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Efficient gene editing in adult mouse livers via adenoviral delivery of CRISPR/Cas9

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ABSTRACT

We developed an adenovirus-based CRISPR/Cas9 system for gene editing in vivo. In the liver, we demonstrated that the system could reach the level of tissue-specific gene knockout, resulting in phenotypic changes. Given the wide spectrum of cell types susceptible to adenoviral infection, and the fact that adenoviral genome rarely integrates into its host cell genome, we believe the adenovirus-based CRISPR/Cas9 system will find applications in a variety of experimental settings.

1. Introduction

Developments in DNA-based methodologies have greatly enhanced our ability to manipulate genetic materials. The CRISPR (clustered regularly interspaced short palindromic repeat)/Cas9 system is the latest development that allows much simplified and efficient gene editing in many settings including cultured human cells and fertilized rodent oocytes [1–7]. Although gene-targeted, even conditionally targeted rodents can now be generated rather efficiently through the use of CRISPR/Cas9 [1,8], the so-generated rodents have still to go through time-consuming breeding procedures in order to generate homozygous animals for phenotypic and functional analyses. With our interests in liver physiology and pathology, we sought to determine if we could perform CRISPR/Cas9-mediated gene editing in adult mouse liver to obtain liver gene knockouts directly without resorting to knockout in the germline first strategy. Therefore, we adapted the CRISPR/Cas9 system into adenoviral vectors (Fig. 1A). We generated two pENTRY vectors, one to express a single sgRNA and the wildtype Cas9, and another to express double sgRNA and D10ACas9 for the double nicking strategy [9]. Cas9 has two activity centers each one of which is responsible for the cutting of one strand of the double-strand DNA. The double nicking strategy makes use of a mutant form of Cas9 which harbors a point mutation that eliminates the activity of one of the activity centers such as the D10A mutant. The mutant Cas9 can only cut one strand now to generate a nick on a double strand DNA molecule. With two sgRNAs targeting to a site, the mutant Cas9 can still generate a double strand break at the site, whereas at off-target sites, it could only make one cut (nick), because it is extremely unlikely that the off-targets of the two sgRNAs would co-exist. Nicks on DNA are rapidly and efficiently repaired through the nuclear excision repair pathway in cells, leaving no change or mutation behind.

Once sgRNA sequences are cloned into these two vectors, the pENTRYs can be cloned into an adenoviral destination vector via the convenient gateway strategy. The resultant vectors were

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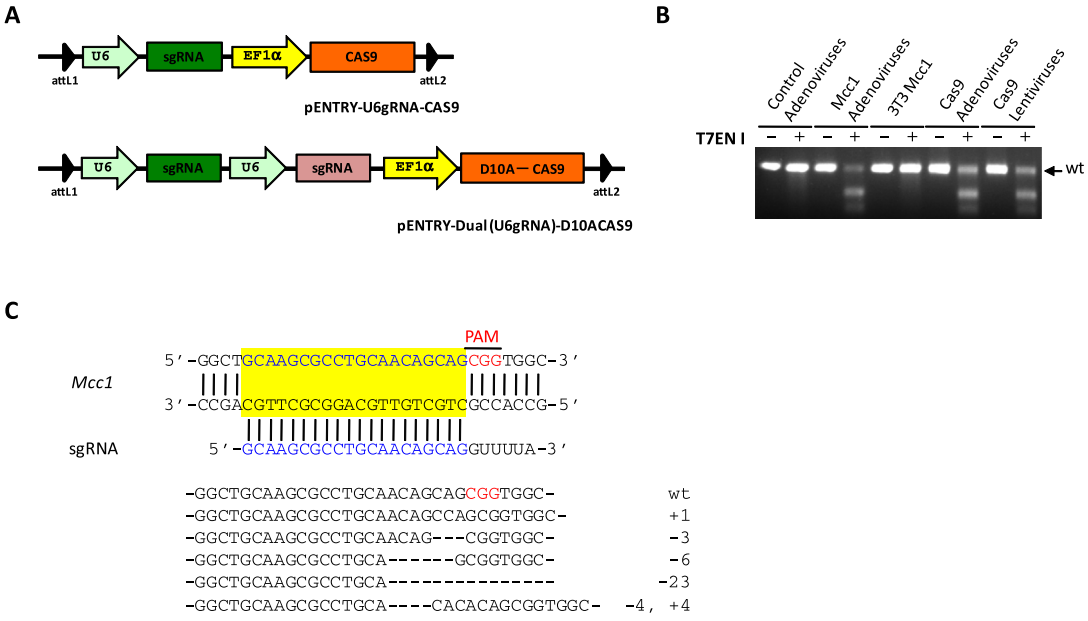

Fig. 1. Adenoviral delivery of CRISPR/Cas9. (A) Diagrams of two pENTRY vectors for cloning of sgRNA sequences. (B) Efficient gene editing with adenoviral CRISPR/Cas9. NIH3T3 cells were infected with adenoCas9 against GFP (control) or Mcc1 (see C for sgRNA sequence) for 4 days and harvested for T7EN I gene editing analysis. In addition, NIH3T3 cells were first infected with lentiviruses (pLKO.1-based) expressing the same sgRNA and then infected with adenoviruses expressing Cas9 or lentiviruses expressing Cas9. 7 days after the infection, the cells were harvested and subjected to T7EN I assay. (C) Illustration of Mcc1 locus with sgRNA and PAM sequences highlighted. 5 edited sequences were also shown.

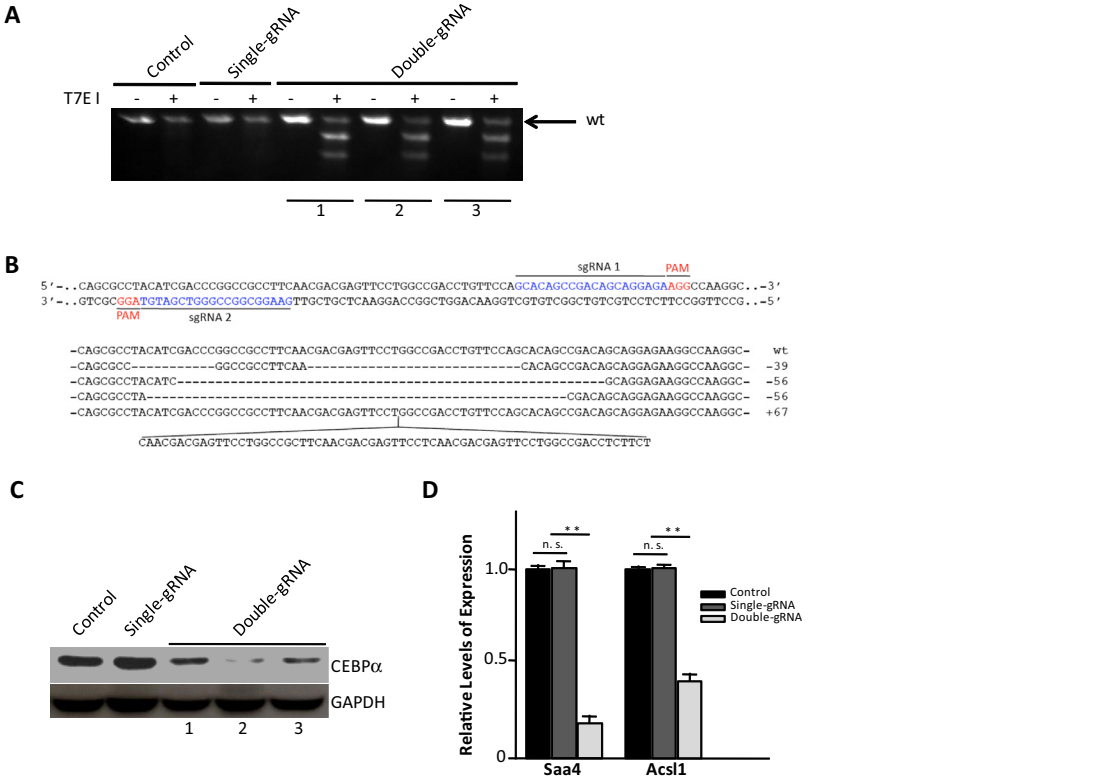

Fig. 2. Gene editing in adult mouse liver. (A) T7EN I assay. AdenoCas9n viruses with single- and double-sgRNA as well as the buffer in which the viruses were suspended for injection (control) were injected into 6-week old male C57BL/6 mice via tail vein. 7 days after the injection, 2/3 of the livers from the injected animals were removed for analyses. (B) Illustration of Cebpα locus with sgRNA and PAM sequences highlighted. 5 edited sequences were also shown. (C) Western blot analysis of CEBPα levels. (D) Quantitative PCR analysis of the expression of Cebpα downstream genes in the livers ($n = 3$). Student t tests were run to determine the statistic significance. n.s., not significant; **, $p < 0.01$.

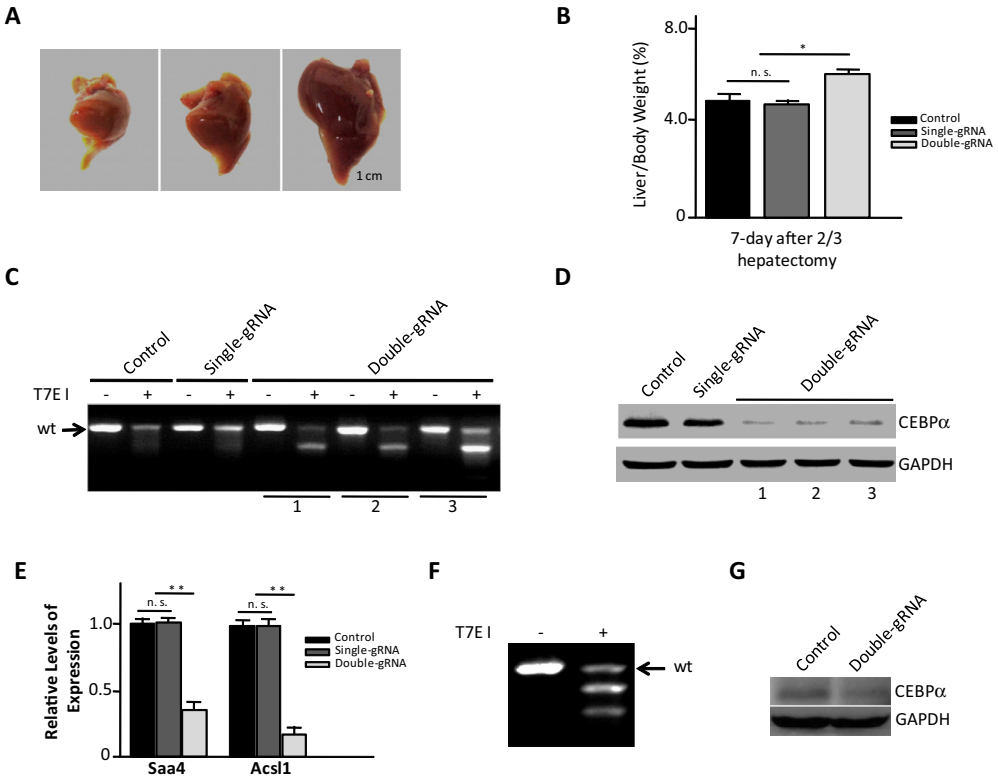

Fig. 3. Gene editing in the liver is maintained after partial hepatectomy. (A) Pictures of the livers 7 days after 2/3 hepatectomy (14 days after the virus injection). (B) Liver over body weight ratios. (C) T7EN I assay. (D) Western blot analysis of CEBPa levels. (E) Quantitative PCR analysis of the expression of Cebpα downstream genes in the livers ($n = 3$). Student t tests were run to determine the statistic significance. n.s., not significant; **, $p < 0.01$. (F) T7EN I assay of the edited *Cebpα* locus 90 days after the injection of the adenoCas9n viruses with double-sgRNA. (G) Western blot analysis of CEBPa levels 90 days after the injection of the adenoCas9n viruses with double-sgRNA. The control was a mouse with adenoCas9 carrying a sgRNA against GFP.

named as adenoCas9 or adenoCas9n (for D10ACas9 nickase) and packaged into adenoviruses in 293A cells. The viral particles were concentrated and purified through gradient centrifugation. We first tested the efficiency of adenoCas9. In NIH 3T3 fibroblasts, we observed near identical gene editing efficiencies against *Mcc1* among 3 different CRISPR/Cas9 delivery systems (Fig. 1B). AdenoCas9 produced expected indels in *Mcc1* (Fig. 1C). In 20 PCR product clones sequenced, we could find only one wildtype. In other words, 95% of the PCR products were indels. There were two scenarios that could explain the sequencing result, assuming that the wildtype and indel alleles were PCR amplified with an equal efficiency and that the cells were diploid. In the first scenario, a 95% indel rate could result from 95% of the cells being −/− ("−" denotes a indel) and 5% +/+. Thus in 20 cells (for the simplicity of calculation), there would be 19 that were −/−, or 38 indel alleles, and 1 wildtype cell or 2 wildtype alleles, giving rise to a indel rate of 95% (38/40). In the second scenario, the same indel rate of 95% could result from 90% of the cells being −/− and 10% +/−. Thus in 20 cells, there would be 18 cells that were −/−, or 36 indel alleles, and 2 cells +/−, or 2 wildtype alleles and 2 indel alleles. The end result is sill the same, 38 indel alleles and 2 wildtype alleles, or an indel rate of 95% (38/40). In reality, the situation is very likely to be somewhere between these two scenarios, but at least 90% (the second scenario) of the cells were homozygous indels.

Having demonstrated the efficiency of AdenoCas9, we wanted to test it in adult mouse liver which is susceptible to adenovirus infection. We chose *Cebpα* as the target. *Cebpα* was among the transcription factors identified in our liver proteome project to be systematically screened for their functions in the liver. It was chosen because the conditional knockout had been described so that we could determine if the knockout in adult mouse liver through CRISPR/Cas9 was feasible. We injected (1×10^{10} virus particles/animal) adenoCas9n targeting Cebpα (and viruses carrying a single sgRNA and Cas9n as well as the buffer in which the viruses were suspended as controls) into mice via tail vein. It is known that the vast majority of adenoviruses injected into tail vein ends up in the liver and other organs and tissues are spared. 7 days after the injection, we sacrificed the animals, isolated the liver, and extracted genomic DNA, total RNA, and proteins for analyses. A 200 bp region of Cebpα encompassing the predicted Cas9 cutting site was amplified from the extracted genomic DNA and subjected to T7EN I mismatch assay, or cloned and sequenced. Efficient editing of Cebpα was observed (Fig. 2A). Sequencing of the cloned PCR products showed typical indels (Fig. 2B). Over 90% of the PCR clones were in mutant forms. According to the calculation performed above, a 90% indel rate could result from the liver cells being 90% −/− and 10% +/+ or 80% −/− and 20% +/− cells. Either way, an effective Cebpα knockout had been achieved with this approach. Indeed, Western blot analysis demonstrated diminished CEBPα levels in the livers (Fig. 2C). CEBPα is a transcription factor important for the expression of metabolic genes in the liver [10–13]. We chose 2 of them, *Saas4* and *Ascl4*, whose expression depends on Cebpα to determine whether their expression was affected in the mice treated with adenoCas9n. As shown in Fig. 2D, their expression indeed decreased greatly, similar to the result obtained with liver-specific *Cebpα* knockouts [10,13]. Taken together, these results demonstrate that our adenoCas9n achieved an effective gene knockout in the liver.

To make sure there are no unwanted editing, we analyzed 5 top off-target sites for each of the two sgRNAs targeting Cebpα (Fig. S1). ~200 bp encompassing the potential off-target sites were amplified through limited numbers of PCR cycles over relatively

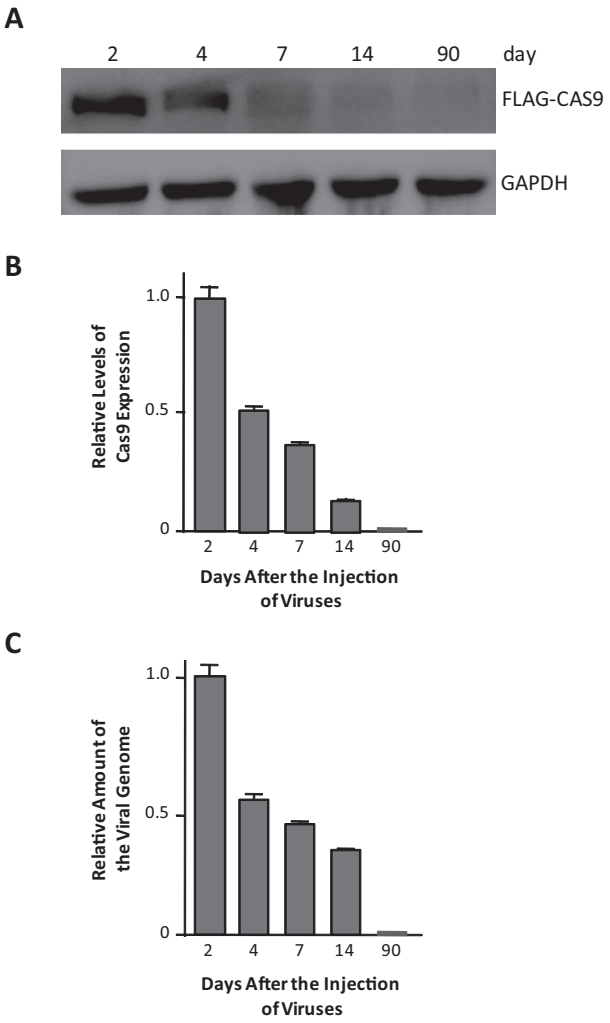

Fig. 4. Adenoviral expression of Cas9 diminishes with time. (A) Western blotting analysis of Flag-tagged Cas9. (B) Q-PCR analysis Cas9 mRNA. (C) Q-PCR analysis of adenoviral genome present in the liver.

large amounts of genomic DNA template. This was to increase the sampling of the off-target sites and to minimize PCR-introduced errors. The PCR products were deep sequenced with each being sequenced close to 2 million times. At all 10 suspected sites, we could not detect any signs of editing.

The mouse liver is remarkable in that it can regenerate from the remaining cells after partial hepatectomy. The CRISPR/Cas9-mediated gene editing is on genomic DNA and should be stable along with cell divisions. To determine whether the editing of Cebpα is stable, we performed partial hepatectomy (2/3 of the liver removed) 7 days after the injection of the viruses. 7 days later (14 days after virus injection), we harvested the livers which had already grown back. Interestingly, the livers from double Cebpα sgRNAs/Cas9-injected mice were much larger than the controls (Fig. 3A and B). The Cebpα locus were still edited as before (Fig. 3C), and CEBPα protein levels were much lower as well in the edited than controls (Fig. 3D). Again, the two downstream genes of CEBPα were expressed at much lower levels in the edited than in controls (Fig. 3E). Moreover, the editing remained evident 3 months after the injection of viruses (Fig. 3F and G). These results demonstrate that Cas9-mediated gene editing is stable in vivo over long term, and even after the extensive regeneration of the liver tissue.

Next, we analyzed the expression of Cas9 after the injection of the viruses. As expected, its expression rapidly decayed at both protein (Fig. 4A) and mRNA (Fig. 4B) levels, most likely due to the immune responses-mediated clearance of the viral genome. Indeed, the viral genome content in the infected livers rapidly decreased with time. By 90 days after the injection, the adenoviral genome content was about 1% of that at 2 days after the injection, but the editing persisted (Fig. 3F and G).

The adenoviral strategy described here opens the door for gene editing in vivo in adult animals, especially in the liver where an effective gene knockout can be achieved. Adenoviruses have been widely used in delivering shRNAs into mouse liver for functional studies [11], but the knockdown effect is transient and is in no comparison to what we obtained here. Our strategy will allow very fast genetic interrogation of gene functions, even genetic screening in the liver, aimed at delineating metabolic and other important functions performed by this vital organ. Further, since liver diseases such as non-alcohol fat liver disease and hepatitis are major health threats worldwide, adenoviral CRISPR/Cas9 systems could be developed into therapeutics against the culprit genes that are behind the diseases. Our study together with the recent reports of the use of CRISPR/Cas9 in the liver, from rapid establishments of animal models [14] to the correction of a disease-causing mutation in mice [15], demonstrate a broad utility of CRISPR/Cas9-mediated gene editing in the liver.

2. Materials and methods

2.1. Plasmids and viruses

Constructs used in the work were generated through standard cloning methods. The human codon optimized Cas9 was reported previously [3]. Packaging of helper-free adenoviruses was done according to published protocols and purification of infectious viral particles was achieved through CsCl gradient centrifugation. The T7EN I gene editing assay was reported previously [3]. All animal protocols were approved by the Animal Care and Use Committee of the Beijing Proteome Research Center. Partial hepatectomy was performed with the established procedure [16].

2.2. DNA/RNA extraction and quantification

Genomic DNA was extracted from cells or liver tissues with an established protocol. In brief, cells or tissues were lysed in 1% SDS/5 mM EDTA/10 mM Tris–HCl pH 8.0/0.1 M NaCl/200 μg/ml Proteinase K (Roche) and digested overnight at 55 °C. The lysates were then extracted with phenol/chloroform and genomic DNA precipitated with ethanol.

Total RNA was extracted from liver tissues frozen in liquid nitrogen using Trizol (Invitrogen). 1 μg of total RNA was used for cDNA synthesis using a reverse transcription kit (Promega).

Quantitative PCR (QPCR) was performed and analyzed with Bio-Rad CFX Connect Real Time system using SYBR Green Master (Rox) (Roche). Primers for QPCR are listed below: *Gapdh*-F, 5′AGGTCG GTGTGAACGGATTTG3′, *Gapdh*-R, 5′TGTAGACCATGTAGTTGAGGT CA3′; *Saa4*-F, 5′CCAGGGCCTCCTAAACCGATA3′, *Saa4*-R, 5′CTCGGG TCGGAAGTGATTGG3′, *Acsl1*-F, 5′TGCCAGAGCTGATTGACATTC3′, *Acsl1*-R, 5′GGCATACCAGAAGGTGGTGAG3′; Cas9-F, 5′CCGCTTACCG GATACCTGTC3′, Cas9-R, 5′TTGGAGCGAACGACCTACAC3′.

To quantify adenoviral genome, genomic DNA (including the viral genome which exists as episome) was subjected to quantitative PCR analysis with primers against actin (on genomic DNA for internal control) and adenoviral backbone (pAd). The primer sequences are: actin-F, 5′AACAGTCCGCCTAGAAGCAC3′, actin-R, 5′CGTTGACATCCGTAAAGACC3′; pAd-F: 5′AAAGCCGTTGATGTTGT GGC3′, pAd-R, 5′TGGGAACAAGACCCGCTTAC3′.

2.3. Western blotting analysis

For Western blotting, fresh liver tissues were homogenated and lysed in RIPA buffer with 2.5 μl/ml protease inhibitor cocktail (Roche) on ice for 15 min. The protein concentrations were determined with Bradford method (Bio-Rad). The primary antibodies for Western blotting included: anti-Cebpα (Boater), anti-FlAG (Sigma), and anti-GAPDH (Santa Cruz).

2.4. Off-target analysis

Potential off-target sequences of each of the two *Cebpα* sgRNAs were identified using the CRISPR design tool (http://tools.genome-engineering.org) and we selected 5 based on the similarity in the seed sequence region (12 nt 5′ to PAM) as well as the ranking scores. For deep sequencing of the off-target sites, each locus (~200 bp) was amplified in 5 separate 50 μl reactions with 5 μg genomic DNA with 20 cycles using KOD FX DNA Polymerase (TOYOBO). The 5 amplification reactions were mixed and gel purified. The purified amplicons were subjected to a second PCR amplification to attach Illumina adaptors and barcodes and sequenced on a MiSeq (Illumina) with a single-end 150 bp run at Beijing Genome Institute (BGI).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.09.008.

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