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Short Communication

Successful *i*-GONAD in Brown Norway Rats by Modification of *in vivo* Electroporation Conditions

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Abstract

Improved-Genome editing via Oviductal Nucleic Acids Delivery (*i*-GONAD) was developed for *in situ* genome editing of the preimplantation embryos present within the oviductal lumen of mice. This method is based on intra-oviductal instillation of genome editing components and subsequent *in vivo* electroporation (EP) in the entire oviduct. Therefore, *i*-GONAD differs from the previous methods (*i.e.*, zygote microinjection and *in vitro* EP) in producing genome-edited mice, which relied on *ex vivo* handling of preimplantation embryos and egg transfer to the recipient females. We have previously demonstrated that *i*-GONAD can be successfully

applied to produce genome-edited rats, including albino Sprague-Dawley and albino Lewis rats (however, not pigmented Brown Norway [BN] rats). We observed that the successful *i*-GONAD was dependent on the mouse strain used; for example, in random-bred mice, such as ICR and C3H/He × C57BL/6, it was successful under relatively stringent electrical conditions but not in the C57BL/6 strain. Under less stringent conditions, *i*-GONAD was successful in the C57BL/6 strain. We speculated that this would also be true for *i*-GONAD using BN rats. On applying a current of >500 mA, we failed to obtain rat offspring (fetuses/newborns); however, *i*-GONAD under a current of 100-300 mA using NEPA21 (NEPA GENE) led to the production of genome-edited BN rats with efficiencies of 75%-100%. Similarly, *i*-GONAD, under a current of 150-200 mA using CUY21EDIT II (BEX Co.) led to the production of genome-edited BN rats with efficiencies of 24%-55%. These experiments suggest the importance of selecting the appropriate current value, depending on the rat strain used, when performing *i*-GONAD.

Keywords
In vivo electroporation; CRISPR/Cas9; *i*-GONAD; ribonucleoprotein; single-guide RNA; Brown Norway rats; mosaicism; indels

1. Introduction

Recent advances in genome editing technologies, exemplified by the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system [1, 2], have produced numerous genome-edited animals, including mice and rats. Most genome-edited animals are derived from *ex vivo* handling of preimplantation embryos (zygote microinjection of genome editing components [GECs] *in vitro* or *in vitro* electroporation [EP] in the presence of GECs) and subsequent egg transfer to the recipient females, which is laborious and time-consuming [3]. For avoiding the *ex vivo* handling of preimplantation embryos, a novel method has been developed that enables *in situ* genome editing by simple intra-oviductal injection of a solution containing GECs and subsequent *in vivo* EP in the entire oviduct. We named this method Genome editing via Oviductal Nucleic Acid Delivery (GONAD) [4] and later renamed it to *improved*-GONAD (*i*-GONAD) [5]. It does not require isolation of zygotes from females for genome editing or egg transfer of the genome-edited embryos to recipients, which simplifies the process of gene-modified mice production. Moreover, we have demonstrated the use of *i*-GONAD in producing gene-modified rats [6, 7]. However, the success rate of *i*-GONAD differed among the rat strains used. For example, when *i*-GONAD targeting the endogenous tyrosinase (*Tyr*) locus was performed in pigmented Brown Norway (BN) females mated with the albino Sprague-Dawley (SD) males, the BN females gave birth to 13 mid-gestation fetuses. Of these, 62% had non-pigmented eyes, indicating the deletion or insertion of mutations (indels) in the *Tyr* genes [6]. However, the BN females successfully mated to BN males failed to produce offspring (fetuses/newborns), despite repeated *i*-GONAD trials (a total of seven times). The reason for this failure remains to be elucidated. A similar problem was found in mice, and the efficiency of *i*-GONAD-mediated mutation varied among the strains used. For example, *i*-GONAD in random-bred mice, such as ICR and C3H/He × C57BL/6, was successfully performed under relatively stringent electrical conditions (40 V/100-200 Ω/~300 mA); however, it

was not successful in the C57BL/6 strain [5]. Subsequently, we noticed that under less stringent conditions (40 V/350–400 Ω/~100 mA), *i*-GONAD was effective in the C57BL/6 strain [5, 8, 9]. We speculated that this would also be true for *i*-GONAD using BN rats.

In this study, we examined whether viable genome-edited fetal offspring of BN rats were obtained when low current values (150-200 mA) were employed in *i*-GONAD.

2. Materials and Methods

2.1 Animals

BN/SsNSlc (BN) rats were purchased from Japan SLC, Inc. (Shizuoka, Japan) (Figure 1A). Adult rats aged 8-10 weeks (female) and 10-12 weeks (male) were used. All rats were maintained under temperature-controlled conditions (24 ±2 °C) with a 12L/12D light-dark cycle (lights on at 7:00 AM). A solid diet and water were provided *ad libitum*. The animal facility was maintained under specific pathogen-free conditions. This study was approved by *the Institutional Animal Care and Use Committee of the Hamamatsu University School of Medicine* (Permission number: 2017064), and animals' experiments were appropriately performed following *the guidelines of the Hamamatsu University School of Medicine Committee on Recombinant DNA Security* (No. 29-16). All animal experiments were performed as per *the Law for the Humane Treatment and Management of Animals* (Japanese Law No. 105), *Standard Relating to the Care and Management of Laboratory Animals and Relief of Pain* (Notice No. 6 of the Prime Minister's Office), and *the Guidelines for Proper Conduct of Animal Experiments* (Science Council of Japan).

2.2 Estrus Cycle Monitoring

The vaginal smears were collected every morning (8:00-9:00) using a cotton-tipped swab wetted with distilled water for determining the estrus cyclicity of each rat using the method described by Takabayashi et al. [6]. Proestrus female rats were mated to sexually experienced male rats at 17:00-18:00. The following morning, the females with sperm in their vaginal smears were considered pregnant and the experimental day was designated as day 0 of pregnancy. For *i*-GONAD experiments, females at day 0.75 of pregnancy corresponding to the late 1-cell stage were used.

2.3 Preparation of i*-GONAD Solutions*

For CRISPR/Cas9-mediated induction of indels, Alt-R® CRISPR-Cas9 crRNAs were designed to recognize target sites (exon 2 of rat *Tyr* [Figure 1B] that matched a 20-bp DNA sequence [crRNA: TTTCC AGGAT TACGT AATAG] just upstream of the protospacer adjacent motif [PAM]). crRNA was synthesized by Integrated DNA Technologies, Inc. (IDT) (IA, USA). Alt-R® CRISPR-Cas9 tracrRNA (#1072533) was also purchased from IDT. Each reagent was dissolved in Opti-MEM® medium (#31985062; Thermo Fisher Scientific, Waltham, MA, USA) at a final concentration of 100 μM and stored at −80 °C until further use. crRNAs and tracrRNA were annealed by mixing equimolar amounts of both (30 μM) and incubating at room temperature for about 10 min to allow the formation of crRNA:tracrRNA duplexes (guide RNA [gRNA]). The gRNA (6 μL) was mixed with Cas9 protein (#1081060; 10 μg/μL; IDT) to form ribonucleoprotein (RNP) complexes in Opti-MEM® medium in a 0.5-mL polymerase chain reaction (PCR) tube. Thus, the final concentrations of GECs

were 30 μM gRNA, 1 μg/μL Cas9 protein, and 0.02% Fast Green FCF (#15939-54; Nacalai Tesque, Kyoto, Japan) dissolved in Opti-MEM® medium.

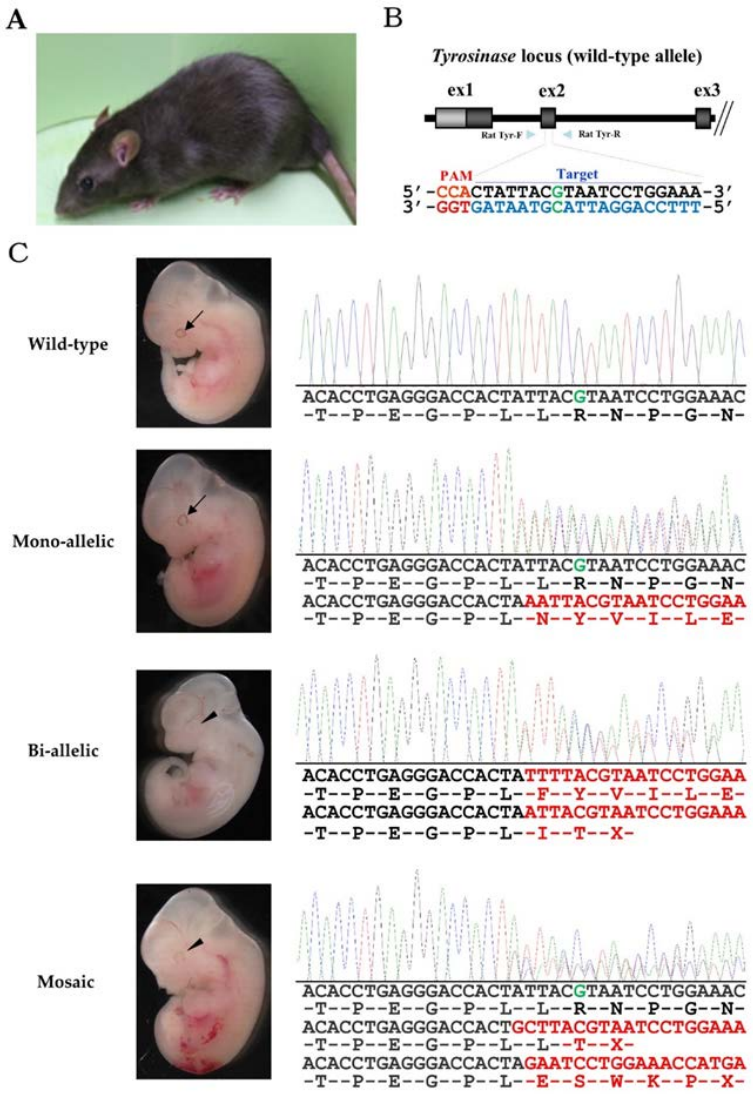

Figure 1 A. BN female. B. Schematic illustration of the wild-type *Tyr* locus. The target sequence of *Tyr* exon 2 (ex2) recognized by guide RNA (gRNA) is overlined, and the protospacer adjacent motif (PAM) sequence is marked in red. The target nucleotide "G" marked in green is a key nucleotide for tyrosinase activity; nucleotide replacement at this position often causes an albino phenotype. C. Offspring (day 14.5 fetuses) obtained after *i*-GONAD (shown on the left side) and direct sequencing of PCR products derived from those fetuses (shown on the right side). Wild-type fetuses exhibited pigmented eyes (arrow) and normal nucleotide "G" (marked in green in *Tyr* exon 2) of the wild-type alleles. Monoallelic fetuses exhibited pigmented eyes (arrow) and possessed one normal and one mutated allele (the beginning of mixed nucleotide peaks is shown in red). Biallelic fetuses exhibited non-pigmented eyes (arrowhead) and possessed two mutated alleles (the beginning of mixed nucleotide peaks is shown in red). Mosaic fetuses exhibited eyes with decreased pigmentation (arrowhead) and possessed mutated alleles (the beginning of mixed nucleotide peaks is shown in red) and a normal allele, probably reflecting a mixture of unedited and edited cells.

2.4 i-GONAD Procedure

We performed *i*-GONAD on day 0.75 of pregnancy (18:00). Surgical procedures were performed according to the method described by Takabayashi et al. [6]. Briefly, approximately 1.5 µL of GECs was injected into the oviductal lumen upstream of the ampulla using a micropipette linked to a mouthpiece under a dissecting microscope. Immediately after the injection, the oviduct regions were covered with a piece of wet paper (KimWipe; Jujo-Kimberly Co. Ltd., Tokyo, Japan) soaked in Dulbecco's modified phosphate-buffered saline (DPBS) and clamped with tweezer-type electrodes (#CUY652-3; NEPA GENE, Chiba, Japan).

EP was performed using a square-wave pulse generator, NEPA21 (NEPA GENE) or CUY21EDIT II (BEX Co. Ltd., Tokyo, Japan). The EP parameters for NEPA21 were as follows: three poring pulse (Pp) (40 V, wavelength: 5 ms, pulse interval: 50 ms, and 10% decay [±pulse orientation]) and three transfer pulse (Tp) (10 V, wavelength: 50 ms, pulse interval: 50 ms, and 40% decay [±pulse orientation]). For CUY21EDIT II, the EP parameters were as follows: driving pulse voltage (PdV): 40 V, Pd on: 5.00 ms, Pd off: 50 ms, pulse cycles: 3, and decay: 10%. Notably, the voltage was set to 40 V (Pp for NEPA21 and PdV for CUY21EDIT II). We obtained the expected current value in all experiments performed using CUY21EDIT II.

After EP, the oviducts were returned to their original position. The treated females were then examined on day 14.5 of pregnancy for the presence or absence of fetuses.

2.5 Treatment of the i*-GONAD-derived Offspring*

Pregnant female rats at day 14.5 of pregnancy were euthanized using isoflurane inhalation overdose. Fetuses were dissected and placed in DPBS. Pigmentation in the eyes was assessed under a dissecting microscope and photographed. Next, tail biopsies were obtained for genomic DNA isolation from the mid-gestation fetuses.

2.6 Analysis of CRISPR/Cas9-induced Mutations

Genomic DNA was isolated from the tail biopsies of mid-gestation fetuses by incubation in 100 µL 50 mM NaOH at 95 °C for 10 min. Approximately 10 µL of 1 M Tris-HCl (pH 8.0) was added to the aliquot and mixed. Crude DNA extract was used as a template for PCR. PCR amplification of an on-target locus (*Tyr*) was performed in 20 µL containing 10 µL 2× PCR buffer for KOD FX, 0.4 mM dNTPs, 1 µL crude lysate, 0.25 µM primer pairs (rat Tyr-F [5'-GCT CAA GGT TTA GTT GGG TAC T-3'] and rat Tyr-R [5'-CTG GCT AGG TTT ACT ATC TCC TTG-3']) [6], and 0.1 U KOD FX (#KFX-101; TOYOBO, Osaka, Japan) under the following cycling conditions: denaturation at 94 °C for 3 min; amplification for 33 cycles at 95 °C for 20 s, 57 °C for 30 s, and 68 °C for 1 min; and a final extension at 68 °C for 5 min. Amplification products (5 µL) of 598 bp in size, spanning exon 2 of *Tyr*, were separated by 2% agarose gel electrophoresis. PCR products were directly sequenced using the dideoxy chain termination method with the BigDye Terminator v.3.1 Cycle Sequencing Kit (#4337455; Thermo Fisher Scientific) and then analyzed on an automated ABI PRISM 3130xl DNA Sequencer (Thermo Fisher Scientific).

3. Results and Discussion

Before exploring the optimal conditions for *i*-GONAD-based genome editing in BN rats, we employed several parameters already used in our previous experiments [6]. These included the use of gRNA, which recognizes the sequence of *Tyr* exon 2, and RNP, a mixture of gRNA and Cas9 protein. For successful injection, GECs (1.5 µL) containing RNP and Fast Green FCF were injected into the ampullae of the oviducts of BN females (that had been naturally and successfully mated to fertile BN males) on day 0.75 of pregnancy (corresponding to late 1-cell stage) and subsequently *in vivo* EP was performed in the entire oviduct. The BN females should have fetuses with pigmented eyes at the mid-gestation stage when no treatment is administered. However, if genome editing is induced at the *Tyr* locus through *i*-GONAD, some fetuses will have non-pigmented eyes owing to indel induction at *Tyr* exon 2.

In this study, we employed two electroporators, NEPA21 and CUY21EDIT II, for performing *in vivo* EP. The former generated an electric pulse under constant voltage, whereas the latter provided constant voltage or current. In this study, we used a constant current mode for CUY21EDIT II. NEPA21 generated a Pp and Tp; the EP parameters were as follows: 3 Pp (40 V, wavelength: 5 ms, pulse interval: 50 ms, and 10% decay [±pulse orientation]) and 3 Tp (10 V, wavelength: 50 ms, pulse interval: 50 ms, and 40% decay [±pulse orientation]). For CUY21EDIT II, the EP parameters were as follows: PdV: 40 V, Pd on: 5 ms, Pd off: 50 ms, pulse cycles: 3, and decay: 10%. Notably, the voltage was set to 40 V (i.e., Pp for NEPA21 and PdV for CUY21EDIT II). For using NEPA21, the resistance value (Ω) must be measured to determine the current value, which can be further calculated using the following formula: I(A) = 40(V)/R (Ω). For measuring the resistance value in this *i*-GONAD system using NEPA21, the exposed oviduct regions of anesthetized females were first covered with a piece of wet paper soaked in DPBS and then clamped between tweezer-type electrodes, according to the method described by Kobayashi et al. [9]. In this case, since the interval between the two electrodes varied from 0 to 2 mm, the resistance measured through this procedure should vary. First, the resistance value was recorded by pressing the Ω button in the NEPA21 electroporator when the oviduct was sandwiched within the electrodes. Next, immediately after measuring the resistance, the start button was pressed to record the current.

The data from the BN females obtained from our previous *i*-GONAD experiment [6] using the NEPA21 electroporator are illustrated in Figure 2. We failed to obtain any viable fetuses (at day 14.5 of pregnancy) when *i*-GONAD was performed under relatively stringent electrical conditions (273-675 mA). To explore the optimal conditions for *i*-GONAD-based genome editing in BN rats, we performed *i*-GONAD using various current values ranging from 100 to 500 mA using the NEPA21 electroporator. Consequently, the oviducts treated with current values between 100 and 500 mA yielded viable fetuses, although oviducts treated with 482 mA produced fetuses with developmental anomalies (Figure 2; Table S1). Among a total of 10 fetuses obtained, six (60%) exhibited indels at the *Tyr* locus (Table S1). Notably, *i*-GONAD performed under current values between 301 and 500 mA yielded three fetuses; however, they were all unedited (Figure 2; Table S1). Current values below 300 mA may be suitable for *i*-GONAD-based genome editing in BN rats. To further confirm whether current values were important for *i*-GONAD, we performed similar experiments using another electroporator, CUY21EDIT II, capable of generating a constant current. We set the constant current to 100, 150, or 200 mA. Under each electrical condition, viable fetuses were obtained; however, those showing genome editing at the *Tyr* locus were derived only from *i*-GONAD performed under

current values between 150 and 200 mA, and the genome editing efficiencies were 24% (5/21) to 55% (11/20) (Figure 2; Table S1).

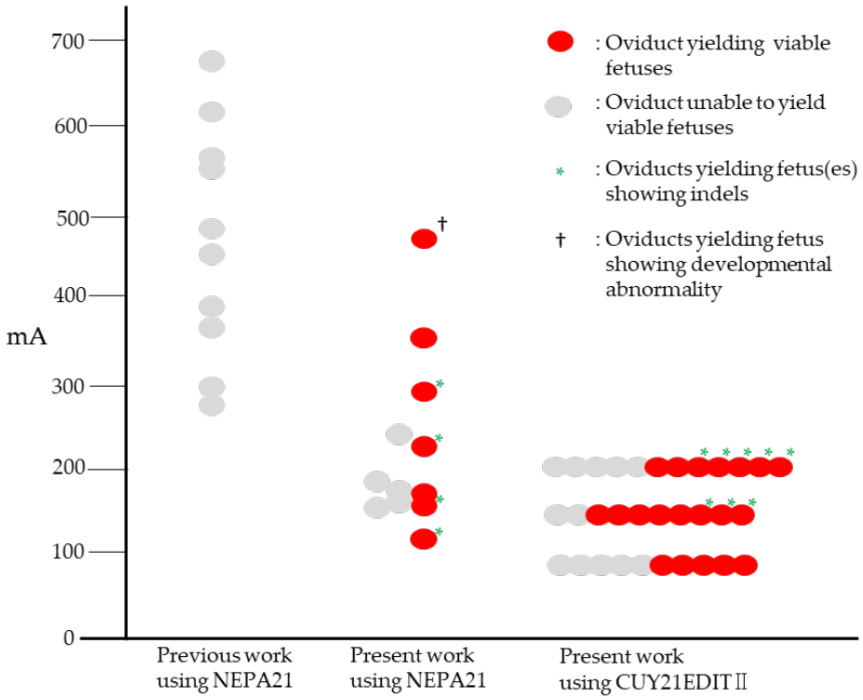

Figure 2 *i*-GONAD for the pregnant BN females under various electrical conditions using two electroporators (NEPA21 and CUY21EDIT II). Before *i*-GONAD, Brown Norway (BN) females are mated to BN males. BN females at day 0.75 of pregnancy are subjected to *i*-GONAD under various current values. On day 14.5 of pregnancy, oviducts yielding viable fetuses (shown in red-filled circles) or those yielding no fetus (shown in gray-filled circles) are plotted. Oviducts with genome-edited fetuses are marked with asterisks. † indicates oviducts, with fetuses showing developmental abnormalities.

In our previous *i*-GONAD experiment to produce genome-edited rats using the NEPA21 electroporator, the *in vivo* EP conditions (50 V/100–200 Ω/~400 mA) were effective for various rat strains, including SD (closed colony) and albino Lewis (inbred) rats with efficiencies of 40% and 41%, respectively [6]. However, the acquisition of genome-edited offspring from the BN females successfully mated to BN males failed under these conditions. This failure was believed to be owing to the employment of relatively stringent EP conditions since we had previously noted a strain difference in the susceptibility to EP in mice when RNP-based *i*-GONAD was applied [6]. Indeed, we obtained genome-edited BN fetuses with relatively high efficiencies (39%; 16/41) when *i*-GONAD was performed using CUY21EDIT II with current values ranging from 150 to 200 mA (see Figure 2 and Table S1). The use of NEPA21 also yielded viable fetuses (86%; 6/7) with knockout (KO) phenotypes when EP was performed under current values between 100 and 300 mA (see Figure 2 and Table S1). The different efficiencies of generating indels between the two electroporator systems may be owing to the voltage difference. A constant 40 V was maintained using the NEPA21, whereas the voltage value using the CUY21EDIT II was variable according to the resistance value. Thus, the balance of current and voltage values may be critical for successful *i*-GONAD. Notably, the rate of occurrence of mosaic mutations was low in our system. The total mosaic rate was 13% (six

fetuses with mosaic mutations of 48 fetuses obtained; see Table S1). Approximately 87% of samples demonstrated clear electrophoretograms, which were frequently seen in samples with homozygous biallelic or heterozygous monoallelic KO (see "Monoallelic" and "Biallelic" in Figure 1C). In contrast, the remaining samples revealed overlapping or erroneous electrophoretograms (see "Mosaic" in Figure 1C).

In our previous experiment, when BN females mated to albino SD males were subjected to *i*-GONAD for targeting the *Tyr* locus, they gave birth to viable mid-gestation fetuses, and 62% of these fetuses had non-pigmented eyes with indels in *Tyr* [6]. In contrast, when similar treatment was performed for BN females successfully mated to BN males, no viable fetuses were obtained [6]. This suggests the involvement of heterosis in tolerating the damage caused by a square pulse-generating electroporator. Further studies should examine whether this principle is also applicable to the mouse C57BL/6 strain, which is sensitive to high stringent EP conditions [5].

4. Conclusions

We succeeded in producing genome-edited knockout BN rats using *i*-GONAD under EP conditions with current values ranging from 150 to 200 mA and efficiencies ranging from 75% (3/4) to 24% (5/21). Our future objective is to test the possibilities of 1) germ-line transmission of genome-edited traits produced through the *i*-GONAD system and 2) knock-in of a sequence into the target locus. We are optimistic that the genome-edited animals, difficult to produce using the currently available *in vivo* EP conditions, may be produced using this technology.

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Author Contributions

T.A., Y.K., and H.T. performed the experiments, especially *i*-GONAD-related work; S.T., E.A., and M.S. conceived and designed the study, drafted and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Competing Interests

The authors have declared that no competing interests exist.

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