

Consistent Production of Mice with Conditional Knockout Alleles by CRISPR/Cas9-Mediated Genome Editing Using Two Guides/Two Oligos Approach

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Received June 30, 2019; revised July 30, 2019; accepted January 18, 2020

Abstract—Gene targeting is extensively used to generate designer mouse mutants and to study gene function in vivo. Knockout mice that harbor a null allele in their germline provide appropriate genetic models of inherited diseases and often exhibit embryonic or early postnatal lethality. To study gene function in adult mice and in selected cell types, a refined strategy for conditional gene inactivation has been developed that relies on the DNA recombinase Cre and its recognition (loxP) sites. This process has traditionally relied on the complex process involving genome editing in embryonic stem (ES) cells despite its limitations, including incorrect targeting or cassette structure, and difficulties with germline transmission of the allele from chimeric mice. CRISPR-Cas9 gene editing technology has considerably facilitated the generation of mouse knockout alleles, relieving many of the cumbersome and time-consuming steps of traditional mouse ES cell technology. However, the generation of conditional knockout alleles remains an important challenge. An earlier study reported up to 16% efficiency in generating conditional knockout alleles in mice using 2 single guide RNAs (sgRNA) and 2 single-stranded oligonucleotides (ssODN), which has been questioned by another report combining data from multiple transgenic cores. With the advent of CRISPR/Cas9 as a mouse genome modification tool, we assessed the efficiency of using this method in creating conditional targeted alleles in three genes, phosphatase and actin regulator 1 (Phactr1), apolipoprotein A-I (ApoA1), and actin-related protein T2 (Actrt2). Even though overall success rate was low—about 2.5%—we show that it's possible to reliably generate conditional knockout alleles using CRISPR/Cas9 on a consistent basis.

Keywords: knockout mouse, CRISPR/Cas9, Cre/loxP

DOI: 10.3103/S0095452720010065

INTRODUCTION

Targeted gene mutation in the mouse is a primary strategy to understand gene function and relation to phenotype. However, according to the International Mouse Phenotyping Consortium (<http://www.mousephenotype.org/>), more than 60% (284/459) of knockout mouse strains (C57BL/6N background) show a prenatal lethality phenotype. To overcome this limitation and study the gene functions in adult mice, a refined knockout strategy termed conditional mutagenesis has been developed that enables to inactivate a target gene only in a selected cell type [1, 2]. The most commonly-used system for conditional knockout is the cell type-specific expression of the DNA recombinase Cre, a site-specific DNA recombinase derived from the PI phage, while the target gene is modified by the insertion of 34-bp recombinase recognition (loxP) sequences [2, 3]. The loxP recognition site for Cre is composed of two 13-bp inverted repeats separated by an 8-bp asymmetric spacer that determines the orientation of the loxP site [4]. Cre-mediated recombina-

tion between two loxP sites results in the reciprocal exchange of DNA strands between these sites. Depending on the orientation and location of the two sequences, different products are obtained. In this system called Cre/lox, a region of interest flanked by two lox sites (floxed) positioned in the same orientation, is deleted or inverted by Cre-mediated recombination, leading to gene knockout only in a Cre-expressing cell. As Cre does not require cofactors or accessory proteins to mediate loxP-specific DNA recombination and exhibits optimal activity at 37°C, the Cre/loxP system is the most widely used recombination tool in ES cells and mice [5]. Today, more than 1.300 strains of Cre mice that show tissue- and stage-specific expression of recombinases are available from bio-resource repositories in several countries (International Mouse Strain Resource; <http://www.findmice.org/index>). By contrast, researchers have to produce a mouse with a floxed allele in a gene of interest in many cases.

Traditionally, flox mice have been obtained by gene targeting in embryonic stem cells followed by produc-

tion of germline chimeric mice. However, generating precise modifications in endogenous genes is very complicated. In addition, it takes about a year or more to obtain flox mice by production of chimeric mice and mating of their offspring. Recently, genome editing using direct injection of engineered endonucleases or RNA-guided nucleases into zygotes has greatly accelerated the production of gene-modified animals. The most popular system, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas), is based on RNA-guided nucleases. The minimal system consists of the Cas9 endonuclease and a target-specific guide RNA (gRNA) [6]. In human cells, Cas9 and gRNA can induce DNA double-strand breaks (DSBs) at target sequences, leading to targeted mutations by non-homologous end joining (NHEJ) [7–10]. Furthermore, direct injection of these components into zygotes generates NHEJ-mediated mutant mice [11–13]. Later it was determined that Cas endonucleases can generate a precise double strand break (DSB) in the DNA under a chimeric single guide RNA (sgRNA) [6]. In an earlier study, a high success rate (16%) of targeting *LoxP* sites in *cis* was reported by using 2 sgRNAs and 2 single-stranded oligonucleotides (ssODN) containing *LoxP* sites (2sgRNA-2ssODN) flanking a targeted critical exon [14]. Nevertheless, the recent study analyzing combined reports from 17 transgenic core facilities shows that results of this approach are inconsistent [15].

Since this method could be a powerful tool to generate flox mice because it is not necessary to construct a knock-in vector via a complicated process, and flox mice can be obtained in a short period of time (e.g., in a month), the goal of our study was to investigate the efficiency of the 2sgRNA-2ssODN method for the generation of several conditional knockout alleles.

MATERIAL AND METHODS

SgRNA Design

Three single guide RNAs (sgRNAs) were designed using proprietary algorithm by CRISPR Core Partnership Program by Sigma Millipore (Merck KGaA, Darmstadt, Germany) per each *LoxP* insertion site (Table 1 shows guide RNA sequences originally selected for our first target gene *Phactr1*). These guide RNAs were then validated via CEL I mutation detection assay (also by Sigma Millipore) and the guide showing the highest activity was chosen for each target site for injections and also to design and synthesize matching single stranded DNA oligo donor (ssDNA, also produced by Sigma Millipore). Cas9 mRNA was also provided by Sigma Millipore (Cat #CAS9MRNA). Concentrations and site of injection for Cas9 mRNA, sgRNA, and template repairs for each locus are indicated in Table 2. Cas9 mRNA, sgRNAs, and oligonucleotides were mixed immediately prior to injection in TE buffer, and centrifuged for 10 min at 14000 rpm.

Mouse Strains and Husbandry

B6.Cg-Tg(Sox2-cre)1Amc/J (Sox2-Cre, stock 8454) mice were purchased from the Jackson Laboratory (Bar Harbor, ME); CrI:CD1(ICR) and C57BL/6NCrI (C57BL/6N) mice were purchased from Charles River (Wilmington, MA). Mice were housed in the AAA-LAC-accredited barrier animal facility at TIGM, and were maintained under 12/12 h light cycle with food and water provided *ad libitum*. All studies were reviewed and approved by the Institutional Animal Care and Use Committee of the Texas A&M University, and followed National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals.

Mouse Zygote Microinjection

Three to five week-old C57BL/6N females were superovulated by intraperitoneal injection of Pregnant Mare Serum Gonadotropin (5IU, ProSpec-Tany TechnoGene Ltd., Israel) followed by intraperitoneal injection of Human Chorionic Gonadotropin hormone (5IU, Sigma-Aldrich, Inc.) 48 h later. Superovulated females were mated with 8 to 20 week-old stud males. The mated females were euthanized next day and the zygotes were collected from their oviducts in microinjection medium (5% FBS added to HEPES-buffered D-MEM (ThermoFisher Scientific, Waltham, MA)). Injections were performed under an inverted microscope, associated micromanipulator, and the microinjection apparatus, Eppendorf Transjector 5246, with in-house pulled glass capillaries. Fertilized oocytes were injected into either the pronuclei or cytoplasm with the prepared CRISPR/Cas9 reagents using air-regulated compensation and an injection pressure of 90–115 psi in order to create a continuous flow of reagents [16]. Due to the continuous flow of reagents, fertilized oocytes with injections into the pronuclei received CRISPR/Cas9 reagents in both pronuclear and cytoplasmic regions. Injected zygotes were incubated at 37°C, 5% CO₂ until transplantation. Approximately nine zygotes were surgically transferred into each oviduct of the pseudo-pregnant ICR recipient females or cultured overnight at 37°C and then transferred into recipients at the 2-cell stage of development.

Genotyping

Mice were screened prior to weaning at the age of 12–17 days. For genotyping, tail snip DNA was extracted using tail lysis procedure with Proteinase K. The lysis (L) buffer contained 25 mM Tris-HCl pH 7.5, 100 mM NaCl, 50 mM EDTA, 1% Sarkosyl, 5 mM DTT, 0.05 mM Spermidine and 2 µl proteinase K (Sigma) per 100 µl of the buffer. Mouse tails in buffer L + proteinase K (300–400 µL/tail) were incubated overnight at 65°C, diluted 1 : 40, heated at 95°C for 5 min and used for PCR reactions. Primers were designed to amplify the regions encompassing the

Table 1. Single Guide RNA design and validation results for Phacrl1 knockout

sgRNA Name	Target Sequence	CRISPR RNA Sequence	On-target*	1 Mismatch**	2 Mismatches***	3 Mismatches****	Cell %
Upstream- 1_0_49_CCT	CCTGACCCACGCCAGTGAATCG	CGATTACACTGCCGTGGGTCAGG	1	0	0	8	3.9
Upstream-1_0_111_CC A	CCACCCCGACTTGGTGTGCCAA	TTGGCACACCAAGTCGGGGTGG	1	0	0	11	5.2
Upstream- 1_0_299_CCC	CCCTTTAACAAACGGCAAATAIG	CATATTGCCGTTGTTAAAGGG	1	0	0	8	4.5
Upstream- 1_0_277_CCT	CCTGTCAGCTGAAAAGAACGTGT	ACACGTTCTTTCAGCTGACAGG	1	0	0	8	5.2
Downstream- 1_0_464_AGG	TAACGTTATATCCGTA TAGG	TAAACGTATATCCGTA TAGG	1	0	0	12	7.1
Downstream- 1_0_23_CGG	AGTTTTAAGAGACATATAGCGG	AGTTTTAAGAGACATATAGCGG	1	0	1	10	5.7
Downstream- 1_0_57_CC A	CCATTGTCAAGCTACCTGAGAA	TTCTCAGGTAGCTTGACAATGG	1	0	2	9	3.2

* Number of Perfect Matches in the Genome.
 ** Number of Sites in Genome that are 1 base pair different from target sequence.
 *** Number of Sites in Genome that are 2 base pair different from target sequence).
 **** Number of Sites in Genome that are 3 base pair different from target sequence).

Table 2. Single Guide RNA and Single Stranded Oligonucleotide DNA sequences, their concentrations and targeting genomic regions (in bp) used in this study

Gene	5' guide RNA	3' guide RNA	5' donor sequence	3' donor sequence	Concentrations (in ng/ μ l)			Exons Floxed	Distance
					Cas9	SgRNAs	ssODNs		
<i>Phactr1</i>	5'- CCACCCCGACT TGGTGTGC- CAA-3'	5'- TAACGTGTATA TCCGTAAGG- 3'	5'- ATAACCCCTGACCCACGCAGTGTAAATC- GGTCATGAGTGGACCCACACAGCCCA- GCAACCATGCTCACCCCAATAACTT- CGTATAATGTATGCTATACGAAGTTAT- GAATCCCCCGACTTGGTGTGCCAA- AGTCACATTCCATAACCACTGAGTCG- CACAAAAAAAACCTGCTCAGTGACAG-3	5'- GCAGACATGCATGTACACACACAGA- CACACACAGGCACACACACAAAAGAG- TAACGTGTATAATCCGTAATAACTTC GTATAATGTATGCTATACGAAGTTATG- AATTCAGGCCATATTTCCCTCAA- CAACATTCAGGTTTGAGAAGGCTG- TGCATGTGTGGTTAGTCATCGTGA-3'	100	50	100	Exon 7	1.6 kb
	<i>Actm2</i>	5'- CCCAAGTTGAG TGCCAGGTC- 3'	5'- CCACCTGAATG CTACTGTTGAA- 3'	5'- TATGAAACCCACCTTTCCAAAGTTCCTT- CATCATCAGGGAAGGAAACTGT- GTCCAGGCTCAGCCCAATAAC-TTC- GTATAATGTATGCTATACGAAGTTATG AATTCAAAGTTGAGTGCCAGGT-CTCA- GAGTAGCATGTGCCTCTGTGTGT- GTGAGGCCATAGTAGGTGGCTCCTC C-3	5'- CTAGGCAAAGTTGAAGAAGAAGATC- CAGCCAAAGGTGTCCTCCATGGGCA- TCACACAGCACAGCTACCAATAACTT- CGTATAATGTATGCTATACGAAGTTAT- GAATTCCTGAATGCTACTGTTGAAT- TGCTTGTCTCAAGATGCTCTGGGT- GCTAAGGCTGGGGCCAGTTCCTCCG- 3'	100	50	100	Entire Gene
<i>ApoA1</i>		5'- CCTTGTAAACCA GCACCGACCAC- 3'	5'- CTAGCCGAGTT TCCAGGTG- GGG-3'	5'- CCCCAGTACTGGTCAGACAGCACCA- AAACAAAAAAAAAAAAAAAAAACAA- AAAAACGGGACTGGCCTATAACTTCG- TATAATGTATGCTATACGAAGTTATGAA- TTCTGTAAACAGCACCCACACATCT- GTTTCTTGGTGCCTTCGGCCATGTG- TACAGAGGAATGTAAAGGGAA-3'	5'- GATGAGAGTGTCTGGCAATAGGACT- GGCTTTGAGACCGCTGGTTCTTGT- GCTAGCCGAGTTTCCAGGTGATAACT TCGTATAATGTATGCTATACGAAGTTA- TGAATTCGGGTCCAGAGGTGAGG- ACGGGAGCCTCTTCCACTCCCTAGA- GCCAGGATTCCTTCAGGAGCCAGGA- AG-3'	100	50	100	Exon 4

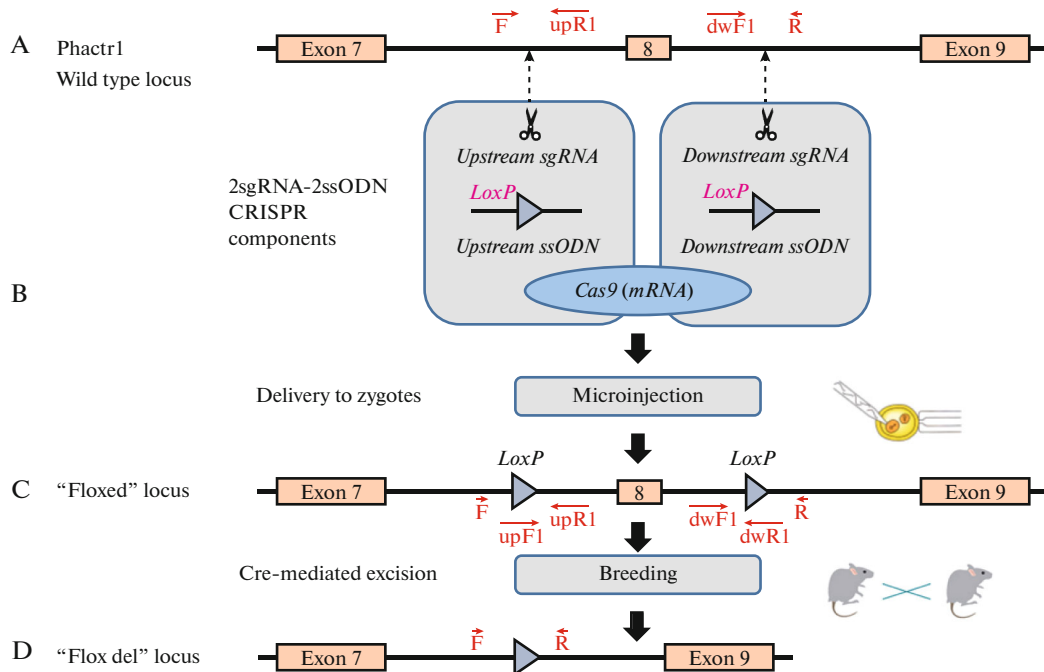


Fig. 1. Schematic process for creation of *Phactr1* conditional knockout allele. (A) Wild type locus showing exons 7, 8 and 9; exon 8 is chosen as a target for inserting *LoxP* sites. gRNAs target introns 7 and 8. (B) CRISPR components—sgRNA, ssODN donors and a Cas9 source—delivered into one-cell stage zygotes via microinjection. (C) The conditional (“floxed”) knockout allele showing target exon 8 with flanking *LoxP* sites. (D) Excision of the target exon 8 following exposure to Cre recombinase (“flox del” locus). Genomic locations of the genotyping primers (F, R, upF1, dwF1, upR1 and dwR1) are also shown.

integrated *LoxP* sequence (Fig. 1). PCR was performed using LongAmp™ Taq Master Mix (New England Biolabs) under standard PCR conditions. Primer Sequences (5' to 3') and expected PCR bands for the *Phactr1* knockout allele were as follows:

Upstream *LoxP* PCR: upF1 + upR1, 208 bp

Phactr1 upF1 GCT CAC CCC CAA TAA CTT CGT ATA

Phactr1 upR1 GTCCAACCTCACCTCCACTGA

Downstream *LoxP* PCR: dwF1 + dwR1, 216 bp

Phactr1 dwF1 GGC AAG TTC CAG GTC AGT GA

Phactr1 dwR1 GGCCTGAATTCATAACTTCG-TATAGC

Upstream *LoxP* to Downstream *LoxP* PCR: upF1 + dwR1, 1572 bp

PCR confirming the floxed fragment excision: F+R, 2063 bp (wt)/2144 bp (floxed) + 557 bp (flox del)

Phactr1 F GCAGCCTTCCTGCTGCAGCTTTCT

Phactr1 R GCTTTGCTTCCAGTACCCTGATCT

Similar strategies were designed for the other two alleles, *ApoA1* and *Actr2*. The PCR products were then purified with a PCR Clean-Up System kit according to the manufacturer’s instructions. Sanger sequencing was performed in Texas A&M Sequencing Core facility. The entire regions encompassing both the guide cleavage sites were amplified to assess for

integrity of the *LoxP* sites and presence of unintended modifications between the cleavage sites.

RESULTS

We report the construction and preliminary analysis of several conditional knockout mouse strains (*Phactr1*, *ApoA1*, *Actr2*). All three alleles were generated using standard procedures following the same scheme described for *Phactr1* (Fig. 1). The first step of the process was design of sgRNAs and their validation. Three guide RNAs were selected per each target site based on their uniqueness and specificity, of which only one was chosen for further work based on results of the Cel I validation assay (Table 1). Only then the ssDNA donors would be designed and synthesized following several basic principles: 70 nt for each homology arm and *LoxP* cassette inserted between guide RNA sequence and Protospacer Adjacent Motif (PAM) (Fig. 2A and Table 2).

Injections were performed according to standard methods with the aim to deliver material both inside the nucleus and cytoplasm. The number of surviving embryos varied from experiment to experiment, but would normally stay in 55–75% range and the embryo to live offspring conversion rate was in the range of 12–22% (Table 3).

Original genotyping PCR screen would identify single *LoxP* insertions followed by long—distance

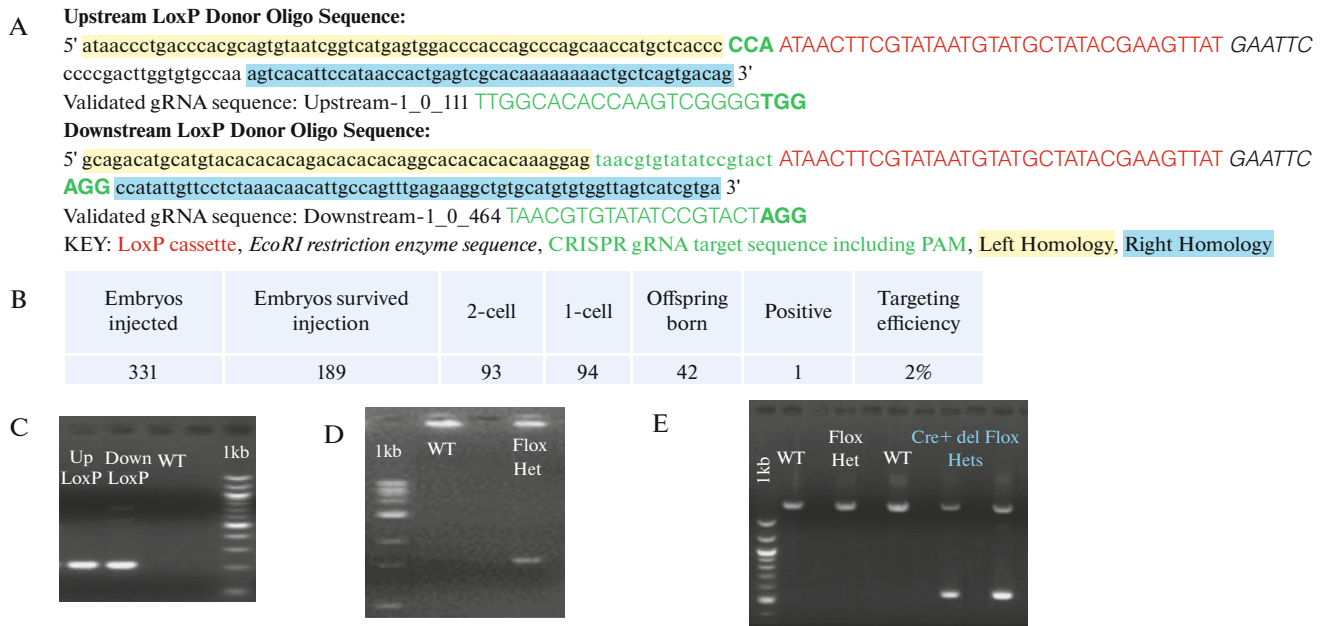


Fig. 2. Creation of *Phactr1* conditional knockout allele. (A) sgRNA and ssODN donor sequences for inserting *LoxP* sites. (B) Microinjection results. (C) Genotyping confirmation of the correct *LoxP* sites insertions. (D) Genotyping confirmation that the *LoxP* sites are inserted in *cis*-orientation (same strand). (E) Genotyping confirmation of the floxed fragment excision following exposure to Cre recombinase.

PCR to confirm *cis*-orientation of the *LoxP* sites on the chromosome. As expected, most of the founders contained single *LoxP* insertions as well as NHEJ-based deletions between guide RNA targets, and only a few mice carried correct insertions at both sites. Interestingly, all 6 founders with both *LoxP* site insertions produced for these 3 projects carried them in *cis*-orientation, which contradicts recent reports of regular insertions in *trans* [15]. Further sequencing of the genomic DNA surrounding *LoxP* site insertions revealed no unintended mutations in the founders, also contrasting recent reports of finding numerous mutations at those sites by other labs [15]. The overall efficiency of correct editing events occurring simultaneously at both sites was approximately 2.5% (Fig. 2B, Table 3).

Founder males or females were bred to C57BL/6 females or males for germline transmission of the mutant alleles. A germline transmission event was

scored when any F0 founder produced progeny with the correct genomic locations of the *LoxP* sites. The progeny were analyzed for transmission of the mutation using site-specific PCR protocols (Figs. 2C and 2D). The F1 progeny heterozygous mutants revealed no visible phenotype. To continue with the functional characterization of these tissue-specific knockouts we obtained homozygous (flox/flox) *ApoA1* and *Actr12* alleles and started breeding with tissue-specific Cre strains. Correct removal of the targeted genomic fragment was confirmed by appropriate genotyping PCR (Fig. 2E shows results of the Cre-mediated deletion for *Phactr1* following breeding with Sox2-Cre line). Homozygous mice were produced at the expected Mendelian rate and didn't display any gross abnormalities, further suggesting that these knockouts do not carry any unwanted genome modifications and can be used as intended.

Table 3. Detailed count of the numbers of zygotes microinjected, transferred, live pups born, correctly targeted and non targeted

Gene	Lethality	Zygotes injected	Zygotes transferred	Live born pups	Correctly targeted	Incorrectly targeted in 5'	Incorrectly targeted in 3'	Site of injection*
<i>Phactr1</i>	Unknown	331	187	42	1	<i>LoxP</i> = 3	<i>LoxP</i> = 5; Deletions = 6	C and N
<i>Actr12</i>	Unknown	1013	562	70	3	<i>LoxP</i> = 7	<i>LoxP</i> = 4	C and N
<i>ApoA1</i>	None	645	456	68	2	<i>LoxP</i> = 9	<i>LoxP</i> = 4	C and N

* C: cytoplasmic only, N: nuclear only C+N: both.

DISCUSSION

CRISPR-Cas9 technology has greatly facilitated the generation of mouse lines containing knockout or knock-in alleles. Over the past several years TIGM has created 20 mouse strains carrying various site-specific genome modifications using this approach. However, the generation of conditional alleles remains a challenge using traditional ES cells and CRISPR-Cas9 gene editing technologies. An earlier paper demonstrated success approaching 16% efficiency with 2 chimeric sgRNAs and 2 single-stranded oligonucleotides donor approach to produce conditional alleles in mouse [14].

To evaluate the efficiency of the method, we performed experiments on production of 3 different conditional alleles. Although we observed numerous single LoxP site insertions and indels at the cleavage sites, the method was also successful in generating two LoxP sites in *cis* for all three alleles (Table 3). We noted the efficiency to insert the two LoxP sites simultaneously to be the best predictor of the likelihood of success in this approach. In fact, we haven't found any *trans* locations whenever both LoxP site insertions were observed so far. We also noted a low success rate overall (~2.5%, representing the number of correctly edited mice to the total number of live offspring born for a particular project) to generate a conditional allele (Table 3). These results are comparable with previous reports demonstrating an important disparity in success rate varying from 0 to 7% of mice harboring two LoxP sites insertions in *cis* by microinjection [17–20]. We also have noted a number of deletions at the target sites following DNA cleavage [19]. Another possibility to improve the efficiency of the method is to avoid recombination between the target sites by placing the LoxP sites over hundreds of kb apart. This was reported previously for a success rate varying from 0 to 18% for 6 loci [21]. A recent report found the successful use of sequential introduction of the LoxP sites to improve its efficiency as much as 3 to 10 fold and avoid recombination between alleles [17], though it should be noted that such an approach requires a more protracted period of time to completion. Additional work has demonstrated over 5 fold improvement in targeting using a long ssODN [20, 22].

In conclusion, even though overall success rate is low—about an average of 300 zygotes were needed to generate 1 correctly targeted animal — it is possible to generate floxed alleles using CRISPR-Cas9 gene editing technology with 2sgRNA-2ssODN. We believe that Phactr1, ApoA1, Actrt2 conditional KO mice strains will serve as a valuable tool for researchers studying appropriate genetic models.

ACKNOWLEDGMENTS

We would like to thank Dr. Christopher Lemke and Dr. Nicola van der Walt of CRISPR Core Partnership Pro-

gram (Sigma Millipore/Merck KGaA, Darmstadt, Germany) for help with design and validation of CRISPR/cas9 materials.

FUNDING

This work was supported by Texas AgriLife Research (SS).

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare no conflict of interest or financial disclosure statements. This article does not contain any studies involving animals or human participants performed by any of the authors.

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