Experimental Animals
Exp. Anim. 71(2), 214–223, 2022

Original

The versatile electric condition in mouse embryos for genome editing using a three-step square-wave pulse electroporator

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Abstract: Technique for Animal Knockout system by Electroporation (TAKE) is a simple and efficient method to generate genetically modified (GM) mice using the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) systems. To reinforce the versatility of electroporation used for gene editing in mice, the electric condition was optimized for vitrified-warmed mouse embryos, and applied to the fresh embryos from widely used inbred strains (C57BL/6NCr, BALB/cCrSlc, FVB/NJcl, and C3H/HeJJcl). The electric pulse settings (poring pulse: voltage, 150 V; pulse width, 1.0 ms; pulse interval, 50 ms; number of pulses, +4; transfer pulse: voltage, 20 V; pulse width, 50 ms; pulse interval, 50 ms; number of pulses, ±5) were optimal for vitrified-warmed mouse embryos, which could efficiently deliver the gRNA/Cas9 complex into the zygotes without zona pellucida thinning process and edit the target locus. These electric condition efficiently generated GM mice in widely used inbred mouse strains. In addition, electroporation using the electrode with a 5 mm gap could introduce more than 100 embryos within 5 min without specific pretreatment and sophisticated technical skills, such as microinjection, and exhibited a high developmental rate of embryos and genome-editing efficiency in the generated offspring, leading to the rapid and efficient generation of genome editing mice. The electric condition used in this study is highly versatile and can contribute to understanding human diseases and gene functions by generating GM mice more easily and efficiently.
Key words: CRISPR/Cas9, electroporation, frozen-thawed embryos, genome editing

Introduction

Genetically modified (GM) mice with specific genes altered have significantly contributed to understanding human diseases and gene functions and are indispensable as a powerful tool in various research fields [1–3]. The conventional GM mice generation method using embryonic stem (ES) cells spends a lot of time and effort [4]. Recently, the development of genome-editing technologies, such as zinc finger nucleases [5], transcription activator-like effector nucleases [6], and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system [7–9], has made it possible to generate GM mice with specific genes altered faster and easier than the conventional method using ES cells. In particular, the CRISPR/Cas9 system is the most widely used genome-editing tool with the simplest protocol and high genome-editing efficiency [10]. This system consists of two components: Cas9 protein and guide RNA (gRNA), a complex of crRNA

(Received 18 July 2021 / Accepted 8 November 2021 / Published online in J-STAGE 7 December 2021)
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Supplementary Figure and Tables: refer to J-STAGE: https://www.jstage.jst.go.jp/browse/expanim

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and tracrRNA [10]. Microinjection is generally used to delivery these components into pronuclear (PN)-stage embryos. Although microinjection is used in fresh and/or vitrified-warmed embryos and can be adapted with many mice and/or rat strains and various species [8, 9, 11–14], this method has low throughput and requires special equipment and skills [15, 16].

In recent years, several groups have reported the generation of GM mice by CRISPR/Cas9-mediated gene editing using electroporation as an alternative to microinjection [15–18]. Although electroporation of double-strand DNA (dsDNA) donors used in microinjection did not allow an efficient targeted integration of large dsDNA via homology-directed repair into genomic loci of intact zygotes [19], simultaneous electroporation of a large number of intact zygotes is rapid and simple, and has higher throughput than microinjection. In addition, the application of vitrified-warmed embryos to these electroporation methods enables to carry out the gene editing of embryos and generate GM mice more efficiently and systematically. However, GM mice generation by the electroporation method using vitrified-warmed embryos showed a low development rate and/or required pretreatment of embryos before freezing [20]. In addition, there are no reports on the gene editing of inbred mouse strain embryos using electroporation, except for C57BL/6.

Technique for Animal Knockout system by Electroporation (TAKE) has enabled high genome-editing efficiency and reduces damage to embryos by dividing the perforation of the zona pellucida and the cytoplasmic membrane of embryos and delivery of CRISPR/Cas9 components into three electric pulses [15, 16]. Furthermore, the TAKE method can prevent the influence of individual experimental skills because it does not require pretreatment of embryos, such as the thinning of the zona pellucida using acidified Tyrode's solution [15, 16]. However, the TAKE method was not optimized for the electric condition in vitrified-warmed embryos. This study optimized the electric condition of the TAKE method for vitrified-warmed embryos using 5 mm gap electrodes that can deliver CRISPR/Cas9 components into many embryos at once by arranging them in multi-rows. In addition, GM mice were generated using fresh embryos from widely used inbred mouse strains, C57BL/6NCr, BALB/cCrSlc, FVB/NJcl and C3H/HeJJcl, using this optimized electric condition.

Materials and Methods

Ethical statement

All animal experiments were approved by the President of the National Center for Global Health and Medicine (NCGM) following consideration by the Institutional Animal Care and Use Committee of the NCGM (approval ID no. 20041) and were carried out in accordance with institutional procedures, national guidelines, and the relevant national laws on the protection of animals.

Animals

B6D2F1, C57BL/6NCr (B6N), and BALB/cCrSlc (BALB/c) mice were purchased from Japan SLC (Hamamatsu, Japan). C57BL/6JJcl (B6J), FVB/NJcl (FVB), C3H/HeJJcl (C3H/HeJ), and Jcl:ICR (ICR) were purchased from CLEA Japan (Tokyo, Japan). All mice were housed in an air-conditioned animal room at 23 ± 2°C with a relative humidity of 40–60% under specific pathogen-free conditions, with a 12 h light/dark cycle (08:00–20:00/20:00–08:00). All mice were fed a standard rodent CE-2 diet (CLEA Japan) and had ad libitum access to water.

In vitro fertilization (IVF) and preparation of frozen fertilized eggs

Sperm were collected from the cauda epididymis of male mice 12 weeks or older and precultured in HTF medium (ARK Resource, Kumamoto, Japan) for 1 h. Female mice 8–12 weeks old were super ovulated by administering pregnant mare serum gonadotropin (ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) and 48 h later human chorionic gonadotropin (hCG; ASKA Pharmaceutical). At 16 h after administration of hCG, female mice were sacrificed by cervical dislocation, and eggs were collected from the ampulla of the uterine tube. The collected eggs were fertilized with preincubated sperm in HTF medium. After 7–8 h, fertilized eggs were cryopreserved by a simple vitrification method [21]. Cryopreserved mouse embryos were thawed with 0.25 M sucrose (ARK Resource) and cultured for 1 h before electroporation. At 3–4 h after IVF, B6N, BALB/c, FVB, and C3H/HeJ fresh embryos were used in this study.

In vitro transcription of mRNA and single gRNA (sgRNA)

Using the pmCherry-N1 plasmid (Takara Bio, Inc., Shiga, Japan) as a polymerase chain reaction (PCR) template, the T7 promoter sequence was added to the N terminus of mCherry sequence by PCR. The pX330-U6-Chimeric BB-CBh-hSpCas9 plasmid was obtained from Addgene (Watertown, MA, USA). hSpCas9 was digested with *Xba*I and *Eco*RI (Takara Bio), and the T7 promoter sequence was added to the N terminus of hSpCas9 by PCR (T7-hCas9). T7-hCas9 was cloned into pCR-BluntII-TOPO (Thermo Fisher Scientific, Waltham,

MA, USA) and used as a template for *in vitro* transcription. mCherry and hSpCas9 were transcribed *in vitro* using mMESSAGE mMACHINE T7 ULTRA Transcription Kit (Thermo Fisher Scientific). The *Psmb11*[22], tyrosinase (*Tyr*) [23], and *Rosa26* [24] target sequences were designed using CRISPOR (http://crispor.tefor.net). sgRNA was transcribed *in vitro* using the MEGAshortscript T7 transcription kit (Thermo Fisher Scientific). The recombinant Cas9 (rCas9) protein and Psmb11 crRNA and tracrRNA were purchased from Integrated DNA Technologies (Coralville, IA, USA). The chemically synthesized single-strand oligo-DNA (ssODN, Exigen, Tokyo, Japan) was used for introducing the loxP allele on the *Psmb11* locus. All RNA and ssODN were dissolved in Opti-MEM medium (Thermo Fisher Scientific). The mCherry mRNA was used at 50 ng/μl. Cas9 mRNA, sgRNA, and ssODN were used at 50, 80, and 500 ng/μl, respectively. The rCas9 protein (100 ng/μl), gRNA (75 ng/μl; complex of crRNA and tracrRNA), and ssODN (500 ng/μl) were preincubated at 37°C for 15 min and used for electroporation. All primer sets used in this study, ssODN sequence and target sequences of gRNA, are listed in Supplementary Table 1.

Electroporation

This study used the super-electroporator NEPA21 (NEPA GENE, Chiba, Japan) and Petri dish platinum plate electrodes (length: 10 mm, width: 3 mm, height: 15 mm, gap: 5 mm; NEPA GENE; Fig. 1A, top). The electroporator has two types of square-wave pulses: the poring pulse for forming pores in the zona pellucida and cell plasma membrane and the transfer pulse for delivering RNA and/or ssODN into the cytoplasm (Fig. 1B). The electric condition of transfer pulses was fixed as follows: pulse voltage, 20 V; pulse width, 50 ms; pulse interval, 50 ms; and pulse number, ±5. The poring pulse interval and number were fixed at 50 ms and +4 times, and the voltage and width of poring pulses were examined. PN-stage embryos were arranged in multiple rows between the electrodes (Fig. 1A, bottom) and introduced mCherry mRNA or the CRISPR/Cas9 components with or without ssODN. The viability of embryos was calculated by dividing the number of surviving embryos after electroporation from the number of embryos used in the experiments. Gene delivery efficiency was evaluated by the relative fluorescence intensity of mCherry at 16–24 h after electroporation using the FV1000 confocal laser scanning biological microscope (Olympus, Tokyo, Japan) and ImageJ software [25]. All experiments were performed twice or more in total by two different technicians. All electric conditions used in this study are summarized in Supplementary Table 2.

Embryos transfer and gene mutation analyses

Two-cell stage embryos were transferred into the oviduct of pseudopregnant ICR mice anesthetized using the mixture of 0.75 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol during operation [26]. Genome-editing efficiency was calculated by the detection of targeted gene mutation (s) as follows. At 13 days of pregnancy, fetuses were collected by cesarean section, and DNA was extracted by standard phenol/chloroform method [27]. PCR was performed using Ex-taq DNA polymerase (Takara Bio) with the target gene-specific primer sets (Supplementary Table 1). These PCR products were purified using ExoSAP-it (Thermo Fisher Scientific) and used as a template for direct sequence analysis. Sequencing analysis was performed using the BigDye Terminator version 3.1 Cycle Sequencing kit (Thermo Fisher Scientific) and ABI 3130xl Genetic Analyzer (Thermo Fisher Scientific) [27].

Statistics

Data were expressed as the mean ± SE and analyzed using Fisher's exact test and Student's *t*-test. Statistical analysis was performed using js-STAR (1.1.2j) software. $P<0.05$ was considered statistically significant.

Results

Optimization of poring pulse electric condition using vitrified-warmed mouse embryos

Because the conventional electric condition efficiently generates GM animals in fresh mouse and rat embryos [15] (Supplementary Table 3), the genome-editing efficiency was assessed in vitrified-warmed and fresh mouse embryos from B6D2F1 and BALB/c, respectively. Although the survival rates of vitrified-warmed B6D2F1 embryos after electroporation were relatively high (71.0–89.7%), the developmental rates of transferred embryos into offspring were very low (0–11.5%) in vitrified-warmed B6D2F1 embryos using the same electric condition compared to fresh embryos (Supplementary Table 3). Relatively low survival rate and lower genome-editing efficiency were also observed in fresh embryo from BALB/c. The TAKE method consists of two types of electric pulses: poring pulses and transfer pulses. Poring pulses have higher voltages than transfer pulses, which are thought to strongly influence the viability and developmental rate of embryos after electroporation. Therefore, the optimization of poring pulses to reduce embryo damage is required to improve the developmental rate and genome-editing efficiency in vitrified-warmed mouse embryos. To optimize the electroporation protocol for delivering CRISPR/Cas9 re-

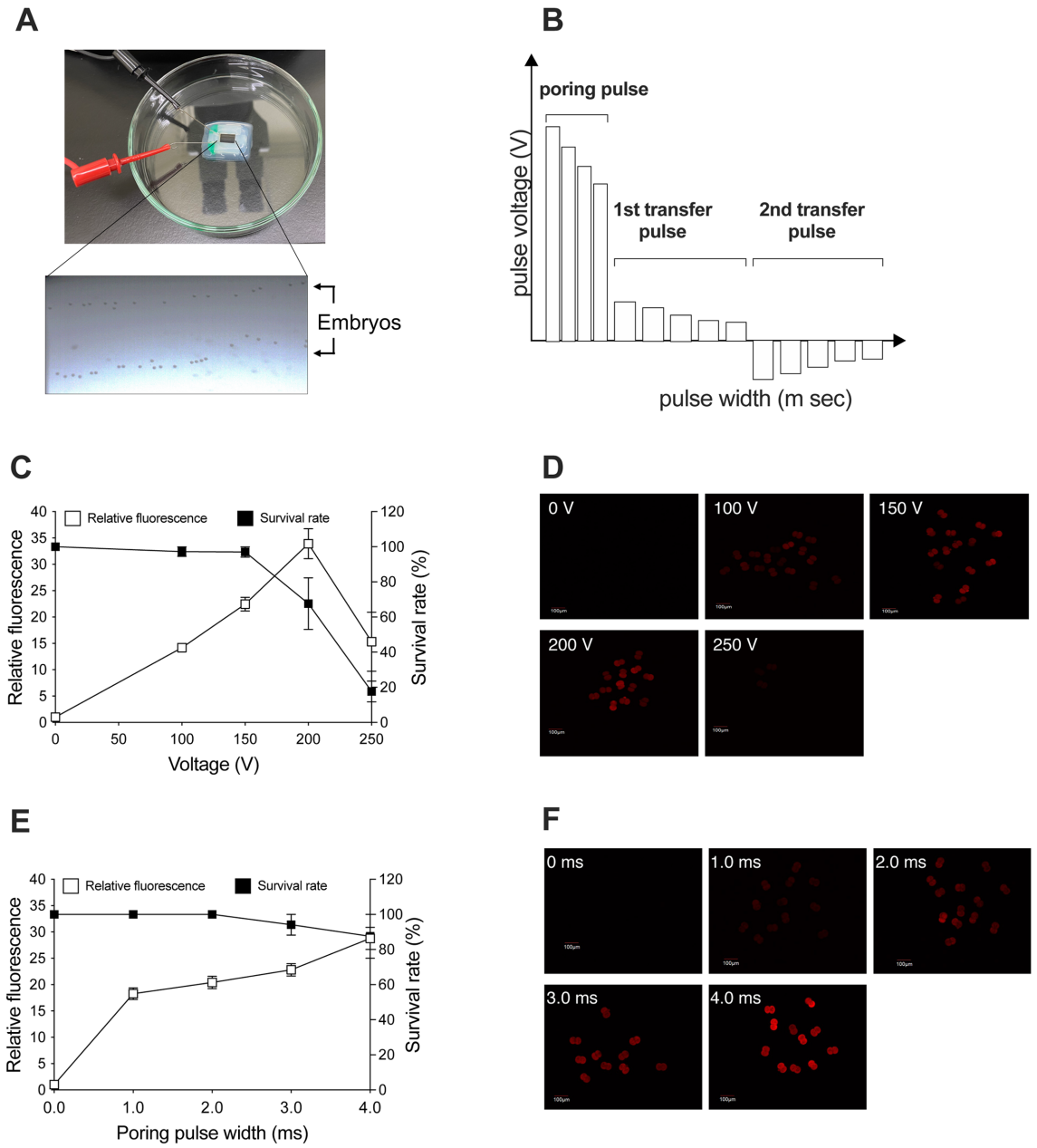

Fig. 1. Optimization of electric conditions for vitrified-warmed embryos in the Technique for Animal Knockout system by Electroporation (TAKE) method. (A) Petri dish platinum plate electrodes with 5 mm gap (top) and microscopic view of mouse embryos arranged in two lines between electrodes (bottom). (B) Schematic of the three-step electric square-wave pulse system of the TAKE method. The relative fluorescence intensity and survival rate of embryos after electroporation at each voltage (C) and each poring pulse width (D). Representative fluorescence microscope images at each voltage (E) and each poring pulse width (F). Scale bar, 100 μm.

agents into zygotes with intact zona pellucida, the mCherry mRNA delivery efficiency and survival rate of embryos were examined at different poring pulse voltages (0–250 V) and poring pulse widths (0–4.0 ms). The number and interval of poring pulses were fixed at four repeats and 3.0 ms, respectively. The introduction efficiency of mRNA into vitrified-warmed mouse embryos was evaluated by measuring the fluorescence intensity of mCherry after electroporation [17]. The relative fluorescence intensity of mCherry increased in proportion to the poring pulse voltage and reached the peak (33.9 ± 2.8) at 200 V at the poring pulse width of 3.0 ms (Figs. 1C, 1D, and Supplementary Fig. 1A). The relative fluorescent intensity at 250 V decreased by ~45% (15.3 ± 5.6) compared to 200 V. The survival rate of embryos was decreased at 200 V (67.6 ± 14.7%) and 250 V (17.6 ± 5.9%), indicating the high poring pulse voltage negatively affected the development of vitrified-warmed embryos after electroporation (Figs. 1C, 1D, and Supplementary Fig. 1C). These results indicated that the poring

pulse voltage at 150 V was optimal for vitrified-warmed mouse embryos because of both their viability and mRNA delivery efficiency.

The mCherry mRNA delivery efficiency and survival rate of embryos were examined at different poring pulse widths (0–4.0 ms) at the poring pulse voltage at 150 V. The relative fluorescence intensity of mCherry increased in proportion to the poring pulse width and reached the maximum at 4.0 ms (4.0 ms: 28.8 ± 2.1 vs. 1.0 ms: 18.3 ± 1.1 and 2.0 ms: 20.4 ± 1.2; [P<0.01] vs. 3.0 ms: 22.8 ± 1.2; [P<0.05]). The relative fluorescence intensity at 3.0 ms was significantly higher than that of 1.0 ms (P<0.01; Figs. 1E, 1F, and Supplementary Fig. 1B), although there was no difference between 1.0 and 2.0 ms. The developmental rate of embryos at the two-cell stage at 1.0 and 2.0 ms was 100%, whereas those at 3.0 and 4.0 ms were 94.1 ± 5.9% and 87.5 ± 12.5%, respectively (Figs. 1E, 1F, and Supplementary Fig. 1D). Furthermore, the mCherry mRNA delivery efficiency with transfer pulses (Tp+) and without transfer pulses (Tp−) was compared (Figs. 2A and B). An increased fluorescence intensity of mCherry of 2.3-fold was seen at Tp+ (Tp+: 18.9 ± 1.5 vs. Tp−: 8.1 ± 0.5; P<0.01; Figs. 2C–E), suggesting that transfer pulses facilitate the delivery of CRISPR/Cas9 reagents into zygotes with intact zona pellucida and ensure genome editing after electroporation. Based on these findings, the poring pulse at a voltage of 150 V and pulse width of 1.0 or 2.0 ms with transfer pulses was more suitable for the efficient introduction of mRNA into vitrified-warmed mouse embryos.

Generation of GM mice using vitrified-warmed mouse embryos

Whether two poring pulse conditions (voltage, 150 V; pulse width, 1.0 or 2.0 ms) in vitrified-warmed B6D2F1 embryos could efficiently generate GM mice was investigated. The results showed that 50.5% and 25.5% of the embryos microinjected with Cas9 mRNA, sgRNA, and ssODN developed into two-cell stage and offspring, respectively, and 91.7% of these offspring had edited *Psmb11* locus (Table 1). In contrast, 98.3% and 97.8% of the embryos electroporated with Cas9 mRNA, sgRNA, and ssODN with a pulse width of 1.0 or 2.0 ms, respectively, developed into the two-cell stage, and 32.8% and 12.2% of the transferred embryos successfully developed into offspring, respectively. Both electric conditions showed markedly higher viability of embryos than the microinjection method (microinjection: 50.5% vs. 1.0 ms: 98.3%; P<0.01 vs. 2.0 ms: 97.8%; P<0.01). Although there was no significant difference in the development rate of the two-cell stage between two electric conditions, the developmental rate of offspring at 1.0 ms was significantly higher than 2.0 ms (1.0 ms: 32.8% vs. 2.0 ms: 12.2%; P<0.01; Table 1). The *Psmb11* edited rates of these offspring were 89.5% at 1.0 ms and 90.9% at 2.0 ms, which were comparable to that of microinjected embryos (Table 1). There were no differences in the genome-editing efficiency and loxP knock-in rate on the *Psmb11* locus between two electric conditions. These results indicated that the electric pulse settings (poring pulse: voltage, 150 V; pulse width, 1.0 ms; pulse interval, 50 ms; number of pulses, +4; transfer pulse: voltage, 20 V; pulse width, 50 ms; pulse interval, 50 ms; number of pulses, ±5) are optimal for vitrified-warmed mouse embryos.

Given the good usability and highly efficient generation of knock-in mice carrying a functional gene cassette using cloning-free CRISPR/Cas9 system of the rCas9 protein combined with chemically synthesized dual crRNA-tracrRNA [28], this study attempted to deliver Cas9-dual RNA ribonucleoprotein (RNP) that could edit *Psmb11, Tyr,* and *Rosa26* locus into vitrified-warmed B6D2F1, B6N, and B6J mouse embryos using the optimal electric condition. The optimal electric condition also showed markedly high viability of the embryos electroporated with Cas9-dual RNA RNP in vitrified-warmed embryos from B6D2F1, B6N, and B6J (Table 2), which is comparable with that of the embryos electroporated with sgRNA-Cas9 mRNA (Table 1). The developmental rates of embryos into two-cell stage and offspring of Cas9-dual RNA RNP-mediated genome editing were almost identical to those using sgRNA-Cas9 mRNA in vitrified-warmed B6D2F1 mouse embryos (Tables 1 and 2). The *Psmb11, Tyr,* and *Rosa26* edited rates of these offspring were high (55.6%–100%), indicating that Cas9-dual RNA RNP complexes were efficiently delivered into zygotes with intact zona pellucida. Using vitrified-warmed embryos with the optimal electric condition, Cas9-dual RNA RNP-mediated genome editing allows for a more rapid and efficient generation of knockout alleles.

Application of electric condition optimized for vitrified-warmed embryos to fresh embryos of inbred mice

To reinforce the versatility of electric condition optimized for vitrified-warmed embryos, the *Psmb11* locus with widely used inbred mouse zygotes from B6N, BALB/c, FVB, and C3H/HeJ was edited using the optimal electric condition for vitrified-warmed mouse embryos, and their production efficiencies were compared. Although the developmental rate of the embryos from BALB/c was relatively low compared to other strains, more than 90% of the embryos from B6N, FVB, and

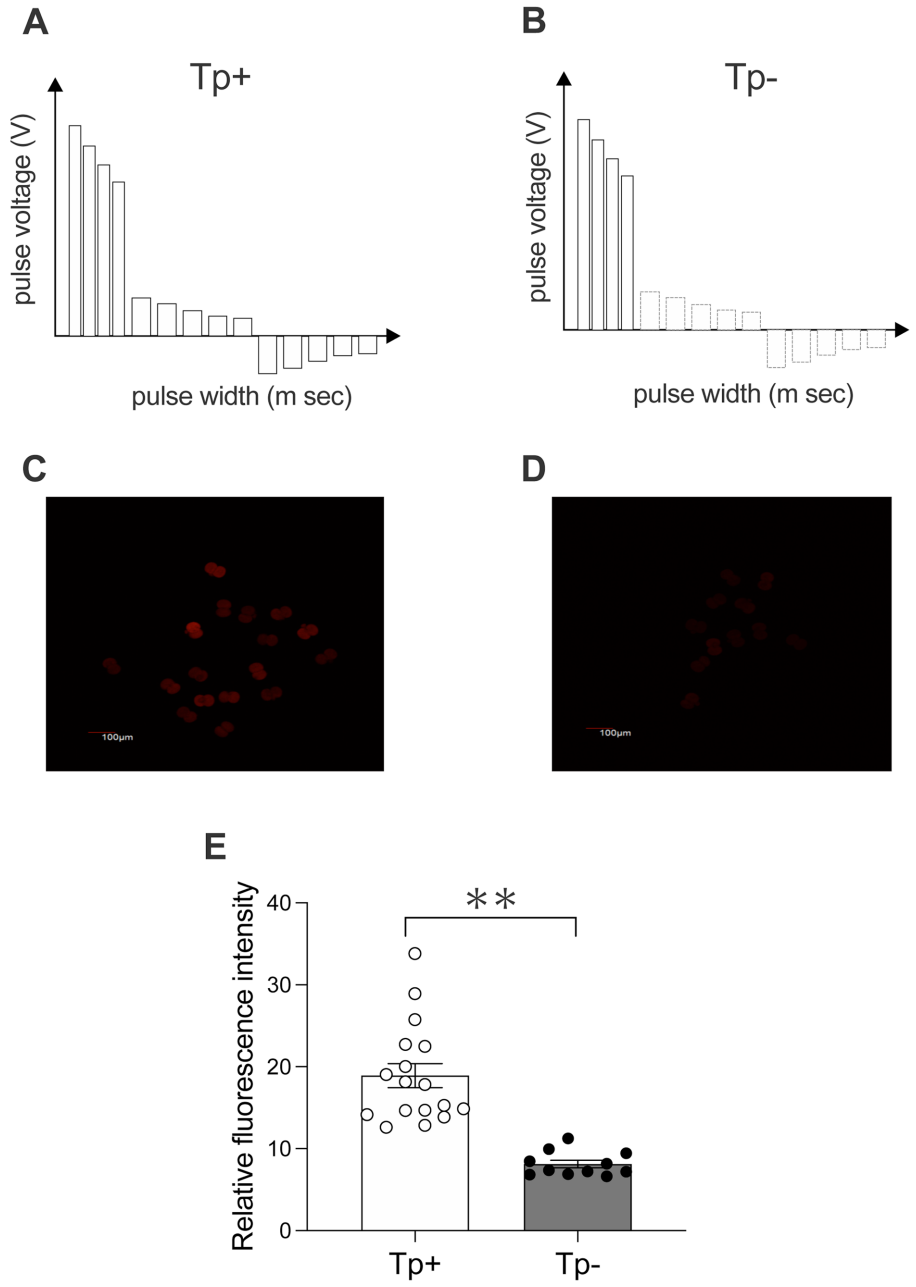

Fig. 2. Effect of transfer pulses (Tp) on the efficacy of mCherry mRNA delivery. Schematic of the three-step electric square-wave pulse system with (A) and without (B) transfer pulses. Dotted lines indicate disappeared transfer pulses. Representative fluorescence microscopic image with (C) and without (D) transfer pulses. Scale bar, 100 μm. (E) Relative fluorescence intensity of embryos. Tp+ and Tp− indicate electroporation with and without transfer pulses, respectively. Data were analyzed by Student's *t*-test (**P<0.01).

C3H/HeJ electroporated with Cas9 mRNA/sgRNA or dual RNA/Cas9 protein and ssODN developed into two-cell stage (Table 3). When using the Cas9-dual RNA RNP, 100% of these offspring had an edited *Psmb11* locus; however, the loxP knock-in efficiencies using Cas9 protein were much lower than those using Cas9 mRNA (BALB/c, Cas9-dual RNA RNP: 7.7% vs. Cas9 mRNA/sgRNA: 72.5%), which was thought to be because the Cas9 protein functions at different times during the stage of PN fertilized eggs. The optimal electric condition efficiently generates GM mice in at least four widely used inbred mouse strains.

Discussion

To generate GM mice more easily with high reproducibility, methods that introduce CRISPR/Cas9 components into mouse embryos by electroporation have re-

Table 1. Development and CRISPR/Cas9 mediated genome editing of vitrified-warmed embryos from B6D2F1

Methods	Target gene	Voltage (V)	Pulse width (ms)	No. embryos	No. (%) transferred embryos	No. (%) offspring	No. analyzed pups	No. (%) mutants*	No. (%) of loxP knock-in mice
TAKE method	*Psmb11*	150	1.0	59	58 (98.3)	19 (32.8)	19	17 (89.5)	7 (36.8)
	Psmb11	150	2.0	92	90 (97.8)	11 (12.2)	11	10 (90.9)	3 (27.3)
Microinjection	*Psmb11*	—	—	93	47 (50.5)	12 (25.5)	12	11 (91.7)	4 (33.3)

*GM mice with the specific gene altered.

Table 2. Generation of target gene knock-out mice by recombinant Cas9 protein combined with crRNA/tracrRNA in vitrified-warmed mouse embryos

Strain	Target gene	Voltage (V)	Pulse width (ms)	No. embryos	No. (%) transferred embryos	No. (%) offspring	No. analyzed pups	No. (%) mutants*
B6D2F1	*Psmb11*	150	1.0	80	79 (98.8)	30 (38.0)	30	30 (100)
	Tyr	150	1.0	80	78 (97.5)	28 (35.9)	28	21 (75.0)
	Rosa26	150	1.0	82	81 (98.8)	27 (33.3)	27	15 (55.6)
C57BL/6N	*Psmb11*	150	1.0	111	109 (98.2)	13 (11.9)	13	13 (100)
C57BL/6J	*Tyr*	150	1.0	65	63 (96.9)	18 (28.6)	18	18 (100)
	Rosa26	150	1.0	90	89 (98.9)	27 (30.3)	27	20 (74.1)

*GM mice with the specific gene altered.

Table 3. Development and CRISPR/Cas9-mediated gene editing of fresh embryos from various strains

Strain	Target gene	Voltage (V)	Pulse width (ms)	Cas9/gRNA	No. embryos	No. (%) transferred embryos	No. (%) offspring	No. analyzed pups	No. (%) mutants*	loxP knock-in mice (%)
C57BL/6	*Psmb11*	150	1.0	Cas9 mRNA/sgRNA	136	131 (96.3)	30 (22.9)	30	30 (100)	13 (43.3)
BALB/c	*Psmb11*	150	1.0	Cas9 mRNA/sgRNA	120	96 (78.7)	23 (24.0)	22	20 (90.9)	16 (72.7)
			1.0	Cas9 protein/crRNA + tracrRNA	59	43 (72.9)	13 (30.2)	13	13 (100)	1 (7.7)
FVB	*Psmb11*	150	1.0	Cas9 protein/crRNA + tracrRNA	125	113 (90.4)	40 (35.4)	38	38 (100)	2 (5.3)
C3H/HeJ	*Psmb11*	150	1.0	Cas9 protein/crRNA + tracrRNA	125	115 (92.0)	44 (38.3)	43	43 (100)	2 (4.7)

*GM mice with the specific gene altered.

cently been energetically studied [15–18]. However, most of these studies have used fresh mouse embryos, and the strains used in these studies are limited to B6D2F1 or B6 mice [15–18]. This study established the new electric condition that can be used in vitrified-warmed mouse embryos and at least four inbred mouse fresh embryos by optimizing the electric condition of the TAKE method.

Although the method for generating GM mice by electroporation in vitrified-warmed B6 embryos was previously reported [20], this method required pretreatment before freezing the embryos. The established protocol in this study is so simple because it does not require pretreatment of embryos and can efficiently deliver CRISPR/Cas9 reagents into zygotes with intact zona pellucida and edit the targeted locus. Another electroporation method that does not require pretreatment of embryos, such as the thinning of the zona pellucida using acidified Tyrode's solution by using seven repeats of a square repeats of 25V or 30V, 3-msec pulse with 1-mm gap electrode, has already been reported [17, 29]. However, our method has the following two advantages. (1) Compared with 1 mm gap electrodes, using the electrode with a 5 mm gap allows us to deliver CRISPR/Cas9 components into more than twice embryos (100 or more) at one time, resulting in higher throughput. Simultaneous electroporation of a large number of intact zygotes leads to the efficient production of a variety of genome-edited mice. (2) It is a highly versatile electric condition in which GM mice can be generated even in fresh embryos from widely used inbred mouse strains (Table 3).

We selected the wildly used inbred strains for this study due to the following reason. BALB/c mice are known to more easily induce Th2 responses after infection or immunization than B6 mice [30, 31]. These mice have been widely used in the research fields of oncology

[32, 33], inflammation [34, 35], and autoimmune disease [36, 37]. FVB mice are highly sensitive to renal glomerular disease [38–40] and useful for searching for nephropathy-related genes [39] and studies of renal disease [38, 40]. C3H/HeJ mice are often used in oncology research [41, 42]. In addition, these mice have a single-point mutation in *Tlr4* [43], leading to a defective TLR4 protein, resulting in high susceptibility to *Mycobacterium tuberculosis* [44], *Salmonella enterica* [45], and mouse hepatitis virus type 1 [46]. Therefore, this strain is also widely used in infectious disease studies. This study reinforced the versatility of the electric condition optimized for vitrified-warmed embryos to generate GM mice and fresh embryos from B6N, BALB/c, FVB, and C3H/HeJ. This study demonstrated that GM mice could be generated with an efficiency of 90% or more even in at least four inbred mice using optical electric conditions (BALB/c used the Cas9 protein, FVB, and C3H/HeJ: 100% and BALB/c used Cas9 mRNA: 90.9%; Table 3). Based on these findings, it is possible to directly generate GM mice in each strain without repeated backcrossing using the optimal electric condition.

It was unexpected that loxP knock-in efficiencies using the Cas9 protein were much lower than Cas9 mRNA in the fresh embryos from inbred strain (BALB/c, Cas9 mRNA: 72.7% vs. Cas9 protein: 7.7%; $P<0.01$; Table 3). This reason was thought to be due to the difference in the stage of PN-stage embryos in which the Cas9 protein functions. The PN-stage of fertilized eggs is classified into five stages: PN1 to PN5 [47]. PN1–PN2, PN3–PN4, and PN5 stages correspond to the G1, S, and G2 phases of the cell cycle, respectively. Double-strand breaks in DNA are repaired by two major repair mechanisms: nonhomologous end joining (NHEJ) and homologous recombination (HR) [48, 49]. NHEJ is active in all cell cycles [50], whereas HR is active only in the S and G2 phases [48, 49]. In addition, several research groups have reported that the delivery of CRISPR/Cas9 components and knock-in vector into embryos during PN3-PN4 (corresponding to the late G1 to S phases) significantly improved the knock-in efficiency [51, 52]. In this study, the Cas9 protein was introduced into fresh embryos during PN1-PN2 at 3–4 h after IVF, whereas the Cas9 protein was introduced into vitrified-warmed embryos during PN3-PN4 at 8–9 h after IVF, corresponding to the G1 and S phases, respectively. Therefore, it is considered that most of the genome-edited locus cleaved by the Cas9 protein was repaired by NHEJ in fresh embryos, resulting in low knock-in efficiency. In addition, Cas9 mRNA takes 4 h from being delivered into cells to be translated into a protein, and translated Cas9 proteins need the additional 4 h to cleave the target genome locus (a total of 8 h after introduction) [53]. Cas9 mRNA was introduced using electroporation into embryos during PN1-PN2. Cas9 mRNA is translated into the protein, and the Cas9 protein cleaves the target genome during the S phase at about 12 h after IVF. The HR is active, resulting in the efficient production of the loxP knock-in allele using Cas9 mRNA compared to the Cas9 protein. These results suggested that vitrified-warmed embryos are more suitable for generating knock-in alleles using Cas9-dual RNA RNP than fresh embryos produced by IVF.

In conclusion, this study established the versatile electric condition for vitrified-warmed mouse embryos and fresh embryos from a widely used inbred strain. Genome editing was feasible under the same electric condition for vitrified-warmed and fresh embryos. Furthermore, the TAKE method using the electrode with a 5 mm gap could introduce more than 100 intact embryos within 5 min without specific pretreatment and sophisticated technical skills, such as microinjection, and exhibited a high developmental rate of embryos and genome-editing efficiency in the generated offspring, leading to the rapid and efficient generation of GM mice. This method may contribute to understanding human diseases and gene functions by generating GM mice more easily and efficiently.

Acknowledgments

We wish to thank our lab members for their helpful discussions. This work was supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT); Grants-in-Aid for Scientific Research, KAKENHI (20K15708) to KN, and was partially supported by Grants-in-Aid for Research from the National Center for Global Health and Medicine (29-1001, 20A1019 and 21A1018) to TO and KN.

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