Chapter 7

Genome Editing in Mouse and Rat by Electroporation

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Abstract

Many knock-out/knock-in mouse and rat strains have been produced by genome editing techniques using engineered endonucleases, including zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), or clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9. Microinjection of engineered endonucleases into pronuclear-stage embryos is required to produce genome-edited rodents and the development of easy, rapid, and high-efficiency methods that do not require special skills such as microinjection is needed. This chapter presents a new technique called Technique for Animal Knockout system by Electroporation (TAKE), which produces genome-edited rodents by direct introduction of engineered endonucleases into intact embryos using electroporation.

Key words Mouse, Rat, ZFN, TALEN, CRISPR/Cas9, Cas9 protein, Knock-out, Knock-in, Electroporation, Intact embryos

1 Introduction

Engineered endonucleases, including zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), or clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9, are powerful tools for genome editing in animals [1–6]. Knock-out and knock-in animals can be rapidly produced by genome editing because these animals can be easily produced without using embryonic stem cells or induced pluripotent stem cells [7–9]. However, conventional microinjection is still routinely used to introduce engineered endonucleases into pronuclear stage embryos [Chapter 9]. Although this technique is the gold standard, it requires a high skill level to minimize cell damage during injection. Furthermore, injecting into embryos one by one is time consuming.

Electroporation is another method that can introduce nucleases into embryos. However, the current protocols damage embryos and require the zona pellucida to be weakened by treatment with Tyrode's acid solution before electroporation [10]. Recently, we developed a new electroporation system that reduces

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damage to embryos by using a three-step electrical pulse system [11]. Knock-out/knock-in mice and rats can be produced by introducing engineered endonucleases into intact embryos using this electroporation system [12]. This technique, which is called Technique for Animal Knockout system by Electroporation (TAKE), can introduce engineered endonucleases into 100 intact embryos within 5 min. It is possible to produce genome-edited animals without specialist skills, such as conventional pronuclear microinjection. The TAKE method is also applicable to gene editing in various species. The easy, rapid, and high-efficiency production of genome-edited animals using the TAKE method will contribute to numerous research fields.

2 Materials

2.1 Collection of Mouse and Rat Pronuclear Stage Embryos

1. Mature male and female mice or rats.
2. Human tubal fluid (HTF) for mouse embryo manipulation. *See* Table 1 for individual components. Adjust pH to 7.4. Sterilize using a 0.22 μm disposable filter. Store at 4 °C.
3. Modified Krebs-Ringer bicarbonate (mKRB) medium for rat embryo manipulation. *See* Table 1 for individual components. Adjust pH to 7.4. Sterilize using a 0.22 μm disposable filter. Store at 4 °C.

Table 1
Components of HTF and mKRB media

	HTF	mKRB
Components	**mg/100 mL (mM)**	**mg/100 mL (mM)**
NaCl	594 (101.6)	553 (94.6)
KCl	35 (4.7)	36 (4.8)
$CaCl_2$	23 (2.0)	19 (1.7)
$MgSO_4 \cdot 7H_2O$	5 (0.2)	29.3 (1.2)
KH_2PO_4	5 (0.4)	16 (1.2)
$NaHCO_3$	210 (25.0)	211 (25.1)
Na-lactate (60% syrup)	0.34 mL	0.19 mL
Na-pyruvate	4 (0.3)	6 (0.5)
D-glucose	50 (2.8)	100 (5.6)
Penicillin G	7	7
Streptomycin	5	5
Bovine serum albumin (BSA)	400	400

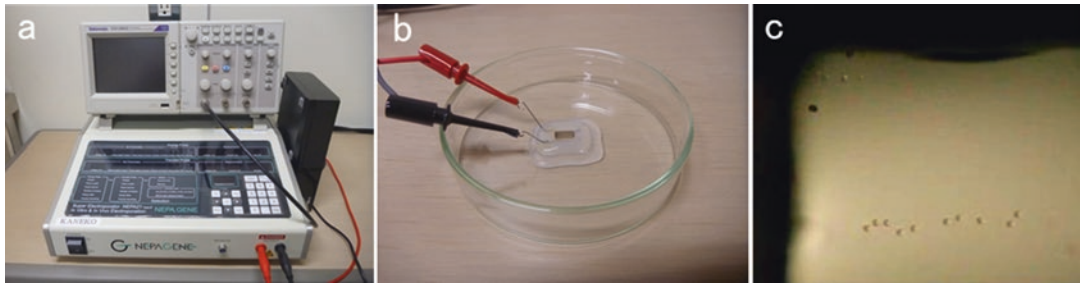

Fig. 1 (**a**) Electroporator NEPA 21. (**b**) Petri dish platinum plate electrodes. (**c**) Embryos are introduced between the electrodes filled with RNA solution

4. Sterile mineral oil.
5. 30–60 mm plastic culture dish.
6. CO_2 incubator.
7. Pregnant mare serum gonadotropin (PMSG).
8. Human chorionic gonadotropin (hCG).
9. Syringe (1 mL) with 30 G steel needle.
10. Pair of small scissors.
11. Fine tipped forceps.
12. Glass capillary pipettes.

2.2 Electroporation of mRNAs into Intact Pronuclear Stage Embryos (TAKE Method)

1. Electroporator (NEPA 21: NEPA GENE Co. Ltd., Chiba, Japan) (Fig. 1a).
2. Petri dish platinum plate electrodes (CUY520P5: NEPA GENE Co. Ltd.) (Fig. 1b).
3. ZFNmRNA, TALEN mRNA, Cas9 mRNA or protein, gRNA and ssODN (*see* **Note 1**).
4. Phosphate buffered saline (PBS) buffer without calcium and magnesium.
5. Opti-MEM (Thermo Fisher Scientific, Waltham, MA, USA).
6. Glass capillary pipettes.

2.3 Embryo Transfer

1. Mature female mice or rats.
2. Vasectomized male mice or rats.
3. Isoflurane for anesthesia: 1%, 0.8 L/min for mouse, 2%, 1 L/min for rat.
4. Pair of small scissors.
5. Fine tipped forceps.
6. Glass capillary pipettes.
7. 30 G steel needle.
8. Wound clips.

2.4 Genotyping of Delivered Pups

1. Pair of small scissors.
2. FTA cards.
3. GENEXTRACTOR TA-100 automatic DNA purification system (Takara Bio Inc., Shiga, Japan).
4. PCR system.
5. Electrophoresis system.
6. DNA sequencing system.

3 Methods

3.1 Preparation of Mouse Pronuclear Stage Embryos

1. Prepare mouse pronuclear stage embryos by in vitro fertilization.
2. Prepare two culture dishes with 200 μL drops of HTF medium covered with sterile mineral oil.
3. Pre-warm dishes at 37 °C under 5% CO_2 and 95% air before use.
4. Euthanize a male by CO_2 overdose and cervical dislocation.
5. Remove the cauda epididymides using a small pair of scissors.
6. Squeeze the sperm mass out of the epididymides using sharply pointed forceps.
7. Place the sperm mass in drops of HTF medium (*see* **Note 2**).
8. Capacitate sperm for 60–90 min at 37 °C under 5% CO_2 and 95% air.
9. Induce superovulation in females by intraperitoneal injection of 5–7.5 IU/body PMSG, followed by injection of 5–7.5 IU/body hCG 48 h later.
10. Euthanize the females by CO_2 overdose and cervical dislocation, 13–15 h after hCG injection.
11. Remove the oviducts using a small pair of scissors (*see* **Note 3**).
12. Place the oviducts into mineral oil in another culture dish.
13. Collect the cumulus–oocyte complexes by puncturing the ampulla of oviducts using a steel needle.
14. Transfer the cumulus–oocyte complexes to drops of HTF medium.
15. Add the capacitated sperm suspension into the drops of HTF medium with the oocytes. The final sperm concentration is approximately 1×10^5 cells/mL.
16. Place the culture dish at 37 °C under 5% CO_2 and 95% air.
17. Collect the embryos that appear pronuclear 5 h after insemination using glass capillary pipettes.
18. Place the pronuclear stage embryos in a new drop of HTF medium at 37 °C under 5% CO_2 and 95% air until used for electroporation.

3.2 Preparation of Rat Pronuclear Stage Embryos

1. Prepare rat pronuclear stage embryos by natural mating.
2. Induce superovulation in females by intraperitoneal injection of 150–300 IU/kg PMSG, followed by injection of 75–300 IU/kg hCG 48 h later.
3. After hCG injection, mate females with males overnight.
4. Confirm the presence of vaginal plugs to ensure mating has occurred.
5. Prepare four 50 μL drops of mKRB medium covered with mineral oil in the culture dish.
6. Pre-warm dishes at 37 °C under 5% CO_2 and 95% air before use.
7. Euthanize females with CO_2 overdose and cervical dislocation.
8. Remove the oviducts using a small pair of scissors (*see* **Note 3**).
9. Flush ampulla with mKRB medium using a 1 mL syringe with a 30 G steel needle.
10. Collect pronuclear stage embryos using glass capillary pipettes and transfer to one of the four drops of mKRB medium (*see* **Note 4**).
11. Remove cumulus cells and other debris, and transfer the embryos to another drop of mKRB medium.
12. Place the pronuclear stage embryos in the fourth drops of mKRB medium and place at 37 °C under 5% CO_2 and 95% air until used for electroporation.

3.3 Electroporation of mRNA into Intact Pronuclear Stage Embryos (TAKE Method)

1. The electroporation procedure is the same for mouse and rat embryos.
2. Prepare mRNA solutions at 40 μg/mL for ZFN and TALEN, or at approximately 500 μg/mL for Cas9 and gRNA in PBS or Opti-MEM medium (*see* **Note 5**) [11, 12].
3. Add appropriate ssODN for the desired knock-in strain to the mRNA solution.
4. Introduce 100 μL mRNA solution into the electrode (Fig. 1b).
5. Place pronuclear stage embryos in a line between electrodes on the petri dish (Fig. 1c).
6. Connect the electrodes to the electroporator (Fig. 1a).
7. Set up the poring and transfer pulses on the electroporator (*see* **Note 6**).
8. Electroporate the intact embryos (*see* **Note 7**).
9. Place electroporated embryos into fresh drops of HTF medium for mouse and mKRB medium for rat at 37 °C under 5% CO_2 and 95% air until embryo transfer.

3.4 Embryo Transfer

1. Perform the same embryo transfer procedure in mouse and rat.
2. Mate female mice or rats with vasectomized males on the day before transfer.
3. Confirm the presence of vaginal plugs of females to ensure mating has occurred.
4. Anesthetize a pseudopregnant female.
5. Expose the ovary, oviduct, and part of the uterus through an abdominal incision.
6. Make a small hole in the upper ampulla using a 30 G steel needle.
7. Aspirate 5–10 two-cell embryos into a glass capillary pipette with a few small air bubbles (*see* **Note 8**).
8. Insert this capillary pipette into the hole in the oviduct.
9. Transfer the embryos with air bubbles.
10. Return the ovary, oviduct, and uterus back inside the body cavity and seal the incision with wound clips.
11. Transfer more embryos into the other oviduct.
12. Deliver pups at gestation day 19 for mouse or day 21 for rat.

3.5 Genotyping of Delivered Pups

1. Collect blood from 3-week-old pups.
2. Blot blood samples onto the FTA cards.
3. Extract genomic DNA from blood samples using a GENEXTRACTOR TA-100 automatic DNA purification system.
4. Amplify the target sequences by PCR.
5. Electrophorese the PCR products on agarose gels.
6. Sequence the PCR products to confirm the mutations (*see* **Note 9**).

4 Notes

1. You can purchase these endonucleases as commercial products or transcripts can be generated from custom-designed plasmids.
2. Handle gently to maintain high sperm motility.
3. Collect oviducts immediately after euthanasia to avoid damage to oocytes.
4. If cumulus cells are attached to embryos, remove using 0.1% hyaluronidase.
5. Introduce Cas9 protein into embryos using the same procedure as that used for the introduction of mRNA.

6. The optimal electric pulses for mouse and rat embryos are shown in Table 2.
7. Intact embryos without any chemical treatment, such as zona-thinning using Tyrode's acid solution, can be used for electroporation.
8. Embryo transfer at the 2-cell stage is suitable for subsequent embryo development.
9. Typical results using C57BL/6J mice and F344/Stm rats are shown in Tables 3, 4, 5 and 6. F344/Stm rats are supplied by the National BioResource Project-Rat (Kyoto, Japan, http://www.anim.med.kyoto-u.ac.jp/NBR/Default.aspx). This strain is suitable for gene modification because many embryos can be obtained [13].

Table 2
Optimal values of electric pulses for electroporation of intact mouse and rat embryos using petri dish platinum plate electrodes (CUY520P5)

	Voltage (V)	Pulse width (ms)	Pulse interval (ms)	Number of pulses	Decay rate (%)	Polarity
Poring pulse	225	0.5–2.5	50	4	10	+
Transfer pulse	20	50	50	5	40	+/−

Table 3
Development of rat embryos with ZFN or TALEN mRNAs introduced by microinjection or TAKE method

mRNA	Methods	Pulse width of poring pulse (ms)	No. of embryos examined	No. of 2-cell embryos (%)	No. of offspring (%)	No. of knockout offspring (%)
ZFN	Microinjection	-	93	41 (44)	9 (10)	3 (33)
	TAKE	0.5	61	58 (95)[a]	19 (31)[a]	7 (37)
		1.5	63	57 (91)[a]	15 (24)[a]	11 (73)
		2.5	66	16 (24)[a]	4 (6)	3 (75)
TALEN	Microinjection	-	52	20 (39)	6 (12)	6 (100)
	TAKE	1.5	57	55 (97)[a]	25 (44)[a]	1 (4)
		2.5	57	56 (98)[a]	17 (30)[a]	3 (18)

F344/Stm rat was used
Il2rg gene was targeted
[a] $P < 0.05$ vs. microinjection
The data are reproduced from ref. 11

Table 4
Development of mouse embryos with Cas9 mRNA, gRNA, and ssODN introduced using the TAKE method

Cas9 mRNA (μg/mL)	gRNA (μg/mL)	ssODN (μg/mL)	No. embryos examined	No. (%) of embryos developed to 2-cells	No. (%) of offspring	No. (%) of knock-out offspring	No. (%) of knock-in offspring
400	600	300	100	84 (84)	36 (43)[a]	24 (67)[b]	12 (33)[c]
200	200	200	100	73 (73)	35 (48)[a]	11 (31)[d]	3 (9)[e]
100	100	100	120	117 (98)	28 (24)[f]	9 (32)[d]	5 (18)

C57BL/6J was used
Il2rg gene was targeted
Significant differences at $P < 0.05$; a vs. f, b vs. d, c vs. e
The data are reproduced from ref. 12

Table 5
Development of rat embryos with Cas9 mRNA, gRNA and ssODN introduced using the TAKE method

Cas9 mRNA (μg/mL)	gRNA (μg/mL)	ssODN (μg/mL)	No. embryos examined	No. (%) of embryos developed to 2-cells	No. (%) of offspring	No. (%) of knock-out offspring	No. (%) of knock-in offspring
400	600	300	60	45 (75)	24 (53)	21 (88)[a]	8 (33)[b]
200	200	200	50	49 (98)	19 (39)	7 (37)[c]	1 (5)
100	100	100	89	88 (99)	41 (47)	16 (39)[c]	1 (2)[d]

F344/Stm was used
Il2rg gene was targeted
Significant differences at $P < 0.05$; a vs. c, b vs. d
The data are reproduced from ref. 12

Table 6
Development of rat embryos cointroduced Cas9 protein and gRNA by microinjection or TAKE method

Methods	No. of embryos examined	No. of 2-cell embryos (%)	No. of offspring (%)	No. of knock-out offspring (%)
Microinjection	40	19 (48)	13 (68)	10 (77)
TAKE	25	25 (100)	17 (68)	17 (100)

Unpublished data by Takehito Kaneko
Cas9 protein and gRNA (Integrated DNA Technologies, Inc. Coralville, IA, USA) were used

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