pISSN 1225-4991, eISSN 2288-0178
J. Emb. Trans. (2015) Vol. 30, No. 4, pp. 299~303
http://dx.doi.org/10.12750/JET.2015.30.4.299

Production of Knockout Mice using CRISPR/Cas9 in FVB Strain

Hee Sook Bae, Soo Jin Lee and Ok Jae Koo†

Laboratory Animal Research Center, Samsung Biomedical Research Institute, Suwon 16419, Korea.

ABSTRACT

KO mice provide an excellent tool to determine roles of specific genes in biomedical filed. Traditionally, knockout mice were generated by homologous recombination in embryonic stem cells. Recently, engineered nucleases, such as zinc finger nuclease, transcription activator-like effector nuclease and clustered regularly interspaced short palindromic repeats (CRISPR), were used to produce knockout mice. This new technology is useful because of high efficiency and ability to generate biallelic mutation in founder mice. Until now, most of knockout mice produced using engineered nucleases were C57BL/6 strain. In the present study we used CRISPR-Cas9 system to generate knockout mice in FVB strain. We designed and synthesized single guide RNA (sgRNA) of CRISPR system for targeting gene, Abtb2. Mouse zygote were obtained from superovulated FVB female mice at 8-10 weeks of age. The sgRNA was injected into pronuclear of the mouse zygote with recombinant Cas9 protein. The microinjected zygotes were cultured for an additional day and only cleaved embryos were selected. The selected embryos were surgically transferred to oviduct of surrogate mother and offsprings were obtained. Genomic DNA were isolated from the offsprings and the target sequence was amplified using PCR. In T7E1 assay, 46.7% among the offsprings were founded as mutants. The PCR products were purified and sequences were analyzed. Most of the mutations were founded as deletion of few sequences at the target site, however, not identical among the each offspring. In conclusion, we found that CRISPR system is very efficient to generate knockout mice in FVB strain.

(Key words : CRISPR-Cas9, knockout mice, FVB)

INTRODUCTION

Genetically engineered mice (GEM), especially knockout mice, is regarded as one of very important tool to find the functions of specific genes. For producing knockout mice, specific gene was modified in embryonic stem cell and then chimeric mice were generated by injecting the mutated embryonic stem cells into murine blastocysts. Knockout mice were born from the chimeric mice that the mutated embryonic stem cells were migrated into germ line. Although, numerous knockout mice were already generated using this technique, it is still very time-consuming and labor-intensive process. Recently, new genome engineering technologies such as zinc finger nuclease, transcription activator-like effector nuclease (TALEN), and clustered regulatory interspaced short palindromic repeat (CRISPR)/CRISPR-associated Cas 9 (Cas9) were adopted to generate GEM (Wefers *et al.*, 2012; Yang *et al.*, 2013) and overall efficiency and cost were significantly reduced (Sung *et al.*, 2014). With this technique embryonic stem cells were not necessary and mutations at the target gene were directly generated by injection of the genome engineering elements into the one cell embryo of the mice.

Among the three genome engineering techniques, CRISPR/Cas9 is the most convenient because it is relatively easy to synthesize and activity in the one-cell embryo is very high. The CRISPR/Cas9 system is consists of Cas9 nuclease protein and a single guide RNA (sgRNA). The sgRNA were specifically designed to target 20 base pair of genomic locus and a protospacer adjacent motif (PAM) sequence was followed. The target specific guide sequence was attached to the target sequence and PAM sequence was attached to the Cas9 protein then this complex cleaves the double-stranded DNA at the specific location. Mutations were occurred during the repair process of the double strand break mediated by CRISPR/Cas9 complex (Hara *et al.*, 2015; Nakagawa *et al.*, 2015).

Using the CRISPR/Cas9 system various mutated mouse in-

This study was financially supported by the Cooperative Research Program for Agriculture Science and Technology Development (no. PJ009802), Rural Development Administration, Korea.
† Correspondence : okjae.koo@gmail.com

cluding knockout mice, multiple site knockout mice and even knock-in mice were already generated (Li *et al.*, 2013; Mou *et al.*, 2015; Qihan *et al.*, 2015; Tsuchiya *et al.*, 2015; Young *et al.*, 2015; Zhang *et al.*, 2015). Most of the generated mice were based on C57BL/6 strain because it is most widely used strain in the biomedical research field. Recently, one groups showed that this technique can be successfully used to generate mutated NRG/NSG strain mice and confirmed that this technique can be used in various strain of mouse. The FVB strain is widely used for generating transgenic mice because of the easiness to get large amount of embryos and to manipulated under microscope due to clearly visible pronuclear. However, in our knowledge, mutated mice derived from CRISPR/Cas9 in FVB strain is not confirmed yet.

In the present study, we successfully generated knockout mice in FVB strain. And the mutated sequence in the mice were analyzed.

MATERIALS AND METHODS

1. Design and Synthesize of CRISPR/Cas9

We targeted mouse Abtb2 gene and randomly selected two candidate sequences with more than two mismatches through the whole genome (Fig. 1). The single guide RNA (sgRNA) and Cas9 protein were designed and synthesized by Toolgen Inc (Seoul, Korea).

2. Preparation of *In Vivo* Derived Murine Zygotes

Murine zygotes were isolated by standard PMSG-hCG procedure. Briefly, female FVB mice were IP injected with 5 IU PMSG, followed by 5 IU of HCG after 48 h. The hormone injected mice were mated with male FVB mice overnight. The next day oviducts were surgically removed from the mated female mice and zygotes were isolated from ampulla of the oviducts. The zygotes were washed with M2 medium (Sigma-Aldrich, MO, USA) and used for micromanipulation.

3. Generation of the Mutated Mice using Pronucleus Injection

Mutated mice were generated following the procedures described elsewhere (Sung *et al.*, 2014) with slight modification. Briefly, 10 ng/μl of each sgRNAs were mixed with 20 ng/μL of Cas9 protein. The complex was incubated at RT for 10 minutes and then injected into pronucleus of the prepared FVB zygotes. For the micromanipulation, micromanipulators and microinjectors (Eppedorf, NY, USA) attached to the microscope (Olympus, Tokyo, Japan) were used. After pronuclear injection, the zygotes were move to drop of M16 medium (Sigma-Aldrich) covered with mineral oil and kept in incubator (5% CO_2, 37 ℃) until embryo transfer to the surrogate mother (ICR mice).

4. Analysis of Mutations

For detection of CRISPR-induced mutations, the target locus was amplified from genomic DNA isolated from the tail of the offsprings (DNeasy kit, Qiagen, CA, USA) by nested PCR and subjected to the T7E1 assay. For sequence analysis, PCR amplicons were purified using the Gel Extraction Kit (Qiagen) and cloned into the T-Blunt vector using the T-Blunt PCR Cloning Kit (SolGent, Daejeon, Korea). Cloned plasmids were sequenced using the M13 primer.

RESULTS

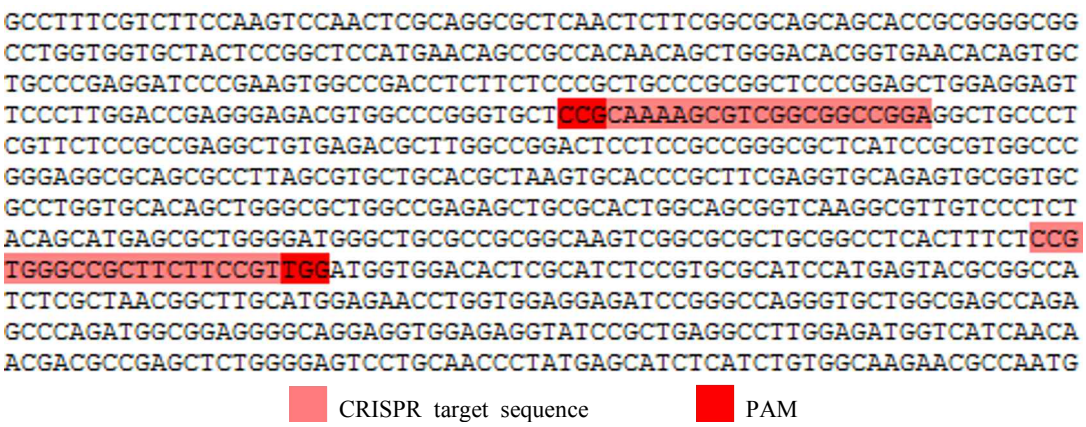

Fig. 1. Design of CRISPR guide RNA sequence used in the study.

1. Production of the Mutated Mice

Total 52 microinjected embryos were transferred to 3 surrogate mice. Among them 2 surrogates delivered 19 offsprings. Among them 4 neonates were dead at first day and the other 15 offsprings were used for further analysis. Picture of mutated mice was shown in Fig. 2.

2. Analysis of Mutation at Target Site

All the live offsprings were analyzed by T7E1 assay. Among the 15 offsprings, 7 mice were founded as mutated mice (Fig. 3). We also analyzed sequence of the target region of the mutated mice and confirmed that the target sequences were deleted (Fig. 4).

Fig. 2. Knockout FVB mice produced using CRISPR/Cas9 system.

DISCUSSION

Knockout mice are excellent tool in biomedical field. Traditionally, knockout mice were generated by homologous recombination in embryonic stem cells. However, it is very time-consuming and labor-intensive procedure. Recently, engineered nucleases, such as zinc finger nuclease, transcription activator-like effector nuclease and clustered regularly interspaced short palindromic repeats (CRISPR), were used to generate knockout mice. This new technology is useful because of high efficiency and ability to generate biallelic mutation in founder mice. Until now, most of knockout mice generated using engineered nucleases were C57BL/6 strain. In the present study we used CRISPR-Cas9 system to generate knockout mice in FVB strain.

In the present study, we successfully produced 19 offsprings (4 were dead) and T7E1 assay showed that 46.7% among the offsprings were founded as mutants. This result is very promising because whole procedure takes less than 2 months to get knockout mice.

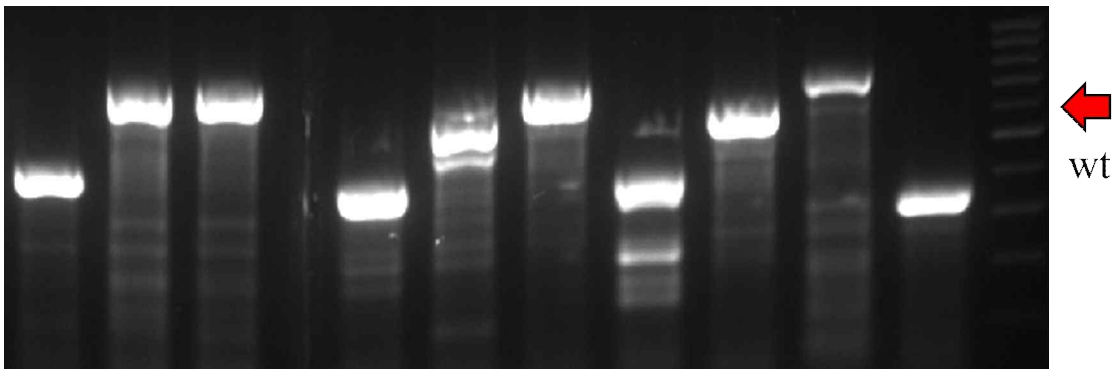

Fig. 3. T7E1 assay for detecting mutation on target site in offsprings.

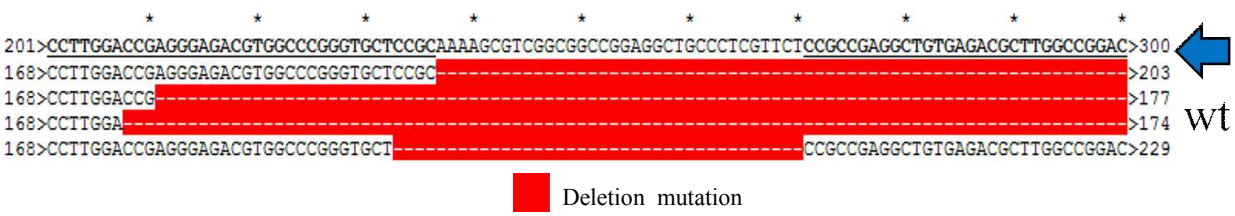

Fig. 4. Analysis of mutation on target sequence in knockout mice.

We also analysis sequence of the target site in the knockout mice. All the mutations founded in the present study were deletion of sequences at the target site, however, not identical among the each offsprings. Interestingly, deletion at the target sequences were relatively larger. It is not clear, however, it might be related with that we simultaneous injected 2 different sgRNA. We found that deletions were founded at the both sgRNA target locus in most case. Furthermore, in some case, sequences between two different target were totally removed and large scale deletion was obtained in knockout mice.

Traditionally, strain of knockout mouse was dependent on the strain of the embryonic stem cell. However, embryonic stem cells were isolated and established from only few mouse strain such as 129 and C57BL/6. However, using genome engineering technique, most of the strain including FVB strain used in the study can be used for generating knockout mice. It is one of the strong point of this new technique compared to the traditional procedure.

On the other hand, we also confirmed that germ line transfer of the mutated sequence and it is well maintained in the further generation of the founder mice (Fig. 5). It is another strong point of this technique compared to traditional method using embryonic stem cells. Because, germ line transfer is frequently time consuming and somewhat difficult point of the traditional embryonic stem cell method.

In conclusion, we produced knockout mice using CRISPR/Cas9 system in FVB strain. It is very efficient to generate knockout mice in this strain and showed successful germ line transfer in the offsprings.

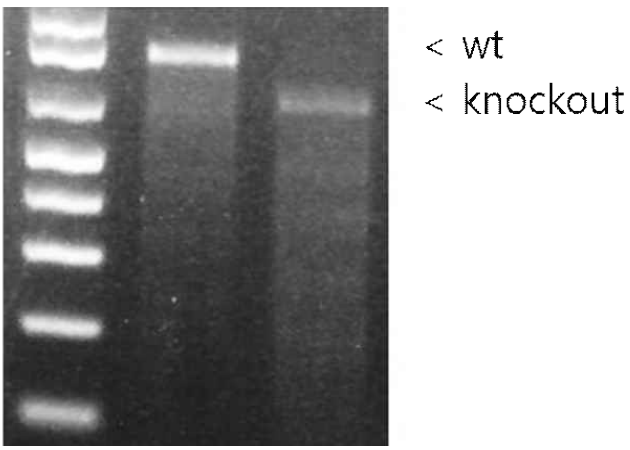

Fig. 5. PCR analysis of target sequence in F_1 mouse derived from mutated founder mouse.

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Received November 25, 2015, Revised December 5, 2015, Accepted December 18, 2015