HHS Public Access

Author manuscript

Arterioscler Thromb Vasc Biol. Author manuscript; available in PMC 2017 May 01.

Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2016 May ; 36(5): 783–786. doi:10.1161/ATVBAHA.116.307227.

CRISPR-Cas9 Targeting of *PCSK9* in Human Hepatocytes *In Vivo*—Brief Report

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Abstract

Objective—Although early proof-of-concept studies of somatic *in vivo* genome editing of the mouse ortholog of proprotein convertase subtilisin/kexin type 9 (*Pcsk9*) in mice have established its therapeutic potential for the prevention of cardiovascular disease, the unique nature of genome-editing technology—permanent alteration of genomic DNA sequences—mandates that it be tested *in vivo* against human genes in normal human cells with human genomes in order to give reliable preclinical insights into the efficacy (on-target mutagenesis) and safety (lack of off-target mutagenesis) of genome-editing therapy before it can be used in patients.

Approach and Results—We used a clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated 9 (Cas9) genome-editing system to target the human *PCSK9* gene in chimeric liver-humanized mice bearing human hepatocytes. We demonstrated high on-target mutagenesis (approaching 50%), greatly reduced blood levels of human PCSK9 protein, and minimal off-target mutagenesis.

Conclusions—This work yields important information on the efficacy and safety of CRISPR-Cas9 therapy targeting the human *PCSK9* gene in human hepatocytes *in vivo*, and it establishes humanized mice as a useful platform for the preclinical assessment of applications of somatic *in vivo* genome editing.

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DISCLOSURES

None.

Keywords

Gene therapy; PCSK9; lipids and lipoprotein metabolism; molecular biology

INTRODUCTION

Clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated (Cas) 9 systems have elicited enormous interest from the biomedical community due to their versatile use in research applications and, perhaps more so, due to their therapeutic potential in addressing human diseases.[1] As with any novel therapeutic approach, before any given CRISPR-Cas9 application can be used in the human body, it will need to undergo extensive preclinical testing. CRISPR-Cas9 and other genome-editing tools present an unusual challenge in that the target is DNA sequence in the human genome. While animal models such as rodents and non-human primates offer opportunities to assess the physiological consequences of *in vivo* therapies, they do not allow for accurate assessment of on-target and off-target mutagenesis by a CRISPR-Cas9 application targeted against a human gene, due to lack of conservation across genomes. What are needed are preclinical models in which the somatic *in vivo* targeting of human genes in normal human cells (i.e., not tumor cells) with human genomes can be performed.

Due to ease of delivery to the organ, as well as the diversity of grievous genetic disorders involving the organ, the liver has emerged as an early target of preclinical genome-editing applications.[2–8] Accordingly, we sought to establish the feasibility of using chimeric liver-humanized mice to assess for on-target and off-target effects of CRISPR-Cas9 *in vivo*. There are several mouse models in which endogenous hepatocytes can be replaced with primary human hepatocytes. Perhaps the best established is the $Fah^{-/-}Rag2^{-/-}Il2rg^{-/-}$ (FRG KO) mouse,[9–11] in which withholding of a specific drug in the diet [2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione; NTBC] results in the death of endogenous mouse hepatocytes from accumulation of a toxic metabolite [due to the *Fah* (fumarylacetoacetate hydrolase) deficiency], and which are immunocompromised ($Rag2^{-/-}Il2rg^{-/-}$) so as to accept transplanted human hepatocytes that can complement the deficient mouse liver function and rescue the animals.

Using the FRG KO mouse model, we targeted the human *PCSK9* gene because it is a prime therapeutic target in the prevention of cardiovascular disease and because the mouse *Pcsk9* gene has previously been the focus of successful *in vivo* genome-editing studies.[5,6] A demonstration of the efficacy and safety of human *PCSK9*-targeting therapy *in vivo* would provide a strong rationale for further preclinical studies with the aim of ultimately bringing to the clinic a one-shot CRISPR-Cas9 “vaccination” for the reduction of low-density lipoprotein cholesterol and cardiovascular risk.

MATERIALS AND METHODS

Materials and Methods are available in the online-only Data Supplement.

RESULTS AND DISCUSSION

Using primary human hepatocytes, we generated FRG KO mice with varying degrees of engraftment at least five months after transplantation (10% to 60% reconstitution of liver with human cells as judged by human serum albumin levels in the mice[11]). We generated adenoviruses bearing *Streptococcus pyogenes* Cas9 and either a guide RNA targeting a sequence in the first coding exon of the human *PCSK9* gene (CRISPR-*PCSK9*) (Figure 1A) or a guide RNA targeting a control sequence (CRISPR-control). Of note, the 20-nt *PCSK9* protospacer has six mismatches with the orthologous sequence in the mouse genome, and no NGG- or NAG-adjacent sequence in the human genome has less than four mismatches with the *PCSK9* protospacer (Supplemental Figures I and II).

We administered CRISPR-*PCSK9* virus ($n = 5$) or CRISPR-control virus ($n = 6$) to FRG KO mice. After four days, we found by Surveyor assay that all of the CRISPR-*PCSK9* mice had substantial mutagenesis at the on-target site (Figure 1B). We performed deep sequencing of PCR amplicons from liver samples of two of the CRISPR-*PCSK9* mice and identified indels in 47% and 42% of the sequence reads. This degree of mutagenesis is quite concordant with previous studies of CRISPR-Cas9 delivered by adenovirus or adeno-associated virus (AAV).[5–7] More than three-quarters of the identified indels were either 1-bp or 2-bp insertions or deletions at the expected CRISPR-Cas9 cleavage site (3 nt upstream of the 3′ end of the protospacer) (Figure 1A, Supplemental Dataset).

Consistent with these DNA-level changes, we found that post-treatment blood levels of human PCSK9 protein—produced and secreted specifically by engrafted human hepatocytes—were reduced on average by 52% compared to pre-treatment levels ($P = 0.007$; Figure 1C and Supplemental Figure III). This is a mildly greater effect than might be expected from the degree of on-target mutagenesis; of note, the mutagenesis detected by sequencing is almost certainly underestimated since next-generation DNA sequencing of small PCR amplicons fails to capture larger indels. Notably, we found that post-treatment blood levels of mouse PCSK9 protein were increased more than two-fold compared to pre-treatment levels ($P = 0.002$; Figure 1C and Supplemental Figure III), suggesting a compensatory mechanism at work within the mouse hepatocytes still present in the transplanted FRG KO mice. Presumably as a consequence, total cholesterol levels were not significantly changed (data not shown). Human albumin levels were unchanged (Figure 1C and Supplemental Figure III), confirming the stability of the engrafted human hepatocytes in the mice with CRISPR-*PCSK9* treatment.

We used deep sequencing to assess for off-target mutagenesis in the human genome at eight top candidate off-target sites by sequence similarity identified by the CRISPR Design server[12] and COSMID server[13] (Supplemental Figures I and II). Given the concordance in the degree of on-target mutagenesis observed among the mice in the CRISPR-*PCSK9* cohort by Surveyor assay, we felt that off-target data from two CRISPR-*PCSK9* mice and one CRISPR-control mouse would be representative of the entire cohort. There was no detectable off-target mutagenesis out of the range of background indel rates resulting from errors inherent in PCR amplification and next-generation DNA sequencing (Figure 1D).

Our results indicate that chimeric liver-humanized mice can be used as a platform to assess for on-target and off-target mutagenesis from CRISPR-Cas9 delivered to human hepatocytes *in vivo* by a somatic approach. Of note, AAV is regarded as a safer vehicle than adenovirus for therapeutic applications in humans, as it is better tolerated by the immune system, but it has a much more limited cargo size that is less conducive to the use of *S. pyogenes* Cas9 and large, strong, tissue-specific promoters. Although CRISPR-Cas9 has been adapted for use in AAV by means of a smaller Cas9 protein from *Staphylococcus aureus*,[6,7] the AAV serotypes that efficiently transduce mouse hepatocytes *in vivo* do not target human hepatocytes *in vivo* well, which means that AAV will need to be optimized before use in genome-editing applications in human liver, perhaps through the development of novel capsid proteins.[14] At present, the primary advantages of adenovirus for the testing of CRISPR-Cas9 applications in chimeric liver-humanized mice are that it efficiently targets human hepatocytes *in vivo* and that it allows for direct comparisons of safety and efficacy of large proteins intended to improve on-target specificity, such as FokI-Cas9 fusion proteins.[15,16]

Although the chimeric liver-humanized FRG KO mouse, being immunocompromised, does not model the immune consequences of using viral vectors to heterologously express bacterial Cas9 protein in the liver, this could potentially be addressed in the future by double humanization of FRG KO mice with respect to both the liver and the hematopoietic system.[17] Finally, we note the inherent challenge of assessing for off-target mutagenesis throughout the genome in a targeted organ with billions of cells. While we can rule out >0.1% events at a number of candidate off-target sites with deep sequencing, a more sophisticated approach will be needed to assess for rare events across the genome. Unbiased screening methods have been developed for use in cell lines *in vitro*,[6,18–20] and such methods will need to be adapted for use in living animals.

As these various issues are successfully addressed, we anticipate that studies in humanized animals will become an important component of the preclinical assessment of applications of somatic *in vivo* genome editing. Our specific results establish the efficacy of and suggest a favorable safety profile of CRISPR-Cas9 therapy targeting the human *PCSK9* gene in authentic human hepatocytes *in vivo* and support further development of the therapy with the goal of eventual clinical use for long-term protection against cardiovascular disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

SOURCES OF FUNDING

This work was supported by the Howard Hughes Medical Institute Medical Research Fellows Program (A.R.); the Shanghai Institutes for Biological Sciences Fellowship (Y5Y1X41491) (Y.Z.); the American Heart Association (Q.D.); the Hundred Talents Program of the Chinese Academy of Sciences (Q.D.); the Shanghai Pujiang Program (15PJ1409200) (Q.D.); the Harvard Stem Cell Institute (K.M.); and grants R01-HL118744, R01-GM104464, and R01-DK099571 from the United States National Institutes of Health (NIH) (K.M.).

ABBREVIATIONS

CRISPR-Cas9	clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated (Cas) 9
PCSK9	proprotein convertase subtilisin/kexin type 9
AAV	adeno-associated virus

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SIGNIFICANCE

Although early proof-of-concept studies of somatic *in vivo* genome editing in mice highlighted the therapeutic potential of CRISPR-Cas9 to target *PCSK9* and other disease-related genes, the unique nature of genome-editing technology—permanent alteration of DNA sequences—argues for it to be tested *in vivo* against human genes in normal human cells (i.e., not tumor cells) with human genomes in order to give reliable preclinical insights into the efficacy (on-target mutagenesis) and safety (lack of off-target mutagenesis) of a CRISPR-Cas9 therapy before it can be used in patients. We describe the use of chimeric liver-humanized mice as a means to do this for the human *PCSK9* gene in human hepatocytes *in vivo*, and we thereby establish the efficacy of CRISPR-Cas9 in human hepatocytes.

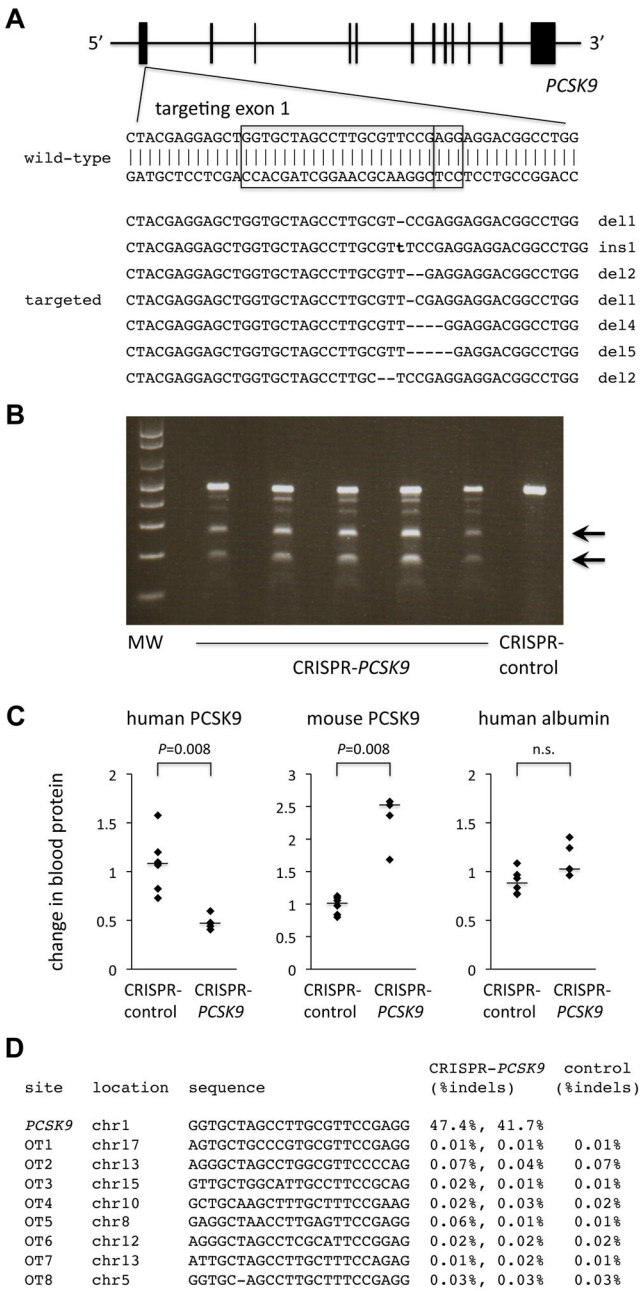

Figure 1.

CRISPR-Cas9 targeting of *PCSK9* in human hepatocytes *in vivo*. **A**, Targeting of a sequence in exon 1 of the human *PCSK9* gene. The boxes indicate the 20-bp sequence matching the protospacer and the 3-bp protospacer-adjacent motif (PAM). The seven targeted sequences shown below the wild-type sequence reflect the seven most common mutations (in descending order of frequency) detected by deep sequencing of the locus in human hepatocytes targeted *in vivo* in chimeric liver-humanized mice (see Supplemental Dataset for more information). **B**, Surveyor assays performed with genomic DNA from liver samples taken from mice 4 days after receiving an adenovirus expressing Cas9 and the *PCSK9* guide

RNA (CRISPR-*PCSK9*) or a control adenovirus (CRISPR-control). Arrows show the cleavage products resulting from the Surveyor assays; the intensity of the cleavage product bands relative to the uncleaved product band corresponds to the mutagenesis rate. **C**, Relative changes in blood human PCSK9 protein levels, blood mouse PCSK9 protein levels, and blood human albumin levels (post-treatment divided by pre-treatment levels) in chimeric liver-humanized mice receiving CRISPR-control virus ($n = 6$ mice) or CRISPR-*PCSK9* virus ($n = 5$ mice). The bars indicate the median values for the relative changes within the groups. The Mann–Whitney U test was performed to compare the relative changes in the two groups. **D**, Indel rates at on-target and off-target sites from next-generation DNA sequencing of liver samples from post-treatment mice.