



CRISPR: a promising tool for lipid physiology and therapeutics

Mia Furgurson and William R. Lagor

Purpose of review

The purpose is to review recent progress in applying the CRISPR/Cas9 system to lipid metabolism and therapeutics.

Recent findings

The CRISPR/Cas9 system has been used to generate knockout animals for lipid genes in multiple species. Somatic genome editing with CRISPR/Cas9 can efficiently disrupt genes in adult animals, including a new strategy for generating atherosclerosis. Refinements to the CRISPR/Cas9 system including epigenetic modulators and base editors offer new avenues to manipulate gene expression. The recent report of germline genome editing in humans highlights the promise as well as perils of this technology.

Summary

CRISPR/Cas9 is a transformative technology that will help advance our understanding of lipid metabolism and physiology. Somatic genome editing is a particularly promising approach for editing genes in tissues of live organisms, and represents a new means of addressing unmet therapeutic challenges in humans. Educational outreach, public debate, and consideration of ethics and safety must guide the use of genome editing in humans.

Keywords

adeno-associated virus, CRISPR/Cas9, germline genome editing, lipid metabolism, somatic genome editing

INTRODUCTION

Genome engineering has become routine molecular biology practice with the development of the CRISPR/Cas9 system. This article reviews recent advances in applying CRISPR/Cas9 to the study of lipid metabolism – both for research and therapeutic purposes.

CRISPR/Cas9 stands for clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9. Cas9 is a programmable nuclease that generates double strand breaks in DNA in a complex with a guide RNA (gRNA). The gRNA is engineered by the user with the first ~20–23 nucleotides as an exact match to the target, based on the requirement for a protospacer adjacent motif (PAM) immediately downstream. The two most commonly used CRISPR/Cas9 systems from *Streptococcus pyogenes* and *Staphylococcus aureus* use PAM sequences of NGG and NNGRRT ($R = G/A$) respectively. The requirement for a PAM does limit, which sequences in the genome are targetable. However, many other CRISPR/Cas9 systems have recently been developed [1,2]. These efforts have been quite successful in either broadening the targetable PAM sequences of the Cas9 nuclease [3,4], or conversely further enhancing its specificity [5,6].

Following the creation of a double stranded break, the host cell's DNA repair mechanisms dictate

the types of DNA modifications that can be introduced. Nonhomologous end joining (NHEJ) is the preferred DNA repair pathway in postmitotic cells and involves error-prone ligation of blunt-ended DNA. Repeated cycles of NHEJ repair generate small insertion or deletion (indels) of bases at the DSB. The majority of these indels will shift the reading frame, effectively knocking out a gene. NHEJ can also be used to delete entire exons or larger sequences with two gRNAs [7], or to insert foreign DNA in a homology-independent manner [8]. In contrast, homology-directed repair (HDR) is a far more precise repair mechanism that only occurs during the S/G2 phase of the cell cycle. Performing HDR requires that the

Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas, USA

Correspondence to William R. Lagor, PhD, Department of Molecular Physiology and Biophysics, Baylor College of Medicine, One Baylor Plaza BCM 335, Houston, TX 77030, USA. Tel: +1 713 798 8666; e-mail: lagor@bcm.edu

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KEY POINTS

- CRISPR/Cas9 is a powerful tool for studying lipid metabolism and atherosclerosis.
- CRISPR/Cas9 enables the use of genetic models in species that were not previously targetable.
- Somatic genome editing with CRISPR/Cas9 allows for rapid interrogation of genes in adult organisms and has tremendous potential for human gene therapy.
- Base editing is a rapidly emerging field that allows for more precise editing without double-strand breaks.
- Safety and ethical concerns are important considerations for translation of this technology to human patients.

user supply a donor template that has homology to the 5' and 3' regions flanking the cut site. The sequence between the homology arms of the donor replaces the sequence between the homology arms at the endogenous locus [9,10[■],11[■]]. This method allows the user to precisely repair mutations or introduce completely new sequences.

CRISPR/Cas9 can be injected directly into fertilized oocytes [12], reducing the time and effort needed to make knockout animals. Cas9 cutting does not occur solely at the single cell stage, and the resulting animals are generally mosaic – composed of a mixture of cells that are either unedited or have one of several different mutations. Founders with the desired modifications can then be backcrossed and bred together to generate homozygous animals. This approach has been used to knockout genes in multiple species, many of which were previously impossible. Recently, Zhao *et al.* used CRISPR to delete *Ldlr* and *ApoE* in rat zygotes and implanted them into pseudopregnant rats. The *Ldlr* and *ApoE* double knockout rats had high levels of LDL-cholesterol (LDL-C) and developed atherosclerosis when placed on high-fat diet [13[■]]. Huang *et al.* used somatic cell nuclear transfer (SCNT) to generate a line of rabbits that were susceptible to atherosclerosis. The group used donor nuclei from a CRISPR-modified pig embryonic fibroblast cell line in which *ApoE* and *Ldlr* were knocked out. The resulting piglets showed an increase in LDL-C, total cholesterol, triglycerides, and APOB [14[■]]. CRISPR/Cas9 was recently used to generate a hamster line deficient in LDLR. Heterozygous hamsters showed increased plasma cholesterol when placed on chow diet. Homozygous mutants spontaneously developed atherosclerosis on chow diet, and surprisingly, died prematurely because of atherosclerosis when maintained on a high-fat/high-cholesterol diet

[15[■]]. Lu *et al.* generated *Ldlr* knockout rabbits through zygote microinjection of CRISPR/Cas9 to model familial hypercholesterolemia. Knockout rabbits spontaneously developed atherosclerosis and hypercholesterolemia on a normal chow diet [16[■]]. These are likely only the first examples of how germline genome editing with CRISPR/Cas9 will be applied to generate new animal models for lipid research.

Somatic genome editing (SGE) involves the manipulation of the genome in any cells of an organism other than the germline. SGE has tremendous potential for human gene therapy of lipid disorders, where edits to the patients' own DNA could provide lasting correction. The first application of SGE to lipid metabolism involved disruption of Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9), a secreted protein that promotes the degradation of LDL receptor. Ding *et al.* [17] used an Adenoviral vector to deliver *Strep. pyogenes* Cas9 (SpCas9) and a gRNA targeting the *Pcsk9* gene to mouse liver *in vivo*. They observed increased LDLR protein expression and 35–40% lower plasma cholesterol levels 3–4 days after injection. Studies by the same group in 2016 showed that this approach can specifically reduce human PCSK9 levels through adenoviral delivery of CRISPR/Cas9 to human liver chimeric mice [18]. In the same year, Ran *et al.* identified a smaller Cas9 ortholog from *Staph. aureus* (SaCas9) [19] that fits easily into adeno-associated virus (AAV) vectors, currently the leading delivery platform for tissue-directed gene therapy in humans. The authors found that SaCas9 uses a more restrictive PAM than SpCas9, limiting targeting options, but also reducing the likelihood of off-target mutagenesis. Robust editing of murine *Pcsk9* (>40%) was achieved in the liver with the use of a single AAV vector. It is clear that PCSK9 is a superb target for LDL-lowering, bolstered by the success of several monoclonal antibodies in humans. The concept of a 'one shot' treatment to eliminate PCSK9 from the majority of the liver [20[■]] is appealing for patients with heterozygous familial hypercholesterolemia. It remains to be determined if the potential benefits of this approach outweigh the risks, and if such a strategy will be commercially viable. Somatic disruption with CRISPR/Cas9 could be applied to other dyslipidemias, particularly, triglyceride-lowering. As an example, *Angptl3* is a promising new candidate, which was recently disrupted *in vivo* through base editing [21[■]].

SGE is also a powerful tool to study lipid metabolism and physiology. Jarrett *et al.* [22[■]] used AAV8 vectors to deliver gRNA targeting the *Ldlr* to SpCas9 transgenic mice. They found that liver-directed

disruption of *Ldlr* was sufficient to produce hypercholesterolemia and atherosclerosis, and that deletion of *ApoB* provided protection from disease. Follow-up work developed an all-in-one AAV8 vector for liver-directed disruption of *Ldlr* using the SaCas9 nuclease [23[■]]. The authors compared this approach to PCSK9 overexpression as an alternative means of generating atherosclerosis. They found that a single injection of the AAV-CRISPR vector was sufficient to produce severe hypercholesterolemia and atherosclerosis, and that this was superior to PCSK9 overexpression in male mice. Although neither AAV-PCSK9 nor AAV-CRISPR are likely to replace the time-tested germline *Ldlr* knockout mice, these new strategies would be particularly useful in situations where complex genotypes are involved. Most recently, Fedoseienko *et al.* [24[■]] investigated the role of the COMMD-CCDC22-CCDC93 (CCC) complex in LDLR endosomal trafficking in the liver. In this article, the authors used SpCas9 transgenic mice treated with an adenoviral vector expressing gRNAs targeting the *Ccdc22* gene, which encodes a core component of the CCC complex. Efficient knockdown of *Ccdc22* was obtained, which was accompanied by reductions in the associated COMMD proteins, and a 35% increase in plasma cholesterol levels, establishing the importance of the CCC complex in hepatic lipoprotein clearance. Taken together, these studies show the utility of SGE for studying lipid metabolism, which may be a valuable alternative to antisense oligonucleotides and liver-specific knockout mice.

CRISPR/Cas9 can also be used for epigenetic regulation. Thakore *et al.* [25[■]] developed an AAV vector that expresses a catalytically dead SaCas9 fused to a Krüppel-associated box epigenetic repressor motif (KRAB domain). This was co-delivered into mice with another AAV expressing a gRNA targeting promoter elements of *Pcsk9*. This dual vector system succeeded in transcriptional silencing of *Pcsk9* in the liver, which significantly lowered plasma cholesterol and PCSK9 protein levels, albeit less efficiently than disruption approaches [17,19]. Interestingly, the dSaCas9-KRAB protein did appear to elicit an immune response based on gene expression profiles in the liver. Transcriptional activation with CRISPR/Cas9 has been difficult to achieve *in vivo*, at least partially owing to delivery challenges. To address this limitation, Liao *et al.* [26[■]] engineered truncated gRNAs (dgrRNAs) containing aptamers that can recruit MS2 domain-containing proteins fused to transcriptional activation domains. This approach uses wild type Cas9, as the shorter dgrRNAs (~15nt) do not allow for DNA cleavage, and the transactivation domains are separately recruited via the dgrRNA-aptamer backbone. The authors showed that

in-vivo target gene activation could rescue disease in the *Mdx* mice with overexpression of *Klotho* and *Utrophin*, and promote the transdifferentiation of liver cells to insulin producing cells with *Pdx1* activation. Although the authors delivered SpCas9 with an AAV9 vector, the vast majority of the work involved the SpCas9 transgenic mice, as efficient delivery of full length SpCas9 with AAV remains challenging. Nonetheless, this work is an impressive example of the power of CRISPR/Cas9 for target gene activation *in vivo*, and could be applied to lipid genes for therapeutic benefit. In addition to epigenetic regulation at the genomic level, there is also considerable interest in CRISPR effectors for RNA targeting. Konermann *et al.* [27[■]] developed a compact RNA-specific nuclease from *Ruminococcus flavefaciens* XPD3002, termed CasRx. A catalytically dead version of CasRx was fused to a fragment of the negative splicing factor hnRNPa1 to mediate exon skipping in a gRNA-dependent manner. By targeting *cis*-acting elements in the premRNA of *MAPT* for exon exclusion, the authors succeeded in reducing the 4R/3R tau ratios to a normal level in human-induced pluripotent stem cell-derived cortical neurons. Although delivery of large CRISPR-based epigenetic regulators is challenging, it is clear that this is an exciting avenue for further innovation and discovery.

Single nucleotide variants (SNVs) are the most common cause of monogenic diseases in humans. Although correcting SNVs with CRISPR/Cas9 is possible with HDR approaches, these are limited by the requirement for cell division, and still involve the generation of DSBs, which can introduce unwanted modifications. David Liu's group developed the first base editor in 2016 – a novel approach to make more precise single nucleotide changes [28]. Base editors allow for the specific conversion of a single base without generating a DSB. A dead Cas9 or Cas9 nickase is fused to a deaminase on its N-terminus, often in conjunction with a uracil glycosylase inhibitor on its C-terminus. Base editors can bind DNA in a gRNA-dependent manner and convert a cytidine to uridine, facilitating a C-T transition [28], or an adenine to an inosine, facilitating an A-G transition [29[■]], within a small editing window. The occurrence of off-targets with base editing is believed to be higher than that of traditional CRISPR (<https://www.biorxiv.org/content/early/2018/11/27/480145>). In order to reduce the amount of off-targeting by base editors, several modifications have been made to expand the range of targetable sites and reduce the amount of off-target base switching [3[■],29[■],30[■],31[■],32[■]]. As base editors are so large in size, they have been delivered into animals by adenovirus or with AAV using a split-intein system

[33[□]]. Base editors have been used to successfully treat hearing loss [34[□]], and dyslipidemias in adult mice [21[□],35[□]] and in utero [36[□]].

In 26 November 2018, Dr He Jiankui announced that he had created the first two genetically engineered human beings [37[□]]. CRISPR/Cas9 was used to disrupt the *CCR5* gene in early human embryos with the use of in-vitro fertilization. The *CCR5* gene encodes the major receptor for HIV, and individuals with loss of *CCR5* are protected from HIV infection. Two of the manipulated embryos were implanted into the female donor, and the pregnancy resulted in a pair of twin girls. On the basis of data presented at the *Second International Summit on Human Genome Editing* in Hong Kong, one of the twins completely lacks *CCR5*, whereas the other has at least one normal copy of the gene. Dr Jiankui's announcement provoked widespread condemnation from the scientific community. Many expressed concern that there were simpler ways to prevent HIV transmission, and there were not sufficient grounds to justify this risky procedure. Questions have been raised about the trial itself, and whether regulatory approval and informed consent were properly obtained. Genome editing experts have criticized the secretive manner in which the work was done, particularly the general disregard for the ethical norms of the scientific community.

These developments highlight a problem with emerging technologies like genome editing. The field is moving very fast, easily outpacing the public's understanding of what is happening. Editing the human germline represents a significant departure from everything that has been done up until this point in medicine. By changing our genetic code, we are changing the very essence of what it means to be human. The first and most fundamental question is whether this should be done at all? How can we be certain that germline genome editing is well tolerated, and that debilitating side effects will not occur? How could we deal with the daunting problem of mosaicism, where different edits happen in different cells? What about off-targets? Costly genetic treatments may widen the socioeconomic gap because only the wealthy will be able to prevent their children from having life-altering disease. When does genome editing become enhancement rather than therapeutic? The concern is that this technology will allow us to engineer designer babies with all of our preferred skills and attributes. In its extreme form, this would create two classes of human beings – those who are genetically engineered and those who are not. It is important to realize that everyone has a stake in this issue, not just the patients or families participating in a trial, or scientists passionate about advancing the technology.

In summary, CRISPR/Cas9 is a powerful research tool for studying lipid metabolism and physiology. If important safety and ethical concerns can be addressed, it has tremendous potential for the treatment of lipid disorders and cardiovascular disease. We can expect exciting discoveries and further developments in the coming years from this transformative technology.

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Conflicts of interest

There are no conflicts of interest.

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- of special interest
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