COMMENTARY

Stepping toward therapeutic CRISPR

Keith T. Gagnon[a,1] and David R. Corey[b,1]

[a]Department of Biochemistry and Molecular Biology, University of Southern Illinois, Carbondale, IL 62901; and [b]Departments of Pharmacology and Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX 75390-9041

Most new technologies for manipulating gene expression in mammalian cells are accepted at a relatively slow pace. Occasionally, however, a new technology is so robust and fills such a critical niche that its adoption is widespread and rapid. Fifteen years ago, duplex RNAs were such a technology. RNA interference (RNAi) in mammalian cells was first demonstrated in 2001 (1) and within 2 y RNAi was a commonly used tool throughout industry and academia. RNAi is making its way into clinical trials as a potential therapeutic as challenges in delivery to relevant tissues begin to be overcome (2–4).

More recently, another revolution in biology appears to be emerging, powered by bacterial type II clustered, regularly interspaced, short palindromic repeats (CRISPR)-associated (Cas) systems. In PNAS, Rahdar et al. take a step toward a strategy that combines genetic and synthetic approaches for delivery of active CRISPR-Cas in vivo (5).

CRISPR-Cas is based on a natural bacterial defense mechanism for controlling pathogens (6). The realization that CRISPR-Cas can efficiently direct cleavage of double-stranded DNA in diverse biological systems has rapidly transformed it into a deft tool for genome editing (7–9). The most popular CRISPR-Cas system makes use of the Cas9 endonuclease from *Streptococcus pyogenes*. Cas9 binds a short 42-nt-long CRISPR RNA (crRNA) and an 80-nt transactivating crRNA (tracrRNA). The crRNA has a variable guide sequence that directs Cas9 endonuclease activity to sequence-specifically cut both strands of a DNA target. Cleavage typically introduces insertions or deletions through errors in natural DNA repair mechanisms. The presence of an appropriate donor DNA can also result in accurate insertion of new sequences through homology-directed repair. By this method, permanent changes to the genome are accomplished. Thus, CRISPR-Cas represents a powerful research tool for understanding gene function (10).

The widespread adoption of CRISPR-Cas provides objective evidence for its reliable use as a tool to investigate the basic biology of cellular processes. It is clear that, like duplex RNA for gene silencing, laboratory applications for CRISPR-Cas will proliferate (10–12). Moving beyond such applications, the potential for applying CRISPR-Cas to therapeutic development is less certain (13).

Traditional small-molecule synthetic drugs are usually below 500 Da in molecular weight. In contrast, CRISPR-Cas is a large complex formed by a protein and two RNA molecules. Present technologies do not offer straightforward solutions for direct entry of such complexes into tissues and cells if administered to patients. Antibodies form an increasingly successful class of drugs, but this success has been facilitated by the fact that, unlike CRISPR-Cas, they function by binding targets outside of cells and do not need to be internalized.

One option would be to use gene therapy to introduce vectors designed to express the endonuclease domain and a fusion of the crRNA and tracrRNA domains, called a single guide RNA (sgRNA) (13). One problem for solely relying on gene therapy to deliver CRISPR components is that, unlike synthetic drugs, where administration can be stopped, once expressed inside cells a fully functional CRISPR-Cas complex might be difficult to turn off, especially if the Cas9 and sgRNA genes are incorporated into the genome. Gene therapy to deliver all CRISPR components may ultimately prove to be a successful
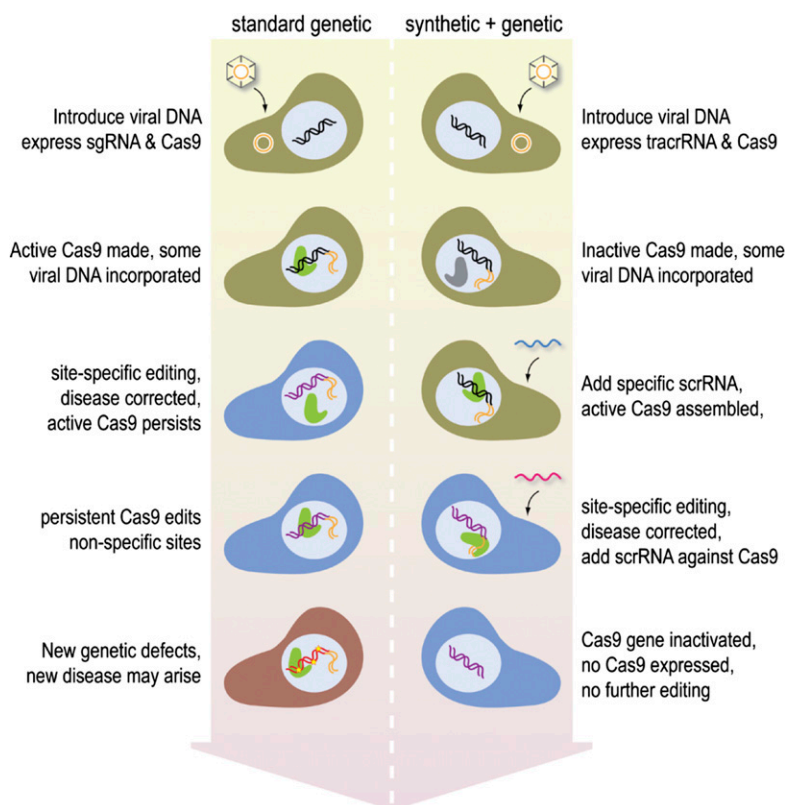

Fig. 1. Potential approach to synthetic CRISPR-Cas9 therapeutics envisioned by Rahdar et al. (5).

approach for some applications, but the lack of control may prove limiting in some cases.

One strategy to increase control over the activity of a CRISPR drug would be to introduce one of the components as a synthetic molecule. This compound would be more like a traditional drug and therefore be administered as needed. Because the Cas9 endonuclease is a large protein that must act inside cells, it is a poor candidate for direct introduction as an intact protein. Methods for introducing synthetic RNA oligonucleotides into cells in vivo are more advanced, opening the possibility of delivering crRNA alone or in combination with the tracrRNA domain as a chimeric sgRNA.

A synthetic 100-nt sgRNA with partial chemical modification was recently demonstrated to successfully guide gene editing by CRISPR-Cas9 (14). Although efficient long-RNA chemical synthesis techniques are available and useful for laboratory research, 100-mer oligonucleotides are cumbersome to synthesize and unlikely to be viable drug development candidates in the near term. In addition, the ability of such large negatively charged molecules to enter cells in vivo and function effectively is unknown. In contrast, the crRNA domain that guides recognition of DNA is just 42 nucleotides in length, not much longer than antisense oligonucleotides (ASOs) or duplex RNAs that are currently being developed for therapeutic use.

ASOs have been the focus of drug development for almost three decades (15). Those who do not follow the field closely could be forgiven for expressing substantial skepticism toward the approach. ASOs are large molecules, greater that 3,000 Da, and have many negative charges. These properties differentiate ASOs from typical successful small-molecule drugs. Progress bringing ASOs through the Food and Drug Administration approval process has been slowed by the need to develop a new pharmacological science to guide their synthesis, development, and delivery. Recently, however, one ASO drug has been approved for systemic administration (16) and several ASO or duplex RNA drugs are showing promise in clinical trials. These successes highlight the value of basic scientific advances and their application to nucleic acid drug development. The lessons learned from clinical application of ASOs provide a toolbox for developing crRNAs for use in vivo.

Rahdar et al. (5) reason that, because the endonuclease domain would need to be delivered by gene therapy, the vector might also encode the tracrRNA. Expression of these genes would provide an inactive CRISPR-Cas complex. Activation would only require programming with a short crRNA, and this could be achieved through introduction of a synthetic crRNA (scrRNA) designed to guide the complex to a target DNA of choice. The authors envision a combined genetic and synthetic system that could prove safer and more reversible

Rahdar et al. take a step toward a strategy that combines genetic and synthetic approaches for delivery of active CRISPR-Cas in vivo.

compared with a genetic-only approach (Fig. 1).

Unmodified synthetic RNA is not well-suited for drug development because it is unstable, subject to digestion by nucleases, and has poor pharmacokinetic properties (17). Therefore, Rahdar et al. (5) introduced chemical modifications into scrRNA. These modifications included phosphorothioate (PS) nucleotide linkages that resist nuclease digestion and improve pharmacokinetics. The authors also tested modifications to improve affinity for target DNA, including 2′-O-methyl (2′-O-me) RNA, 2′-fluoro (2′-F) RNA, and constrained ethyl (cET) RNA. The challenge posed by introducing these modifications is that they must be compatible with preservation of CRISPR-Cas function.

Initial experiments showed that scrRNAs altered with PS and 2′-O-Me modifications were more efficient at genome editing than unmodified crRNA. However, efficiency was lower than a standard sgRNA produced by transcription within cells. To achieve improved editing efficiency, Rahdar et al. (5) systematically tested chemical modifications at differing positions within the 42-base scrRNAs. These modifications were introduced into guide regions that recognize DNA targets or tracrRNA-interacting regions, and they improved the efficiency of gene disruption up to 75% of that achieved by expressed sgRNA. Additional modification schemes and truncation minimized the size of the scrRNA to as little as 29 nucleotides, with activity equal to or greater than the benchmark sgRNA. At 29 bases, the scrRNAs are not much bigger than standard ASOs being tested in the clinic (~20 bases) and, unlike duplex RNAs currently in clinical use, they contain just one nucleic acid strand.

These proof-of-principle experiments are one critical step toward the eventual development of CRISPR-Cas systems for therapeutic application. Like gene therapy or nucleic acid therapeutics, the development CRISPR-Cas as a treatment strategy will not happen overnight. Much needs to be learned about designs to optimize and improve potency while reducing off-target effects (18, 19), in addition to the challenges of in vivo delivery (20). Because development is unlikely to be easy, success will require testing many different options. The semisynthetic/semigenetic option outlined by Rahdar et al. (5) provides an attractive path forward that can build on the growing foundation of existing gene therapy and nucleic acid therapeutics.

1 Elbashir SM, et al. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411(6836):494–498.
2 Borna H, Imani S, Iman M, Azimzadeh Jamalkandi S (2015) Therapeutic face of RNAi: In vivo challenges. *Expert Opin Biol Ther* 15(2):269–285.
3 Kubowicz P, Żelaszczyk D, Pękala E (2013) RNAi in clinical studies. *Curr Med Chem* 20(14):1801–1816.
4 Kanasty R, Dorkin JR, Vegas A, Anderson D (2013) Delivery materials for siRNA therapeutics. *Nat Mater* 12(11):967–977.
5 Rahdar M, et al. (2015) Synthetic CRISPR RNA-Cas9–guided genome editing in human cells. *Proc Natl Acad Sci USA*, 10.1073/pnas.1520883112.
6 Wiedenheft B, Sternberg SH, Doudna JA (2012) RNA-guided genetic silencing systems in bacteria and archaea. *Nature* 482(7385):331–338.
7 Jinek M, et al. (2012) A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *Science* 337(6096):816–821.
8 Mali P, et al. (2013) RNA-guided human genome engineering via Cas9. *Science* 339(6121):823–826.
9 Cong L, et al. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339(6121):819–823.
10 Sternberg SH, Doudna JA (2015) Expanding the biologist's toolkit with CRISPR-Cas9. *Mol Cell* 58(4):568–574.
11 Riordan SM, Heruth DP, Zhang LQ, Ye SQ (2015) Application of CRISPR/Cas9 for biomedical discoveries. *Cell Biosci* 5:33.
12 Kennedy EM, Cullen BR (2015) Bacterial CRISPR/Cas DNA endonucleases: A revolutionary technology that could dramatically impact viral research and treatment. *Virology* 479-480:213–220.
13 Kaulich M, Dowdy SF (November 5, 2015) Combining CRISPR/Cas9 and rAAV templates for efficient gene editing. *Nucleic Acid Ther*, 10.1089/nat.2015.0545.
14 Hendel A, et al. (2015) Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. *Nat Biotechnol* 33(9):985–989.
15 Deleavey GF, Damha MJ (2012) Designing chemically modified oligonucleotides for targeted gene silencing. *Chem Biol* 19(8):937–954.
16 Rader DJ, Kastelein JJ (2014) Lomitapide and mipomersen: Two first-in-class drugs for reducing low-density lipoprotein cholesterol in patients with homozygous familial hypercholesterolemia. *Circulation* 129(9):1022–1032.
17 Braasch DA, et al. (2003) RNA interference in mammalian cells by chemically-modified RNA. *Biochemistry* 42(26):7967–7975.
18 Kim S, Kim D, Cho SW, Kim J, Kim JS (2014) Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Res* 24(6):1012–1019.
19 O'Geen H, Yu AS, Segal DJ (2015) How specific is CRISPR/Cas9 really? *Curr Opin Chem Biol* 29:72–78.
20 Peng R, Lin G, Li J (November 4, 2015) Potential pitfalls of CRISPR/Cas9-mediated genome editing. *FEBS J*, 10.1111/febs.13586.