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—Original Article—

Production of genome-edited mice by visualization of nucleases introduced into the embryos using electroporation

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Abstract. Genome editing technology contributes to the quick and highly efficient production of genetically engineered animals. These animals are helpful in clarifying the mechanism of human disease. Recently, a new electroporation technique (TAKE: Technique for animal knockout system by electroporation) was developed to produce genome-edited animals by introducing nucleases into intact embryos using electroporation instead of the microinjection method. The aim of this study was to increase the efficiency of production of genome-edited animals using the TAKE method. In the conventional protocol, it was difficult to confirm the introduction of nucleases into embryos and energization during operation. Using only embryos that introduced nucleases for embryo transfer, it will lead to increased efficiency in the production of genome-edited animals. This study examined the visualization in the introduction of nucleases into the embryos by using nucleases fluorescent labeled with ATTO-550. The embryos were transfected with Cas9 protein and fluorescent labeled dual guide RNA (mixture with crRNA and tracrRNA with ATTO-550) targeted tyrosinase gene by the TAKE method. All embryos that survived after electroporation showed fluorescence. Of these embryos with fluorescence, 43.7% developed to morphologically normal offspring. In addition, 91.7% of offspring were edited by the tyrosinase gene. This study is the first to demonstrate that the introduction of nucleases into embryos by the TAKE method could be visualized using fluorescent-labeled nucleases. This improved TAKE method can be used to produce genome-edited animals and confirm energization during operation.
Key words: CRISPR/Cas, Electroporation, Embryos, Genome editing, Mouse

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Genetically engineered mice have been recently used to clarify the mechanisms of human diseases [1–3]. These mice, including genome-edited strains, are generally produced by microinjection of nucleases into pronuclear stage embryos [4]. This method requires a high skill level to operate the micromanipulator. Furthermore, simultaneous assessment of several cells is not convenient because the nucleases must be injected into embryos successively using a micromanipulator. Recently, a new technology by electroporation has been invented to produce genome-edited animals. The technology for animal knockout system by electroporation (TAKE) method is a simple and effective technique to produce genome-edited animals using engineered endonucleases, including zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system [5, 6]. This method realized the introduction of nucleases and a high survival rate into intact embryos using a new 3-step electrical pulse program [7]. This 3-step electrical pulse program constructed the first pulse, called a poring pulse, to create a hole in the zona pellucida and oolemma by a high-voltage electrical pulse, and transfer pulse to transfer nucleases into embryos as the second and third pulses.

The introduction of nucleases was clear in the microinjection method because it was injected directly into the embryos using a thin glass pipette [4]. In the conventional TAKE method protocol, however, it is difficult to confirm the introduction of nucleases into embryos and energization during operation. It takes at least 3 months or more to produce genetically modified mice or other animals for use in research. Using only embryos that introduced nucleases for embryo transfer, it will lead to an increase in the production efficiency of genome-edited animals. The aim of this study was to further improve the production efficiency of genome-edited animals using the TAKE method. This study demonstrated visualization of nucleases by fluorescent labeling for introduction into embryos using the TAKE method.

Materials and Methods

Animals

C57BL/6J male and Crlj:ICR female mice were purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan). Males older than 11 weeks and females aged 8 to 16 weeks were used as sperm and oocyte donors, respectively. Crlj:ICR female mice aged 10–16 weeks were used as recipients for embryo transfer. All animals were maintained in an air-conditioned (temperature 23 ± 5°C, humidity 50 ± 10%) and light-controlled room (lights on from 0700 to 1900 h). All animal care and procedures performed in this

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study conformed to the Guidelines for Animal Experiments of Iwate University and were approved by the Animal Research Committee of Iwate University.

Collection of pronuclear stage embryos

Crlj:ICR female mice were induced superovulation by intraperitoneal injection of 10 IU/body pregnant mare serum gonadotropin (PMSG; ASKA Animal Health, Tokyo, Japan), followed by intraperitoneal injection of 10 IU/body human chorionic gonadotropin (hCG, ASKA Animal Health) 48 h later. These females were mated with C57BL/6J male mice overnight. Pronuclear stage embryos were collected by flushing the oviducts by PB1 [8] on the day after mating. The embryos were collected and cultured in KSOM [9] until electroporation.

Preparation of Cas9 protein and guide RNA

Cas9 protein and dual guide RNA were purchased from IDT (Integrated DNA Technologies, Coralville, IA, USA). Dual guide RNA mixture with crRNA and tracrRNA was used as guide RNA. Guide RNA was designed to target the tyrosinase gene of the C57BL/6 mouse (5′-GGGTGGATGACCGTGAGTCC-3′) that participate in melanin biosynthesis [10]. This gene is specifically expressed in retinal pigment epithelial cells of the eye, choroidal melanocytes, and hair follicle melanocytes in mammals [11]. It is possible to discriminate the result of genome editing from the fetal eye color without genetic analysis by knocking out the tyrosinase gene. tracrRNA with or without ATTO-550 labeled at the 5′ end was used in this study.

The nuclease solution contained 200 ng/μl Cas9 protein, 15 μM crRNA, and 15 μM tracrRNA (conducted 0, 7.5, or 15 μM tracrRNA-ATTO550) in Opti-MEM (Thermo Fisher Scientific, Waltham, MA, USA). The nuclease solution was prepared just before electroporation.

Introduction of Cas9 protein and guide RNA into pronuclear stage embryos using the TAKE method

The TAKE method was carried out to intact pronuclear stage embryos which collected at 22–24 h after hCG injection according to a previously described protocol [7]. Super electroporator NEPA21 (NEPA GENE, Chiba, Japan) was used to introduce nucleases into embryos. The poring pulse was set to voltage: 40 V, pulse length: 3.5, 2.0 or 0.5 msec, pulse interval: 50 msec, number of pulses: 4, decay rate: 10%, polarity: +. The transfer pulse was set to a voltage: 15 V, pulse length: 50 msec, pulse interval: 50 msec, number of pulse: 5, decay rate: 40%, Polarity: +/–. The nuclease solution (5 μl) was filled between metal plates of 1 mm gap electrodes on a glass slide (CUY501P1-1.5; NEPA GENE). The embryos placed in line between the electrodes were then discharged. The nuclease solution was exchanged for two operations to avoid dilution of the solution. After electroporation, the embryos were transferred into KSOM.

Measurement of fluorescent intensity in embryos after electroporation

Fluorescence of electroporated embryos was observed using an inverted microscope (Fig. 1). The fluorescent intensity of embryos electroporated nucleases, including 7.5 μM tracrRNA-ATTO550 at 0.5 or 3.5 msec pulse length was measured using ImageJ (https://imagej.nih.gov/ij/). The fluorescent intensity of each embryo was

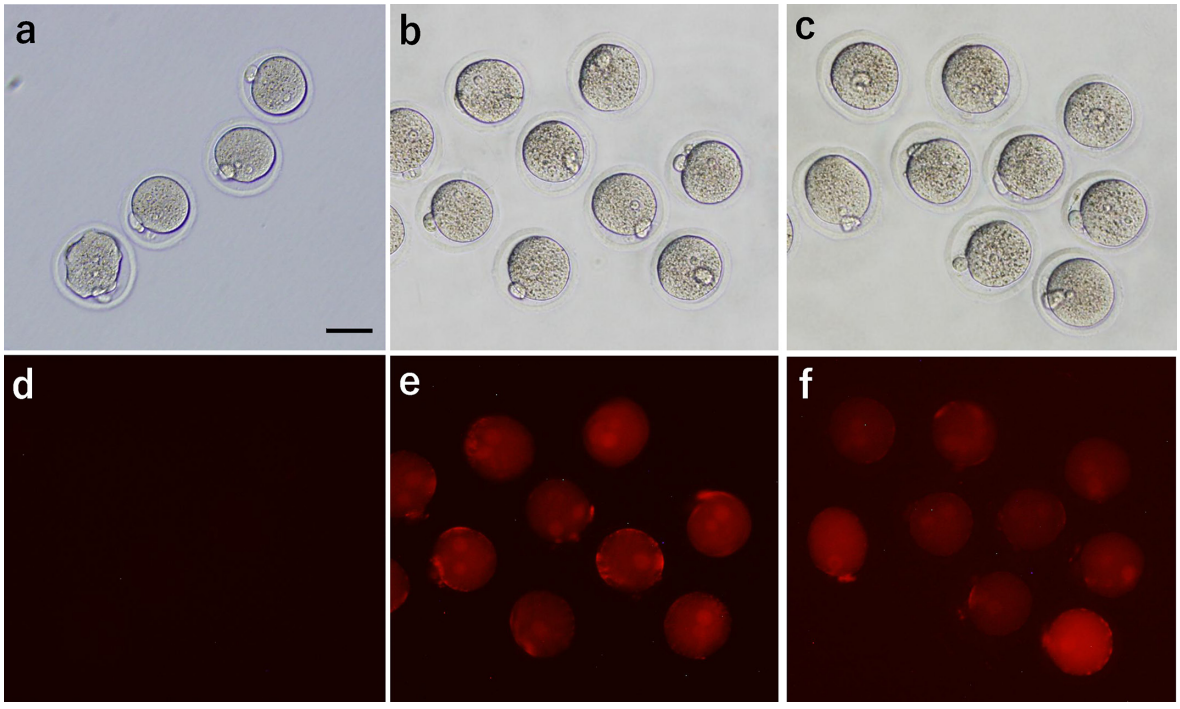

Fig. 1. Fluorescence of embryos electroporated nucleases including 7.5 μM tracrRNA-ATTO550. Without electroporation (a, d). Electroporated at 0.5 (b, e) or 3.5 msec (c, f) of pulse lengths. Scale bar was 50 μm.

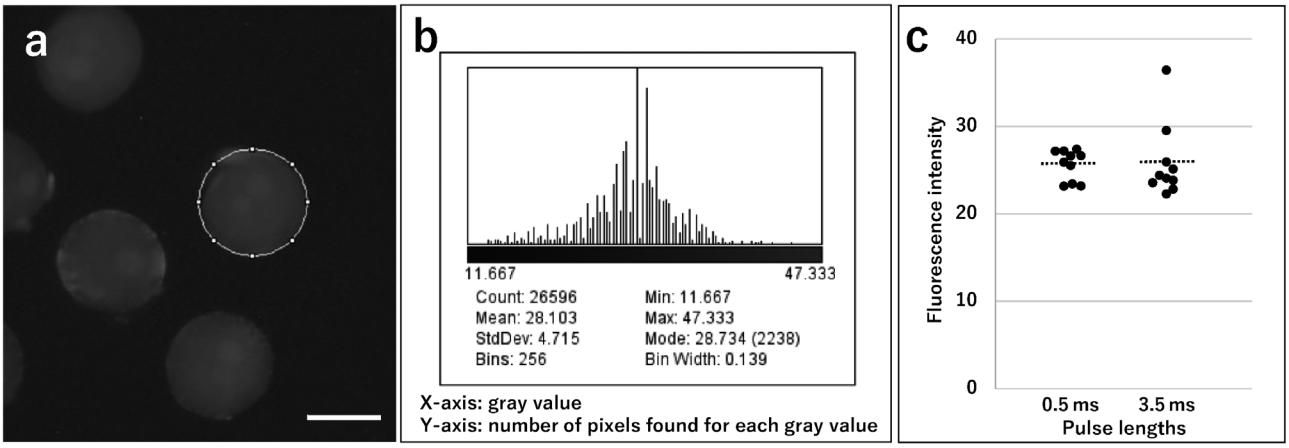

Fig. 2. Measurements of embryos with fluorescence using ImageJ. The fluorescent intensity of embryo surrounded white circle were measured (Scale bar was 50 μm) (a). The histogram of fluorescent intensity in an embryo (b). Mean gray values of fluorescent intensity in each embryo. The broken line was average value (n = 10) (c).

measured (Fig. 2a) and then created histogram of gray value (Fig. 2b). The mean gray value in each embryo were plot and compared (Fig. 2c). The embryos with fluorescence were further cultured in KSOM at 37°C under 5% CO_2 in air for embryo transfer.

Embryo transfer and genome editing in the offspring

Embryos with fluorescence that developed to the 2-cell stage after electroporation were transferred into the oviducts of pseudopregnant females that mated with vasectomized males on the day before embryo transfer. The number of offspring was counted at 14 days after embryo transfer. Genome editing of offspring was estimated by differences in eye color.

Data analysis

Experiments were repeated 3 times for each group. The development and genome editing rates of embryos after electroporation was analyzed using chi-square test followed by a multiple comparisons test using Ryan's method. The fluorescent intensity of embryo was analyzed using the Student's *t*-test.

Results

The development and genome editing rates of embryos electroporated nucleases, including tracrRNA-ATTO550 at different concentrations, are shown in Table 1. Over 97% of the embryos survived after electroporation. All the embryos survived after electroporation showed fluorescence. No significant differences were observed in the development to 2-cell stage of embryos that introduced nucleases, including 7.5 μM tracrRNA-ATTO550, compared with that of 0 μM. However, there was a significant decrease in the number of embryos that introduced nucleases, including 15 μM tracrRNA-ATTO550. Although, no significantly differences were obtained in the rate of knockout offspring derived from embryos introducing 0, 7.5 or 15 μM tracrRNA-ATTO550, it was high (91.7%) in the embryos introducing 7.5 μM tracrRNA-ATTO550.

Table 2 shows the development and genome editing rates of embryos electroporated nucleases, including 7.5 μM tracrRNA-ATTO550, using different electrical conditions. In these experiments, all embryos survived after electroporation and fluorescence was observed (Fig. 1e and f). More than 86% of the embryos developed to the 2-cell stage without significant differences. No significant differences were observed in the development to offspring from pulse length of 0.5 to 3.5 msec of poring pulse. However, the genome editing rate significantly increased with increasing pulse length.

The fluorescence intensity of each embryo electroporated at 0.5 or 3.5 msec of pulse length was measured (Fig. 2a and b). No significant differences were observed in the means of fluorescent intensity at 0.5 msec (25.6) or 3.5 msec (25.8) of pulse length (Fig. 2c).

Table 1. Development and genome editing rates of embryos electroporated nucleases, including tracrRNA-ATTO550 at different concentrations

Conc. of tracrRNA-ATTO550 (μM)	Conc. of tracrRNA (μM)	No. embryos electroporated	No. (%) of embryos survived after electroporation	No. (%) of embryos with fluorescence	No. (%) of embryos developed to 2-cell stage and transferred	No. (%) of offspring	No. (%) of knockout offspring
0	15	35	35 (100.0 ± 0.0)	-	32 (90.0 ± 12.2) [a]	15 (50.7 ± 15.1)	7 (46.7 ± 8.2)
7.5	7.5	29	29 (100.0 ± 0.0)	29 (100.0 ± 0.0)	26 (91.0 ± 11.0) [a]	11 (43.7 ± 16.3)	10 (91.7 ± 10.2)
15	0	84	82 (97.6 ± 6.5)	82 (100.0 ± 0.0)	20 (21.7 ± 16.3) [b]	4 (10.0 ± 3.67)	1 (16.7 ± 40.8)

The pulse length of the poring pulse was set to 3.5 msec. Percentages were showed as the mean ± SEM. a *vs.* b. Significant differences at $P < 0.05$.

Table 2. Development and genome editing rates of embryos electroporated nucleases included 7.5 μM tracrRNA-ATTO550 under different electrical conditions

Pulse lengths (ms)	No. embryos electroporated	No. (%) of embryos survived after electroporation	No. (%) of embryos with fluorescence	No. (%) of embryos developed to 2-cell stage	No. of embryos transferred	No. (%) of offspring	No. (%) of knockout offspring
0.5	27	27 (100.0 ± 0.0)	27 (100.0 ± 0.0)	27 (100.0 ± 0.0)	27	12 (44.3 ± 0.8)	5 (53.3 ± 40.8) [a]
2.0	56	56 (100.0 ± 0.0)	56 (100.0 ± 0.0)	51 (86.7 ± 11.4)	31	8 (25.3 ± 18.8)	4 (36.7 ± 44.9)
3.5 *	29	29 (100.0 ± 0.0)	29 (100.0 ± 0.0)	26 (91.0 ± 11.0)	26	11 (43.7 ± 16.3)	10 (91.7 ± 10.2) [b]

* Same results were shown as Table 1. Percentages were showed as the mean ± SEM. a *vs*. b. Significant differences at $P < 0.05$.

Discussion

We recently developed a new method, named TAKE, that could produce genome-edited animals by electroporation instead of the microinjection method [5–7]. Many kinds of knockout and knock-in mice and rats have already been produced by TAKE method using ZFN, TALEN, CRISPR-Cas system [12–17], and other nucleases [18]. Furthermore, this method has widely been applied for the production of genome-edited strains in other animals [19, 20]. Although this method is easy and simple to operate, it is difficult to confirm the introduction of nucleases into embryos and energization during operation. Visualization of nucleases by labeling fluorescent dye was used as one of the methods to confirm introduction of nucleases into embryos after electroporation [21]. This study visualized nucleases introduced into the embryos by using nucleases fluorescent labeled with ATTO-550. The development to the 2-cell stage and offspring, and rate of knockout offspring in the embryos electroporated nucleases with 15 μM tracrRNA-ATTO550 was strongly inhibited (Table 1). Fluorescent dyes such as Hoechst 33342 inhibit DNA synthesis and mutation [22]. It was thought that ATTO550 was also toxic to the early development of embryos. However, this toxicity greatly reduced the dilution of concentration, and no effects were observed in the visualization of nucleases into the embryos (Table 1, Fig. 1e and f).

The aim of this study was to select embryos by visualizing the introduction of nucleases for efficient production of genome edited animals. Fortunately, all embryos showed fluorescence after electroporation (Table 1 and 2, Fig. 1e and f). These results demonstrated that the TAKE method could be introduced sufficiently nucleases into the embryos as well as the microinjection method.

No significant differences were observed in the rate of development to offspring from pulse length of 0.5 to 3.5 msec of poring pulse (Table 2). It was demonstrated that the electricity used in the TAKE method caused no damage to the embryos and subsequent development. The genome editing rate significantly increased with increasing pulse length (Table 2). However, no significant differences were observed in the average of fluorescent intensity at 0.5 or 3.5 msec of pulse length (Fig. 2c). This suggests that the amounts of Cas9 protein and crRNA into the embryos were different under each electrical condition. Thus, further studies on the measurement of these nucleases in the embryos after electroporation are required.

To the best of our knowledge, the present study is the first to demonstrate visualization of nucleases into embryos by introducing fluorescent-labeled nucleases using the TAKE method. This improved TAKE method can be used to produce genome-edited animals and confirm energization during operation.

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