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

Andrei Golovko, John Adams, Huiping Guo, Johnathan Ballard, and Ben Morpurgo

Texas A&M Institute for Genomic Medicine, College Station, TX 77843

ABSTRACT

CRISPR-Cas9 technology represents a significant improvement of genome editing tools, reaching a new level of targeting, efficiency, and ease of use, thereby relieving many steps of traditional mouse ES cell technology when it comes to generation of mouse knockout alleles. However, production of conditional knockouts remains an important challenge. Several studies provided contradictory reports regarding efficiency in generating conditional knockout alleles in mice using 2 single guide RNAs (sgRNA) and 2 single-stranded oligonucleotides (ssODN) [1]. We assessed the efficiency of using this method in creating conditional targeted alleles in a set of 11 mouse genes. Even though overall success rate was low – about 3.4% - and not all genes have been correctly targeted, we show that it's possible to generate conditional knockout alleles using the CRISPR/Cas9 two guides/two oligos approach on regular basis.

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. 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 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 for Cas9 mRNA, sgRNA, and template repairs for each locus varied from injection to injection and the ranges are indicated in Table 1. Cas9 mRNA, sgRNAs, and oligonucleotides were mixed immediately prior to injection in TE buffer and centrifuged for 10 min at 14,000 rpm.

Mouse strains and husbandry. CD1(ICR) and C57BL/6N mice were produced from in house colonies, B6.Cg-Tg(Sox2-cre)1Amc/J (stock 8454) mice were purchased from the Jackson Laboratory. Mice were housed in the AAALAC-accredited barrier animal facility at TIGM and were maintained under 12/12 hr 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 IP injection of PMSG (5IU, ProSpec-Tany TechnoGene Ltd, Israel) followed by IP injection of HCG hormone (5IU, Sigma-Aldrich, Inc) 48 hours later. Superovulated females were mated with 10 - 30-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 [2]. Due to the continuous flow of reagents, fertilized oocytes with injections into the pronuclei received CRISPR/Cas9 reagents in both pronuclear and cytoplasmic regions. Approximately nine zygotes were surgically transferred into each oviduct of the pseudo-pregnant ICR recipient females or cultured overnight at 37ºC 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. Primers were designed to amplify the regions encompassing the integrated LoxP sequence (Figure 1). PCR was performed using LongAmp™ Taq Master Mix (New England Biolabs) under standard PCR conditions. 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

All alleles were generated following the same scheme described for one of the genes, Phactr1 (Figure 1). 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 PAM (Figure 2A). 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 60-75% range and the embryo to live offspring conversion rate was in the range of 6-22% (Table 2). Original genotyping PCR screen would identify single LoxP insertions followed by long—distance PCR to confirm cis-orientation of the LoxP sites on the chromosome. 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. Germline was successfully obtained for only 5 alleles (Phactr1, Actrt2, ApoA1, RanBP3l and Grk3). Correct removal of the floxed genomic fragments was confirmed by appropriate genotyping PCR and sequencing in offspring produced after breeding with the Sox2-Cre line (Figure 2E shows results of the Cre-mediated deletion for Phactr1 allele only).

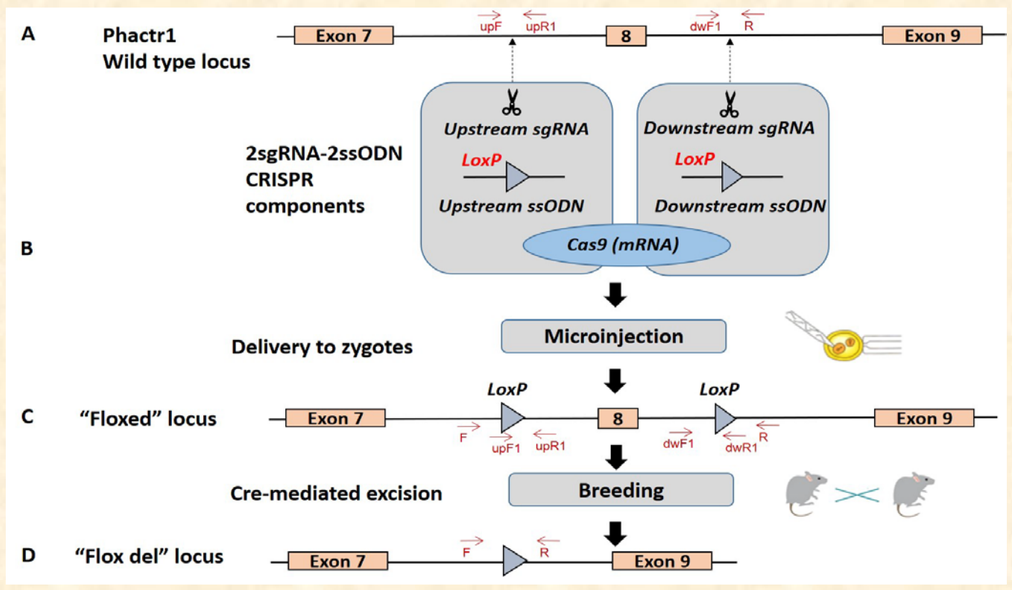
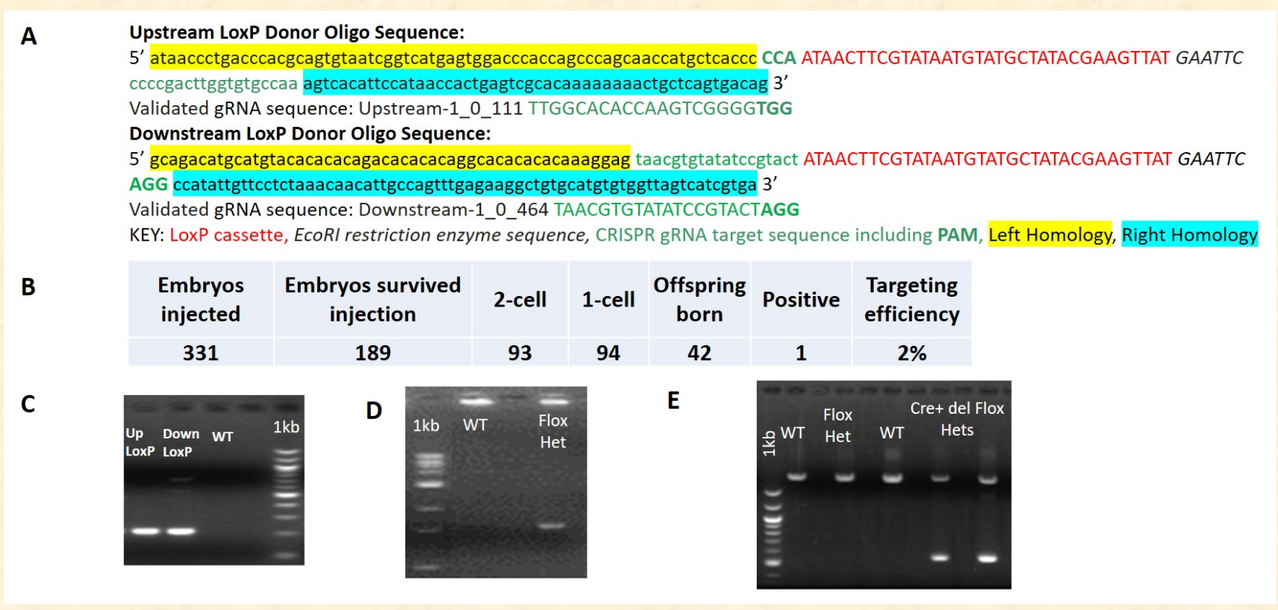


Figure 1: Schematic process for creation of Phactr1 conditional knockout allele. (A) Wild type locus showing exons 7, 8 and 9; exon 8 is chosen as a target for inserting LoxP sites. gRNAs target introns 7 and 8. (B) CRISPR components - sgRNA, ssODN donors and a Cas9 source – delivered into one-cell stage 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 are also shown.



Embryos injected	Embryos survived injection	2-cell	1-cell	Offspring born	Positive	Targeting efficiency
331	189	93	94	42	1	2%

Figure 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.

Gene	concentrations (in ng/µl)			Exons floxed	distance between targets
	Cas9	SgRNAs	ssODNs		
Phactr1	100	50	100	Exon 7	1.6 kb
Actrt2	100	50	100	Entire Gene	2.6 kb
ApoA1	100	50	100	Exon 4	1.6 kb
Clasp2	100-160	20-40	160-300	Exons 1-2	1.9 kb
Mertk	100-200	25-50	150-300	Exon 9	1.2 kb
RanBP3l	100	25	100-325	Exons 2-5	6.8 kb
Cox7a2l	100-200	25-50	200-375	Exon 2	1 kb
Slc9a3r1	50-200	25-50	150-410	Exons 2-3	2.1 kb
Grk3	160-200	40-50	300-440	Exon 6	2.5 kb
Zfp281	200	50	200	Entire Gene	6.3 kb
Plagl1	100	25	150-300	Exon 7	2.6 kb

Table 1: Concentrations of Single Guide RNA and Single Stranded Oligonucleotide DNA sequences, targeting genomic regions (in bp) used in this study.

Gene	Lethality	Zygotes injected	Zygotes transferred	Live born pups	Correctly targeted	Incorrectly targeted			
						Both LoxPs in trans	5' LoxP insertions only	3' LoxP insertions only	Deletions between 5' and 3' sites
Phactr1	Unknown	331	187	42	1		3	5	6
Actrt2	Unknown	1013	562	70	3		7	4	
ApoA1	None	645	456	68	2		9	4	
Clasp2	None	534	420	63	2*		6	5	2
Mertk	Possible	883	573	38	0	1	4	6	11
RanBP3l	Unknown	761	478	37	2		3	3	3
Cox7a2l	None	450	337	32	1		1	2	20
Slc9a3r1	Possible	941	699	102	1*	2	5	10	15
Grk3	None	556	400	32	5		5	3	10
Zfp281	Possible	487	357	23	0			9	2
Plagl1	Possible	249	202	23	0		1	1	3

Table 2: Detailed count of the numbers of zygotes microinjected, transferred, live pups born, correctly (both LoxPs in cis) and correctly targeted. *3' LoxP had mutations

CONCLUSIONS

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 8 out of 11 alleles (Table 2). 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 many trans locations whenever both LoxP site insertions were observed. We also noted a low success rate overall (varying between 1 and 15% with an average of ~ 3.4%, 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 2). 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 [3-5]. We also have noted a number of deletions at the target sites following DNA cleavage as also reported before [19]. In conclusion, even though overall success rate is low – about an average of 270 zygotes were needed to generate 1 correctly targeted animal – and not all genes can be successfully targeted, it is possible to generate floxed alleles using CRISPR-Cas9 gene editing technology with Two Guides/Two Oligos approach.

REFERENCES

1. Gurumurthy, C., et al. Reproducibility of CRISPR-Cas9 methods for generation of conditional mouse alleles: a multi-center evaluation. Genome Biology, v 20, Article number: 171 (2019), doi:10.1186/s13059-019-1776-2.
2. Behringer R., et al., Manipulating the Mouse Embryo: a Laboratory Manual. Cold Spring Harbor Laboratory Press, 2014, Cold Spring Harbor, NY.
3. Horii, T., et al., Efficient generation of conditional knockout mice via sequential introduction of lox sites. Sci Rep, 2017, vol. 7(1), p. 7891.
4. Bishop, K.A., et al., CRISPR/Cas9-Mediated Insertion of loxP Sites in the Mouse Dock7 Gene Provides an Effective Alternative to Use of Targeted Embryonic Stem Cells. G3 (Bethesda), 2016, vol. 6(7), pp. 2051-61.
5. Kueh, A.J., et al., An update on using CRISPR/Cas9 in the one-cell stage mouse embryo for generating complex mutant alleles. Cell Death Differ., 2017, vol. 24(10), pp. 1821-1822.
6. Lanza, D.G., et al., Comparative analysis of single-stranded DNA donors to generate conditional null mouse alleles. BMC Biol., 2018, vol. 16(1), p. 69.