# Chapter 19

# Generation of Rabbit Models by Gene Editing Nucleases

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## Abstract

Due to the lack of germline transmitting pluripotent stem cells (PSCs) cell lines and the extreme difficulty of somatic cell nuclear transfer (SCNT) in rabbit, the gene targeting technology in rabbit was lagging far behind those in rodents and in farm animals. As a result, the development and application of genetically engineered rabbit model are much limited. With the advent of gene editing nucleases, including ZFN, TALEN, and CRISPR/Cas9, it is now possible to produce gene targeting (i.e., knockout, knockin) rabbits with high success rates. In this chapter, we describe a comprehensive, step-by-step protocol for rabbit genome editing based on gene editing nucleases with specific emphasis of CRISPR/Cas9.

**Key words** Rabbit, Genome editing, Engineered endonucleases, CRISPR/Cas9, Microinjection

## 1 Introduction

Rabbit is a classic animal model, and is increasingly becoming a translational model of choice, serving to bridge the gap between rodent models and larger animal models [1–4]. Rabbits are phylogenetically closer to primates than rodents [5, 6]. It is much easier to perform surgical procedures, multiple blood sampling (in larger volumes), tissue and organ sampling and analysis of organ function in vitro (e.g., heart perfusion) in rabbit. Technology or equipment developed for human infants could be easily tested in adult rabbits without modification due to their weight and size. Unlike pigs or rhesus monkeys, rabbits are relatively inexpensive and can be easily adapted to research facilities/institutions. They are easy to breed and handle and are recognized by the scientific and regulatory communities as a well-established model species. In addition, rabbit is a species that can tolerate restraint, which enables imaging technologies like magnetic resonance imaging (MRI) to be applied in an awake and conscious state with minimal preparation and habituation [7].

Since the first transgenic rabbit produced by Hammer and his colleagues [8], transgenic rabbit models have been generated by

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different laboratories worldwide for different research purposes, such as studies of lipid metabolism and atherosclerosis [4, 9–13], oncology [14, 15], acquired immunodeficiency syndrome (AIDS) [16–18], and bioreactors for the production of pharmaceutical proteins [19, 20]. Most of these rabbit models are produced by pronuclear microinjection of foreign DNA, which is subjected to position effects, including variations in the expression levels of the transgene caused by variation in the copy number of the transgenes or lack of crucial regulator elements related to the integration site, and potentially disrupting endogenous gene function through insertional mutagenesis. A technology that can insert or delete specific modifications into the rabbit genome more precisely is desired to study human genetic diseases in rabbit model.

The ascent of the mouse as genetic model organism is largely attributed to the development of gene targeting technology in embryonic stem cells (ESCs) that relies on the spontaneous homologous recombination (HR) to introduce defined modifications into sequences of interest in the genome. The gene targeted mice ESCs are capable of integrating into germline in chimeric mouse to produce gene targeted mouse model. In non-rodent animals where germline transmitting PSCs are not available, somatic cell nuclear transfer (SCNT) technology enabled gene targeting in species including pigs [21–23], sheep [24, 25], and cattle [26]. In these cases, gene targeting was performed in the somatic cells, which were then used as nuclear donor for animal cloning.

Unfortunately, germline competent rabbit PSCs are still not available since the first attempt in 1966 [27]. The SCNT efficiency, especially when using fibroblast cells as nucleus donors, is extremely inefficient in rabbit [28, 29]. Thus, production of gene targeted (KO/KI) rabbits has remained an extreme challenge for many years which has limited the application of this invaluable animal model in biomedical research.

Recently, Zinc Finger Nuclease (ZFN), Transcription Activator-Like Effector Nuclease (TALEN), and Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein-9 (CRISPR/Cas9) have emerged as powerful means for genome editing [30, 31]. These engineered endonucleases are efficient in generating double-strand breaks (DSB) in the specific genomic loci that can be repaired by error-prone non-homologous end joining (NHEJ) leading to a functional knockout of the targeted gene or used to integrate a DNA sequence at a specific locus through homologous recombination (HR). Thanks to the very high targeted DSB rates, knockout /knockin animals can be now readily derived by direct injection of the engineered endonucleases together with other elements (e.g., donor DNA) into pronuclear stage embryos, bypassing the need for germline transmitting PSCs [32, 33].

The early generations of nucleases, i.e., ZFNs and TALENs, employ custom-designed, locus-specific proteins fused to a sequence-independent nuclease (e.g., FokI) domain to generate targeted mutations. For each new target DNA sequence, the protein motifs of ZFN or TALEN need to be redesigned, synthesized, and validated. Despite these tedious molecular biology works, ZFN and TALEN each has brought breakthroughs over prior technologies in the gene editing field upon its introduction. That being said, there is no doubt that CRISPR/Cas9 has outperformed these two nucleases in many aspects. To name a few, first, CRISPR/cas9 uses so called guide RNA (gRNA) complementation to recognize the target sequence. The only requirement of a targetable sequence is the specific protospacer adjacent motif (PAM) that varies depending on the bacterial species of the Cas9 origin. For example, a NGG PAM is required for SpCas9 target. So for each target sequence, one can almost always find targetable sequence, and only need to synthesize the gRNA (vs protein motifs in ZFN or TALEN). Furthermore, the RNA guided Cas9 nuclease is much more efficient in generating double-strand DNA breaks than ZFN and TALEN. These favorable features have made CRISPR/Cas9 the first choice for gene editing in many applications nowadays [34, 35].

Here we describe comprehensive, step-by-step protocols for producing genetically modified rabbit models using the CRISPR/Cas9 technology. We have developed optimal conditions for superovulation of female New Zealand White (NZW) rabbits, for Cas9 components microinjection, and for embryo transfer into recipient females. The same procedure could also be used in ZFN or TALEN-mediated rabbit gene targeting, with the only difference being the preparation of the nuclease reagents. In general, generation of gene-edited founder rabbits by following this protocol takes as few as 2 months, and homozygous gene-edited rabbit model could be established within 15 months (Figs. 1 and 2, **Note 20**).

## 2 Materials

### 2.1 Equipment

1. Inverted microscope with fluorescent light source and digital camera.
2. Vibration Isolation Platform.
3. Thermo Plate for microscope stage.
4. Micromanipulator.
5. Microinjector.
6. Stereomicroscope.
7. Micropipette puller.
8. Microforge.

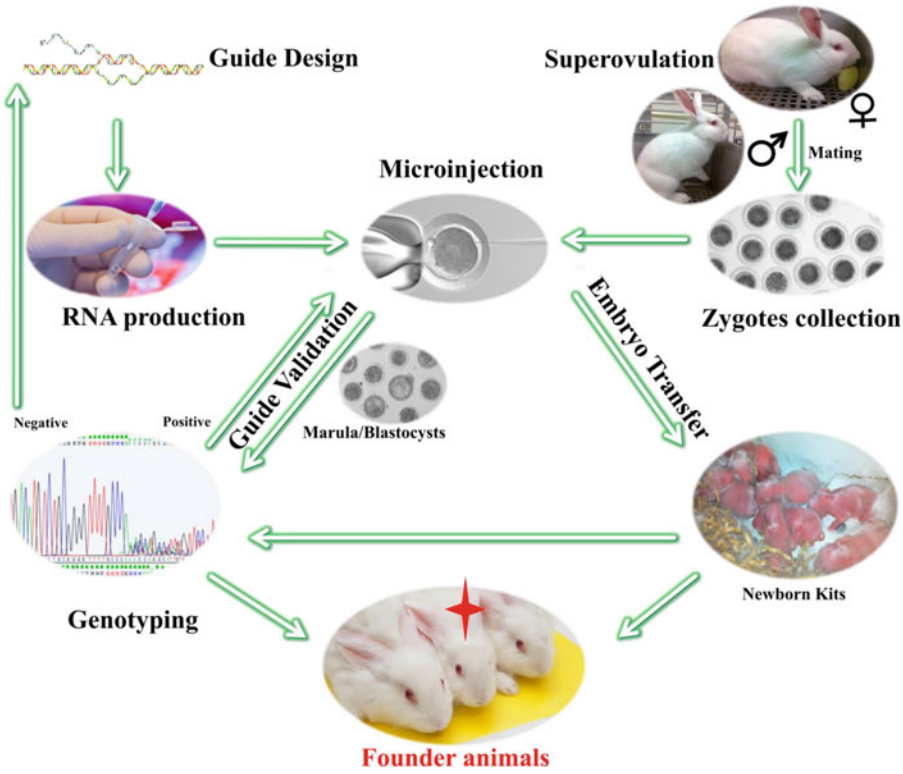


**Fig. 1** Procedure of rabbit genome editing using CRISPR/Cas9

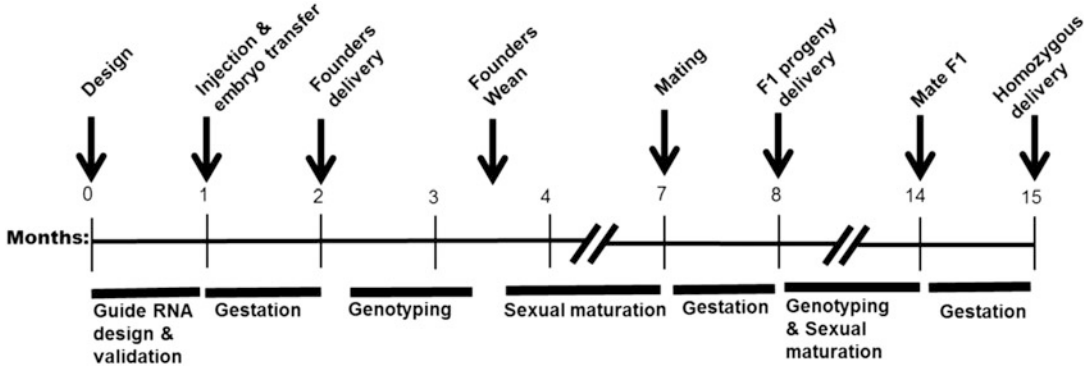


**Fig. 2** Timeline for rabbit genome editing

9. Ceramic Tile for scoring glass.
10. CO2 incubator.
11. Alcohol lamp.
12. Embryo moving pipette (pulled Pasteur Pipette) assembled into a hand or mouth operated system.
13. Autoclave-steam sterilizer.

14. Anesthesia Machine.
15. TPR Temperature/Pulse Oximeter/Respiration System.
16. Water heated mat.
17. Vacuum Clipper System: Clipper connected to vacuum.
18. Skin Stapler Handle.
19. Skin Staple Remover.
20. DNA thermal cyclers.
21. Refrigerated Microcentrifuge.
22. Spectrophotometer.
23. Horizontal Gel Electrophoresis System.
24. Ultra-low Freezer.
25. Surgery pack: Thumb Tissue Forceps 5.5″ 1 × 2 teeth, Operating Scissors 6.5″ Sharp/Sharp Straight, Operating Scissors 4.5″ Blunt/Blunt Straight, Forceps Thumb Dressing 5.5″, Adson Dressing Forceps 4.75″, Iris Scissors 3 1/2″ Straight, Scalpel Handle Stainless Steel #3—for #10–15 Blades, Towel Clamps 5.5″, Needle Holder 5.25″, Crile Forceps 5.5″ Straight, Thumb Dressing Forceps 8″.

### 2.2 Consumables

1. Glass capillary: Thin wall borosilicate tubing with filament, 0.78 mm inner diameter, 1 mm out diameter.
2. Injection slide: Glass Chamber Slide System; 1-well, chamber removed.
3. 4-Well Dish.
4. Petri dish, 35 mm, 60 mm.
5. Centrifuge tubes, sterile, 15 ml, 50 ml.
6. Microcentrifuge Tubes, 0.5 ml, 1.5 ml, Certified RNase- and DNase-free.
7. Syringes, 1 cc, 5 cc Luer Slip.
8. Embryo transfer tube: Tom Catheter 3.5Fr × 4.5″, Sterile.
9. Scalpel Blades #10 Stainless Steel.
10. Powder Free Surgeon Glove, Sterile.
11. Surgery Drape: Poly-lined Sterile 18″ × 26″.
12. Suture #4-0 3/8 Circle Rev. Cut 19 mm/36″.
13. Surgery Gown: XLarge 47″ Non-Reinforced Sterile with Towel.
14. Staple Cartridges Surgi-Close 6 Cartridges × 20 Staples.
15. Soft-E Collar XSmall (Cat/Dog 5-9#).

### 2.3 Media and Reagents

1. HM: 25 mM HEPES, 10% fetal bovine serum in Medium 199 with Hanks's salts.

2. EBSS-complete medium: 1× MEM nonessential amino acid, 1× BME amino acid, 1 mM L-glutamine, 0.4 mM sodium pyruvate, 10% FBS in Earle's Balanced Salt Solution with calcium, magnesium, and phenol red.
3. Mineral oil: BioReagent, suitable for embryo cell culture.
4. FSH (Follicle-stimulating hormone): 10 mg/ml in saline.
5. HCG (Human chorionic gonadotropin): 200 IU/ml in saline.
6. Euthasol (Euthanize solution): Sodium pentobarbital 390 mg/ml, sodium phenytoin 50 mg/ml.
7. Anesthetic: Isoflurane Inhalant Anesthetic.
8. Analgesic: carprofen Injectable: 50 mg/ml.
9. NP40 LYSIS buffer: 1% Tergitol-type NP-40, 1 μg/ml Proteinase K in Taq Buffer.
10. Digestion buffer for DNA extraction: 5 mM EDTA, pH 8.0, 200 mM NaCl, 100 mM Tris–HCl, pH 8.0, 0.2% sodium dodecyl sulfate (SDS).
11. RNase-Free 0.1× TE Buffer: 1 mM Tris–HCl pH 7.4, 0.1 mM EDTA in DNase/RNase-Free Distilled Water.
12. Molecular biology Kits: T7 Standard RNA IVT Kit, T7 Standard mRNA Production System.

PCR Purification Kit, RNA purification Kit, Whole genome amplification kit.

### 2.4 Animals

Embryo Donor: 4 months to 2 years old New Zealand White (NZW) does.

Fertile males: 6 months to 2 years old NZW buck rabbits. They should be replaced every 1–2 years.

Recipients: 5 months to 2 year old NZW does.

## 3 Methods

### 3.1 Prepare the Engineered Nucleases Reagents

1. Design guide RNA and donor DNA according to project aims (*see* **Notes 1** and **2**).
2. To produce Cas9 mRNA and guide RNA in lab, we use the Cas9 expression plasmid JDS246 and guide RNA expression plasmid PX330 from Addgene as template (*see* **Note 3**).
3. Linearize plasmid JDS246 with PmeI: 5 μg plasmid JDS246 DNA, 10 μl 10× CutSmart® buffer, 1 μl PmeI (10 U/μl), add distilled water to a total volume of 100 μl. Incubate at 37 °C for 2–3 h.
4. Verify the complete digestion by running 2 μl of the cut DNA on a 1% agarose gel.

**Table 1**
**Primers for guide RNA synthesis (*see* Note 1)**

| Primer | Sequence (5′–3′, NNs stand for customized 20nt guide) |
|---|---|
| T7-sgRNA_F | TGTAATACGACTCACTATAGGNNNNNNNNNNNNNNNNNN gttttagagctagaaatag |
| T7-sgRNA_R | AAAAGCACCGACTCGGTGCC |

5. Clean up the digested DNA using a PCR Purification kit.
6. Measure DNA concentration using a spectrophotometer. Continue to the next step or store the purified DNA at −20 °C.
7. In vitro transcribe, cap, and polyadenylate Cas9 mRNAs from the linearized template using the T7 Standard mRNA Production kit following the manufacturer's protocol.
8. Clean up the resulting mRNA using the RNeasy Mini Kit.
9. Elute the purified mRNA in RNase-free 0.1× TE buffer.
10. Measure Cas9 mRNA concentration using a spectrophotometer.
11. Dilute Cas9 mRNA in RNase-free 0.1× TE Buffer, centrifuge at 20,000 × *g* at 4 °C for 30 min.
12. Carefully remove the top 2/3 of diluted RNA to a new RNase-free tube and store in −80 °C freezer in 10 μl aliquots.
13. Order primers in Table 1 and use PX330 as PCR template to produce guide RNA in vitro transcription template by PCR amplification. PCR condition: 50 °C, 38 cycle, 2–8 ng plasmid as template in 200 μl PCR reaction. Use Taq DNA polymerase with higher fidelity.
14. Clean up the PCR product using a PCR Purification kit.
15. In vitro transcribe guide RNA by using T7 Standard RNA IVT Kit following the manufacturer's protocol.
16. Clean up guide RNAs using RNeasy Mini Kit. Elute the purified RNA in RNase-free 0.1× TE buffer.
17. Measure the concentration of the guide RNA with Spectrophotometer, and dilute in RNase-free 0.1× TE buffer.
18. Centrifuge at 20,000 × *g* at 4 °C for 30 min.
19. Carefully remove the top 2/3 of diluted RNA to a new RNase-free tube.
20. Store guide RNA in −80 °C freezer in 10 μl aliquots.
21. Before microinjection, thaw the RNA samples on ice, and keep it on ice.

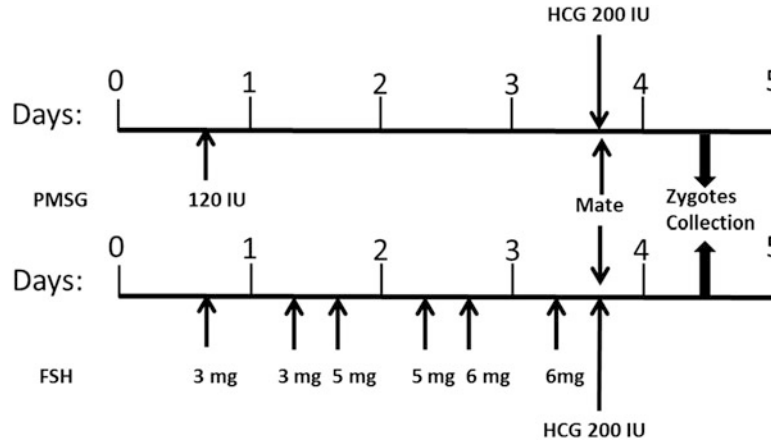


**Fig. 3** Time schedule for rabbit superovulation

22. For knockin project, dilute the donor DNA (ssDNA oligo or dsDNA plasmid) in RNase-free 0.1× TE buffer.
23. Centrifuge at 20,000 × *g* for 30 min.
24. Carefully remove the top 2/3 of diluted DNA to a new RNase-free tube.
25. Store DNA in 4 °C fridge.

### 3.2 Superovulation

1. Subcutaneously inject sexually matured NZW female rabbits with FSH twice/day with a dosage of 3 mg for the first two injections, 5 mg for the next two injections, and 6 mg for the last two injections. Alternatively, PMSG can be used instead of FSH. Intramuscularly inject 150 IU of PMSG in single dosage (*see* Fig. 3 and **Notes 4** and **5**).
2. At 72 h after the first FSH injection, or PMSG injection, inject 200 IU HCG intravenously to induce ovulation (*see* Fig. 3).
3. Put a superovulated doe into a male's cage immediately after hCG injection.
4. Observe and record the successful mating. It will happen within minutes.
5. Optionally, to ensure successful fertilization, put the female to more male's cages to record at least 2–3 successful mating.
6. Put the female back to her cage.

### 3.3 Zygotes Collection

1. Prepare two 30 mm petri dishes with 2 ml HM medium covered by mineral oil. Keep the dishes on a heated stage at 38.5 °C.
2. Prepare two 50 ml centrifuge tubes with 25 ml HM medium and warm in 37 °C water bath.

3. At 17–19 h after hCG injection, euthanize the superovulated rabbits with an overdose intravenous injection of Euthasol (150 mg/kg). Recover the oviducts with a part of uterus in 25 ml HM medium.
4. Wash the oviducts in a new 25 ml HM medium. Use an iris scissors to remove the fat tissue around the oviduct to make it straight, hold the uterine side with one pair of tissue forceps, insert a blunt needle connected to a syringe filled with 5 ml of HM medium, and flush the oviduct from uterus to infundibulum.
5. Collect the flushed media in a 60 mm dish. Swirl the dish gently to gather eggs in the center.
6. Collect eggs under a stereomicroscope, wash the eggs in the 2 ml pre-warmed HM dish prepared in **step 1**, and kept in the second 2 ml HM dish on a 38.5 °C heated stage (*see* **Note 6**).

### 3.4 Embryo Microinjection

1. The day before microinjection, prepare a 4-well dish containing 500 μl EBSS-complete medium in each well, cover with 400 μl mineral oil (for embryo culture). Prepare a 30 mm dish with 4 × 80 μl drops of EBSS-complete medium covered with mineral oil (for embryo wash after injection). Place the dishes in 5% $CO_2$ incubator at 38.5 °C overnight.
2. Prepare holding pipette by hand pulling a piece of the borosilicate glass capillary tubes in the flame of an alcohol lamp. The outside diameter of the drawn-out region of the pipette should be 120–200 μm (i.e., close to the diameter of the zona pellucida). Break the pipette at ~1–2 cm from the shoulder of the pipette by scoring the glass lightly with a ceramic tile at the desired position. Fire-polish the pipette tip with microforge until the pipette shrinks to an inner diameter of ~40 μm. Bend the pipette ~20° angle by approaching the side of the pipette to the heating glass bead on the microforge.
3. Before microinjection, prepare the microinjection platform by placing a 20 μl drop of HM medium on a 1-well chamber slide that has the media chamber removed. Cover with 1.5 ml mineral oil and place on the 38.5 °C heated microscope stage of the inverted microscope (*see* Fig. 4a, b).
4. Place holding pipette into the left arm of the micromanipulator and position into the drop of manipulation medium. Adjust the holding pipette angle to make the tip paralleled to the stage (*see* Fig. 4b).
5. Fabricate injection micropipettes by heating and pulling borosilicate glass capillary tubes in Sutter P-1000 micropipette puller with parameter as following: Heat = 526, Pull = 80, Vel. = 70, Delay = 80, Pressure = 200, Ramp = 516, Delay Mode, Safe heat.

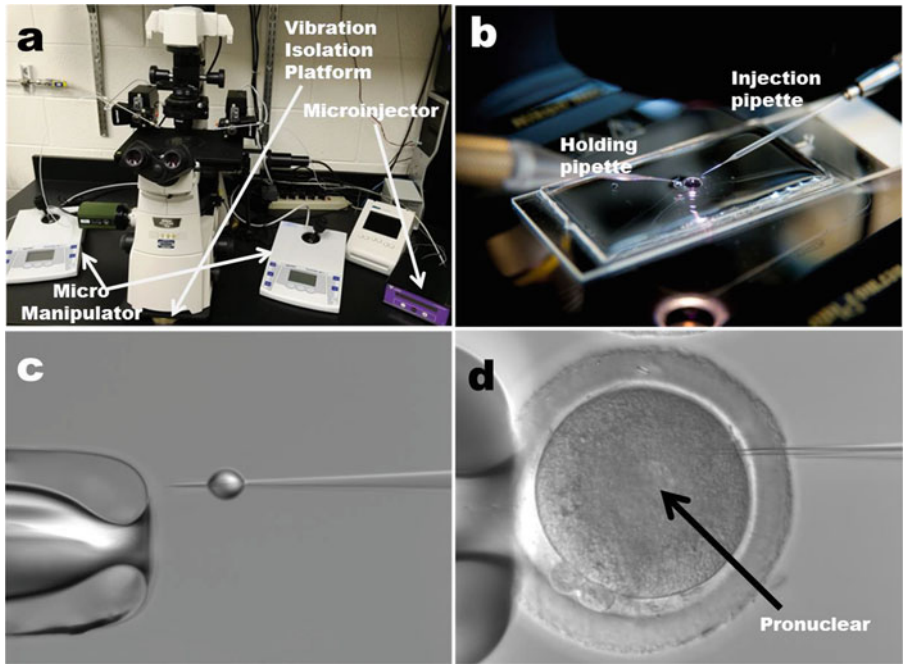


**Fig. 4** Rabbit Embryo Microinjection. (**a**) Microinjection setup. Inverted microscope standing on a vibration isolation platform, the microscope was equipped with a Differential Interference Contrast (DIC) device, and a thermoplate. Microinjector was connected with pressured air supply. Holding arm and injection arm were controlled by micromanipulators. (**b**) Microinjection slide: 20 μl drop of HM medium on a 1-well chamber slide that has the media chamber removed, covered with 1.5 ml mineral oil and placed on the 38.5 °C heated microscope stage. (**c**) Opening of the injection pipette. In mineral oil, apply and hold clear pressure (80 psi) on microinjector, open the tip of the injection pipette (right) by gently tapping it against the holding pipette (left). The RNA solution formed a droplet (arrow) on the injection tip once it is opened. (**d**) Injection of rabbit embryo. Avoid pronuclear (arrow) when advancing the injection pipette tip into the embryo

6. Thaw the RNA aliquots on ice, mix Cas9 mRNA and guide RNA in a final concentration of 150/50 ng/μl in Rnase-free 0.1× TE Buffer, and keep on ice. Add donor template DNA in final concentration of 100–200 ng/μl for knockin project (*see* **Note 7**). Drop 1 μl of the injection mixture on the back opening of the injection pipette. A siphon effect will fill the tip of the injection pipette immidiately.
7. Place injection pipette into the right arm of the micromanipulator, and position it into the drop of manipulation medium with ~15° angle to the stage (*see* Fig. 4b).
8. Turn on the compressed air supply that is connected to a microinjector and set up the microinjector. We use the following parameters on our microinjector (Tritech research, MINJ-D): holdP = 2 psi, InjP = 20 psi, ClearP = 80 psi, Injtime = manual.

9. Transfer embryos (30–50) to the micromanipulation drop on the platform.
10. Move the stage to leave the tips out of the medium into oil, apply clear pressure (80 psi), and open the tip of the injection pipette by gently tapping it against the holding pipette. You will see the injection solution forms droplets on the injection tip when it is opened (*see* Fig. 4c).
11. Back to the medium, use the holding pipette to capture an embryo and align the injection needle, embryo and holding pipette along the *x*-axis.
12. Under 400× magnification, advance the injection pipette through the embryo. Be careful to avoid the nucleus. Once the cell membrane is pierced, press the foot pedal to inject. After injection, withdraw the needle. Release the embryo and repeat till all the embryos in the drop are injected (*see* Fig. 4d).
13. Wash the injected embryos three times in EBSS-complete medium, and incubate at 38.5 °C, 5% $CO_2$ in air for 1–2 h or overnight before embryo transfer (*see* **Note 7**).
14. For ex vivo validation of guide RNA, culture the injected embryos in EBSS-complete medium in vitro for 3–4 days until they reach morula or blastocyst stage.

### 3.5 Ex Vivo Validation of Guide RNA

1. For each guide RNA to be validated, collect 5–10 morula or blastocyst stage embryos, put embryos in 10 μl NP40 LYSIS buffer in a PCR tube. Incubate for 0.5–1 h at 55 °C, and 10 min at 95 °C, keep in 4 °C and use as PCR template (*see* **Note 8**).
2. PCR amplify the target sequence using PCR primer pair located upstream and downstream of the target site.
3. PCR products are then purified and Sanger sequenced with a sequencing primer.
4. A successful guide RNA will direct Cas9 to cut the target DNA and results in insertion/deletions (indels) after NHEJ repair, which can be manifested on the sequencing chromatography as overlapping peaks starting around the target site (*see* **Note 9**).
5. More accurate estimation of the efficiency can be calculated with an online software (TIDE https://tide.nki.nl/).
6. Guide RNAs with indels efficiency higher than 30% is recommended for use to produce embryos for embryo transfer (*see* **Note 10**).

### 3.6 Embryo Transfer

Surgery is performed under sterile conditions, with all surgical tools, media, supplies, etc. sterile. For each animal, a fresh, sterile, surgical pack, containing sterile drapes and tools is used. Personnel involved in the surgical procedures are scrubbed, gloved, gowned,

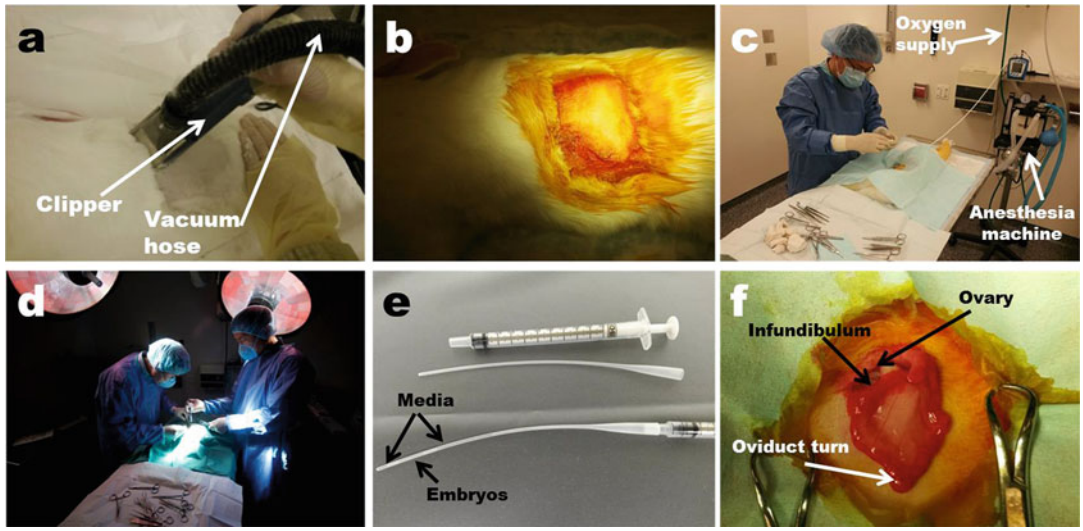


**Fig. 5** Rabbit embryo transfer surgery. (**a**) A clipper connected with a vacuum was used to remove the hairs around incision site. (**b**) Incision site was prepared by three cycles of antiseptic scrub, followed by antiseptic solution and wipe with a sterile gauze sponge. (**c**) Surgery room setup. Anesthesia machine connected with oxygen supply and scavenger system. Rabbit was monitored by a TPR System. (**d**) Surgery ongoing. (**e**) Embryo transfer tube assembly. Tom Catheter connected with a 1 ml syringe. The transfer tube is loaded in the following order: 2 cm HM medium (right black arrow), 1–2 cm HM medium with zygotes, and 0.5 cm HM medium (left black arrow) separated by two 0.5 cm air bubbles. (**f**) For embryo transfer, the transfer tube is inserted through the infundibulum into the oviduct. Make sure the tube passed the turn of the oviduct and unload the embryos inside

capped, and masked. The rabbit is placed on the surgical table on a water heated mat maintained at 37 °C, and covered with a sterile drape (*see* Fig. 5).

1. Select female NZW rabbit in good health, weighing approx. 3–4 kg and at least 5 months of age. Check vagina to select does with red or pink vulva color (*see* **Note 11**).
2. To prepare recipients, inject 100 IU of hCG intravenously at same time when donor was mated and injected with hCG (*see* **Notes 12** and **13**).
3. Before surgery, weigh the rabbit and inject 4 mg/kg carprofen subcutaneously as an analgesic (*see* **Note 14**).
4. Connect Anesthesia Machine to oxygen supply and scavenging system (*see* Fig. 5c).
5. Restrain rabbit on its neck and back firmly, put on an anesthesia mask connected with the Anesthesia Machine to the rabbit mouth (*see* **Note 15**).
6. On the Anesthesia machine, adjust flowmeter at 100–200 ml/min and vaporizer at 5% to induce anesthesia. Squeeze the foot

pad to check for lack of pedal reflex which is an indication of adequate anesthesia (*see* **Note 16**).

7. Adjust the evaporator to 2–5% to maintain anesthesia. Monitor temperature, pulse, and respiration during procedure and adjust vaporizer as needed.
8. Clip the rabbit's flanks with a vacuum clipper system (*see* Fig. 5a).
9. Put the rabbit under anesthesia on a water heated mat in lateral recumbence.
10. Wash the shaved flank with antiseptic scrub (Pivodine Scrub) three times, followed by antiseptic solution (70% alcohol), and wipe with a sterile gauze sponge (*see* Fig. 5b).
11. The surgical incision site can be approximated by drawing an imaginary equilateral triangle over the flank with vertices at the wing of the ilium, the greater trochanter, and the ovary.
12. Make a 2–3 cm horizontal (anterior-posterior) skin incision with a scalpel blade over the anticipated location of the ovary. Incise the external and then internal abdominal oblique muscles.
13. Gently elevate the peritoneum with Crile forceps and sharply incise.
14. Grasp the fat pad attached to the oviduct with Thumb Dressing forceps to pull the ovary and oviduct out of the peritoneal cavity.
15. Under a stereomicroscope, 10–15 embryos are loaded into an embryo transfer tube (Tom Catheter) attached to a 1 ml syringe with HM medium (*see* Fig. 5e).
16. Insert the embryo transfer tube through the infundibulum into oviduct of recipients. Make sure the tube passed the big turn of the oviduct and unload the embryos inside. Withdraw the transfer tube gently and carefully (*see* **Note 17** and Fig. 5f).
17. Put the ovary and oviduct back to the peritoneal cavity, close the peritoneum and muscle layer, and then subcutaneous fascia layer with absorbable suture (e.g., Vicryl). Close the skin with Skin Staples.
18. Apply the same procedure to the other side to transfer another batch of 10–15 embryos (*see* **Note 18**).
19. At the end of the surgery, turn vaporizer and gas supply off, place animal in recovery area with thermal support until fully recovered.
20. Put Elizabethan Soft-E Collar on the neck to prevent the rabbit from biting the wound.

21. After surgery, inject carprofen subcutaneously at 4 mg/kg/day as an analgesic for at least 5 days.
22. At 10–14 days post operation, remove the Soft-E Collar and remove the skin staples with a skin staple remover.
23. 1–2 week before the expected delivery date, move the recipient rabbit into a breeding cage with nesting materials (*see* **Note 19**).

### 3.7 Genotyping Analysis of Founders by PCR and Sequencing

1. For ear skin tissue biopsy from 1-3 week old kits, hold the kit ear firmly with one hand and cut off a tiny piece of ear skin (around 2–5 mm) with a scissors in the other hand.
2. Digest the ear skin tissue with 300 μl digestion buffer with 1 mg/ml proteinase K in a 1.5 ml Eppendorf tube at 55 °C overnight.
3. Add 1 ml of 100% ethanol into each tube, mix completely. Centrifuge at 13,000 × *g* for 12 min, and then pour out the ethanol.
4. Wash the DNA pellets by adding 0.8 ml of 70% ethanol into the tubes, then centrifuge at 13,000 × *g* for 8 min.
5. Pour out the ethanol from the tube, and then suck the remaining ethanol with pipette.
6. Dissolve the DNA pellets with 300 μl TE buffer or distilled water.
7. Store the DNA at 4 °C or −20 °C and use as template for PCR.
8. For KO project, PCR amplify the target locus and sumit for Sanger sequencing using the same procedures as in Subheading 3.5 (*see* **Note 9**).
9. For knockin project using single-stranded oligo donor (ssODN) as template to introduce a point mutation or short tag sequences, identify the knockin kits by deep sequencing or TA cloning and sequencing of the PCR products from **step 8**.
10. For large fragment knockin project, identify the knockin kits by two PCR primer pairs spanning each of the homologous arms.

## 4 Notes

1. There are dozens of free online tools for guide RNA design. The popular tools include CRISPR Design tool from MIT (http://crispr.mit.edu/), and Breaking Cas from Spain. (http://bioinfogp.cnb.csic.es/tools/breakingcas/?gset=2x1_GENOMES_Ensembl_85). To order T7-sgRNA-F in table 1, please note a 5'-GG was added at the begining to facilitate T7 transcription, so remember to skip the 5' -G or -GG if present in the customized 20nt guide.

2. New Zealand White Rabbit is not a pure inbred line. There are some single-nucleotide polymorphisms (SNPs) in their genome. We recommend PCR sequencing your target region before you design your guide RNA and donor template to exclude SNPs or sequencing mistakes.
3. Cas9 mRNA and guide RNA are also commercially available.
4. NZW rabbits as early as 12 weeks of age are old enough to be stimulated for superovulation with either FSH or PMSG treatments, but the embryo development may be compromised (unpublished data). We recommend using donor rabbit older than 16 weeks.
5. In rabbits, PMSG and FSH have been commonly used for superovulation. Though the PMSG program is simpler and less laborious, FSH program is more reliable [36].
6. It is not necessary to remove cumulus cells using hyaluronidase, since most of the cumulus cells will be detached from the rabbit oocytes at the time zygotes are recovered. For the same reason, the rabbit zygotes are collected by oviduct flush but cannot be released by ampulla tearing as in mice.
7. Donor DNA could be either ssODN or circular plasmid DNA vector. It is not recommended to linearize the plasmid DNA donor due to concerns of higher random insertion risks. All the contents should be injected in cytoplasm, since injection of 100–200 ng/μl DNA in pronuclear will kill the embryos. The survival rate of embryos cultured for 2–3 h after injection is between 90% and 100%. For knock-in project using plasmid donor carrying long homologous arms as template, which depends on homologous recombination, we recommend culture the embryos after injection for 20 h in EBSS-complete medium containing 7.5 μM RS-1 to stimulate Rad51 activity [37].
8. For single-embryo PCR, we recommend using REPLI-g® Mini Kit (Qiagen, Germantown, MD) to amplify genomic DNA before PCR reaction. Briefly, collect embryos individually in 2 μl distilled water, add 1.75 μl Buffer D2 (include in the kit) to each embryo, mix by vortexing, centrifuge briefly, and incubate on ice for 10 min. After that, add 1.75 μl Stop Solution (include in the kit), mix by vortexing, and centrifuge briefly. Add 2 μl of the mixture (denatured DNAs) to 8 μl master mix (include in the kit) and incubate at 30 °C for 10–16 h. Heating at 65 °C for 3 min, keep in 4 °C fridge, and use as PCR template.
9. Since New Zealand White Rabbit is not a pure inbred line, T7E1 and Surveyor mismatch cleavage assays are not always reliable due to potential SNPs. In Sanger sequencing chromatography, the PCR products of the mutant samples will show

different sequencing diagram depending on the type of the mutation. A typical mono-allelic indel mutation will show an "peaks on peaks" starting around the target site on the sequencing diagram, with wildtype sequence still readable. If you cannot read a wild-type sequence from the double curve, it may indicate a hetero double allelic indels. For homo double allelic indels, the sequencing diagram will be as clean as wild-type, but you can read the indels out. Please always keep in mind that these reading from Sanger sequencing data is not accurate. You need a cloning sequencing or targeted deep sequencing to confirm the exact mutant genotype. In practice, many rabbit embryos resulted from microinjection are mosaic, which means there are more than two peaks on the sequence diagram starting around the targeting site.

10. Thanks to the high KO efficiency of CRISPR/Cas9 tools, it is possible to combine 3–4 KO projects in one embryo transfer experiment to reduce the number of recipients. Divide the embryos into 3–4 groups, and inject with different guide RNAs for each group. Mix the embryos from all the groups for embryo transfer. In this way, one or two of the recipients may deliver all the KO kits you need [38].
11. Female rabbits do not have an estrus cycle and are classified as induced ovulators. A doe is considered to be receptive when she accepts service [39]. In recipient preparation, however, we cannot test its receptivity by mating. The color of the vulva is instead used as a strong indication of receptivity. It is reported that higher success rates were achieved using does with red and pink colored vulva in artificial insemination experiments, compared with that using does with white and purple colored vulva [40].
12. Rabbit ovulation is normally induced by the stimuli associated with coitus and occurs 10–12 h after mating. By mimic the natural mating process, ovulation can be induced artificially by mechanical stimulation of the vagina, alternatively, I.V. injection of hCG could also induce ovulation. In our experience, hCG injection is more reliable than mechanical stimulation.
13. Recipients induced the same day as mating the donors, or one day after mating the donors are both acceptable in rabbit embryo transfer, with similar success rates.
14. Pre-operative fasting in rabbit embryo transfer is not recommended, as the risk of aspiration due to vomiting in rabbit is negligible because rabbits lack the vomiting reflex, and are physically almost incapable of regurgitation. Feeding rabbit before surgery helps the gastrointestinal (GI) tract remain

active, which will speed recovery. Rabbit may suffer liver damage when the GI tract is empty [41].

15. Because rabbits lack the vomiting reflex, intubation is not necessary for anesthesia [42].
16. Rabbit can tolerate restraint, it is not necessary to use injectable anesthetics to induce anesthesia in rabbit.
17. Rabbit embryo will form a thick mucin layer around the zona pellucida during their passage through the oviduct. Without proper mucin layer development, their implantation and subsequent viability are adversely affected [43, 44]. It is desirable to transfer early stage embryos into the recipient oviducts, not uterus, to allow the mucin layer formation.
18. In rabbits, the embryos never move from one uterine horn to the other. Bilateral embryo transfer is necessary to fully utilize the recipients. The average rabbit litter size is 7–8 in natural mating. The litter size in gene editing project may vary depending on both the experiences of microinjectionist and the influence of the target gene. A minimum of two implanted embryos seems to be necessary to carry a pregnancy to term [45]. In our experience, when the litter size is larger than 10, the survival rate of the kits will be reduced greatly.
19. The average gestation period in rabbit is 30–32 days. Newborn kits weigh 30–100 g at birth, are hairless, and have closed eyelids. The doe normally nurses her kits once a day, usually in the morning. Kits open their eyes at about 10–12 days, and begin to eat solid food at about 3 weeks. Rabbit kits are weaned at 4–6 weeks.
20. A video demonstration of production of ApoCIII KO rabbits using Zinc Finger Nucleases is available at https://www.jove.com/video/50957/production-apolipoprotein-c-iii-knock out-rabbits-using-zinc-finger [46].

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