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Short Communication

Electroporation of Cas9 protein/sgRNA into early pronuclear zygotes generates non-mosaic mutants in the mouse

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ABSTRACT

The CRISPR/Cas9 system is a powerful tool for elucidating the roles of genes in a wide variety of organisms including mice. To obtain genetically modified embryos or mice by this method, *Cas9* mRNA and sgRNA are usually introduced into zygotes by microinjection or electroporation. However, most mutants generated with this method are genetically mosaic, composed of several types of cells carrying different mutations, which complicates phenotype analysis in founder embryos or mice. To simplify the analysis and to elucidate the roles of genes involved in developmental processes, a method for producing non-mosaic mutants is needed. Here, we established a method for generating non-mosaic mouse mutant embryos. We introduced Cas9 protein and sgRNA into *in vitro* fertilized (IVF) zygotes by electroporation, which enabled the genome editing to occur before the first replication of the mouse genome. As a result, all of the cells in the mutant carried the same set of mutations. This method solves the problem of mosaicism/allele complexity in founder mutant embryos or mice generated by the CRIPSR/Cas9 system.

1. Introduction

Genetically modified mice are a powerful and widely used tool for elucidating the roles of genes in development and disease. However, conventional methods involving homologous recombination in embryonic stem cells are costly and time-consuming. The development of the CRIPSR/Cas9 system dramatically improved this situation (Cong et al., 2013; Gaj et al., 2013). Because of its high efficiency, the CRISPR/Cas9 system can elucidate the functions of genes directly in the founder generation (F0).

A common approach for generating mutant embryos or mice using the CRISPR/Cas9 system is to inject *Cas9* mRNA and sgRNA, or their expression plasmid into zygotes (Wang et al., 2013; Yang et al., 2013; Yasue et al., 2014). Alternatively, we and other groups recently established electroporation methods for introducing *Cas9* mRNA and sgRNA into zygotes (Hashimoto and Takemoto, 2015; Kaneko et al., 2014; Qin et al., 2015). However, most of the mutants that develop from the injected or electroporated zygotes are genetically mosaic, that is, individual cells in a mutant carry different sets of insertions or deletions (indels) (Mizuno et al., 2014; Oliver et al., 2015; Yen et al., 2014). Typically, four or more different mutant alleles are observed in a mutant, indicating that genome editing occurred after the first genome replication. This genetic mosaicism complicates the phenotype analysis. Thus, to elucidate the roles of genes in the F0 generation, a method for producing biallelic and non-mosaic mutants is needed.

In this study, we generated biallelic and non-mosaic mutant mouse embryos by transferring Cas9 protein and sgRNA into *in vitro* fertilized (IVF) zygotes by electroporation. This method will greatly ease studies of gene function using genome-edited F0 mice.

2. Material and methods

2.1. Animals

All of the animal care and experiments were carried out in accordance with the Guidelines for Animal Experiments of Tokushima University and of Osaka University, and were approved by the Ethics Committee of Tokushima University for Animal Research (Approval number: 14022) and the Animal Care and Use Committee of Osaka University (Approval number: FBS-15-001).

2.2. mCherry mRNA, sgRNA, Cas9 protein, and ssODN preparation

For pCS2-mCherry plasmid construction, the coding region of mCherry cDNA was amplified by PCR using pmCherry vector (Clontech) as a template. The amplified fragment was ligated

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between BamHI and EcoRI sites of pCS2 vector. *mCherry* mRNA was synthesized using the mMessage mMachine SP6 Kit (Ambion) and the NotI digest of pCS2-mCherry as a template.

A pair of oligonucleotides targeting the *Fgf10* or *Brachyury* gene was annealed and inserted into the *Bsa*I site of the pDR274, a plasmid vector to create a sgRNA by *in vitro* transcription using T7 promoter (Hwang et al., 2013). pDR274 was a gift from Dr. Keith Joung (Addgene plasmid # 42250). The target sequences were as follows: Fgf10 (5′-GGAGAGGACAAAAAACAAGA-3′), Brachyury1 (5′-TTGCGAGACCGGTTGCCG-3′) and Brachyury2 (5′-TTAAACCCTGCCGGCATA-3′). After digestion with DraI, sgRNAs were synthesized using the MEGAshortscript T7 Transcription Kit (Ambion). The synthesized sgRNAs were purified by phenol-chloroform-isoamylalcohol extraction and isopropanol precipitation. The precipitated RNA was dissolved in Opti-MEM I (Thermo Fisher Scientific) at 2–4 μg/μl, and stored at −20 °C until use. RNAs were quantified by absorption spectroscopy and agarose gel electrophoresis. Cas9 protein was purchased as a component of the Guide-it™ Complete sgRNA Screening System (Takara Bio Inc.). The ssODNs were purchased from Sigma in dry form, dissolved in Opti-MEM I to 1 μg/μl, and stored at −20 °C until use. The sequences of the ssODNs were: Fgf10XbaI (5′-TCGTCATGGGGAGGAAGTGAGCAGAGGTGTTTTTCCTTCTAGATGTTTTTTGTCCTCTCCTGGGAGCTCCTTTTCCATTCAAT-3′) and Fgf10EcoRI (5′-TGGATCGTCATGGGGAGGAAGTGAGCAGAGGTGTTTTTCCGAATTCTTGTTTTTTGTCCTCTCCTGGGAGCTCCTTTTCCATT-3′).

2.3. Mice, and egg and embryo collection and transfer

B6D2F1 (C57BL/6NCr × DBA/2Cr F1) mice were used in this study. Fertilized eggs were collected at E0.25 (8:00) from the oviducts of naturally intercrossed females. The covering cumulus cells were removed by incubation in 1% hyaluronidase/M2 medium. The fertilized eggs were incubated in KSOM medium until electroporation. IVF was performed following a standard protocol using the B6D2F1 strain (Behringer, 2014). After a 3-h culture of oocytes and sperm, the eggs were removed from the sperm and cultured for 2 h until electroporation. The electroporated embryos were cultured in KSOM medium, and transferred next day to the oviducts of pseudopregnant females on the day of the vaginal plug (Behringer, 2014). All the media were prepared following a standard protocol (Behringer, 2014).

2.4. Electroporation

CUY21EDIT II or Genome Editor electroporator and LF501PT1-10 platinum plate electrode (length: 10 mm, width: 3 mm, height: 0.5 mm, gap: 1 mm) (BEX Co. Ltd., Tokyo, Japan) were used for electroporation.

The electrode was connected to the electroporator and was set under a stereoscopic microscope. 30–40 zygotes prepared by natural breeding (NB) or IVF were subjected to electroporation at one time. The collected zygotes cultured in KSOM medium were washed with Opti-MEM I three times to remove the serum in the medium, placed in a line in the electrode gap filled with 5 μl of Opti-MEM I containing *mCherry* mRNA or mixture of Cas9 protein and sgRNA, and subjected to electroporation. The electroporation conditions were 30 V (3 msec ON +97 msec OFF) 7 times in most experiments. After electroporation, the zygotes were immediately collected from the electrode chamber and subjected to four washes with M2 medium followed by two washes with KSOM medium. The eggs were then cultured in KSOM medium at 37 °C and 5% CO_2 in an incubator until the two-cell stage.

2.5. Fluorescent signal detection and analyses

The mCherry fluorescence of the electroprorated zygotes cultured *in vitro* was detected by an EM-CCD camera equipped to the inverted microscope and Nipkow-disc confocal unit. The data was analyzed using the ImageJ software. Relative fluorescent intensity was calculated as the average intensity of E0.75-EP embryos at E1.5 was regarded as 100%. Average intensity was calculated using five embryos and plotted.

2.6. Genome editing of Fgf10 and Brachyury

At the indicated time points (Fig. 2b), 50 ng/μl Cas9 protein and 200 ng/μl sgRNAs targeting the *Fgf10* or *Brachyury* gene were introduced into zygotes by electroporation. For the HDR-mediated knock-in study, 400 ng/μl ssODN was introduced together with Cas9 protein and sgRNA. The surviving 2-cell-stage embryos were transferred to the oviducts of pseudopregnant females on the day of the vaginal plug. The mice were dissected at E16.5 or E17.5 (*Fgf10*) or E9.5 (*Brachyury*), and the embryos were collected.

To investigate the CRISPR/Cas9-mediated mutations in the *Fgf10 or Brachyury* gene, genomic DNA was prepared from the yolk sac of the embryos. The genomic regions flanking the sgRNA target were amplified by PCR using specific primers: Fgf10 Fwd (5′-TGACTCTTCTGTTGTTAGCGTTG-3′) and Rev (5′-ACATCCAAAGCCTTCCTTCC-3′), Brachyury Fwd (5′-ACTGGAATGACCAGGTTTGC-3) and Rev (5′-CTGCTTCCCACAGATGGTCT-3′). The PCR amplicons of *Fgf10* or *Brachyury* were cloned into the pMD20 (Takara Bio Inc.) vector. More than ten plasmids for each embryo were isolated, and the genomic region was sequenced. Sequencing was performed using the BigDye terminator Cycle Sequencing Kit ver. 3.1 and the ABI 3500 Genetic Analyzer (Applied Biosystems).

2.7. Deep-sequencing analysis

Fgf10 and *Brachyury* target sites were amplified from the genomic DNA extracted from entire embryos. The PCR amplicons were sequenced using the MiSeq Reagent Kit Nano v2 (500 cycles) and the MiSeq sequencer (Illumina). MiSeq reads were analyzed using Excel software.

2.8. Immunostaining

The immunostaining was done as described previously (Takemoto et al., 2011). The embryos were fixed overnight with 4% PFA in PBS, immersed in 15% and 25% sucrose in PBS in sequence for 2 h each step, and embedded in OCT compound. Cryosections with thickness of 10 μm were prepared, and treated at 105 °C for 15 min in antigen unmasking solution (Vector Laboratories) using an autoclave. The sections were reacted with 10% normal donkey serum for 30 min, then with the goat anti-Sox2 antibody (AF2018, R&D Systems) at 1:200 dilutions overnight at 4 °C. After several washes, the sections were incubated with Alexa Fluor 488-labelled anti-goat IgG (A-11055, Molecular Probes) secondary antibody at 1:200 dilutions for 1 h at ambient temperature. After several washes the samples were stained with 0.5 μg/ml Hoechst 33342, and mounted in Permafluor (Thermo Fisher Scientific). Fluorescence images were taken using a DMI4000B inverted microscope (Leica Microsystems) with an ORCA-Flash4.0 V2 sCMOS camera (Hamamatsu Photonics).

3. Results

3.1. Translation of exogenously introduced mRNA in zygotes occurs after the first genome replication

Mutant embryos or mice are usually generated with the CRISPR/Cas9 system by introducing *Cas9* mRNA and sgRNA into