL levels in mice but not in humans, (2) modulation of intestinal de-novo lipogenesis of human but not murine PCSK9[19], and (3) effects on triglyceride and lipoprotein(a) levels in humans[6] but not in mice[18]. Thus, targeted PCSK9 perturbation in humans may have additional effects of lipid metabolism beyond LDL-C reduction.

As a note of caution for the applicability of the current study to human disease management, the authors used an adenovirus to carry out the delivery of the CRISPR/Cas9 system to the liver, a method not applicable for human treatment because it triggers a host immune response. Other appealing non-viral approaches for the delivery of the CRISPR/Cas9 construct include: (1) lipid nanoparticles[20] (2) hydrodynamic tail injection[12] and (3) ultrasound micro-bubbles[21].

Another theoretical limitation of the CRISPR/Cas9 system is the possibility of off-target mutagenesis. In the small-scale experiment presented by Ding *et al*., no off-site mutagenesis was detected in a list of 10 selected genes that were most likely to be affected based on the CRISPR guiding sequence and similarities to the target gene. To turn the CRISPR/Cas9 into a valid therapeutic application, methods to detect and minimize off-target mutagenesis at the level of the whole genome are needed. More importantly, as this method involves permanent alteration of the genome, which unlike a drug cannot be undone, the safety and tolerability of life-long gene deletion must be confirmed for each target. Although beneficial loss-of-function mutations are rare, permanent partial disruptions of gene expression may have several other therapeutic applications, such as to gain protection against malaria via introduction of the sickle cell trait, or protection against HIV infection via deletion of CCR5[22]. Equally intriguing is the possibility of utilizing permanent gene perturbation in adult animal models for basic research purposes. Current techniques to modulate gene

expression *in vivo* include RNA inhibition, with the limitation of looking only at acute and transient effects, and gene knockout or overexpression, with the limitation of heavy financial burden and long periods required to generate, breed and maintain animals. The use of the CRISPR/Cas9 system as an efficient, tissue-specific delivery strategy with minimal to no off-target effects has the potential to become a new standard for introducing genomic changes in mice and with the tremendous advantage of being equally applicable to any other experimental model.

In conclusion, this study should serve as an introduction to our field of a revolutionary technology that holds the enormous promise to change the face of modern medicine. The study itself simply shows that PCSK9 is targetable with this novel approach, leading to all the expected and desirable consequences of PCSK9 inhibition or loss. The larger point is that one day we may be able to correct hypercholesterolemia permanently with a one-time injection of a biologic agent. The nearer future likely will show a massive adoption of this new technology in the basic sciences.

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References

1. Stone, Neil J., JR; Lichtenstein, Alice H.; Noel Bairey Merz, C.; Blum, Conrad B.; Eckel, Robert H.; Goldberg, Anne C.; Gordon, David; Levy, Daniel; Lloyd-Jones, Donald M.; McBride, Patrick; Sanford Schwartz, J.; Shero, Susan T.; Smith, Sidney C., Jr; Watson, Karol; Wilson, Peter WF. 2013 ACC/AHA Guideline on the Treatment of Blood Cholesterol to Reduce Atherosclerotic Cardiovascular Risk in Adults: A Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. Circulation published online November 12, 2013 2013. 2013 ACC/AHA Blood Cholesterol Guideline.
2. Abifadel M, Varret M, Rabes JP, et al. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. Nat Genet. 2003; 34:154–6. [PubMed: 12730697]
3. Seidah NG, Awan Z, Chretien M, Mbikay M. PCSK9: a key modulator of cardiovascular health. Circulation research. 2014; 114:1022–36. [PubMed: 24625727]
4. Zhao Z, Tuakli-Wosornu Y, Lagace TA, et al. Molecular characterization of loss-of-function mutations in PCSK9 and identification of a compound heterozygote. American journal of human genetics. 2006; 79:514–23. [PubMed: 16909389]
5. Hampton EN, Knuth MW, Li J, Harris JL, Lesley SA, Spraggon G. The self-inhibited structure of full-length PCSK9 at 1.9 A reveals structural homology with resistin within the C-terminal domain. ProcNatlAcadSciUSA. 2007; 104:14604–9.
6. Stein EA, Raal F. Reduction of low-density lipoprotein cholesterol by monoclonal antibody inhibition of PCSK9. Annu Rev Med. 2014; 65:417–31. [PubMed: 24422577]
7. Mitchell T, Chao G, Sitkoff D, et al. Pharmacologic Profile of the Adnectin BMS-962476, a Small Protein Biologic Alternative to PCSK9 Antibodies for LDL Lowering. The Journal of pharmacology and experimental therapeutics. 2014
8. Lindholm MW, Elmen J, Fisker N, et al. PCSK9 LNA antisense oligonucleotides induce sustained reduction of LDL cholesterol in nonhuman primates. MolTher. 2012; 20:376–81.
9. Fitzgerald K, Frank-Kamenetsky M, Shulga-Morskaya S, et al. Effect of an RNA interference drug on the synthesis of proprotein convertase subtilisin/kexin type 9 (PCSK9) and the concentration of serum LDL cholesterol in healthy volunteers: a randomised, single-blind, placebo-controlled, phase 1 trial. Lancet. 2014; 383:60–8. [PubMed: 24094767]

10. Ding Q, Strong A, Patel KM, et al. Permanent Alteration of PCSK9 With In Vivo CRISPR-Cas9 Genome Editing. Circulation research. 2014
11. Cong L, Ran FA, Cox D, et al. Multiplex genome engineering using CRISPR/Cas systems. Science (New York, NY). 2013; 339:819–23.
12. Yin H, Xue W, Chen S, et al. Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. Nature biotechnology. 2014; 32:551–3.
13. Niu Y, Shen B, Cui Y, et al. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. Cell. 2014; 156:836–43. [PubMed: 24486104]
14. Tavori H, Fan D, Blakemore JL, et al. Serum proprotein convertase subtilisin/kexin type 9 and cell surface low-density lipoprotein receptor: evidence for a reciprocal regulation. Circulation. 2013; 127:2403–13. [PubMed: 23690465]
15. Sasaki M, Terao Y, Ayaori M, et al. Hepatic overexpression of idol increases circulating protein convertase subtilisin/kexin type 9 in mice and hamsters via dual mechanisms: sterol regulatory element-binding protein 2 and low-density lipoprotein receptor-dependent pathways. Arterioscler Thromb Vasc Biol. 2014; 34:1171–8. [PubMed: 24675665]
16. Tavori H, Giunzioni I, Linton MF, Fazio S. Loss of Plasma Proprotein Convertase Subtilisin/Kexin 9 (PCSK9) After Lipoprotein Apheresis. Circulation research. 2013
17. Kosenko T, Golder M, Leblond G, Weng W, Lagace TA. Low-density lipoprotein binds to proprotein convertase subtilisin/kexin type-9 (PCSK9) in human plasma and inhibits PCSK9-mediated LDL receptor degradation. J Biol Chem. 201310.1074/jbc.M112.421370
18. Rashid S, Curtis DE, Garuti R, et al. Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. Proc Natl Acad Sci U S A. 2005; 102:5374–9. [PubMed: 15805190]
19. Rashid S, Tavori H, Brown P, et al. PCSK9 Promotes Intestinal Overproductin of Triglyceride-Rich Apolipoprotien-B Liporptoein Throug Both LDL-Receptor Dependent and Independent Mechanisms. Circulation. 201410.1161/CIRCULATIONAHA.113.006720
20. Shi Q, Liu P, Sun Y, et al. siRNA delivery mediated by copolymer nanoparticles, phospholipid stabilized sulphur hexafluoride microbubbles and ultrasound. Journal of biomedical nanotechnology. 2014; 10:436–44. [PubMed: 24730239]
21. Unger E, Porter T, Lindner J, Grayburn P. Cardiovascular drug delivery with ultrasound and microbubbles. Advanced drug delivery reviews. 2014; 72c:110–26. [PubMed: 24524934]
22. Niu J, Zhang B, Chen H. Applications of TALENs and CRISPR/Cas9 in Human Cells and Their Potentials for Gene Therapy. Molecular biotechnology. 2014

Nonstandard Abbreviations and Acronyms

CRISPR	clustered regularly interspaced short palindromic repeats
Cas	CRISPR-associated
ELISA	enzyme-linked immunosorbent assay
FAS	fumaryl-acetoacetase hydrolase
FH	familial hypercholesterolemia
LDL-C	low-density lipoprotein cholesterol
LDLR	low-density lipoprotein receptor
PCSK9	proprotein convertase subtilisin/kexin type 9