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 challenges of traditional mouse E3 cell technology when generation of knockout mouse models is concerned. Moreover, production of conditional knockouts is an important challenge. Several studies provided contradictory results 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 were designed using proprietary algorithm by CRISPR Core Partnership Program by Sigma Millipore (Merck KGaA, Darmstadt, Germany) per each Loxp insertion site. The guide RNAs were designed using the CRISP TAR tool. 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) by Sigma Millipore. Constructions of Cas9, sgRNA, and template repair for each locus varied from injection to injection and the ranges are indicated in Table 1. Cas9, sgRNA, ssDNA, 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, bred in a temperature controlled and humidified room, and maintained on a 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. To five-week-old C57BL/6N females were superovulated by IP injection of PMSG (STU, Spec-Pro-Tac TechnoGene, Ltd. Israel) followed by IP injection of HCG hormone (STU, 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 micromanipulation medium (FI, Fisher). Fertilized zygotes were injected into the one-cell stage using a Pico-Pette (Dagan). Zygotes were put into 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 micromanipulation apparatus, Eppendorf Transjector 5246, with in-house pulled glass capillaries. Fertilized oocytes were injected into the pronucleus or cytoplasm, with the prepared CRISPR/Cas9 reagents using air-regulated micromanipulation (150-215 psig). Injected zygotes were 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 genes were generated following the same scheme described for one of the genes, Phactr1 (Figure 1). The ssDNA donors were 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 both into 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 only. Five single-stranded oligonucleotides (ssODNs) [1] were also synthesized. The ssODN donors would be designed and synthesized following the same scheme described for one of the genes, Phactr1 (Figure 1). The ssDNA donors would be designed and synthesized following the same scheme described for one of the genes, Phactr1 (Figure 1). The ssDNA donors would be designed and synthesized following the same scheme described for one of the genes, Phactr1 (Figure 1). The ssDNA donors would be designed and synthesized following the same scheme described for one of the genes, Phactr1 (Figure 1).

Although we observed numerous single Loxp site insertions and indels at the cleavage stages, 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 any success in generating both Loxp site insertions when less than 1 in 10 single Loxp site insertions were observed. We also noted a notable decrease in efficiency of 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 fixed alleles using CRISPR/Cas9-genome editing technology with Two Guides/Two Oligos approach.

CONCLUSIONS