

Amount of Cas9 protein introduced into mouse embryos via electroporation affects the genome-editing rate

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Abstract. Genetically engineered animals can be produced quickly using genome editing technology. A new electroporation technique, technique for animal knockout system by electroporation (TAKE), aids in the production of genome-edited animals by introducing nucleases into intact embryos using electroporation instead of microinjection. It is difficult to confirm nuclease delivery into embryos after electroporation using the conventional TAKE method. We previously reported the successful visualization of fluorescently-labeled tracrRNA in embryos after electroporation Cas9 paired with the crRNA:tracrRNA-ATTO550 duplex. However, the amount of fluorescence signal from labeled tracrRNA in embryos did not correlate with the genome editing rate of the offspring. This study examined the visualization of Cas9 protein in embryos after electroporation and its correlation with the genome editing rate of the offspring using a fluorescent Cas9 fusion protein. The fluorescent Cas9 protein was observed in all embryos that survived following electroporation. We found that the efficiency of Cas9 protein delivery into embryos via electroporation depended on the pulse length. Furthermore, we demonstrated that the amount of fluorescent Cas9 protein detected in the embryos correlated with the genome editing efficiency of the embryos. These data indicate that the TAKE method using fluorescently-labeled nucleases can be used to optimize the delivery conditions and verify nuclease delivery into individual embryos prior to embryo transfer for the efficient production of genome-edited animals.

Key words: CRISPR/Cas, Electroporation, Embryos, Genome editing, Mouse

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Genetically engineered mice, including genome-edited strains, have recently been used to study human diseases [1–3]. These mice are generally produced by the introduction of nucleases into pronuclear stage embryos via microinjection [4]. However, the microinjection method is inconvenient because of the high skill level required to operate the micromanipulator. Furthermore, nucleases must be successively injected into embryos using a micromanipulator. Recently, genome-edited animals have been produced by a new technique using electroporation, known as the technology for animal knockout system by electroporation (TAKE). It could produce simply and effectively genome-edited animals using zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) systems [5, 6]. This method can effectively introduce nucleases into intact embryos with a high survival rate using a new three-step electrical pulse program [7].

Microinjection can be used to reliably introduce nucleases via a direct injection into embryos using a thin glass pipette [4]. However, it is difficult to confirm nuclease delivery into embryos after electroporation using the conventional TAKE method. We have previously demonstrated the successful visualization of nucleases in embryos

following electroporation using fluorescently-labeled tracrRNA as part of the guide RNA [8]. In that study, the genome-editing rate had significantly increased with increasing pulse length; however, no significant differences were observed in the average fluorescence intensities at the different pulse lengths. This suggests that the amount of Cas9 protein, not fluorescent tracrRNA, delivered into the embryos affects the genome editing rate of embryos after electroporation. This study examined the visualization of Cas9 protein in embryos after electroporation and its correlation with the genome editing rate of the offspring using a fluorescent Cas9 fusion protein.

Materials and Methods

Animals

C57BL/6J male and ICR female mice (Charles River Laboratories Japan Inc., Yokohama, Japan) were used in this study. Males older than 11 weeks and females aged 8–16 weeks were used as sperm and oocyte donors, respectively. ICR female mice, aged 10–16 weeks, were used as recipients for embryo transfer. All animals were maintained in an air-conditioned (temperature, 23 ± 3°C; humidity, 50 ± 10%) and light-controlled room (lights on from 0700 to 1900 h). Animal Research Committee of Iwate University approved that all animal care and procedures performed in this study conformed to the Guidelines for Animal Experiments of Iwate University.

Production of pronuclear stage embryos

Pronuclear stage embryos were produced using *in vitro* fertilization. Sperms collected from the cauda epididymis of C57BL/6J male mice were pre-cultured in human tubal fluid (HTF) medium [9] for 1 h

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at 37°C under 5% CO₂ to induce capacitation. Superovulation was induced in ICR females via an intraperitoneal injection of 10 IU/body pregnant mare serum gonadotropin (ASKA Animal Health Co., Ltd., Tokyo, Japan), followed by an intraperitoneal injection of 10 IU/body human chorionic gonadotropin (hCG; ASKA Animal Health Co., Ltd.) 48 h later. Cumulus-oocyte complexes were collected from the oviducts of females 16 h after hCG injections. The cumulus-oocyte complexes and capacitated sperms (1×10^5 cells/ml) were then co-cultured at 37°C under 5% CO₂. Pronuclear stage embryos were collected in fresh HTF medium 5 h after insemination. Embryos were maintained at 37°C under 5% CO₂ until electroporation.

Preparation of Cas9 protein and guide RNA

Cas9-green fluorescence protein (GFP) (cat no.10008100), crRNA, tracrRNA (cat no.1072533), and ATTO550-labeled tracrRNA (cat no. 1075928) were obtained from Integrated DNA Technologies Inc. (Coralville, IA, USA). crRNA was designed to target the tyrosinase gene of C57BL/6 mice (5'-GGGTGGATGACCGTGAGTCC-3'), which participates 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 results of genome editing from the eye color of offspring derived from C57BL/6 \times ICR embryos without genetic analysis by knocking out the tyrosinase gene. The nuclease solution for embryo electroporation contained 200 ng/ μ l Cas9-GFP, 15 μ M crRNA, 15 μ M tracrRNA or a mixture solution with 7.5 μ M tracrRNA and 7.5 μ M tracrRNA-ATTO550 in Opti-MEM (Thermo Fisher Scientific Inc., MA, USA) [8] was prepared just before electroporation.

Introduction of nucleases into embryos using the TAKE method

Nucleases were introduced into pronuclear stage embryos 22–24 h after hCG injection using the TAKE method [7]. A super electroporator NEPA21 (NEPA GENE Co., Ltd., Chiba, Japan) was used to introduce the nucleases. The nuclease solution (5 μ l) was placed between metal plates of 1 mm gap electrodes on a glass slide (CUY501P1-1.5; NEPA GENE Co., Ltd.). Embryos were placed in a line between the electrodes. The poring pulse was set to voltage: 40 V, pulse length: 0.5 or 3.5 msec, pulse interval: 50 msec, number of pulses: 4, decay rate: 10%, and polarity: +. The transfer pulse was set to voltage: 15 V, pulse length: 50 msec, pulse interval: 50 msec, number of pulses: 5, decay rate: 40%, and polarity: +/- . Embryos were then discharged and transferred into the HTF medium. The nuclease solution was exchanged for two operations to avoid dilution. Embryos placed in the nuclease solution without electroporation were used as controls.

Measurement of fluorescence intensity in embryos after electroporation

The fluorescence of electroporated embryos was observed using an inverted microscope (Figs. 1 and 3). The fluorescence intensity inside each embryo was measured using the ImageJ software (<https://imagej.nih.gov/ij/>) (Fig. 1) [8]. The mean gray values for each embryo were plotted and compared (Figs. 2B and 4B). The embryos were further cultured to the 2-cell stage in HTF medium at 37°C under 5% CO₂ for embryo transfer.

Embryo transfer and genome editing in the offspring

Two-cell embryos were transferred into the oviducts of pseudo-pregnant ICR females that were mated with vasectomized males the

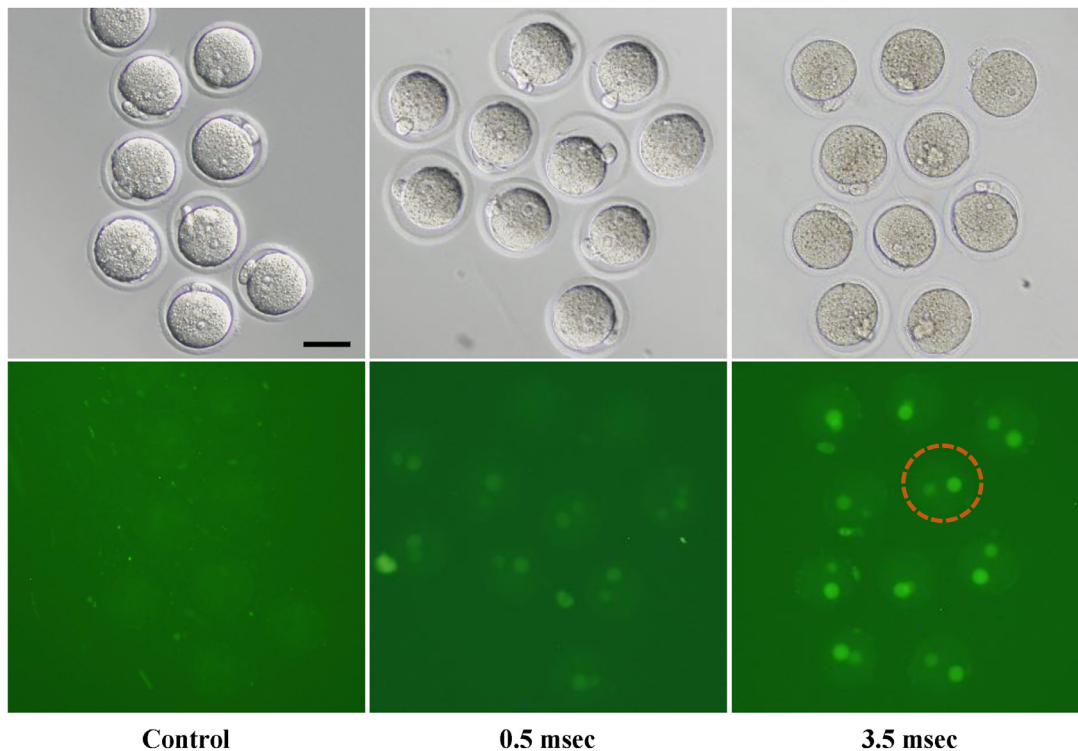


Fig. 1. Visualization of fluorescence in embryos after Cas9-GFP delivery via electroporation using either 0.5 or 3.5 msec pulse length (Scale bar, 50 μ m). Embryos placed in the nuclease solution without electroporation were taken as the control. The dotted circle of fluorescence in embryos was the area used to measure the fluorescent intensity using the ImageJ software.

day before embryo transfer. The number of offspring was counted 19 days after embryo transfer. Genome editing of the offspring was estimated based on the differences in eye color (Fig. 5).

Data analysis

The experiments were repeated 3 times for each group. The fluorescence intensity of the embryos was analyzed using the Student's *t*-test. The development and genome editing rates of the embryos after electroporation were analyzed using Fisher's exact test.

Results

Nucleases were introduced into pronuclear stage embryos using

the TAKE method with a pulse length of either 0.5 msec or 3.5 msec for the poring pulse. After electroporation, 99% of the embryos survived, and all surviving embryos showed GFP fluorescence at either pulse length. No GFP fluorescence was observed in embryos placed in the nuclease solution without electroporation (Fig. 1). No significant differences were observed in the development of embryos to offspring or the rate of knockout in offspring using a pulse length of either 0.5 msec (52 and 81%, respectively) or 3.5 msec (40 and 97%, respectively) (Fig. 2A). The fluorescence intensity of each embryo electroporated using a 0.5 or 3.5 msec poring pulse was measured. Significant differences were observed in the mean gray values of fluorescence intensity using a pulse length of 0.5 msec (40.8) or 3.5 msec (43.1) (Fig. 2B).

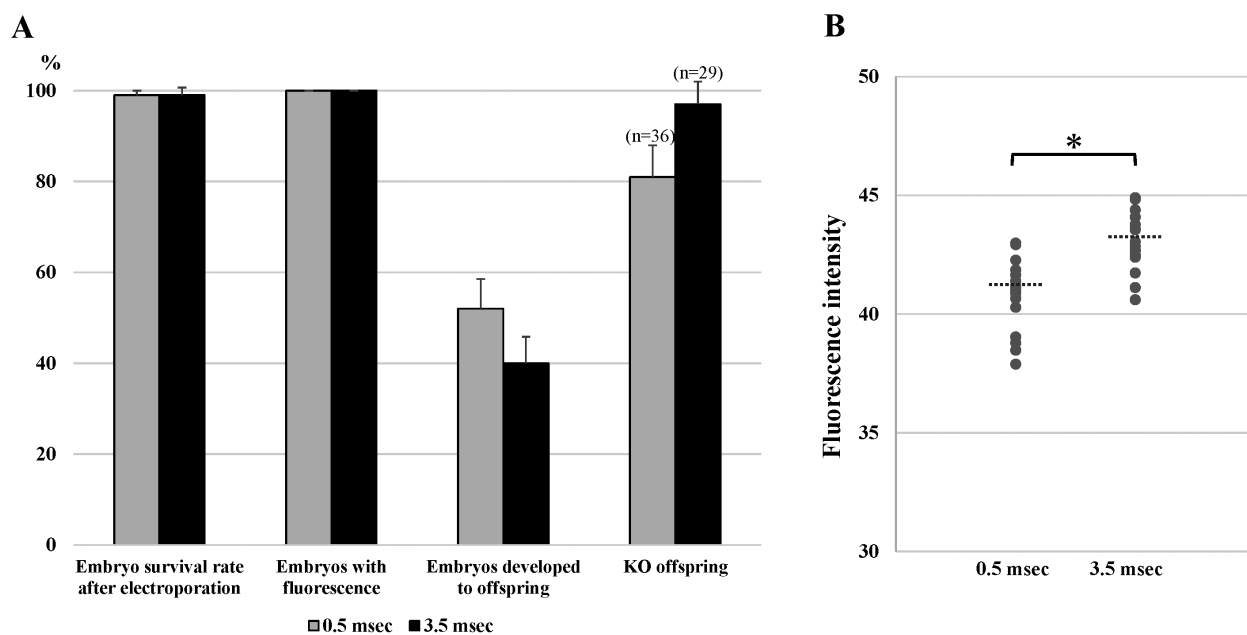


Fig. 2. A) Development and genome editing rates of embryos after Cas9-GFP delivery via electroporation using a pulse length of 0.5 (n = 99) or 3.5 (n = 95) msec (Error bar was standard error [SE]). B) Mean gray values of fluorescence intensity of GFP in each embryo. Broken line represents the average value (n = 20). * Significant differences at P < 0.05.

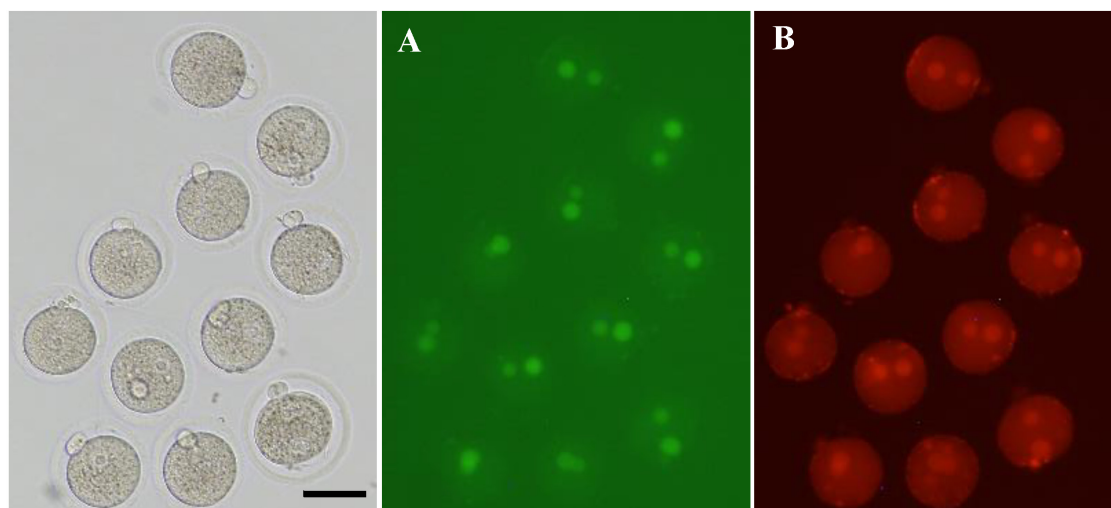


Fig. 3. Visualization of fluorescence in embryos after delivery of Cas9-GFP paired with crRNA:tracrRNA-ATTO550 via electroporation using a pulse length of 3.5 msec. (A) GFP (B) ATTO550 (Scale bar, 50 μm).

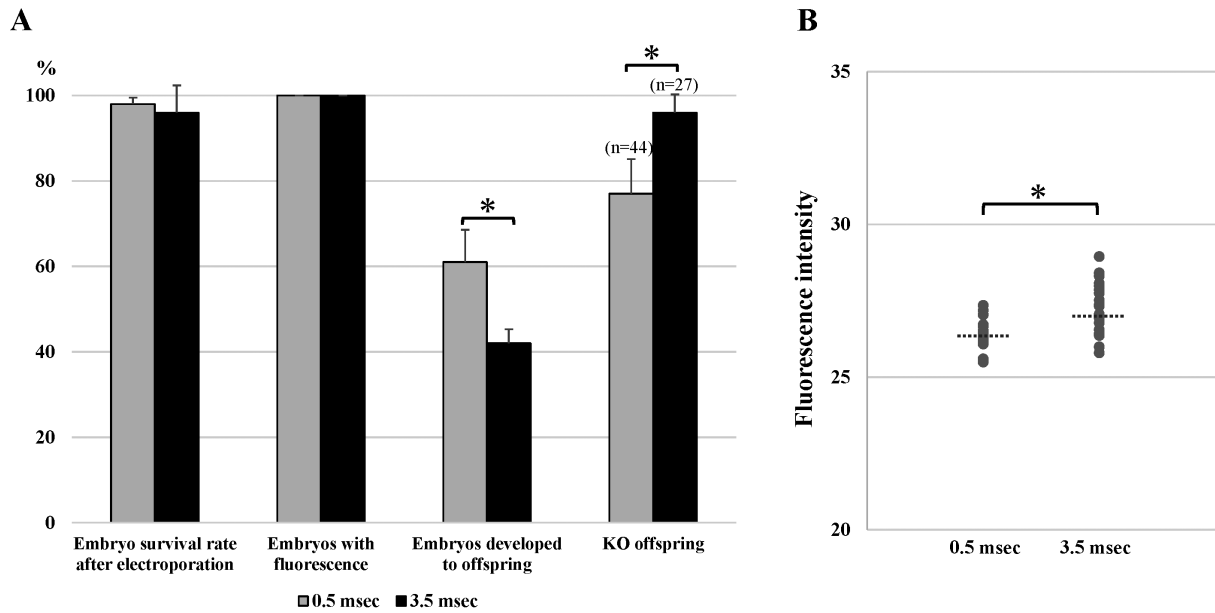


Fig. 4. A) Development and genome editing rates of embryos after delivery of Cas9-GFP paired with crRNA:tracrRNA-ATTO550 via electroporation using a pulse length of 0.5 (n = 125) or 3.5 (n = 128) msec (Error bar was SE). B) Mean gray values of fluorescence intensity of GFP in each embryo. Broken line represents the average value (n = 20). * Significant differences at $P < 0.05$.

To confirm the introduction of tracrRNA, including crRNA, with Cas9-GFP in the embryo, the nuclease solution containing 200 ng/ μ l Cas9-GFP, 15 μ M crRNA, and mixture solution with 7.5 μ M tracrRNA and 7.5 μ M tracrRNA-ATTO550 in Opti-MEM was introduced into pronuclear stage embryos by TAKE method using 0.5 or 3.5 msec pulse length for the poring pulse. After electroporation using a pulse length of 0.5 or 3.5 msec, 98 or 96% of embryos had survived. All embryos that survived had fluorescence of GFP and ATTO550 after electroporation using a pulse length of 0.5 or 3.5 msec (Fig. 4A). Significant differences were observed in the development of embryos to offspring and in the rate of knockout after electroporation using a pulse length of 0.5 msec (61 and 77%, respectively) or 3.5 msec (42 and 96%, respectively) (Fig. 4A). The mean gray values of fluorescence intensity of GFP in embryos electroporated using a pulse length of 0.5 msec (26.5) or 3.5 msec (27.3) also showed significant differences (Fig. 4B).

Discussion

The TAKE method is an easy and simple method for producing genome-edited animals [5–7]. This method has been widely applied to genome editing using the ZFN, TALEN, and CRISPR-Cas systems in mice [12–18]. This method has also been used to produce genome-edited strains in other animals [19, 20].

It is difficult to confirm nuclease entry into embryos after electroporation using the conventional TAKE method. This problem was overcome by the successful visualization of nucleases in embryos after electroporation using fluorescently-labeled tracrRNA as part of the guide RNA [8]. This study further examined the visualization of the Cas9 protein in embryos after electroporation using a Cas9-GFP fusion protein. We demonstrated successful visualization of Cas9 protein in embryos after electroporation (Figs. 1 and 3). In addition, all embryos surviving electroporation showed fluorescence (Figs. 2A and 4A). In a previous study using tracrRNA-ATTO550, no significant differences were observed in the average fluorescence



Fig. 5. Offspring with tyrosinase gene knocked out (left) and wild-type (non-genome edited) offspring (right) (Scale bar, 1 cm).

intensity at different pulse lengths, although the genome-editing rate had significantly increased with increasing pulse length [8]. However, this study demonstrated that the average fluorescence intensity of Cas9 protein and genome editing rate had significantly increased with increasing pulse length (Figs. 2B and 4B). These results indicate that the efficiency of Cas9 delivery into embryos via electroporation depends on the time duration of the use of poring pulse. Furthermore, it was suggested that the genome editing efficiency of embryos depend on the amount of Cas9 protein introduced into embryos via electroporation.

In this study, the fluorescence intensity of the embryos was directly measured after electroporation. Fluorescence was observed in the cytoplasm; however, there was clear localization of fluorescence in

the male and female pronuclei of embryos. The Cas9 protein used in this study had a nuclear localization signal. In microinjection method, nuclease solution, including nucleases, is directly injected into the pronuclei of embryos for efficient genome editing [21]. Horii *et al.* reported that injection of RNA into the cytoplasm was the most efficient method in terms of the number of viable blastocyst stage embryos, full-term pups generated, and knockout efficiency [22]. This study also demonstrated that Cas9-GFP with a nuclear localization signal introduced into the cytoplasm of embryos promptly transitioned into the pronuclei. This study demonstrated that the TAKE method can be reliably used to introduce nucleases into mouse embryos as all electroporated embryos had observable fluorescence that correlated with genome-editing rates. Furthermore, fluorescently labeled nucleases can be used to optimize delivery conditions and verify nuclease delivery into individual embryos prior to embryo transfer for the efficient production of genome-edited animals.

Conflict of interests: The authors declare no conflicts of interest. C.A.V. and S.E.G. are employees of Integrated DNA Technologies (IDT), which sold the reagents used in this study. C.A.V. holds equity in Danaher Corporation, which owns IDT.

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