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

Xiao Wang, Avanthi Raghavan, Tao Chen, Lyon Qiao, Yongxian Zhang, Qiu-Rong Ding, Kiran Musunuru

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 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 (Cas) 9 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. (Arterioscler Thromb Vasc Biol. 2016;36:783-786. DOI: 10.1161/ATVBAHA.116.307227.)

Key Words: gene therapy • liver • molecular biology • mutagenesis • subtilisins

Clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated (Cas) 9 systems have elicited enormous interest from the biomedical community because of their versatile use in research applications and, perhaps more so, because of their therapeutic potential in addressing human diseases. 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. Although 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 because of 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 (ie, not tumor cells) with human genomes can be performed.

Because of 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. 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, in which withholding of a specific drug in the diet [2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexadiene] results in the death of endogenous mouse hepatocytes from accumulation of a toxic metabolite [because of 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
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, there is a strong rationale for further preclinical studies with the virus (AAV).5–7 More than three quarters of the identified CRISPR-Cas9 samples of 2 of the CRISPR-Cas9 mice and identified performed deep sequencing of PCR amplicons from liver tissue-specific promoters. Although CRISPR-Cas9 has been adapted for use in AA V by means of a smaller Cas9 protein from Streptococcus pyogenes,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 sequencing of small PCR amplicons fails to capture larger indels. Notably, we found that posttreatment blood levels of mouse PCSK9 protein were increased more than 2-fold compared with pretreatment levels (P=0.002; Figure [C] and Figure III in the online-only Data Supplement), 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 [C] and Figure III in the online-only Data Supplement), 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 8 top candidate off-target sites by sequence similarity identified by the CRISPR Design server12 and COSMID server13 (Figures I and II in the online-only Data Supplement). 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 2 CRISPR-PCSK9 mice and 1 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 [D]).

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, AA V 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 AA V by means of a smaller Cas9 protein from Staphylococcus aureus,5,6 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. Although 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, 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
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 (ie, not tumor cells) with human genomes 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 in vivo, and we thereby establish the efficacy of CRISPR-Cas9 in human hepatocytes in vivo.
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SUPPLEMENTAL MATERIAL

Detailed Methods

**Screening of guide RNAs.** Candidate guide RNAs were designed by visual inspection of the sequences of exon 1 of the human PCSK9 gene. Guide RNAs were screened for on-target activity in HEK 293T cells as previously described. The guide RNA used (protospacer 5'-GGTGCATAGCCTTGCGTTCCG-3') was chosen because of its combination of high on-target activity in vitro and favorable off-target profiling (see Supplemental Figures I and II). An irrelevant guide RNA was used as a control (protospacer 5'-GTGCTTGATTGAGCAACCTC-3').

**Generation of adenoviruses.** The *Streptococcus pyogenes* CRISPR-Cas9 system and the guide RNA protospacers were inserted into the Adeno-X vector (Clontech) as previously described. The Penn Vector Core at the University of Pennsylvania used these vectors to generate recombinant adenoviral particles (designated CRISPR-PCSK9 and CRISPR-control).

**Animal studies.** All procedures used in animal studies were approved by the pertinent Institutional Animal Care and Use Committees at Harvard University and were consistent with local, state, and federal regulations as applicable. *Fah−/− Rag2−/− Il2rg−/−* (FRG KO) breeder mice on the C57BL/6 background were obtained from Yecuris Corporation. Mice were maintained on NTBC (also called Nitisinone; Yecuris) prior to transplantation according to the manufacturer's instructions. Twenty-four hours prior to transplantation, mice that were one to three months of age underwent intraperitoneal injection with $1 \times 10^9$ pfu of adenovirus expressing the secreted form of urokinase-type plasminogen activator (Yecuris). For transplantation, $1 \times 10^6$ primary hepatocytes (HEP10 Pooled Human Cryopreserved Hepatocytes; Thermo Fisher Scientific) were injected into the lower pole of the spleen. During the surgery, 1%-2% inhaled isoflurane was used for anesthesia, and 0.05-0.1 mg/kg subcutaneous buprenorphine was used as needed for analgesia in the perioperative and postoperative periods. Following transplantation, NTBC was gradually withdrawn over several weeks according to the manufacturer's instructions, and human albumin levels in the blood were monitored on a monthly basis using the Human Albumin ELISA Quantitation Set (Bethyl Laboratories) according to the manufacturer's instructions, with the levels used to estimate % reconstitution with human hepatocytes.

Chimeric liver-humanized mice that were 8 to 11 months of age (at least 5 months following transplantation) were used for experiments. After initial blood samples were collected, mice were administered $1 \times 10^{11}$ particles each via retro-orbital injection. 1%-2% inhaled isoflurane was used for anesthesia at the time of the injections. A total of five mice were given CRISPR-PCSK9 virus, and six mice were given CRISPR-control virus. As much as possible, the mice in the two groups were matched with respect to age and, to some degree, % reconstitution with human hepatocytes, though the mice with the best % reconstitution were preferentially used for the CRISPR-PCSK9 group. After four days, the mice were sacrificed by carbon dioxide asphyxiation after overnight fasting. Whole liver samples were harvested for DNA analysis, and terminal blood samples were collected. Pre-treatment and post-treatment human PCSK9 levels in the blood were measured using the Human Proprotein Convertase 9/PCSK9 Quantikine ELISA Kit (R&D Systems) according to the manufacturer’s instructions. Pre-treatment and post-treatment mouse PCSK9 levels in the blood were measured using the Mouse Proprotein Convertase 9/PCSK9 Quantikine ELISA Kit (R&D Systems) according to the manufacturer’s instructions. Pre-treatment and post-treatment human albumin levels in the blood were measured using the Human Albumin ELISA Quantitation Set according to the manufacturer’s instructions.
**On-target and off-target mutagenesis analyses.** To analyze the PCSK9 exon 1 on-target site, liver genomic DNA samples were isolated and PCR amplicons for the on-target site were subjected to Surveyor assays as previously described.\(^1\) PCR amplicons from two of the CRISPR-PCSK9 mice were subjected to next-generation DNA sequencing at the Massachusetts General Hospital CCIB DNA Core (CRISPR Sequencing service; [https://dnacore.mgh.harvard.edu/new-cgi-bin/site/pages/crispr_sequencing_main.jsp](https://dnacore.mgh.harvard.edu/new-cgi-bin/site/pages/crispr_sequencing_main.jsp)).

Off-target sites were predicted using the CRISPR Design server ([http://crispr.mit.edu/](http://crispr.mit.edu/))\(^3\) and the COSMID server ([https://crispr.bme.gatech.edu](https://crispr.bme.gatech.edu)).\(^4\) Seven of the top eight sites (all with a score > 0.1) from the list generated by the CRISPR Design server (Supplemental Figure I) as well as the single predicted off-target site from the COSMID server (Supplemental Figure II) were PCR amplified from two of the CRISPR-PCSK9 mice and one of the CRISPR-control mice and subjected to next-generation DNA sequencing at the Massachusetts General Hospital CCIB DNA Core. The remaining site of the top eight sites from the list generated by the CRISPR Design server proved to have an extremely high background sequencing error rate and so was not analyzed for this study.

On-target and off-target mutagenesis rates were determined as previously described.\(^5\) In brief, sequencing data were processed according to standard Illumina sequencing analysis procedures. Processed reads were mapped to the expected PCR amplicons as reference sequences using custom scripts; reads that did not map to reference were discarded. Frequencies of on-target and off-target indels were determined as follows. The reads were analyzed using custom scripts to identify indels by matching reads against reference, with indels involving any portion of the sequence within 15 nt upstream or downstream of the predicted CRISPR-Cas9 cleavage site (3 nt upstream of the 3' end of the protospacer) considered to be possible on-target or off-target effects. Reads for which there was any 18-nt sequence with more than 2 mismatches with the corresponding 18-nt portion of the reference sequence, either upstream or downstream of a candidate indel, were discarded as errors.

**Primer sequences.** Genomic amplification of on-target site: PCSK9 exon 1: 5', CACGGCCTCTAGGTCTCCT; 3', GCCTCCCATCCCTACACC. Genomic amplification of off-target sites: OT1: 5', GGGAGGAAGAGCTGTGTGG; 3', AGCATCCTGGGTCATCAGAC. OT2: 5', CCAAAAATGCCTTGAGCCTA; 3', GGGGTATATGGCTTGGGAAC. OT3: 5', GGCATTAGAGGCGAGTTGGA; 3', TATCACAGCTCTGGCCACAA. OT4: 5', GCTGTTTGTTCAGCAAAATG; 3', GACCCCTTCTGGAATGTGA. OT5: 5', GGGACCTACTGCACCTCTTCCG; 3', CCTGGTGACAGAGCGAGACT. OT6: 5', GACAGGGAGCAACAGCTTC; 3', TAAATGGTTGGCGGTCTCAT. OT7: 5', CATCTTCCACTCCACACCT; 3', CGATGGGCCTATTTCTGAGCT. OT8: 5', TGATCCTCTGACCCACACCA; 3', CAGATGGGCAACCCACACATT.

**Supplemental References**


Supplemental Figure I. Output from CRISPR Design server.
**Supplemental Figure II.** Output from COSMID server.

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Supplemental Figure III. Pre-treatment and post-treatment human PCSK9 protein, mouse PCSK9 protein, and human albumin levels in the blood. A, Pre-treatment human PCSK9 and albumin levels in individual mice are highly correlated. \( r \) is the Pearson correlation coefficient. B, Pre-treatment and post-treatment levels in individual mice in the two treatment groups. \( P \) values were calculated with the paired t-test.