Methods in
Molecular Biology 1630

Springer Protocols

Izuho Hatada *Editor*

Genome Editing in Animals

Methods and Protocols

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Methods and Protocols

Edited by

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ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-4939-7127-5 ISBN 978-1-4939-7128-2 (eBook)
DOI 10.1007/978-1-4939-7128-2

Library of Congress Control Number: 2017943506

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Preface

Conventional gene targeting is widely used to generate knockout mice. However, the conventional method depends on a rare event, spontaneous homologous recombination. This makes it impossible to produce gene-targeted mice directly from fertilized eggs, which in any case are limited in numbers for use in experiments. Consequently, for conventional gene targeting, embryonic stem cells (ESCs) are used instead of eggs because the former can be easily propagated. However, because generation of germline-competent ESCs is extremely difficult, conventional gene targeting is only possible for mice and rats, for which ESCs are available. This problem was solved by the advent of genome editing technologies, which enable specific cleavage of targeted loci, frequently followed by non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ results in a small deletion or insertion at the target locus, whereas HDR can yield a knock-in at the target locus if a donor DNA is provided. There are three genome editing technologies: zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system.

Genome Editing in Animals: Methods and Protocols aims to collect protocols that can be used for the generation of knockout animals. The first three chapters introduce basic protocols for three genome editing technologies that can be applied to all animals. Chapter 4 introduces target design tools that can also be used in all animals. Starting in Chapter 5, specific protocols for each animal are provided: mouse (Chapters 5–8), rat (Chapter 9), rabbit (Chapter 10), pig (Chapter 11), monkey (Chapter 12), chicken (Chapter 13), zebra fish (Chapter 14), medaka (Chapter 15), *Xenopus* (Chapter 16), silkworm (Chapter 17), cricket (Chapter 18), sea squirt (Chapter 19), and *C. elegans* (Chapter 20).

I thank all of the authors for their outstanding contributions to this volume. On behalf of all of us, I hope that our effort to make these methods accessible will prove useful to genome editing aficionados around the world.

Maebashi, Gunma, Japan ***Izuho Hatada***

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Chapter 1

Construction and Evaluation of Zinc Finger Nucleases

Hiroshi Ochiai and Takashi Yamamoto

Abstract

Zinc-finger nucleases (ZFNs) are programmable nucleases that have opened the door to the genome editing era. The construction of ZFNs recognizing a target sequence of interest is laborious, and has not been widely used recently. However, key ZFN patents are expiring over the next 2–4 years, enabling a wide range of deployments for clinical and industrial applications. This article introduces a ZFN construction protocol that uses bacterial one-hybrid (B1H) selection and single-stranded annealing (SSA) assay.

Key words Zinc-finger nuclease, Genome editing, Bacterial one-hybrid system, Single-stranded annealing assay, PCR-based assembly

1 Introduction

Zinc-finger nucleases (ZFNs) are programmable nucleases that have opened the door to the genome editing era [1]. ZFN is an artificial protein consisting of a C_2H_2 zinc-finger array (ZFA) DNA binding domain and the nuclease domain of the FokI restriction enzyme. When a pair of ZFNs binds to DNA in a tail-to-tail configuration, the two FokI nuclease domains dimerize and induce a DNA double-stranded break (DSB) (Fig. 1a). Since a single zinc finger domain can recognize a three-nucleotide sequence, a pair of three-finger (three-domain) nucleases can recognize an 18-nucleotide sequence. This recognition ability means that the pair could recognize one sequence out of 6.8×10^{10} bp, implying the ability to target a unique sequence in a eukaryotic genome. As reviewed elsewhere, DSBs introduced by programmable endonucleases are repaired through non-homologous end joining (NHEJ) or homology-directed repair (HDR). Exploiting these endogenous repair pathways, researchers can edit genomic sequences [2].

The construction method for sequence-specific ZFNs is laborious, and has not been widely used in recent years. ZFN proteins are more compact in size than transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short

Izuho Hatada (ed.), *Genome Editing in Animals: Methods and Protocols*, Methods in Molecular Biology, vol. 1630, DOI 10.1007/978-1-4939-7128-2_1, © Springer Science+Business Media LLC 2017

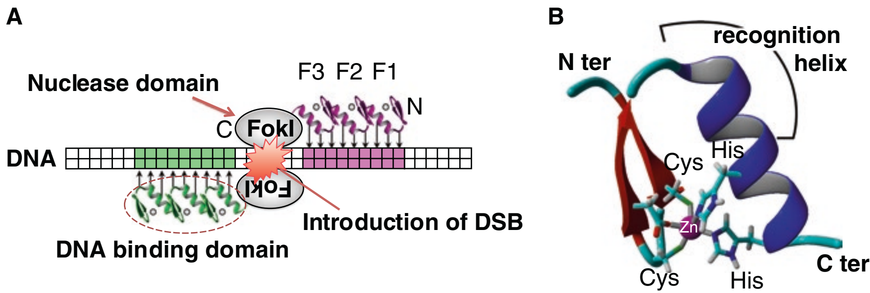

Fig. 1 Introduction of DNA double-stranded break by ZFNs. (**a**) Schematic representation of ZFNs. Each ZF domain is referred to as F1, F2, or F3, depending on its position. (**b**) Schematic representation of a C_2H_2 ZF domain

palindromic repeats (CRISPR) associated protein 9 (Cas9). Furthermore, key ZFN patents are expiring over the next 2–4 years; these expirations may encourage a wide range of deployments for clinical and industrial applications [3]. This article introduces a ZFN construction protocol that uses bacterial one-hybrid (B1H) selection and single-stranded annealing (SSA) assay [4].

The C_2H_2 zinc finger domain is a peptide comprised of ~30 amino acids (aa). It contains two β-strands and one α-helix (Fig. 1b) [5, 6]. It has been well known that amino acids beside α-helix (−1 to +6 aa from the start aa of α-helix) recognize a 3-nucleotide sequence (subsite) (Fig. 1). Therefore, the α-helix is referred to as the recognition helix. To engineer a ZFA to recognize a sequence of interest, ZF libraries with partially randomized recognition helices are constructed (Fig. 2). Next, by using a bacterial one-hybrid system, a ZFA binding specifically to the target sequence is selected (see below).

A randomized library is constructed using a long degenerate base-containing oligo (Random + BbsI-L) and a primer (Random + BbsI-S) (Fig. 3). The degenerate base-containing oligo is converted to double-stranded DNA by primer extension. Next, using appropriate restriction enzymes, three randomized libraries are constructed (Fig. 3). Each library corresponds to one randomized ZF for the F1, F2, or F3 position. Next, the randomized libraries are transformed into US0Δ*hisB*Δ*pyrF*Δ*rpoZ* bacteria containing pH3U3 reporter plasmid (Fig. 4). The US0Δ*hisB*Δ*pyrF*Δ*rpoZ* bacterial strain does not have functional *HisB*, which encodes an enzyme essential for histidine synthesis; the strain cannot survive on a histidine-free medium. Meanwhile, the pH3U3 reporter plasmid contains yeast-derived *His3* with a basal promoter and an upstream zinc-finger target site. The bacteria express the randomized library, producing fusion proteins of RNA-polymerase ω subunit and ZFA with a randomized finger. If a ZFA-RNA polymerase ω subunit binds to the target site, the bacteria express HIS3 and

Fig. 2 Partial randomization of recognition helix. Primary sequence of a ZF domain. *Blue* and *pink letters* represent helix residues participating in zinc coordination and nucleotide recognition, respectively. Residues in the recognition helix are subjected to partial randomization. X is one of all possible amino acid residues except for C, F, Y, or W; this randomization is achieved by using codon VNS

Fig. 3 Construction of randomized ZF library. Schematic representation of randomized ZF library construction

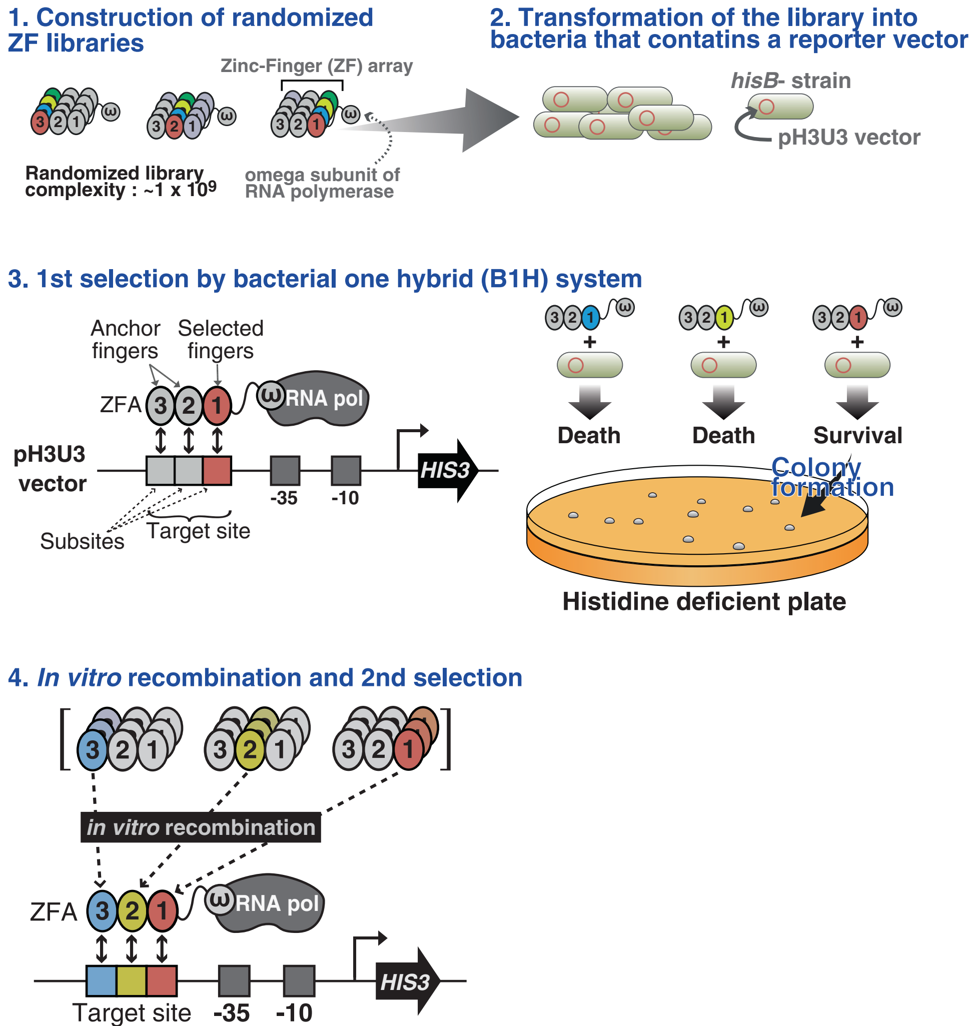

Fig. 4 Directed domain shuffling and bacterial one-hybrid system. Schematic representation of functional ZFA selection using a bacterial one-hybrid system

survive on histidine-free medium (Fig. 4). By using the HIS3-specific inhibitor 3-amino-1,2,4-triazole (3-AT), the selection intensity can be adjusted. In the first selection, three sub-selected ZF libraries are obtained. Using PCR-based assembly, sub-selected ZF libraries are combined. Next, using this new library, a second selection with a higher selection pressure is performed, to obtain a three-finger ZF recognizing the target sequence.

To test the function of ZFNs containing ZFA DNA binding domains selected by B1H, single-stranded annealing (SSA) assays are performed (Fig. 5). At first, ZFN expression and SSA reporter vectors are transfected into HEK293T cells. The reporter plasmid contains ZFN target sites between two split and inactive parts of the luciferase gene, with overlapping repeated sequences. Following

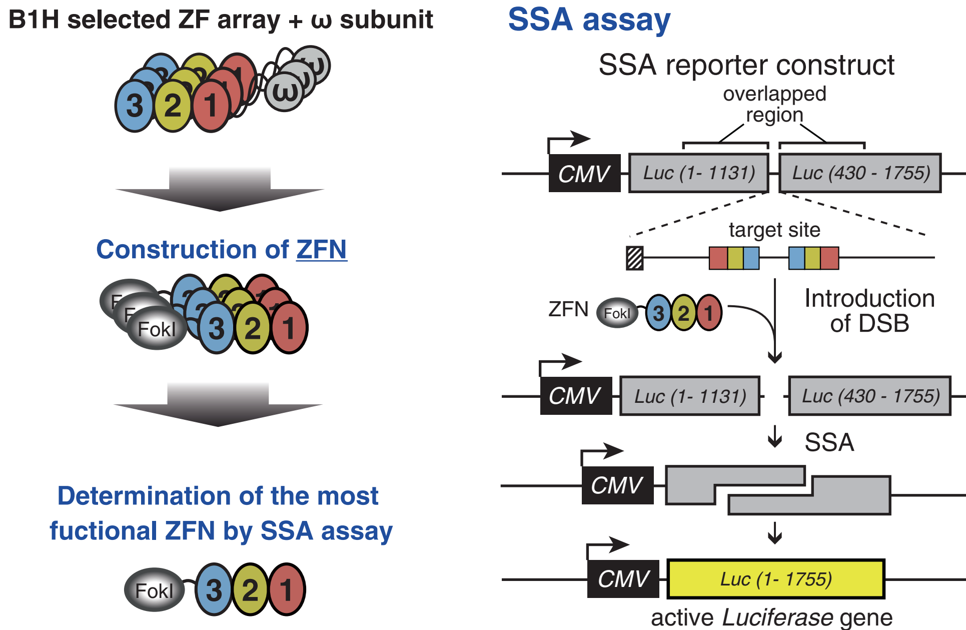

Fig. 5 SSA assay. Schematic representation of SSA assay

a DSB caused by a functional ZFN, a functional luciferase gene is generated by an SSA reaction (Fig. 5). Zinc finger nuclease functionality can therefore be quantified by the luciferase activity.

2 Materials

2.1 Plasmids and Bacterial Strains

1. pB1H2ω2-Z1B [4]: low copy plasmid, ampicillin resistant.
2. pB1H2ω2-Z2B [4]: low copy plasmid, ampicillin resistant.
3. pB1H2ω2-Z3B [4]: low copy plasmid, ampicillin resistant.
4. pH3U3-mcs (Addgene plasmids 12609): low copy plasmid, kanamycin resistant.
5. pB1H2ω2 + BbsI [4]: low copy plasmid, ampicillin resistant.
6. pSTL plasmid [4]: ampicillin resistant.
7. pSTL-ZFA36 [7]: ampicillin resistant.
8. pGL4-SSA [4]: ampicillin resistant.
9. pGL4-SSA-ZFA36 [7]: ampicillin resistant.
10. pRL-CMV (Promega): ampicillin resistant.
11. XL1-Blue Electroporation-Competent Cells (Agilent, 200228).
12. US0Δ*hisB*Δ*pyrF*Δ*rpoZ*: [F′ *lacIqZ* Δ*MI5* *Tn10(Tetr)*] (Addgene plasmids 18049): tetracycline resistant.
13. HEK293T cell.

2.2 Primers

1. Random + BbsI-S: 5′- GCGATCATGATCATGGAAGAC -3′.
2. Random + BbsI-L: 5′- TGGTGCAGCGTACTAGAAGACG TGTGTACGCTGGTGSNBSNBAAGSNBSNBSNBSNB GCTAAAACTCTTTCCACCAGTCTTCCATGATCAT GATCGC -3′.
3. HIS3 rev seq: 5′- GATCGAGTGCTCTATCGCTA -3′.
4. ZF1-forward: 5′- AAGGTCGTGCGGCCGGAAGACAAGC CTTACAAATGCCCAGAA -3′.
5. ZF1-reverse: 5′- CAGGACACTTATAGGGTTTTTCCCCGG TATGTGTA -3′.
6. ZF2-forward: 5′- GAAAAACCCTATAAGTGTCCT -3′.
7. ZF2-reverse: 5′- CCGGGCACTTGTATGGCTTCTCCCCG GTATGTGTA -3′.
8. ZF3-forward: 5′- GAGAAGCCATACAAGTGCCCG -3′.
9. ZF3-reverse: 5′- GACTTGTCGGCCTTGAAGACGTCTCTA GTGTGCAGAGGATCCACGCAGG -3′.
10. pB1H2ω2-seq-F: 5′- GATCCTCGACGTTCGCGAAC -3′.
11. pB1H2ω2-seq-R: 5′- GCTGCGCAACTGTTGGGAAG -3′.
12. T7 promoter primer: 5′- TAATACGACTCACTATAGGG -3′.
13. S418-R: 5′- ATTTCTGGCAATTTCAATTAATTC -3′.

2.3 Enzymes

1. KOD -Plus- (TOYOBO).
2. Restriction enzymes: BbsI, EcoRI, XmaI, XbaI, BsgI, BsaI, KpnI, SacI.
3. T4 DNA ligase.
4. rAPid Alkaline Phosphatase (Roche).

2.4 Instruments and Kits

1. Dialysis membrane.
2. Gene Pulser Electroporator (BIORAD).
3. 1-mm Electroporation cuvette.
4. 150 × 15–mm round culture dish.
5. 1 mm glass beads.
6. Gel Indicator DNA Extraction Kit (Biodynamics).
7. Ultrafiltration filter, Amicon Ultra-0.5, 10k (Millipore).
8. QIAGEN Plasmid Midi Kit (QIAGEN).
9. QIAGEN gel extraction kit (QIAGEN).
10. QIAGEN PCR purification kit (QIAGEN).
11. NuSieve 3:1 agarose (Lonza).
12. NuSieve GTG agarose (Lonza).
13. Agarose.

14. Ligation-Convenience Kit (NIPPON GENE).
15. Dual-Glo luciferase assay system (Promega).
16. 96-well flat-bottom cell culture plate.
17. 96-well solid white flat-bottom polystyrene plate.
18. Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific).

2.5 Medium and Buffers

1. 0.5× TBE.
2. 3 M sodium acetate (pH 5.2).
3. Ethanol.
4. Phenol:Chloroform:Isoamyl Alcohol 25:24:1 saturated with 10 mM Tris, pH 8.0, 1 mM EDTA.
5. Chloroform.
6. Autoclaved double-distilled water (ddH_2O).
7. 10% glycerol.
8. 25 mg/mL ampicillin.
9. 30 mg/mL kanamycin.
10. 5 mg/mL tetracycline in EtOH.
11. 50 mg/mL carbenicillin.
12. Transformation buffer for chemically competent cell preparation: Dissolve 0.6 g of PIPES and 3.72 g KCl into 170 mL ddH_2O. Adjust the solution to pH 6.7 using 5 N KOH. Add ddH_2O up to 190 mL, and autoclave the solution. Dissolve 0.528 g $CaCl_2 \cdot 2H_2O$ and 2.616 g $MnCl_2 \cdot 4H_2O$ into 12 mL ddH_2O. Sterilize the solution using a 0.22 μm filter. Add 10 mL of $CaCl_2$/$MnCl_2$ solution into autoclaved solution. Store at 4 °C.
13. DMSO.
14. 10× annealing buffer [8]: 400 mM Tris-HCl (pH 8), 200 mM $MgCl_2$, 500 mM NaCl.
15. 2× YT: 16 g/L Tryptone, 10 g/L yeast extract, 5 g/L NaCl; adjust pH to 7.0 ± 0.2 (25 °C) with 10 N NaOH (for 500 mL 2× YT medium preparation, ~200 μL of 10 N NaOH is required). Autoclave to sterilize.
16. LB: 10 g/L Bacto-tryptone, 5 g/L yeast extract, 10 g/L NaCl; adjust pH to 7.5 with 10 N NaOH. Sterilize by autoclaving.
17. SOB: 20 g of Bacto-tryptone, 5 g of yeast extract, 0.5 g of NaCl, 2.5 mL of 1 M KCl, ddH_2O to 1000 mL; adjust pH to 7.0 with 10 N NaOH; autoclave to sterilize. Add 2 mL of 1 M $MgCl_2$.
18. SOC: 20 g of Bacto-tryptone, 5 g of yeast extract, 0.5 g of NaCl, 2.5 mL of 1 M KCl, ddH_2O to 1000 mL; adjust pH to

7.0 with 10 N NaOH; autoclave to sterilize. Add 20 mL of sterile 1 M glucose and 2 mL of 1 M $MgCl_2$.

19. NM medium [9]: 1× M9 salts (see below), 1× amino acid mixture containing 17 of the 20 amino acids (see below), 40 mg/mL glucose, 10 μg/mL thiamine, 200 μM uracil (*see* **Note 1**), 200 μM adenine (*see* **Note 1**), 20 μM $ZnSO_4$, 100 μM $CaCl_2$, and 1 mM $MgSO_4$. Filter-sterilize through a 0.22 μm filter (Corning bottle-top vacuum filter system). NM medium can be stored at 4 °C for ~6 months.
20. 20× M9 salts: Dissolve 13.6 g $Na_2HPO_4 \cdot 7H_2O$, 6 g KH_2PO_4, 1 g NaCl, and 2 g NH_4Cl, sequentially, in 90 mL distilled water. Make up the volume to 100 mL with distilled water. Sterilize by autoclaving, and store at room temperature.
21. 33.3× amino acid mixture [9]: Amino acid mixture containing 17 of the 20 amino acids (omitting His, Met and Cys). Prepare the following six solutions (all percentages are wt/vol). Mix equal volumes of all six solutions to get 33.3× amino acid mixture. Filter-sterilize through a 0.22 μm filter (Corning bottle-top vacuum filter system), and store at 4 °C for up to 6 months.

    - Solution I (200×): dissolve 0.99 g Phe (0.99%), 1.1 g Lys (1.1%), and 2.5 g Arg (2.5%) in water (100 mL final volume).
    - Solution II (200×): dissolve 0.2 g Gly (0.2%), 0.7 g Val (0.7%), 0.84 g Ala (0.84%), and 0.41 g Trp (0.41%) in water (100 mL final volume).
    - Solution III (200×): dissolve 0.71 g Thr (0.71%), 8.4 g Ser (8.4%), 4.6 g Pro (4.6%), and 0.96 g Asn (0.96%) in water (100 mL final volume).
    - Solution IV (200×): add 9.1 mL 36.5% HCl to 80 mL water, and subsequently dissolve 1.04 g Asp (free acid, 1.04%) and 14.6 g Gln (14.6%) into the solution (100 mL final volume).
    - Solution V (200×): dissolve 18.7 g potassium-Glu (18.7%) in 80 mL water, subsequently add 0.36 g Tyr (0.36%) and 4 g NaOH pellets, and stir to dissolve (100 mL final volume).
    - Solution VI (200×): dissolve 0.79 g Ile (0.79%) and 0.77 g Leu (0.77%) in water (100 mL final volume).

22. 3-AT selection plate (500 mL) [9]: Autoclave 9 g Bacto agar in 438 mL ddH_2O with a stir bar. Stir agar while cooling to ~55 °C. Combine the solutions in the recipe below in order and add to the agar; 25 mL of 20× M9 salt solution, 10 mL of 20% (wt/vol) glucose, 5 mL of 20 mM adenine, 15 mL of 33.3× amino acid mixture, 5 mL of 20 mM uracil, 0.5 mL of 0.1 M $CaCl_2$, 0.5 mL of 1 M $MgSO_4$, and 0.5 mL of 10 mM $ZnSO_4$. Next, add 50 μL 100 mM IPTG (10 μM final concentration), 5–10 mL

of 1 M 3-AT (depending on the final desired 3-AT concentration), 0.5 mL of 25 mg/mL kanamycin (50 μg/mL final concentration), and 1 mL 50 mg/mL carbenicillin (100 μg/mL final concentration) to the agar while stirring (500 mL final volume). Pour 50 mL into each 150 × 15–mm round culture dish. AT selection plates are good for only ~3 weeks when stored at 4 °C.

23. Opti-MEM I Reduced Serum Medium (Thermo Fisher Scientific).
24. Growth medium: D-MEM with 10% Fetal Bovine Serum (FBS).

3 Methods

3.1 Construction of the Randomized ZFN Library

3.1.1 Primer Extension

1. For primer extension, assemble the following reaction in a PCR tube: 15 μL of 2 mM dNTPs, 15 μL of 10 × Buffer for KOD -Plus-, 60 μL of 50 μM Random + BbsI-S, 20 μL of 50 μM Random + BbsI-L, 6 μL of 25 mM $MgSO_4$, 3 μL of KOD -Plus-, and 31 μL of ddH_2O. Reaction conditions are 95 °C, 5 min, 50 °C, 5 min, and 68 °C, 30 min.
2. Add an appropriate amount of loading dye to the reaction solution. Load all of the solution into ten separate wells of a 3% NuSieve GTG agarose gel, and run the gel at 100 V for 35 min. Stain DNA with suitable DNA staining solution; collect the band of interest. Purify DNA fragments using electroelution, as described below (*see* **Note 2**).

3.1.2 Fragment Purification by Electroelution

1. Place the excised gel in a dialysis membrane tube. Next, add 800 μL 0.5× TBE (or 1× TAE).
2. Close the dialysis membrane tube with clips. Remove the air as much as possible.
3. Put the tube in an electrophoresis tank containing 0.5× TBE or 1× TAE buffer. Electrophorese at 100 V for 40 min to elute DNA fragments.
4. Carefully remove the clip on one side, recover the DNA solution using a pipette, and transfer it to a 1.5 mL tube.
5. Centrifuge the tube at 18,000 × *g*, 22 °C for 5 min to pellet residual agarose gel particles.
6. Aliquot 400 μL of supernatant into each of two 1.5 mL tubes, add 1/10 volume of 3 M sodium acetate, and mix well. Add 800 μL of 100% ethanol to each tube, and mix well. Incubate the tubes on ice for 10 min.
7. Centrifuge at 16,000 × *g*, 4 °C, for 15 min.
8. Discard the supernatant, and carefully wash the DNA pellet with ice-cold 70% ethanol.

9. Dry the pellet in vacuum for 5 min. Dissolve it in 20 μL of ddH_2O, and quantify the DNA concentration. Store at −20 °C.

3.1.3 BbsI Treatment

1. Treat the purified fragment with BbsI by using the following reaction composition: 40 μL of DNA solution, 10 μL of NEBuffer 2.1, 25 U of BbsI, and ddH_2O up to 100 μL.
2. Incubate at 37 °C for 3 h or more (overnight, if possible).
3. Add an appropriate amount of loading buffer to the reaction solution. Next, electrophorese the entire solution in 10 separate lanes of a 3% NuSieve GTG agarose gel. Electrophorese Random + BbsI-L oligo in one lane of the same gel. After electrophoresis and appropriate DNA dye staining, excise the band of interest (47 bp randomized fragment). DNA fragments are purified by electroelution, as above (*see* **Note 2**).
4. Treat plasmid vectors for the insertion of the randomized fragment (pB1H2ω2-Z1B, pB1H2ω2-Z2B, and pB1H2ω2-Z3B) [4] by BbsI as follows. Mix 30–50 μg of plasmid vector, 10 μL of NEBuffer 2.1, 25 U of BbsI, and ddH_2O up to 100 μL. Incubate the mixture at 37 °C for 3 h or more (overnight, if possible).
5. Electrophorese reaction on a 1% agarose gel, and purify backbone fragments using a QIAGEN gel extraction kit. Next, perform ethanol precipitation, and dissolve the precipitate in ddH_2O, to 100 ng/μL. In addition, excise the smaller fragments, and recover them by electroelution (Subheading 3.1.2). The smaller fragments can be used as a positive control at the ligation step.

3.1.4 Small-Scale Ligation

1. Find the optimal concentration ratio of vector and insert in ligation reactions. Prepare four reaction solutions as described by Table 1.
2. Incubate at 16 °C for 4 h or more.
3. After the incubation, the reaction solution of each 1 μL is electroporated into 60 μL of XL1-Blue electro-competent cells in a 1 mm cuvette (1.75 kV, 25 μF, 200 Ω in a Gene Pulser Electroporator). Immediately transfer the electroporated cells to 940 μL of 37 °C pre-warmed SOC, and culture with shaking at 37 °C for 1 h. Prepare sixteen 1.5 mL tubes (4 tubes for each culture), each containing 45 μL of LB medium. After incubation, take 5 μL of cultured medium, transfer to an LB containing tube, and mix well. Next, transfer 5 μL of the diluted culture to another LB-containing tube; mix well. Repeat this procedure for the remaining two tubes. Plate 5 μL each of diluted culture onto 10 cm LB agar plates containing ampicillin. Incubate overnight at 37 °C. Process other samples in the same way.

Table 1
Ligation reaction

	Negative control (μL)	Sample 1 (μL)	Sample 2 (μL)	Sample 3 (μL)
pB1H2ω2-Z(1–3)B (100 ng/μL)	1	1	1	1
randomized fragment (10 ng/μL)	0	0.5	1	2.5
10× ligation buffer	1	1	1	1
T4 DNA ligase	0.2	0.2	0.2	0.2
ddH_2O	7.8	7.3	6.8	5.3

4. On the following day, count the number of colonies. Depending on the dilution ratio (2×10^{-3}, 2×10^{-4}, 2×10^{-5}, or 2×10^{-6}), determine the transformation efficiency. The transformation efficiency of a ligation product containing a randomized fragment should be at least tenfold that of empty vector (negative control). The ideal transformation efficiency of ligation product with insert is 10^4–10^5. If the number of colonies for ligation product with a randomized fragment is about the same as it is for the negative control, then it is possible that the randomized fragment preparation is not going well. In this case, perform a ligation reaction with the positive control fragment prepared in Subheading 3.1.3. If this positive control exhibits an increase in the number of colonies, but the randomized fragment insert does not, the randomized fragment preparation failed. In this case, prepare new randomized fragments.

3.1.5 Large-Scale Ligation

1. At the optimum concentration ratio of vector and insert, perform the ligation on a large scale, as follows: 100 μL of pB1H2ω2-Z(1–3)B (100 ng/μL), appropriate amount of randomized fragment (50 ng/μL), 20 μL of 10× ligation buffer, 10 μL of T4 DNA ligase, and ddH_2O up to 200 μL.
2. Incubate at 16 °C overnight.
3. Add 20 μL of 3 M sodium acetate, and mix well. Next, add 220 μL of Phenol:Chloroform:Isoamyl Alcohol; vortex for 30 s. Centrifuge at 14,000 × *g* at room temperature for 5 min.
4. Carefully collect aqueous phase and transfer to a new 1.5 mL tube. Add 220 μL of chloroform; vortex for 30 s. Centrifuge for 5 min at 14,000 × *g* and room temperature.
5. Repeat **step 4**.

6. Carefully collect aqueous phase and transfer to a new 1.5 mL tube. Add 500 μL of 100% ethanol; mix well. Incubate at −20 °C for at least 20 min. Centrifuge for 10 min at 14,000 × *g* and 4 °C.
7. Discard the supernatant, and carefully wash the DNA pellet with ice-cold 70% ethanol.
8. Dry the pellet in vacuum for 5 min. Dissolve it in 24 μL of ddH_2O. Store at −20 °C.

3.1.6 Electroporation

1. Add 24 μL of the purified ligation product into 1.2 mL of XL1-Blue electrocompetent cells. Gently mix the tube, and put it on ice. Transfer 60 μL of this mixture into an ice-cold electroporation cuvette, and perform electroporation as described above (Subheading 3.1.4). Immediately transfer the electroporated cells into pre-warmed 1 mL SOC. Repeat this procedure 20 times.
2. Transfer all electroporated cells to the 500 mL flask containing 100 mL pre-warmed SOC. Agitate for 1 h at 80 rpm and 37 °C.
3. Using 20 μL of culture, predict the number of total transformants (*see* Subheading 3.1.4).
4. Add 150 mL of 2× YT medium, and ampicillin to a final concentration to 100 μg/mL; agitate for 3 h at 80 rpm and 37 °C. As described above, using 20 μL of culture, predict the number of expanded transformants (*see* Subheading 3.1.4). The number of transformants should proliferate by five to tenfold compared to that before 3 h incubation.
5. Dispense bacterial cultures into two prechilled 50 mL tubes. Centrifuge at 4,000 × *g*, at 4 °C for 15 min. As possible, discard the supernatant.
6. Add 25 mL each of remaining bacterial cultures into individual tubes. Centrifuge at 1,200 × *g*, at 4 °C for 10 min. As possible, discard the supernatant.
7. Add 10 mL of ice-cold 15% glycerol to one of the tubes. Gently pipet to completely suspend the cells. Flash-freeze the tube in liquid nitrogen, and store it at −80 °C.
8. Purify plasmids from the other tube using a QIAGEN Plasmid Midi Kit. Adjust the plasmid concentration to 100 ng/μL in ddH_2O. The obtained plasmid solution can be stored at −20 °C. (Keep in mind that pB1H2ω2 is a low copy-number plasmid. The typical amount of purified plasmid is ~8 μg.)

3.2 First B1H Selection

3.2.1 Target Sequence Determination

1. Determine a target sequence. The target sequence should contain a 6 bp spacer sequence. Each ZF binding subsite should have the motif RNN; that is, the target sequence should satisfy the pattern 5′-NNYNNYNNYNNNNNNRNNRNNRNN-3′ [10].

3.2.2 Construction of pH3U3-Target Vector

1. In the first randomized libraries, nonrandomized ZF domains are derived from ZFA36, whose binding sequence is GAAGATGGTc [4, 11, 12]. Therefore, in an F3 library, the target sequence should satisfy the pattern XXXGATGGTc, where XXX is the target subsite of the final target sequence.
2. A three-finger ZFN contains a ZFA and a nuclease domain at its N and C termini, respectively. Fingers from N terminus are referred to as F1, F2, and F3, respectively. The F3 and F1 ZF domains recognize subsites at the 5′ and 3′ sides, respectively (Figs. 1 and 6).

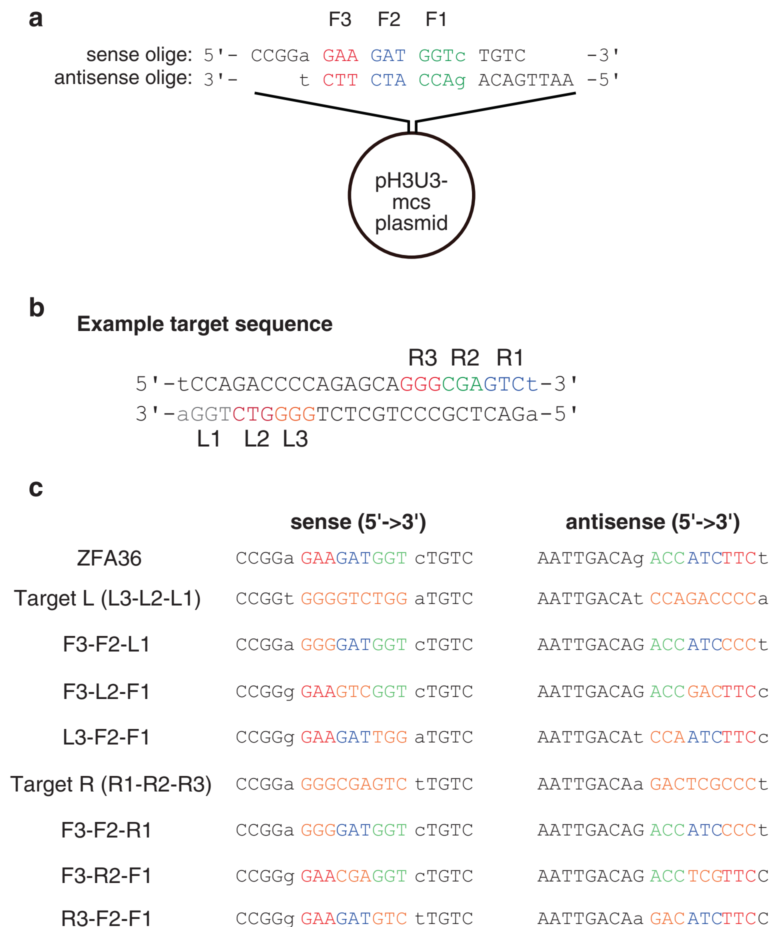

Fig. 6 Construction of pH3U3-target vector. (**a**) Schematic representation of construction of pH3U3-target vector. The vector can be prepared by insertion of annealed oligos into EcoRI/XmaI digested pH3U3-mcs. (**b**) Example target sequence. (**c**) List of oligos for preparation of ZFAs targeting the example sequence shown in (**b**)

3. Individual ZF domains tend to recognize not only their corresponding subsite, but also a nucleotide adjacent to the 3′ side of the subsite [5]. Therefore, the target subsite for F1 ZF should be XXXx (XXX and x are the final target subsite, and the additional 3′ nucleotide, respectively).
4. As described in Fig. 6, prepare oligos containing ZF target sites.
5. Sequentially digest 200 ng of pH3U3-mcs by EcoRI and XmaI. After electrophoresis, excise the band of interest (~5.8 kbp), and purify the DNA using a QIAGEN gel extraction kit. Extract DNA using 30 μL of 0.1× EB buffer. Before centrifugation, incubate the tube at 65 °C for 2 min.
6. Prepare designed oligos from the appropriate manufacturer. Prepare reaction mixture as follows: 1 μL of 10× annealing buffer, 1 μL of B1H sense oligo (50 μM), 1 μL of B1H antisense oligo (50 μM), and 7 μL of ddH_2O.
7. Anneal oligos using the following conditions: 95 °C for 2 min, followed by decreasing the temperature to 25 °C, at a ramp rate of −1 °C/1.16 min.
8. Dilute extracts of pH3U3-mcs XmaI/EcoRI to 1/50. Add 1 μL of annealed oligo solution and 1 μL of diluted plasmid solution to a PCR tube. Next, add 2 μL of Ligation-Convenience Kit; gently mix. Incubate the reaction at 16 °C for 30 min. Next, transform the reaction into chemically competent cells (e.g., XL1-Blue), and plate onto LB plates containing 30 μg/mL kanamycin.
9. On the following day, perform colony PCR. Prepare the following master mix: 5 μL of 10× PCR buffer, 2 μL of 2 mM dNTP, 1 μL of His3-rev primer (10 μM), 1 μL of the sense oligo (10 μM) used in **step 6**, 0.25 μL of Taq polymerase, and 36.5 μL of ddH_2O.
10. Aliquot 6 μL of the master mix into each tube of an 8-strip PCR tube. To each cold PCR tube containing the PCR reaction, add a small amount of colony. Use a fine yellow pipette tip attached to a pipettor (set at 10 μL to avoid adding air to the PCR reaction), transfer the subset of colony by touching a replica plate, and pipetting up and down to mix. The amount of cells should be small; just a touch will do. Sufficient mixing will result in complete cell lysis and high yields. The reaction conditions are: 95 °C for 5 min; 35 cycles of 15 s at 95 °C, 15 s at 55 °C, and 20 s at 72 °C; 20 s at 72 °C; and 4 °C until sample collection. Incubate the replica plate at 37 °C for about 5 h.
11. Electrophorese the PCR reactions in 2% agarose gels. If the ligation was successful, you should detect a ~ 200-bp band.
12. Inoculate a colony with positive results into 3 mL of LB containing 30 μg/mL kanamycin; incubate overnight with agitation.

13. On the following day, purify the plasmids. Plasmids can be stored at −20 °C.
14. To confirm the success rate, we recommend preparing a negative control sample (without insert).

3.2.3 Electrocompetent Cell Preparation

1. To prepare chemically competent US0 strain, thaw glycerol stock US0 stain from deep freezer in an ice bath. Inoculate a small amount of the bacterial solution onto LB plate containing 10 μg/mL tetracycline; incubate at 37 °C overnight.
2. On the following day, inoculate one culture tube of 3 mL of LB containing 10 μg/mL tetracycline with 2–3 colonies from the plate, and incubate at 37 °C with agitation overnight.
3. Prepare 200 mL of SOB without antibiotics in a 500 mL flask. Aliquot 500 μL of SOB medium from the flask into a 1.5 mL tube, for use when measuring bacterial turbidity. Add 3 mL of the overnight culture to the 500 mL flask of SOB medium, and incubate at 18 °C with agitation, until OD_{600} reaches 0.6. (It will take ~24 h to reach this OD, depending on the bacterial strain. The turbidity should be checked repeatedly.)
4. Incubate on ice bath for 20 min.
5. Transfer bacterial solution into four ice-cold 50 mL tubes. Centrifuge at 1,200 × *g*, at 4 °C for 10 min. Discard the supernatant.
6. Add 16 mL each of ice-cold Transformation Buffer into individual tubes. Gently pipet to completely suspend the cells. Incubate on ice for 10 min.
7. Centrifuge at 1,200 × *g*, at 4 °C for 10 min. Discard the supernatant.
8. Add 4 mL of ice-cold Transformation Buffer to each tube. Gently pipet to completely suspend the cells.
9. Add 300 μL of DMSO to each tube. Because of the increment in temperature, gently mix well. Incubate 10 min on ice.
10. Aliquot 100 μL of each in to PCR tubes. Flash-freeze in liquid nitrogen; store at −80 °C.
11. Transform constructed pH3U3-target vector into a chemically competent US0 strain. Plate on LB plate with 30 μg/mL kanamycin. On the following day, inoculate a colony into 3 mL LB medium containing 30 μg/mL kanamycin; incubate at 37 °C with agitation (*see* **Note 3**).
12. Prepare a 200 mL flask containing 50 mL of 2× YT medium and 30 μg/mL kanamycin. Aliquot 500 μL of 2× YT medium from the flask into a 1.5 mL tube, for use when measuring bacterial turbidity. Transfer 1 mL of bacterial medium into the 200 mL flask containing the 2× YT medium. Culture at 37 °C with agitation, until the OD_{600} reaches 0.4 (~2–3 h).

13. Transfer the culture to four 50 mL tubes; cool on ice for 20 min. For homogenous cooling, gently shake at 5 min intervals. Take care to keep the cells close to 0 °C.
14. Centrifuge for 10 min at 1,200 × *g* and 4 °C. Discard the supernatant.
15. Add 15 mL of ice-cold ddH_2O into each tube. Gently pipet to completely suspend the cells. Add ice-cold ddH_2O to individual tubes up to 50 mL. Centrifuge for 10 min at 1,200 × *g* and 4 °C. Discard the supernatant.
16. Add 15 mL of ice-cold 10% glycerol into each tube. Gently pipet to completely suspend the cells. Add ice-cold 10% glycerol to individual tubes up to 50 mL. Centrifuge at 2,500 × *g*, at 4 °C for 10 min. Discard the supernatant.
17. Add 1 mL of ice-cold 10% glycerol into each tube. Gently pipet to completely suspend the cells. Transfer the cell suspension to a precooled 1.5 mL tube.
18. Centrifuge for 30 s at 16,000 × *g* and 4 °C. As possible, discard the supernatant.
19. Add 60 μL of ice-cold 10% glycerol. Gently pipet to completely suspend the cells.
20. Flash-freeze the tube in liquid nitrogen, and store at −80 °C.

3.2.4 Electroporation

1. Thaw appropriate electrocompetent cells containing pH3U3-target vector in an ice bath. Add 1 μL of 100 ng/μL appropriate randomized library, and mix gently. Put the tube on ice for 1 min. Transfer the cells into an ice-cold 1 mm gap electroporation cuvette; perform electroporation. Recover the cells in 1 mL SOC, as described above. Incubate at 37 °C with agitation for 1 h.
2. Centrifuge the cells for 10 min at 3,500 × *g* and 22 °C. Discard the supernatant.
3. Add 1 mL of NM medium containing 100 μg/mL carbenicillin and 30 μg/mL kanamycin. Gently pipet to completely suspend the cells. Incubate at 37 °C for 1 h with agitation.
4. Centrifuge for 30 s at 14,000 × *g* and 22 °C. Discard the supernatant.
5. Add 1 mL of ddH_2O into those tubes. Gently pipet to completely suspend the cells.
6. Centrifuge for 2 min at 14,000 × *g* and 22 °C. Discard the supernatant.
7. Add 1 mL of NM medium into those tubes. Gently pipet to completely suspend the cells.
8. Centrifuge for 30 s at 14,000 × *g* and 22 °C. Discard supernatant.
9. Add 1 mL of NM medium to those tubes. Gently pipet to completely suspend the cells.

3.2.5 Plating to Large Plate

1. Using 5 μL of culture, predict the number of total transformants (*see* Subheading 3.1.4).
2. Add ~30 sterile glass beads to 3-AT selection plate (10 mM 3-AT, room temperature) and remaining bacterial solution; homogenously spread the bacteria.
3. Let the surface of the plate dry. After drying, close the lid and invert the plate. Tap the plate and let the beads drop on the lid. To prevent overdrying, seal the plate. Incubate at 37 °C for 1–3 days. Usually, 100–10,000 colonies can be obtained. Stop culturing before individual colonies merge with each other.

3.2.6 Plasmid Purification

1. Invert the plate and let the beads drop on the agarose surface.
2. Add 10 mL of 2× YT medium to the plate, and tilt to coat the surface. Wait for 2–3 min.
3. Tap the side of the plate, and suspend the cell colonies using the glass beads. Collect the cell suspension into a 15 mL tube.
4. Centrifuge for 40 s at 14,000 × *g* and 4 °C. Discard the supernatant.
5. Purify plasmids using a QIAGEN Plasmid Midi Kit.
6. Store purified plasmids at −20 °C.
7. These plasmid pools, obtained from the first selection (sublibrary), are used to construct the second selection library. These sublibraries can be used to construct other libraries.

3.3 PCR-Based in Vitro Recombination

3.3.1 Amplification of Individual ZFs

1. Amplify individual finger domains from each sublibrary, using PCR. Each finger position has a finger-specific sequence [4].
2. Amplify randomized ZF domains using the following primer set. For ZF1 amplification: ZF1-forward and ZF1-reverse; for ZF2 amplification: ZF2-forward and ZF2-reverse; for ZF3 amplification: ZF3-forward and ZF3-reverse.
3. Prepare a PCR reaction by mixing: 6.8 μL of ddH_2O, 1 μL of Buffer for KOD -Plus-, 1 μL of 2 mM dNTP mix, 0.4 μL of 25 mM $MgSO_4$, 0.2 μL each of primers (50 μM), 0.2 μL of sublibrary plasmid, and 0.2 μL of KOD –Plus-. The reaction conditions are: 94 °C for 2 min; 28 cycles of 20 s at 94 °C, 20 s at 60 °C, and 20 s at 68 °C; 20 s at 68 °C; and 4 °C until sample collection.
4. Electrophorese 5 μL of PCR reactions in 2.5% NuSieve 3:1 agarose. Excise bands of interest (ZF1: 124 bp, ZF2: 106 bp, ZF3: 126 bp). Purify DNA fragments using a QIAGEN Plasmid Midi Kit (*see* **Note 2**).

3.3.2 In Vitro Recombination

1. Construct three finger ZFs using extracted ZFs with PCR. Prepare PCR reaction master mix using: 128 μL of ddH_2O, 20 μL of Buffer for KOD -Plus-, 20 μL of 2 mM

dNTP mix, 8 μL of 25 mM $MgSO_4$, 4 μL each of primers (50 μM), 4 μL each of gel-extracted DNA solutions, and 4 μL of KOD -Plus-. Aliquot 50 μL of reaction mix into four PCR tubes. Perform PCR under the following conditions: 94 °C for 2 min; 30 cycles of 20 s at 94 °C, 1 min at 65 °C, and 1.5 min at 68 °C; 2 min at 68 °C; and 4 °C until collection of sample.

2. After PCR reaction, combine PCR products into a 1.5 mL tube. Add 20 μL of 3 M sodium acetate and 500 μL of 100% ethanol. Incubate at −80 °C for 20 min. Centrifuge at 14,000 × *g* at 4 °C for 10 min. Discard the supernatant. Add 200 μL of 70% ethanol and centrifuge at 14,000 × *g* at 4 °C for 5 min. Vacuum dry for 5 min. Dissolve in 10 μL of TE buffer, and add 2 μL of 6× Loading dye (Biodynamics).
3. Electrophorese the sample in 0.8% agarose gel, containing Gel Indicator.
4. Excise bands with intended size (~380 bp). Purify DNA fragments using a QIAGEN gel extraction kit. Extract DNA solution using 30 μL of 1/10 diluted EB buffer.
5. Digest the eluted DNA fragments with BbsI, using ~30 μL extracted DNA, 10 μL of NEBuffer 2.1, 25 U of BbsI, and ddH_2O added to 100 μL.
6. Add 10 μL of 3 M sodium acetate and 250 μL of 100% ethanol. Incubate at −80 °C for 20 min. Centrifuge for 10 min at 14,000 × *g* and 4 °C. Discard the supernatant. Add 200 μL of 70% ethanol and centrifuge for 5 min at 14,000 × *g* and 4 °C. Vacuum dry for 5 min. Dissolve in 10 μL of TE buffer, and add 2 μL of 6× Loading dye (Biodynamics).
7. Electrophorese the sample in 0.8% agarose gel, containing Gel Indicator. At this time, the DNA of interest is less abundant than in previous samples. If the band of interest is too obscure to excise, restain the gel using another DNA staining reagent (*see* **Note 2**).
8. Purify DNA fragment using QIAGEN gel extraction kit. Extract DNA using 100 μL of EB buffer.
9. Add 10 μL of 3 M sodium acetate and 250 μL of 100% ethanol, and mix well. Incubate at −20 °C for 20 min. Centrifuge for 10 min at 14,000 × *g* and 4 °C. Discard the supernatant. Add 70% ethanol and centrifuge for 5 min at 14,000 × *g* and 4 °C. Discard the supernatant and vacuum dry for 5 min. Dissolve in 8.5 μL of ddH_2O.

3.3.3 Ligation

1. Assemble a ligation reaction, using: 2 μL of 10× ligation buffer, 1 μL of T4 DNA ligase, 1 μL of BbsI treated fragment, and 8.5 μL of pB1H2ω2 + BbsI (BbsI treated and gel-purified, 100 ng/μL).

2. Incubate at 16 °C overnight.
3. On the following day, add 80 μL of ddH_2O and 100 μL of Phenol:Chloroform:Isoamyl Alcohol, and vigorously vortex for 30 s. Centrifuge at RT for 5 min. Transfer aqueous phase to a new 1.5 mL tube. Add 100 μL of chloroform, and vigorously vortex for 30 s. Centrifuge at RT for 5 min. Transfer aqueous phase to an ultrafilter (Amicon Ultra-0.5, 10 k, Millipore), and add 400 μL of ddH_2O to fill the container.
4. Centrifuge for 10 min at 22,000 × *g* and RT. Discard filtered liquid. Add 400 μL of ddH_2O to the filter container. Centrifuge at 22,000 × *g* at RT, until ~20 μL of unfiltered liquid remains. Store unfiltered liquid (DNA solution) at −20 °C.

3.4 Second B1H Selection

3.4.1 Construction of pH3U3-Target Vector

1. Prepare pH3U3 vector for the second selection, as described before (*see* Subheading 3.2.2).

3.4.2 Preparation of Electrocompetent US0 (pH3U3) Cells

1. Prepare electrocompetent US0Δ*hisB*Δ*pyrF*Δ*rpoZ* transformed with appropriate pH3U3 vector (*see* Subheading 3.2.3).

3.4.3 Electroporation and Plating to Large Plates

1. Perform electroporation as described in Subheadings 3.2.3–3.2.5. However, use ultrafiltered 5 μL DNA solution for electroporation. Furthermore, adjust the 3-AT concentration to 20 mM for large plates.
2. Usually, transformation efficiency is 10^5–10^6.
3. Culture the plate at 37 °C for 1–3 days until colonies grow large enough to pick. If the number of colonies is lower than 10, the chance of obtaining functional ZFNs is quite low.

3.4.4 Colony PCR

1. Perform colony PCR. Prepare a master mix, using: 42.35 μL of ddH_2O, 5 μL of 10× PCR buffer, 2 μL of 2 mM dNTP, 0.2 μL of pB1H2ω2-seq-F (50 μM), 0.2 μL of pB1H2ω2-seq-R (50 μM), and 0.25 μL of Taq polymerase.
2. Aliquot 6 μL of the reaction mixture into each tube of an 8-strip PCR tube. To each cold PCR tube containing the PCR reaction, add a small amount of colony. To perform these inoculations, use a fine yellow pipette tip attached to a pipettor (set at 10 μL to avoid addition of air into the PCR reaction); transfer the colony subset by touching a replica plate, and pipetting up and down to mix. The amount of cells should be small; just a touch will do. Sufficient mixing will result in complete cell lysis and high yields. Pick colonies in descending order of size. Perform PCR under the following conditions: 95 °C for 5 min;

30 cycles of 15 s at 95 °C, 15 s at 60 °C, and 30 s at 72 °C; 20 s at 72 °C; and 4 °C until collection of sample.

3. Digest unpurified PCR product by BsgI and XbaI, using: 5.8 μL of ddH_2O, 1 μL of CutSmart buffer, 1 μL of 1/40 diluted SAM (supplied with BsgI from NEB), 2 μL of PCR reaction, 0.1 μL of XbaI, and 0.1 μL of BsgI.
4. Incubate at 37 °C for 3 h. Electrophorese in 2.5% NuSieve 3:1 agarose gel. Excise about 256 bp bands.
5. Digest pSTL plasmid by BsgI, using 13.6 μL of ddH_2O, 2 μL of CutSmart buffer, 2 μL of 1/40 diluted SAM (supplied with BsgI from NEB), 4 μL of pSLT (200 ng/μL), and 0.4 μL of BsgI.
6. Incubate at 37 °C overnight. Purify DNA fragments using the QIAGEN PCR purification kit. Elute DNA using 30 μL of 0.1× EB buffer. Before centrifugation, incubate the tube at 65 °C for 2 min.
7. Digest purified pSTL plasmid by XbaI, using 2 μL of CutSmart buffer, 17.6 μL of purified pSLT solution, and 0.4 μL of BsgI.
8. Incubate at 37 °C at least for 3 h. Next, add 1 μL of rAPid Alkaline Phosphatase, and mix well. Incubate at 37 °C at least for 30 min.
9. Electrophorese in 1% agarose gel. Excise about 5.6 kbp bands.
10. Purify the DNA fragments using QIAGEN gel extraction kit. Extract DNA using 30 μL of 0.1× EB buffer. Before centrifugation, incubate the tube at 65 °C for 2 min.
11. Add 1 μL of ZFA fragment solution and 1 μL of pSTL plasmid solution into a PCR tube. Add 2 μL of Ligation-Convenience Kit, and gently mix. Incubate the reaction at 16 °C for 30 min. Next, transform the reaction into chemically competent cells (e.g., XL1-Blue), and plate them onto an LB plate containing 100 μg/mL ampicillin.
12. On the following day, perform colony PCR (*see* Subheading 3.4.4). Use T7 promoter primer and S418-R primer.
13. Electrophorese the PCR reactions in 2% agarose gels. If ligation was successful, you should detect a 479-bp band.
14. Inoculate a colony with positive results into 3 mL LB containing 100 μg/mL ampicillin; incubate overnight with agitation.
15. On the next day, purify transfection grade plasmids using an appropriate kit.
16. Add 10 μL of 3 M sodium acetate (per 100 μL starting material), and mix well. Next, add 110 μL of Phenol:Chloroform:Isoamyl Alcohol and vortex for 30 s. Centrifuge 14,000 × *g* at room temperature for 5 min.
17. Carefully collect aqueous phase and transfer to a new 1.5 mL tube. Add 110 μL of chloroform and vortex for 30 s. Centrifuge at 14,000 × *g* at room temperature for 5 min.

18. Repeat **step 17**.
19. Carefully transfer the aqueous phase to a new 1.5 mL tube. Add 250 μL of 100% ethanol and mix well. Incubate at −20 °C for more than 20 min. Centrifuge at 14,000 × *g*, at 4 °C for 10 min.
20. Discard the supernatant, and carefully wash the DNA pellet with ice-cold 70% ethanol.
21. Dry the pellet in vacuum for 5 min. Dissolve it in 20 μL of ddH_2O. Adjust the concentration to 300 ng/μL. Plasmids can be stored at −20 °C.

3.5 SSA Assay

3.5.1 Construction of pGL4-SSA

1. Prepare oligos containing target sequence, as described in Fig. 7.
2. Digest purified pGL4-SSA plasmid by BsaI, using 1 μL of CutSmart buffer, 2 μL of pGL4-SSA (200 ng/μL), and 0.4 μL of BsaI.
3. Incubate at 37 °C at least for 3 h. Next, add 1 μL of rAPid Alkaline Phosphatase, and mix well. Incubate at 37 °C, for at least 30 min.
4. Electrophorese in 1% agarose gel. Excise about 5.6 kbp bands.
5. Purify the DNA fragments using QIAGEN gel extraction kit. Extract DNA using 30 μL of 0.1× EB buffer. Before centrifugation, incubate the tube at 65 °C for 2 min.
6. Prepare reaction mixture, using 1 μL of 10× annealing buffer, 1 μL each of oligos prepared in **step 1** (50 μM), and 7 μL of ddH_2O.
7. Anneal oligos using the following conditions: 95 °C for 2 min, followed by decreasing temperature to 25 °C at a ramp rate of −1 °C/1.16 min.

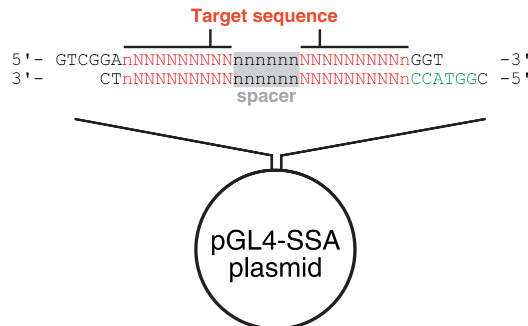

Fig. 7 Construction of pGL4-SSA-target vector. Schematic representation of construction of pGL4-SSA-target vector. Prepare appropriate oligos containing ZFN target sites, and additional adaptor sequence. *Green letters* represent KpnI site that is generated only after insertion of annealed oligos

8. Add 1 μL of annealed oligo solution and 1 μL of purified plasmid solution to a PCR tube. Add 2 μL of Ligation-Convenience Kit, and gently mix. Incubate the reaction at 16 °C for 30 min. Transform the reaction into chemically competent cells (e.g., XL1-Blue), and plate onto an LB plate containing 100 μg/mL ampicillin.
9. On the following day, inoculate 2–3 colonies into separate tubes with 3 mL LB medium containing 100 μg/mL ampicillin; incubate overnight with agitation.
10. On the following day, purify the plasmids using an appropriate kit. Digest purified plasmids by KpnI as follows: 1 μL of CutSmart buffer, 2 μL of plasmid (200 ng/μL), and 0.4 μL of KpnI.
11. Incubate at 37 °C at least for 3 h. Next, electrophorese all reactions on a 1% agarose gel. After electrophoresis and appropriate DNA dye staining, check the DNA fragment size. If appropriately constructed, 3,800 bp and 1,800 bp bands should be visible. Plasmids can be stored at −20 °C.

3.5.2 Luc Assay

1. Culture HEK293T cells in growth medium containing 10% FBS.
2. Prepare the mixture described by Table 2 in wells of a 96-well plate.
3. Dilute 0.7 μL of Lipofectamine LTX with 25 μL of Opti-MEM (prepare master mix); add this mixture to the DNA-Opti-MEM containing wells within 5 min. Incubate at room temperature for 30 min.
4. Prepare HEK293T cell suspension at 5×10^4 cells/100 μL in growth medium by standard trypsinization protocol. Next, add 100 μL of cell suspension to DNA-Lipofectamine mixture-containing wells. Mix by pipetting.

Table 2
Mixture of reagents for SSA assay

	Negative control (μL)	Positive control (μL)	Sample (μL)
pSTL-ZFN (300 ng/μL)	–	–	0.66
pSTL-ZFA36 (300 ng/μL)	–	0.66	–
pGL4-SSA-ZFN target (150 ng/μL)	0.66	0.66	0.66
pRL-CMV (30 ng/μL)	0.66	0.66	0.66
Opti-MEM	23	23	23

5. Incubate for 24 h at 5% CO_2 and 37 °C.
6. Discard 75 μL each of medium from cultures.
7. Add 75 μL of Dual-Glo Luciferase Reagent (Dual-Glo luciferase assay system; Promega). Incubate at room temperature for 10 min. Transfer all samples to a white 96-well plate.
8. Quantify luciferase activities using a plate reader.
9. Add 75 μL of Dual-Glo Stop & Glo Reagent (Dual-Glo luciferase assay system; Promega). Incubate at room temperature for 10 min.
10. Quantify luciferase activities using the plate reader.

4 Notes

1. Although 20 mM uracil and adenine stock solutions can be prepared, precipitate might appear when they are stored at 4 °C. These precipitates can be removed by incubation in a hot bath. Preparation of medium using 20 mM uracil and/or adenine stocks with precipitates will not work appropriately.
2. If gels are stained with DNA-binding fluorescent dye, dye excitation should be minimized as much as possible.
3. It is important that all bacteria possess pH3U3 vector. Therefore, avoid overgrowth by transferring the culture plate to room temperature until inoculating a starter culture. Do not store the culture plate at 4 °C.

Acknowledgment

We thank Dr. Scot Wolfe for providing the pH3U3-mcs reporter vector and US0*ΔhisBΔpyrFΔrpoZ* bacterial strain (Addgene plasmids 12609 and 18049, respectively). This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (15 K18467,16H01407) and by the JST PRESTO program to H.O.

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Chapter 2

Current Overview of TALEN Construction Systems

Tetsushi Sakuma and Takashi Yamamoto

Abstract

Transcription activator-like effector (TALE) nuclease (TALEN) is the second-generation genome editing tool consisting of TALE protein containing customizable DNA-binding repeats and nuclease domain of FokI enzyme. Each DNA-binding repeat recognizes one base of double-strand DNA, and functional TALEN can be created by a simple modular assembly of these repeats. To easily and efficiently assemble the highly repetitive DNA-binding repeat arrays, various construction systems such as Golden Gate assembly, serial ligation, and ligation-independent cloning have been reported. In this chapter, we summarize the current situation of these systems and publically available reagents and protocols, enabling optimal selection of best suited systems for every researcher who wants to utilize TALENs in various research fields.

Key words TALEN, Golden Gate assembly, Pre-assembled library, Serial ligation, Ligation-independent cloning

1 Introduction

The rapid emergence of clustered regularly short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) caused a paradigm shift in genome editing study [1]; however, TALEN is still an important genetic tool for functional genomics study, because various characteristics of these two tools differ from each other [2]. For example, CRISPR-Cas9 normally works as a monomer nuclease, while TALEN works as a dimer. CRISPR-Cas9 produces blunt ends, while TALEN produces cohesive ends. CRISPR-Cas9 consists of a complex of protein and RNA, while TALEN consists of protein only. These differences enable complementary usage of these two technologies.

Functional TALEN protein can be created by the assembly of DNA-binding repeats, in which the specificity of base recognition is defined by repeat-variable di-residue (RVD) [3, 4], into the backbone of TALEN harboring N- and C-terminal domains. Each single repeat consists of 34 amino acids, where the 12th and 13th residues are called RVD. Amino acid composition other than RVD among repeats is basically the same, but some non-RVD variations

Izuho Hatada (ed.), *Genome Editing in Animals: Methods and Protocols*, Methods in Molecular Biology, vol. 1630,
DOI 10.1007/978-1-4939-7128-2_2, © Springer Science+Business Media LLC 2017

on particular positions such as 4th and 32nd residues have been determined to be important for TALEN activity [5]. The lengths and amino acid sequence of N- and C-terminal domains of TALE are also identified as important factors for the activity and specificity of TALENs [6–8]. In addition, other contexts such as promoter, UTRs, poly-A signal sequence, and codon usage of TALEN-coding sequence are also important for the appropriate use in various cells and organisms. Therefore, proper choice of TALEN structure and vector backbone is needed in terms of the best practical application as well as the selection of construction systems described below.

Typically, 15–20 repeats have to be assembled in a defined order to construct functional TALENs. To achieve this, various one- or two-step modular assembly methods using single or multiple repeat libraries have been developed, improved, and applied by many research groups. In this chapter, we describe an overview of the current construction systems of TALEN, providing brief introduction of each system and detailed information of publically available plasmid kits and protocols.

2 Summary of TALEN Construction Kits

Generally, the custom-made TALEN is created using a set of plasmids packaged for its construction. Such plasmid kits can be obtained from Addgene, the nonprofit plasmid repository [9]. Table 1 summarizes a current list of TALEN kits available from Addgene. There are various differences among these kits, including the method of modular assembly, the number of plasmids, and framework of TALEN scaffold. Regarding the construction method, a one-pot cloning strategy called Golden Gate assembly is most widely used. This method enables the simultaneous digestion and ligation of multiple plasmids in a single tube with high efficiency and accuracy, without requiring any special equipment other than a standard thermal cycler. Thus, the current TALEN kits are mostly based on this method. Other methods, however, are adopted in some kits, and they have unique characteristics that might be advantageous for a particular use. The Joung Lab TAL Effector Engineering Reagents are for the simple restriction digestion and ligation of modules in a serial manner, which can commonly be utilized for a low−/middle−/high-throughput generation of custom TALENs. The LIC TAL Effector Assembly Kit is based on a ligation-independent cloning (LIC) method, which does not require a recombinant ligase, but depends on the annealing of relatively long overhangs. The following sections are the detailed information of these methods and systems to provide the best understanding.

Table 1
Summary of TALEN kits available from Addgene

Kit name	Kit ID	Depositor	Assembly method	Number of plasmids	Repeat number
Golden Gate TALEN and TAL Effector Kit 2.0	1,000,000,024	Daniel Voytas and Adam Bogdanove	Golden Gate	86	12–31
Platinum Gate TALEN Kit	1,000,000,043	Takashi Yamamoto	Golden Gate	35	6–21
TALE Toolbox	1,000,000,019	Feng Zhang	PCR/Golden Gate	12	13, 19, 25
Musunuru/Cowan Lab TALEN Kit	1,000,000,034	Kiran Musunuru and Chad Cowan	Golden Gate	834	15
Ekker Lab TALEN Kit	1,000,000,038	Stephen Ekker	Golden Gate	256	15
FusX TALEN assembly system	1,000,000,063	Stephen Ekker	Golden Gate	336	15, 16
REAL Assembly TALEN kit	1,000,000,017	Keith Joung	Serial ligation	32	Any number
LIC TAL Effector Assembly Kit	1,000,000,023	Veit Hornung	Ligation-independent cloning	76	10–19

3 Golden Gate Assembly-Based Systems

3.1 Introduction of Golden Gate Assembly

Golden Gate assembly was first reported in 2008 [10], and soon after the initial publication, the high capacity of simultaneous assembly of many fragments (nine inserts in the acceptor vector) was shown [11]. In this assembly method, type IIS restriction enzymes such as BsaI and BsmBI are used to generate various patterns of cohesive ends at the same time by using a single enzyme, enabling simultaneous ligation of multiple modules in a defined order (Fig. 1). The recognition sequence of the enzyme on the insert plasmid is placed outside the module sequence, while that on the acceptor vector is placed inside the vector backbone sequence. It results in not only a seamless cloning, but also a prevention of re-cutting the assembled products. In addition, a lacZ cassette for the blue/white selection in the cut-out region of the acceptor vector and different antibiotic selection marker on the insert vectors can reduce the false-positive clones. Thus, the Golden Gate assembly method is a very sophisticated protocol for tandem

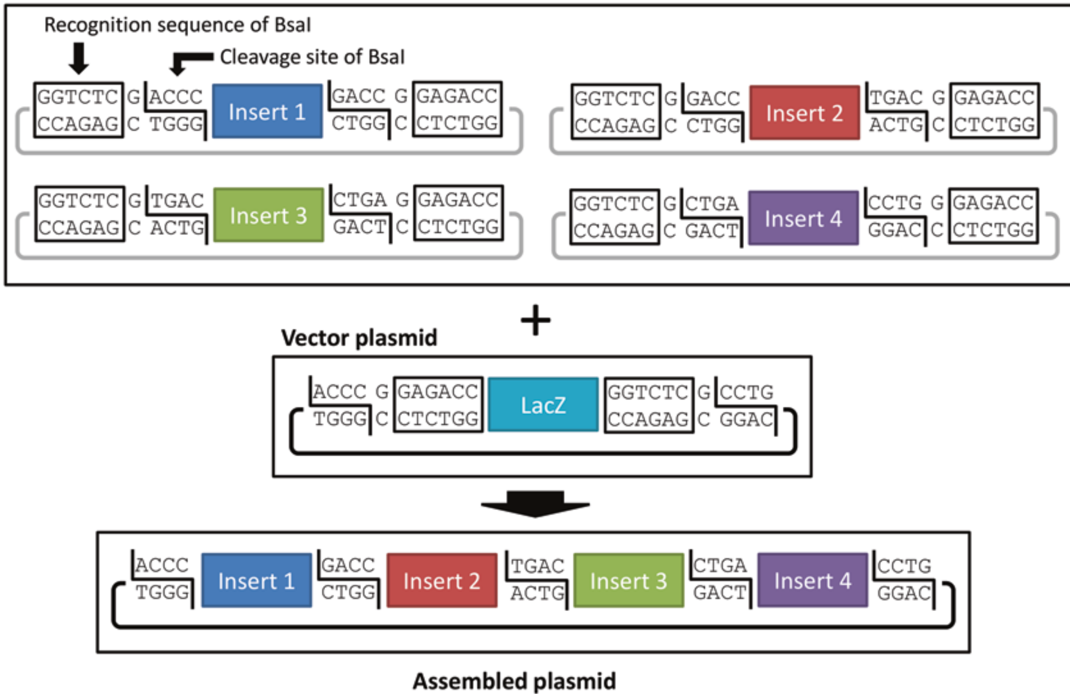

Fig. 1 Example of Golden Gate assembly. The assembly of four inserts mediated by BsaI enzyme is shown. The four inserts are tandemly ligated into the vector plasmid without carrying BsaI recognition sequences

ligation of multiple modules, which is quite suited for the TALE repeat assembly.

3.2 Typical Protocol for the Golden Gate Assembly-Based TALEN Construction

1. Prepare all the plasmids needed and design TALEN target sequences.
2. Mix the insert and vector plasmids with a restriction enzyme and a DNA ligase in a single PCR tube, and perform 1st-step Golden Gate assembly in a thermal cycler. Typically, up to ten modules are tandemly assembled in one intermediate array vector.
3. Perform bacterial transformation with blue/white selection. Screen the intended clones by colony PCR. Culture the clones and extract the plasmid DNA.
4. Perform second-step Golden Gate assembly, transformation, colony screening, and plasmid preparation in a similar way. All the procedures can be conducted in 5 days [12, 13].

3.3 Original Golden Gate Kit from Voytas/Bogdanove Lab

The first TALEN kit appeared in Addgene is the Golden Gate TALEN and TAL Effector Kit, deposited by Daniel Voytas and Adam Bogdanove [12]. Currently, the version 2.0 of the kit is available (Kit #1000000024). The kit contains 86 plasmids,

including 60 module vectors, 5 last repeat vectors, 13 intermediate array vectors, and 6 destination vectors to create TALEN vectors containing 12–31 DNA-binding repeats with no non-RVD variations (Fig. 2a). The functionality of the TALEN vector created using this kit has been widely validated in many cells and organisms; however, the vector often cannot be used directly and needs additional plasmids, because the original kit only contains yeast expression vectors as destination vectors. In addition, the reaction of 10-module assembly is relatively difficult and not always successful. In this regard, the add-on plasmids compatible with the Golden Gate kit have been created and deposited in Addgene by many groups.

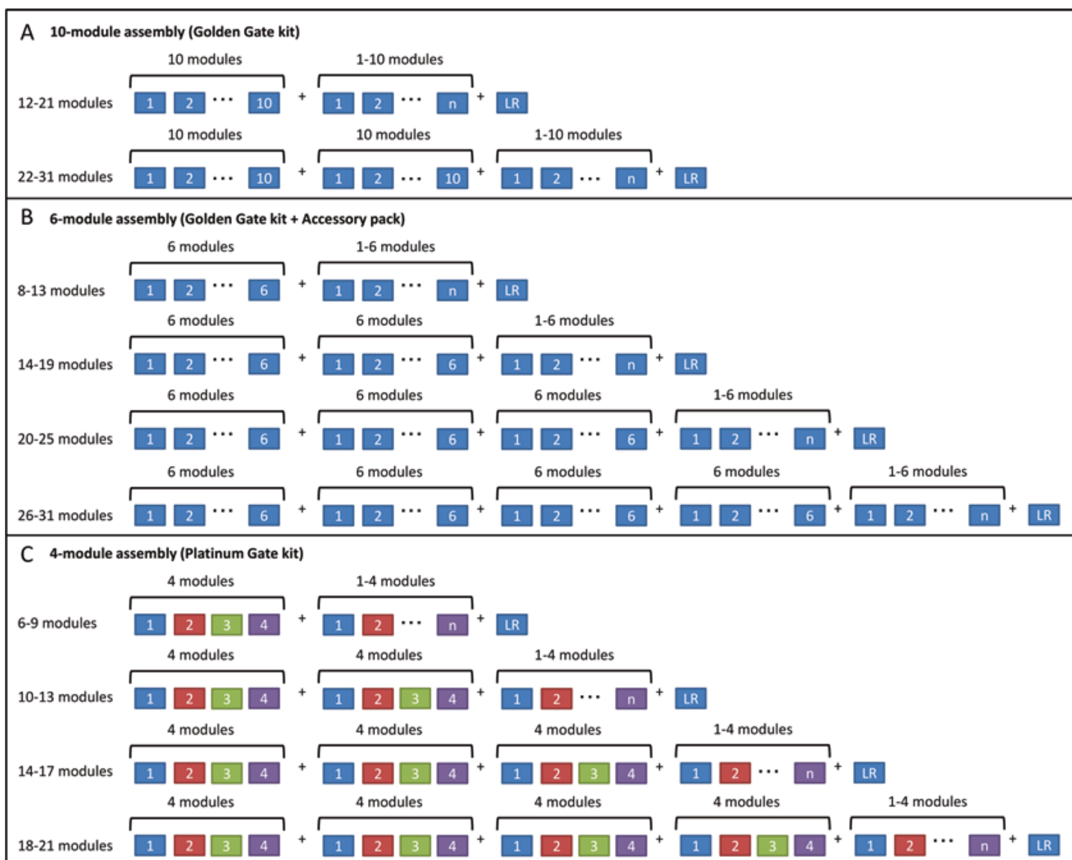

Fig. 2 Summary of 10-, 6-, and 4-module assembly needed for the Golden Gate kit (**a**), the Golden Gate kit with the Accessory pack (**b**), and the Platinum Gate kit (**c**), respectively. Up to 31 (**a**, **b**) or 21 (**c**) modules can be assembled with the 2-step Golden Gate assembly systems. In the systems based on the Golden Gate kit, all the DNA-binding modules consist of the same amino acid composition excluding RVDs (**a**, **b**), whereas those in the TALENs created with the Platinum Gate kit consist of variable repeats (**c**). See the main text for details. *LR* last repeat

3.4 Accessory Plasmids for the Golden Gate Kit

To make the most of the Golden Gate kit, various add-on plasmids have been developed (Table 2). All of them are destination vectors and distributed as single plasmids, excluding the TALEN Construction Accessory Pack (Kit #1000000030), developed by ours [13]. This accessory pack contains nine plasmids including seven intermediate array vectors and two destination vectors. The unique intermediate array vectors are, of course, compatible with the Golden Gate kit, and they enable six-module assembly, which results in efficient and accurate concatemerization of the TALE repeats without any additional cloning steps (Fig. 2b). The destination vectors are for in vitro transcription using T7 RNA polymerase and expression in mammalian cells via CMV or CAG promoters, harboring truncated TALEN scaffolds with enhanced activity and specificity (47 amino acids for C-terminal domain) [7, 13].

Regarding other plasmids, RCIscript-GoldyTALEN and pC-GoldyTALEN (Plasmid #38142 and #38143), pCS2TAL3-DD and pCS2TALE3-RR (Plasmid #37275, #37276, #48636, and #48637), and pCAG-T7-TALEN(Sangamo)-Destination Constructs (Plasmid #37184, #40131, and #40132) are in vitro transcription and expression vectors with CMV or CAG promoters for TALENs. TAL5-BB and pTAL6-BB (Plasmid #36033 and #36034) are yeast expression vectors for TALE-based transcriptional repression. TALE-transcription activation destination vectors (Plasmid #47388 and #47389) are mammalian expression/in vitro transcription vector for TALE-based transcriptional activation. Destination vectors for TALE-mediated Genome Visualization (TGV) (Plasmid #47874 and #47875) are mammalian expression vector to visualize the dynamics of the chromosome. pTAL7a and pTAL7b (Plasmid #48705 and #48706) are destination vectors for the TALEN application in human pluripotent stem cells. pBlue-TAL (Plasmid #49401) is for the generation of germline mutations in *Bombyx mori* and *Drosophila melanogaster*.

3.5 Platinum Gate Kit from Yamamoto Lab

Although the convenience of the Golden Gate kit has become a higher level by utilizing various additional plasmids, the activity of resultant TALENs is not always sufficient, requiring improvement of amino acid composition of the TALE repeats. We previously found that periodically patterned variable repeats can enhance the DSB-inducing activity of TALENs [5, 14]. The Platinum Gate TALEN kit (Kit #100000004) is the only one that highly active TALENs with the variable repeats can be created using Golden Gate assembly method. The versatility of TALENs constructed with this kit, referred to as Platinum TALENs, has been proven in a number of publications, reporting various applications in a wide variety of cells and organism, such as human iPS cells [14, 15], nematodes [16], sea urchins [17], ascidians [18], water flea [19], zebrafish [20], newts [21], frogs [5], mice [22], rats [5], and marmosets [23].

Table 2
Summary of Golden Gate add-ons

Kit/plasmid name	Kit/plasmid ID	Depositor	Contents	Number of plasmids
TALEN Construction Accessory Pack	1,000,000,030	Takashi Yamamoto	Intermediate array vectors and destination vectors for in vitro transcription and mammalian expression	9
RCIscript-GoldyTALEN and pC-GoldyTALEN	38,142, 38,143	Daniel Carlson	Destination vectors for in vitro transcription and expression of TALENs	2
TAL5-BB and pTAL6-BB	36,033, 36,034	Tom Ellis	Destination vectors for yeast expression of TALE-based transcriptional repressors	2
pCS2TAL3-DD and pCS2TALE3-RR	37,275, 37,276, 48,636, 48,637	David Grunwald	Destination vectors for in vitro transcription and expression of TALENs	4
pCAG-T7-TALEN(Sangamo)-Destination Constructs	37,184, 40,131, 40,132	Pawel Pelczar	Destination vectors for in vitro transcription and expression of TALENs	3
TALE-transcription activation destination vectors	47,388, 47,389	Charles Gersbach	Destination vectors for mammalian expression of TALE-based transcriptional activators	2
Destination vectors for TALE-mediated Genome Visualization (TGV)	47,874, 47,875	Maria-Elena Torres-Padilla	Destination vectors for mammalian expression of fluorescent TALE to visualize chromosome dynamics	2
pTAL7a and pTAL7b	48,705, 48,706	Boris Greber	Destination vectors for the application of TALEN technology in human pluripotent stem cells	2
pBlue-TAL	49,401	Michal Zurovec	Destination vector for the generation of germline mutations in *Bombyx mori* and *Drosophila melanogaster*	1

The kit contains 35 plasmids including 16 module vectors, 11 intermediate array vectors, and 8 destination vectors. TALENs with 6–21 repeats can be constructed using 4-module assembly method (Fig. 2c). Fewer numbers of assembled modules compared with previous systems (4 vs. 10 or 6) result in an easy, robust, and efficient assembly reaction. Destination vectors are designed to be applicable in mammalian expression and in vitro transcription, similar to the previous ones. Moreover, two truncation patterns of N- and C-terminal domains of TALE based on different TALE architecture (+153/+47 of PthXo1 and +136/+63 of AvrBs) are included. These two scaffolds have different preferences on the TALEN activity; +153/+47 TALEN is active with limited spacer lengths, while +136/+63 TALEN is active with broad spacer lengths [5]. Users can choose the appropriate scaffold from these two options depending on the intended use. The detailed protocol to construct Platinum TALEN was described in a previous publication [24].

3.6 TALE Toolbox from Zhang Lab (Combined Method of Golden Gate Assembly and PCR Amplification)

Another popular TALEN kit based on Golden Gate assembly is the TALE Toolbox (Kit #1000000019) developed by Feng Zhang [25]. The TALE Toolbox consists of a relatively small number of plasmids: four module vectors and eight destination vectors. Among them, four destination vectors are for TALE-based transcriptional activation; thus, only eight plasmids are needed to create TALENs. However, this kit requires several additional procedures including PCR amplification, purification of PCR products, exonuclease treatment, another round of PCR amplification, and gel extraction and purification. Therefore, although the initial cost and labor is low compared with the other methods, users have to consider the running cost and labor. Two rounds of PCR amplifications might introduce unintended errors. Variable repeat TALENs cannot be created, because there are only four module vectors.

3.7 Multiple Module Library-Mediated Systems from Ekker Lab and Musunuru/Cowan Lab

As described so far, Golden Gate assembly-based construction of TALENs basically depends on the two-step assembly process. However, there are several systems enabling one-step construction of TALEN vectors by using pre-assembled module libraries. Musunuru/Cowan Lab TALEN Kit (Kit #1000000034) contains 834 plasmids including 58 three-module library, 768 four-module library, and 2 destination vectors (pTAL_GFP and pTAL_RFP) [26]. Using these large-scale libraries, 15-repeat TALENs can be constructed with a single cloning step. Stephen Ekker lab first deposited the partial library containing 256 four-module plasmid sets (Ekker Lab TALEN Kit; Kit #1000000038), which is compatible with the Golden Gate kit [27]. This plasmid set can help in creating 15-repeat TALENs, but requires additional construction of intermediate array plasmids. Subsequently, Ekker lab has

developed another system called FusX TALEN Assembly System (Kit #1000000063) [28]. The FusX system contains 336 plasmids, enabling one-step construction of TALENs without additional intermediate constructs. This system is also compatible with the Golden Gate kits; thus, all the accessory plasmids for the Golden Gate kit can also be used. These multiple module library-mediated methods are suitable for high-throughput production of TALEN vectors, while there also are some limitations. First, hundreds of pre-assembled plasmids are needed and have to be maintained. Second, assemblable repeat length is very limited (15 repeats in the Musunuru/Cowan system and 15 or 16 repeats in FusX system). Since the N-terminal domain of TALE recognizes a thymine, this limitation narrows the target range of TALENs. Third, incorporation of repeat variations such as variable repeats and noncanonical RVD repeats [29] is very difficult.

4 Other Systems

4.1 TAL Effector Engineering Reagents from Joung Lab (Serial Ligation Method)

Keith Joung lab has provided the REAL Assembly TALEN kit (Kit #1000000017) that consists of 32 plasmids including 28 module vectors and 4 destination vectors [30]. Since the REAL assembly method depends on the standard serial digestion and ligation procedure, it requires several cloning steps to create functional TALENs. To improve the throughput, the pre-assembled module library can be used, similar to the Golden Gate assembly-based systems (REAL-Fast method). The detailed procedures for creating TALENs using REAL or REAL-Fast method were described in a previous publication [31]. In addition, the fast ligation-based automatable solid-phase high-throughput (FLASH) method can also be applicable with the same reagents [32, 33]. The FLASH system enables automated or manual TALEN assembly in a 96-well format. Moreover, TALENs constructed with these systems contain variable repeats similar to the Platinum TALENs. However, the pre-assembled module library for the REAL-Fast and FLASH systems has not yet distributed via Addgene.

4.2 LIC Assembly Kit from Hornung Lab (Ligation-Independent Cloning Method)

Veit Hornung lab has reported another approach for TALEN assembly. They developed a ligation-independent cloning (LIC) assembly-mediated system (LIC TAL Effector Assembly Kit; Kit #1000000023) [34]. In this system, a unique procedure named chew back reaction is required. The chew back reaction is a programmed DNA end resection mediated by T4 DNA polymerase, resulting in unique 5′ overhangs for the annealing of multiple DNA fragments in a defined order. The kit contains 76 plasmids, creating 10- to 19-repeat TALENs with two assembly steps. The detailed procedures for LIC-based TALEN construction were described in a previous publication [35].

4.3 Other Systems Currently Unavailable from Addgene

Besides the Addgene kits, there are a number of alternative TALEN construction systems reported, such as iterative capped assembly (ICA) system [36], uracil-specific excision reagent (USER) cloning-based methods [37, 38], STAR method based on isothermal assembly [39], synthetic oligonucleotide-mediated system [40]. It is highly desired for these interesting systems to be available via Addgene in the future.

Acknowledgments

This work was partly supported by the Japan Society for the Promotion of Science (16 K18478 to T.S.).

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Chapter 3

CRISPR/Cas9

Izuho Hatada and Takuro Horii

Abstract

CRISPR/Cas9 is a novel method that has become the most widely used genome editing technology around the world. Its widespread adoption is largely due to its simplicity and easy of use. Here, we introduce the construction of vectors and genome editing of the target gene in cells expressing the CRISPR/Cas9 system.

Key words CRISPR/Cas9, Cas9, gRNA

1 Introduction

Clustered regularly at interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) nuclease 9 (CRISPR/Cas9) is a relatively simple genome editing technology that has become widely adopted around the world [1]. The method requires only Cas9)nuclease and a short RNA, called single-guide RNA (gRNA or sgRNA), which includes 20 nucleotides complementary to the target sequence in the genome; when present, these two factors induce cleavage of the target. Earlier genome editing systems, such as zinc-finger nucleases (ZFNs) [2] and transcription activator-like effector nucleases (TALENs) [3], require complicated processes for construction of DNA-binding proteins, making these methods time-consuming and expensive. Thus, CRISPR/Cas9 is the most widely used genome editing technology.

For the design of gRNA, the only restriction is that the locus needs to contain the "NGG" protospacer adjacent motif (PAM) downstream of the target sequence (Fig. 1) [1]. Therefore, the user only needs to design the gRNA and construct the gRNA expression vector. After transfection of cells with the gRNA and Cas9 expression vectors, the Cas9/gRNA) complex binds to the target genomic locus and induces double-stranded breaks (DSBs) (Fig. 1). Cas9-induced DSBs can be repaired by either non-homologous end joining (NHEJ) or homology-directed repai

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DOI 10.1007/978-1-4939-7128-2_3, © Springer Science+Business Media LLC 2017

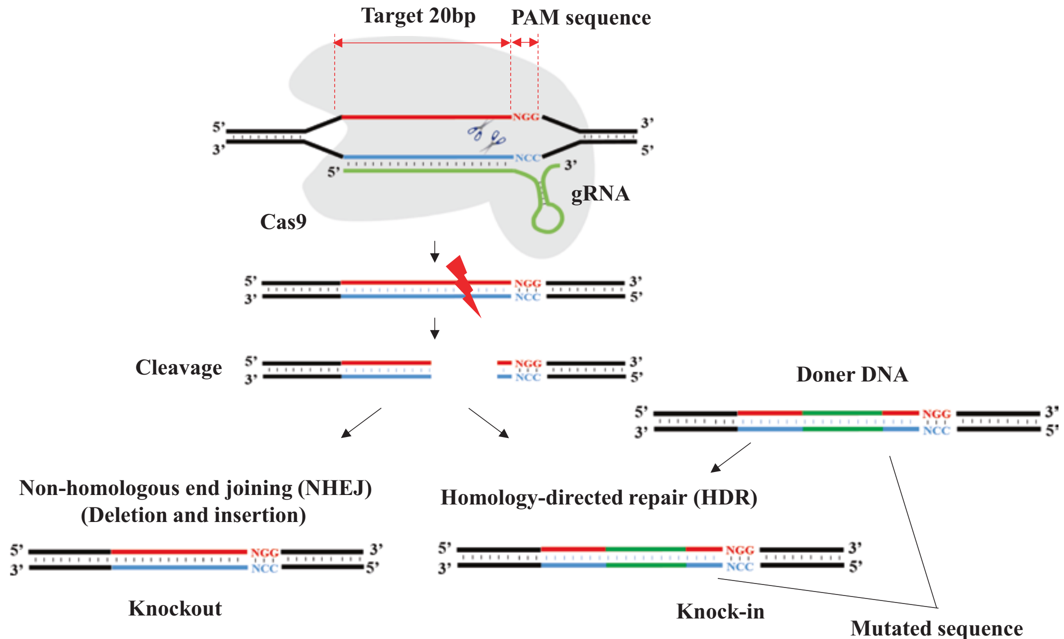

Fig. 1 Schematic of the CRISPR/Cas9 system. The Cas9 endonuclease and gRNA complex cleave the target sequence. The PAM (NGG) adjacent to the 3′ end of the 20 bp target sequence is essential for cleavage. The cleaved target is frequently repaired by spontaneous non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ results in a small deletion or insertion at the targeted locus, whereas HDR generates a knockin at the targeted locus if donor DNA is present

(HDR). NHEJ-mediated repair usually leads to small insertions or deletions (indels) at the targeted site, leading to frameshift mutations (Fig. 1). By contrast, in the presence of a single- or double-stranded DNA)template containing homology to the sequences flanking the DSB, HDR can lead to precise point mutations or DNA insertions (Fig. 1).

Here, we introduce the construction of gRNA vectors and genome editing of target sequences in cells, as well as the validation of editing efficiency.

2 Materials

2.1 Cloning of gRNA

1. CRISPRdirect: http://crispr.dbcls.jp/ [4].
2. UCSC Genome Browser: https://genome.ucsc.edu/cgi-bin/hgGateway.
3. gRNA cloning vector: an empty gRNA expression vector, used to create a gRNA targeting a specific sequence (#41824, Addgene).
4. 2× Phusion High-Fidelity PCR Master Mix with HF Buffer (M0531S, NEB).

5. 2× Gibson Assembly Master Mix (E2611S, NEB).
6. *Afl*II: restriction enzyme used for Gibson assembly cloning.
7. 10× NEBuffer 4: 500 mM Potassium Acetate, 200 mM Tris-acetate, 100 mM Magnesium Acetate, 10 mM Dithiothreitol (supplied with *Afl*II if purchased from NEB; alternatively, a similar buffer can be used for *Afl*II digestion).
8. LB agar with kanamycin: 1.6% Agar, 1% tryptone, 0.5% yeast extract, 1% NaCl, 50 μg/mL kanamycin (add 10 mg/mL stock solution after cooling down the autoclaved mix).
9. DNA sequencing kit.
10. LB: 1% tryptone, 0.5% yeast extract, 1% NaCl, 50 μg/mL kanamycin (add 10 mg/mL stock solution after cooling down the autoclaved mix).
11. Plasmid DNA purification kit.
12. Thermal cycler.

2.2 Validation of gRNA

1. Lipofection reagent: Lipofectamine) 2000 (Thermo Fisher Scientific), etc.
2. pCAG-hCas9: Expression vector for hCas9 (human codon optimized Cas9) under the CAG promoter (#51142, Addgene).
3. PCR reagents.
4. PCR primers flanking the targeted region.
5. Restriction enzyme whose recognition site overlaps with Cas9 cleavage site of the target.

3 Methods

3.1 Cloning of gRNA

1. Obtain exon sequence from genome database such as)UCSC Genome Browser.
2. Find 23 bp target and PAM sites (5′-N_{20}NGG-3′) on the locus that you intend to edit using a CRISPR/Cas9 target design tool such as CRISPRdirect.
3. We usually use the gRNA cloning vector from the Church lab [5], which uses Gibson assembly [6] for cloning (*see* **Note 1**). Combine 20 bp of the target sequence without the PAM into two 60-mer oligonucleotides as indicated below (the regions marked in red and blue are reverse complements of each other). If the 5′ end of the target sequence (opposite site of PAM) is not G in the sense strand (C in antisense strand), change to G and C, respectively (Fig. 2, *see* **Note 2**).

INSERT_S:

5’-TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACC**GNNNNNNNNNN**

NNNNNNNNN-3’

INSERT_AS:

5’-GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC**NNNNNNNNNNNN**

NNNNNNNC-3’

4. Anneal the two oligonucleotides and extend them to make a double-stranded DNA fragment using Phusion polymerase. Add the following to a 0.1 mL Eppendorf tube on ice: 1 μL of 5 pmol/μL INSERT_S, 1 μL of 5 pmol/μL INSERT_AS, 5 μL of 2× Phusion Master Mix, and 3 μL of sterile distilled water. On a thermal cycler, perform one cycle at 98 °C for 30 s, 50 °C for 30 s, and 72 °C for 3 min.
5. Linearize the gRNA cloning vector using *Afl*II (*see* **Note 3**). Add the following to a new 0.1 mL Eppendorf tube on ice: 1 μL of 10× NEBuffer 4, 1 μL of 1 mg/mL BSA, 20 units of *Afl*II, and 1 μg of gRNA cloning vector. Adjust volume to 10 μL with sterile distilled water. Incubate for 3 hr. at 37 °C (digestion) and 20 min at 65 °C (inactivation).
6. Combine the 100 bp annealed DNA fragment with the linearized vector via Gibson assembly. Add the following to a new 0.1 mL Eppendorf tube on ice: 5 μL of 2× Gibson Assembly Master Mix, 0.5 μL of insert mixture (prepared in **step 3**), 1 μL of linearized vector mixture (prepared in **step 4**), and 3.5 μL of sterile distilled water. Incubate at 50 °C for 60 min, and 4 °C until use for transformation)).
7. Perform bacterial transformation (*see* **Note 4**) using 1 μL of combined DNA. Plate some of the transformation onto LB agar plates containing kanamycin. After incubation of the plate overnight at 37 °C, pick a few colonies (around four will be sufficient) and confirm the sequence by conventional DNA sequencing.
8. Culture the colony and purify the plasmid.

3.2 Validation of gRNA

1. Transfect the constructed gRNA vector into cells with an equal amount (μg) of pCAG-hCas9 using a lipofection reagent.
2. Recover transfected cells 2 days after transfection.

Target (20 mer)

Replace to G if not G

Sense

5' TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGNNNNNNNNNNNNNNNNNNN 3'

3' CNNNNNNNNNNNNNNNNNNNCAAAATCTCGATCTTTATCGTTCAATTTTATTCCGATCAG 5'

Antisense

Replace to C if not C

Fig. 2 Design of oligonucleotides used for the construction of gRNA vector. If the 5′ end of the target sequence is not G in the sense strand (C in antisense strand), these residues should be changed to G and C, respectively

3. Extract genomic DNA by a conventional method.
4. Perform PCR using primers flanking the targeted region. Digest the PCR products with a restriction enzyme that specifically cleaves at the Cas9 target site of the non-mutated genomes. Analyze the PCR products by gel electrophoresis to determine mutation efficiency (*see* **Note 5**). The mutated DNA fragment cannot be cleaved by the restriction enzyme, while the non-mutated DNA fragment can be cleaved by the enzyme. Therefore, the mutation rate is calculated as $u/(c + u)$ where c = intensity of cleaved band and u = intensity of uncleaved band.

4 Notes

1. Gibson assembly is not a simple cut-and-paste-based cloning. In other gRNA vector systems, cut-and-paste-based cloning is used [7].
2. It is necessary for the first transcribed base to be guanine (G) because of the transcription initiation requirement of the human U6 promoter.
3. Size-fractionation by agarose gel electrophoresis followed by purification of linearized vector is unnecessary because of the high efficiency of Gibson assembly.
4. Highly efficient competent cells are not necessary because of the high efficiency of Gibson assembly.
5. T7 endonuclease I assay [8] can also be used to determine the mutation rate.

Acknowledgments

This work was supported by grants from the Basic Science and Platform Technology Program for Innovative Biological Medicine from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (MEXT), and The Japan Agency for Medical Research and Development (AMED) to I.H.

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Chapter 4

Computational Prediction of CRISPR/Cas9 Target Sites Reveals Potential Off-Target Risks in Human and Mouse

Qingbo Wang and Kumiko Ui-Tei

Abstract

The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system is a prominent genome engineering technology. In the CRISPR/Cas system, the RNA-guided endonuclease Cas protein introduces a DNA double-stranded break at the genome position recognized by a guide RNA (gRNA) based on complementary base-pairing of about 20-nucleotides in length. The 8- or 12-mer gRNA sequence in the proximal region is especially important for target recognition, and the genes with sequence complementarity to such regions are often disrupted. To carry out target site-specific genome editing, we released the CRISPRdirect (http://crispr.dbcls.jp/) website. This website allows us to select target site-specific gRNA sequences by performing exhaustive searches against entire genomic sequences. In this study, target site-specific gRNA sequences were designed for human, mouse, *Drosophila melanogaster*, and *Caenorhabditis elegans*. The calculation results revealed that at least five gRNA sequences, each of them having only one perfectly complementary site in the whole genome, could be designed for more than 95% of genes, regardless of the organism. Next, among those gRNAs, we selected gRNAs that did not have any other complementary site to the unique 12-mer proximal sequences to avoid possible off-target effects. This computational prediction revealed that target site-specific gRNAs are selectable for the majority of genes in *D. melanogaster* and *C. elegans*. However, for >50% of genes in humans and mice, there are no target sites without possible off-target effects.

Key words CRISPR/Cas9, Target site, Off-target gene, CRISPR direct

1 Introduction

Genome engineering using the clustered regularly interspaced short palindromic repeat/CRISPR-associated (CRISPR/Cas) system has been widely applied in recent years due to its simplicity and wide range of applicability [1–5]. The step-by-step mechanism of the CRISPR/Cas system, which is derived from the adaptive immune system of prokaryotes, is as follows [6–8]:

(a) The Cas protein, an RNA-guided endonuclease, interacts with a single-guide RNA (sgRNA) to form a Cas-sgRNA complex. The sgRNA is a short RNA artificially constructed by

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DOI 10.1007/978-1-4939-7128-2_4, © Springer Science+Business Media LLC 2017

connecting CRISPR RNA (crRNA) with the trans-activating crRNA (tracrRNA).

(b) The Cas protein in the Cas-sgRNA complex recognizes a specific sequence motif called proto-spacer adjacent motif (PAM), 5′-NGG in the case of *Streptococcus pyogenes*, downstream of the target site. If the 20-mer sequence upstream of the PAM is homologous to the guide RNA (gRNA) spacer sequence, the gRNA spacer region pairs with the complementary strand upstream of the PAM (target sequence).

(c) After binding to the target sequence, the Cas protein in the Cas-sgRNA complex cleaves both DNA strands a few bases upstream of the PAM sequence to introduce a double-stranded break (DSB).

(d) The DSB site is repaired by error-prone non-homologous end joining (NHEJ) that often results in a small insertion or deletion. When there is a homologous template DNA, the site is repaired by homology-directed repair (HDR) that results in insertion of a specific DNA sequence.

Genome engineering using the CRISPR/Cas system allows for the introduction of DSBs to induce NHEJ or HDR in the intended genomic regions by designing a target site-specific gRNA sequence. Due to this, the CRISPR/Cas system is advantageous compared to previous genome engineering techniques based on protein engineering technology (such as transcription activator-like effector nucleases "TALENs" and zinc finger nucleases "ZFNs"), which require considerable efforts to design effective proteins [9–11].

However, the stringency of target sequence recognition by the Cas-sgRNA complex is not well understood. Previous studies have revealed that site recognition and cleavage by the Cas protein can occur even when there are gaps or mismatches between the gRNA spacer and target sequences [12–14]. A number of different mismatch patterns have been reported for such nonspecific cleavage ("off-target effect"), but the 8 or 12 nucleotides upstream of the PAM (seed region) are especially important for target site recognition. In many cases, off-target mutations happen at sites where the gRNA seed region has no mismatches but the non-seed region does [3, 15]. To knock out a specific region in the genome, such off-target effects should be avoided. Reducing such risk is especially important when we consider further application of the technology, including therapeutic applications.

In this study, we implemented a computational pipeline to design site-specific gRNAs. Our pipeline enabled evaluation of the off-target risk of each gRNA by calculating the number of seed-matched off-target candidate sequences in the entire genome and allowed for the design of off-target risk-reduced gRNAs. Using the

pipeline, the number of applicable gRNAs was gg (Subheading 3.4. also, *see* **Note 1**). The percentage of genes that had a certain number of such gRNAs was also calculated and compared between four different organisms (Subheadings 3.5 and 3.6).

2 Materials

2.1 Protein-Coding Sequence Preparation

1. All human (hg19, GRCh37 Genome Reference Consortium Human Reference 37), mouse (mm10, Genome Reference Consortium Mouse Build 38), *Drosophila melanogaster* (*D. melanogaster*) (dm3, BDGP Release 5), and *Caenorhabditis elegans* (*C. elegans*) (ce10, Washington University School of Medicine GSC and Sanger Institute WS220) mRNA sequences (protein-coding sequences) were downloaded from RefSeq, through the UCSC Genome Browser (https://genome.ucsc.edu/index.html) and stored as fasta files (Fig. 2, **step 1**).
2. The table describing the relationship between mRNA ID and gene name was also downloaded from the UCSC Table Browser by changing the "output format" to "all fields from selected table." This table was used to map each mRNA to the corresponding gene.

2.2 Software for Genome-Wide Specificity Check

The CRISPRdirect web server [16] (https://crispr.dbcls.jp) was used to conduct genome-wide investigation of off-target sites. This server investigates the entire genome of the organisms of interest for PAM-proximal 20-, 12-, or 8-mer-matched sequences, and lists all the possible gRNA sequences and the number of matched sequences.

2.3 Building Environment for Iteration and Visualization

Bash (GNU bash, version 3.2.51(1)-release) and Python 2.7.5 were used to construct a computational pipeline (Fig. 2) for iterative design of site-specific gRNAs against each gene. "Pandas" [17], an open-source tool for data analysis, was used to parse the data into tab-separated value (tsv) files. The calculation results were visualized using matplotlib [18], a python library for 2D plotting. The entire process, described in the next chapter (from gRNA search to graph visualization), was automated in a single pipeline under the environment.

3 Methods

3.1 Directory Structure Construction

Before beginning the calculation process, the directories for output file storage were structured as shown in Fig. 1 using bash scripts.

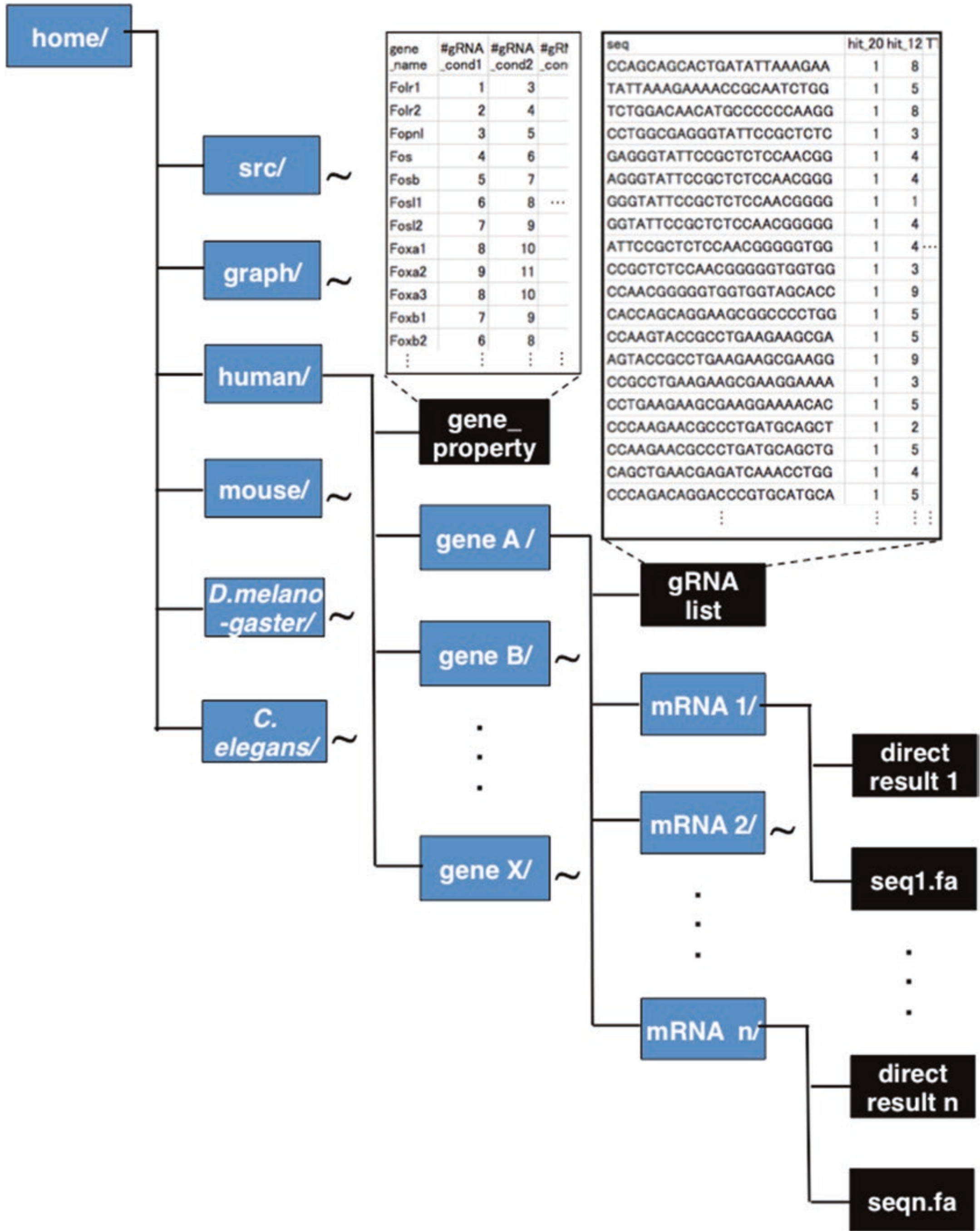

Fig. 1 The directory structure for storage of output files. The home directory contains src/ (where source codes are stored), graph/ (where the visualized results of the calculation are stored), and the directory of each organism. The directories for each of the genes were positioned under the directory of each organism, and followed by mRNA directories. The calculation summary for all the genes of a single organism (*gene_property*) is stored under the directory of each organism. Each directory of a gene contains a list of all the target site-specific gRNAs for the gene (*gRNA_list*). The results of off-target candidate search using CRISPRdirect software (*direct_result n*) and the genomic sequence of each mRNA (*seqn.fa*) are stored under the directory of each mRNA. *Blue boxes* indicate directories. *Black boxes*, tsv or fasta files. "~," the lower directories

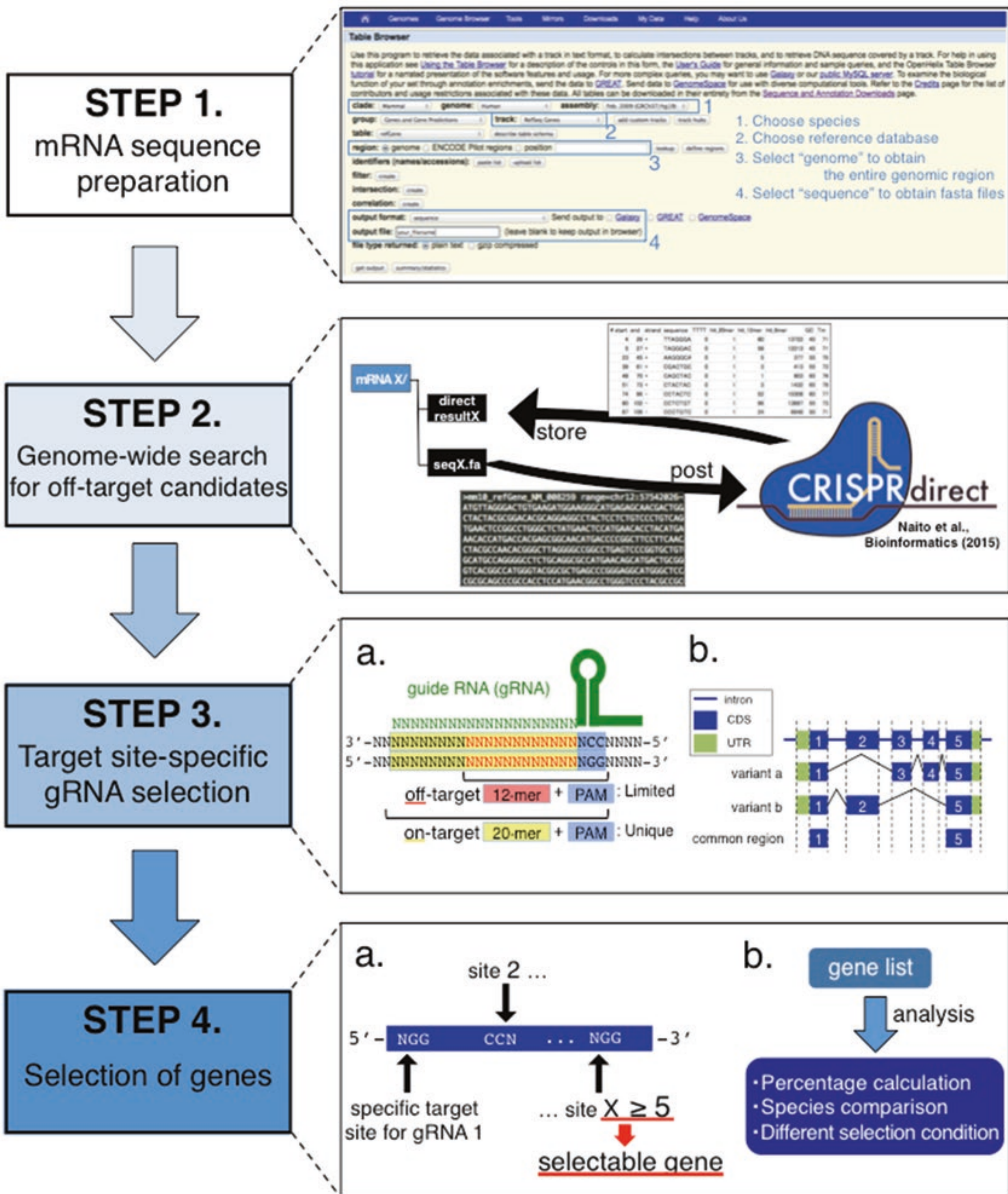

Fig. 2 Workflow of the target site-specific gRNA selection process with a minimum number of off-target hits, and genes that have more than a certain number of selectable gRNAs per gene. *STEP 1*: Preparation of mRNA sequences used. All mRNA sequences (protein-coding mRNA sequences) were downloaded through the UCSC Genome Browser (https://genome.ucsc.edu/index.html). *STEP 2:* Genome-wide search for off-target candidates using CRISPRdirect. Given the query sequence, CRISPRdirect investigates the entire genome for PAM-proximal 20-, 12-, or 8-mer-matched sequences. *STEP 3:* Target site-specific gRNA selection based on STEP2 results. (a) The schematic structure of 20-mer on-target region, 12-mer off-target region, and PAM region used for the evaluation of gRNA specificity. (b) The mRNA regions where gRNAs were designed in this study. *STEP 4:* Selection of genes containing a sufficient number of target site-specific gRNAs based on the *STEP 3* results. (a) A gene containing more than five gRNAs, for example. (b) The selection step of genes under different conditions

The workflow of the calculation pipeline is shown in Fig. 2, and the details of each step are shown below.

3.2 Genome-Wide Search for Off-Target Candidates

Each mRNA sequence, stored in a fasta format, was posted to our software, CRISPRdirect [16], using the API provided by the software (Fig. 2, **step 2**). The list of possible gRNA candidates with the specificity check results were then stored in the local directory as a tsv file.

3.3 Target Site-Specific gRNA Selection

The target site-specific gRNA candidates satisfying the following conditions were selected:

(a) Uniqueness of the target sequence among the entire genome: No perfect match other than the target site (20-mer sequence + PAM) was allowed (Fig. 2, **step 3a**).

(b) Limitation of the number of possible off-target sites with seed (PAM-proximal 12-mer sequence) complementarity among the entire genome: Only a limited number of seed-matched sites was allowed (Fig. 2, **step 3a**).

(c) Absence of a “TTTT” stretch: No “TTTT” stretch (more than three sequential Ts) was allowed in the gRNA sequence.

(d) The target site was positioned in the common exons among all the transcription variants of a target gene (Fig. 2, **step 3b**).

Conditions (a) and (b) were applied to reduce the risk of off-target cleavage, and (c) was applied to avoid the termination of gRNA transcription by RNA polymerase III (the “TTTT” stretch is a known RNA polymerase III termination site). Condition (d) was needed to disrupt the target gene expression regardless of the transcription pattern (*see* **Notes 2–4**).

The gRNAs that did not satisfy any one of four conditions shown above were removed using a filtering operation in Pandas, and the gRNAs that simultaneously satisfied all four conditions were defined as “target site-specific gRNA candidates.”

3.4 Calculating the Number of Target Site-Specific gRNA Candidates per Gene

Given the list of target site-specific gRNA candidates selected in Subheading 3.3, the number of selectable target site-specific gRNA candidates for each gene was calculated in four organisms: human, mouse, *D. melanogaster*, and *C. elegans*. The cumulative fraction distribution of genes (*y* axis) as a function of the number of selectable gRNAs (*x* axis) was shown in two different cases: (1) the case where an unlimited number of seed-matched off-target candidate sites was allowed (Fig. 3a), and (2) no seed-matched site other than the target site was allowed (Fig. 3b). In the former case, *C. elegans* had relatively fewer gRNAs compared to the other three organisms (Fig. 3a). However, the cumulative curves for *C. elegans* and *D. melanogaster* were similar to each other but different from humans and mice when no seed-matched site other than the target site was allowed (Fig. 3b).

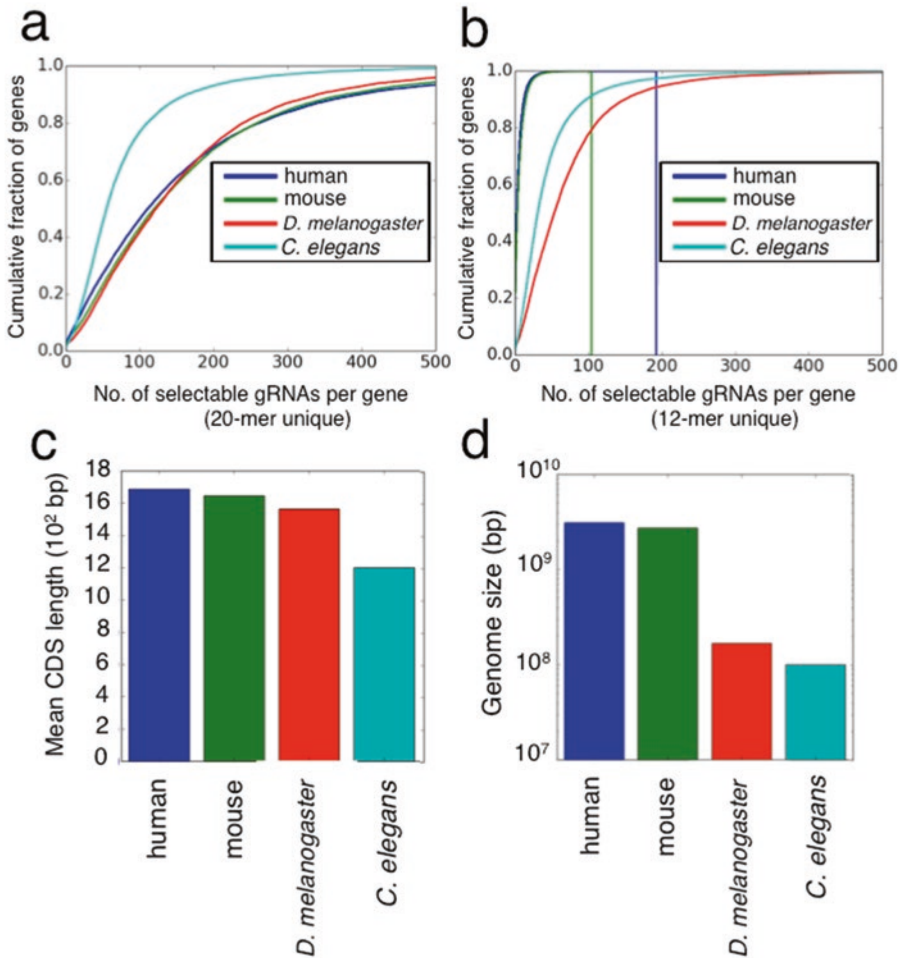

Fig. 3 Comparison of the number of target site-specific gRNA candidates per gene, mean CDS length, and genome sizes of human (*blue*), mouse (*green*), *D. melanogaster* (*red*), and *C. elegans* (*light blue*). (**a**), (**b**) Cumulative distributions of the genes as a function of the number of gRNAs with a 20-mer (**a**) or 12-mer (**b**) completely matched unique site per gene. The *x* axis indicates the number of designable gRNAs per gene, and the *y* axis is the cumulative fraction of genes. The *blue* and *green vertical lines* indicate the cumulative fractions of human and mouse genes reached to 1.0, respectively, in (**b**). The mean CDS length (c) and genome sizes (**d**) of four different organisms used in this research. For CDS length, the mean length of transcription variants was calculated for each gene, and then the mean CDS length was calculated for each species

The results indicated that at least two different parameters may contribute to specificity. First, in our selection conditions, since the target region that we designed gRNAs for was restricted to the coding sequence (CDS), the relatively short average CDS length (Fig. 3c) was likely the reason for the lower abundance of gRNAs in *C. elegans*, especially in the case where an unlimited number of seed-matched sites was allowed. Second, the genome sizes of these four organisms differ by double-digits, and range from 10^9 to 10^7 bp (Fig. 3d). Since the off-target search was performed on a

genome-wide scale, the off-target hits may increase based on genome size. The sharp decrease in abundance of target site-specific gRNAs in human and mouse genomes could be attributed to their large genome sizes. Thus, it may be difficult to select a large amount of target site-specific gRNAs without off-target candidates in organisms with large genomes.

3.5 Selection of Genes with More than Five Target Site-Specific gRNAs for Each Gene

Even when a target site-specific gRNA with a perfectly matched target sequence is selected, there is no guarantee that the target site would be cleaved by the gRNA [19, 20] since the conditions that determine gRNA sequence functionality are not well known (*see* **Note 5**). One of the possible procedures to efficiently induce DSB is to design multiple gRNAs that target different sites of the same gene. For example, in recent large-scale knock-out experiments, five gRNAs were designed for each gene, and numerous gene sets corresponding to fundamental biological processes in mammalian cells were identified [21, 22]. In an analogous way, we used the genes for which more than five target site-specific gRNAs were selectable, and defined them as "5 ≤ gRNA" genes (Fig. 2, **step 4a**). The percentage of such genes was calculated based on the results shown in Fig. 3a and b. Since gRNA abundance is highly dependent on the number of off-target hits that we allowed, we gradually changed the maximum number of seed-matched off-target hits per gRNA and counted the selectable gRNA abundance for each condition. The results revealed that at least five gRNA sequences with only one perfectly complementary site in the whole genome could be designed for more than 95% of genes for all four organisms when neglecting possible off-target risks with 12-mer matched sequence (Fig. 4a). However, the percentage of such genes decreased with the decreased number of possible off-target sites when 12-mer matched sequences were allowed, especially for human and mouse genomes. The results suggest that when considering seed-matched off-target risks, knockout screening experiments using the CRISPR/Cas system would be relatively more feasible for *D. melanogaster* and *C. elegans*, compared to humans and mice.

3.6 Selection of "N ≤ gRNA" Genes

For the human genome, which had the lowest percentage, the minimum number of target site-specific gRNA candidates was changed for each selected gene (i.e., we selected "*N* ≤ gRNA" genes, where N is not restricted to 5, ranging from 1 to 10.) to examine the changes in percentage of selectable genes. The results (Fig. 4b) clearly show that there is a trade-off relationship between the minimum gRNA-abundance threshold *N* (*y* axis) and the maximum number of off-target candidate sites allowed per gRNA (*x* axis): If we strictly select gRNAs that have fewer 12-mer matched off-target candidate sites, we can only select a few gRNAs per gene. In other words, the N parameter must be relatively small to maintain a high percentage of selectable genes.

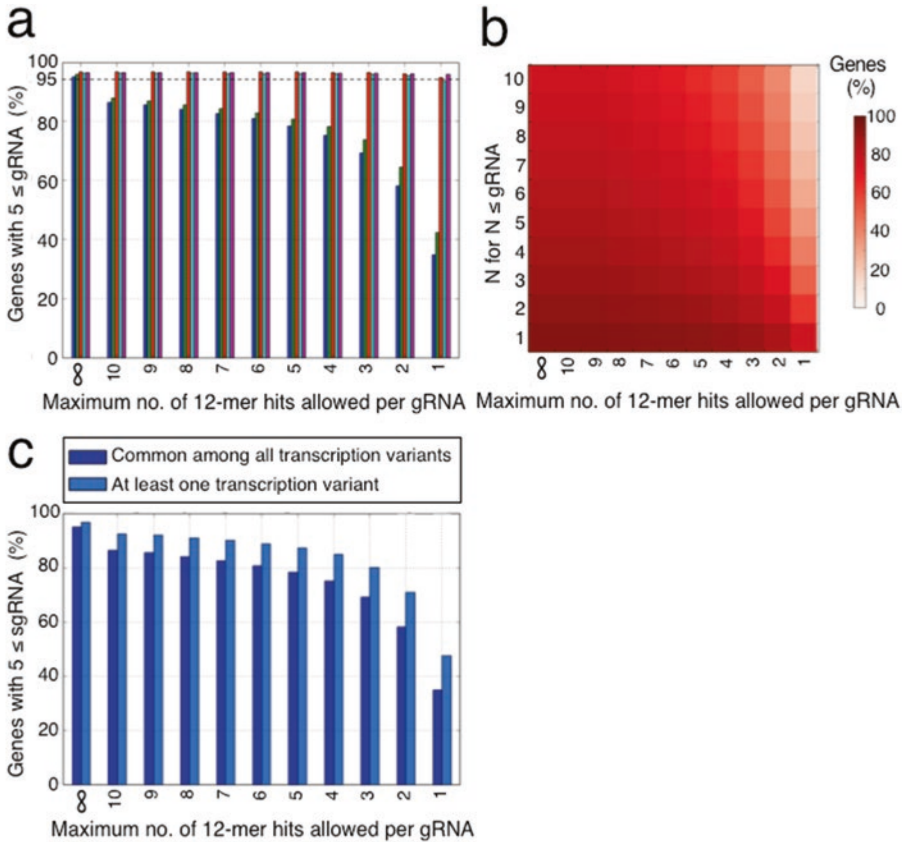

Fig. 4 The percentage of "$N \leq$ gRNA" genes. (**a**) The percentage of genes with more than five target site-specific gRNAs in different organisms (*y* axis), with different numbers of 12-mer matched sequences allowed (*x* axis). The *colors of the bars* correspond to specific organisms: human (*blue*), mouse (*green*), *D. melanogaster* (*red*), and *C. elegans* (*light blue*). Note that the "∞" column of (**a**) corresponds to the intersection of $x = 5$ and each curve in Fig. 3a, and the "1" column of (**a**) to that in Fig. 3b. (**b**) Heat map of the percentage of "$N \leq$ gRNA" genes (*N* ranging from 1 to 10) in the human genome, with different numbers of possible off-target sites with 12-mer matched sequences allowed. *Y* axis, *N* for "$N \leq$ gRNA" gene. *X* axis, the number of 12-mer matched sequences allowed. The *color gradient* indicates the percentage of genes in each condition. (**c**) Comparison of the percentage of genes with more than five target site-specific gRNAs, with and without considering the splice variants of each gene. The *dense blue bar* indicates the result from selecting gRNAs targeting common regions among splice variants, and the *light blue bar* is the result of selecting gRNA not limited to the common region

Overall, our study provided a computational pipeline to design target gene-specific gRNAs, and we quantitatively compared the number of selectable candidate gRNAs per gene among four different organisms under differing selection conditions. This pipeline may be applied to data sets of different organisms that were not analyzed in this research.

4 Notes

1. The time needed to complete the calculation depended mainly on the speed of web communication, rather than the algorithm itself. When the human genome (hg19, 41,845 mRNAs, and 19,132 genes) was used, the whol0e process required 48 hours to complete (Subheading 3.3, Fig. 2, **step 2**).
2. The gRNAs that target exon-exon junctions, the regions without continuous genomic DNA sequences, are eliminated by the CRISPRdirect algorithm (Subheading 3.2), and therefore were not included in this study.
3. In this research, the off-target candidate sites were estimated based on the complementarity to 12-mer, rather than 8-mer, seed sequences to increase prediction accuracy as shown in Subheading 3.3, **step (b)** (Fig. 2, **step 3a**). Although seed match is important for target recognition, different patterns of off-target hits are reported [13]. For example, cell type, chromatin state, or SNP presence are also important factors [23–25]. Revealing more in-depth mechanisms of target site recognition to reduce off-target effects could also be meaningful.
4. If it is not necessary to target the common regions of all transcription variants of a gene, it is possible to eliminate condition (d) in Subheading 3.3 (Fig. 2, **step 3b**). The alteration can be easily achieved by making a minor change in the Python script. Figure 4c shows the comparison of the percentages in the human genome with and without condition (d).
5. As described in Subheading 3.5, the major reason we designed multiple gRNAs for a single gene is because the editing efficiency of each gRNA in the CRISPR/Cas system is not known. Therefore, we did not exclude gRNAs that are likely to show no or weak genome engineering function in this research. Additional research may improve our understanding of both the efficiency and off-target risks of the CRISPR/Cas system, leading us to select truly target-specific and highly efficient gRNAs.

Acknowledgment

We thank Dr. Yuki Naito for valuable discussion and technical advice. The English in this document has been checked by at least two professional editors, both native speakers of English. This work was supported by the grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan to K.U.-T.

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Chapter 5

Genome Editing of Mouse by Cytoplasmic Injection

Takuro Horii and Izuho Hatada

Abstract

CRISPR/Cas enables rapid production of genome-edited animals. The Cas9/gRNA component can be introduced to fertilized eggs in several ways. Here, we provide an instructional guide for the generation of knockout mice by cytoplasmic injection using in vitro transcribed Cas9 and gRNA.

Key words CRISPR/Cas, Cytoplast, In vitro transcription, Microinjection, Mouse

1 Introduction

Novel genome editing technologies using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated proteins (Cas) (CRISPR/Cas) system have emerged as effective tools for generating gene knockouts and knockins in animals. The use of CRISPR/Cas has enabled rapid production of genome-edited mammals including mouse, rat, pig, monkey, and other species. In general, the system entails introduction of two components, Cas9 and guide RNA (gRNA), into fertilized eggs. Currently, Cas9/gRNA can be injected in multiple forms (DNA, RNA, or protein) into various locations within the cell (pronucleus or cytoplasm). The easiest and most direct method is pronuclear injection of plasmid DNA encoding Cas9 and gRNA. Expression vectors can be easily produced from large-scale culture of gene-modified bacteria, and many laboratories have established routine systems for generation of transgenic mice that can be applied to the CRISPR/Cas system without any modifications. However, this method has the risk of vector integration into the chromosomes, albeit at a very low rate (4.3%) [1]. In addition, pronuclear injection causes physical damage to nucleus, decreasing the rate of development both in vitro and in vivo [2]. Therefore, injection into the cytoplasm is an attractive alternative.

Izuho Hatada (ed.), *Genome Editing in Animals: Methods and Protocols*, Methods in Molecular Biology, vol. 1630,
DOI 10.1007/978-1-4939-7128-2_5, © Springer Science+Business Media LLC 2017

We previously showed that the injection of RNA into the cytoplasm is a more efficient method that yields larger numbers of normal blastocyst-stage embryos, full-term pups, and mutant mice [2]. However, cytoplasmic injection of RNA has a few disadvantages. For example, RNA is often degraded due to contaminating RNase; therefore, microinjection must be performed carefully under RNase-free conditions. Moreover, in vitro transcription of RNA may be troublesome and time-consuming for researchers who are not familiar with handling RNA. Fortunately, due to the widespread adoption of the CRISPR/Cas system, in vitro transcribed Cas9 and gRNA are commercially available. These reagents cost approximately USD 300 in total, and the components are delivered to the user within 7–10 days after they are ordered. Thus, RNA injection can be performed as easily as DNA injection.

This chapter serves as an instructional guide for cytoplasmic injection of CRISPR/Cas RNA into fertilized mouse eggs.

2 Materials

2.1 In Vitro Transcription

1. gRNA cloning vector (#41824, Addgene, Cambridge, MA, USA) [3].
2. pCAG-hCas9 (#51142, Addgene) [4].
3. High-fidelity DNA polymerase: e.g., Q5® Hot Start High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA, USA).
4. PCR purification kit: e.g., QIAquick PCR Purification Kit (Qiagen, Hilden, Germany).
5. In vitro transcription kit for gRNA: MEGAshortscript T7 Kit (Life Technologies, Waltham, MA, USA).
6. In vitro transcription kit for Cas9: mMESSAGE mMACHINER T7 ULTRA Kit (Life Technologies).
7. RNA purification kit: MEGAclear Kit (Life Technologies).
8. RNA quantification: e.g., Qubit® Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

(*See* Chapter 4 for construction of gRNA cloning vector and Chapter 5 for design of gRNA.)

2.2 Embryo Collection and Culture

1. Mice: e.g., C57BL/6 J mice, 4 weeks of age (female) and >8 weeks of age (male). B6D2F1 mice, 8–10 weeks of age (female) and >8 weeks of age (male).
2. Pregnant mare’s serum gonadotropin (PMSG).
3. Human chorionic gonadotropin (hCG).

4. M16 embryo culture medium: prepare as reported [5]. Add disodium EDTA (final concentration, 100 μM) before use.
5. M2 embryo culture medium: prepare as reported [5].
6. M2-hyaluronidase medium: M2 medium containing hyaluronidase (300 U/mL).
7. 600 mm cell culture dish.
8. Parafin oil.

2.3 Cytoplasmic Microinjection

1. Micropipet Puller for fabricating micropipets: e.g., P-97/IVF (Sutter Instrument, Novato, CA, USA).
2. Microelectrode Beveler for precision beveling of micropipet tips: e.g., BV-10 (Sutter Instrument).
3. Microforge for bending injection pipet and fire-polishing holding pipet: e.g., MF-900 (Narishige, Tokyo, Japan).
4. Glass micropipet: e.g., Borosilicate Glass B100–75-10-PT (Sutter Instruments).
5. 100 mm cell culture dish.

2.4 Embryo Transfer

1. Pseudopregnant foster mice: e.g., ICR mice, >8 weeks of age.
2. Vasectomized mice: e.g., ICR mice, >8 weeks of age.

2.5 Assay for Genome Modification

1. TA-cloning vector: e.g., TOPO TA Cloning Kit (Thermo Fisher Scientific).

3 Methods

3.1 In Vitro Transcription

3.1.1 In Vitro Transcription of gRNA

1. We use gRNA cloning vectors as templates for in vitro transcription (*see* Chapter 4 for the construction of gRNA cloning vector). Prepare the primer set shown below for in vitro transcription of gRNA. Insert 20 bp of the target sequence (the regions marked in **Bold**) into the forward primer (IVTXgRNA_F). The 5′-end of the 20 bp target sequence should be changed to **G** for highly efficient transcription under the control of the T7 promoter (*see* Chapter 4).

   Target sequence: 20 bp target (**Bold**) + PAM sequence (*Italic*).

   GNNNNNNNNNNNNNNNNNNN*NGG*

   Primer set.

   IVTgRNA_F:

   5′- GCGTTAATACGACT CACTATAGG

G **NNNNNNNNNNNNNNNNNNN**-3′

IVTgRNA_R:

5′- AAAAGCACCGACTCGGTGCC-3′

2. Amplify the template DNA for in vitro transcription. Add the following to a 0.1 mL plastic tube on ice: 0.2 ng of template gRNA cloning vector, 2.5 μL of 10 μM forward primer (final, 0.5 μM), 2.5 μL of 10 μM reverse primer (final, 0.5 μM), 10 μL of 5X Q5 Reaction Buffer, and 0.5 μL of Q5 Hot Start High-Fidelity DNA Polymerase. Adjust volume to 50 μL with nuclease-free water. Perform PCR on a thermal cycler under the following conditions: 98 °C for 30 s; 20–25 cycles of 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s; and 72 °C for 2 min. Analyze 3 μL of PCR products by agarose gel electrophoresis (*see* **Note 1**).
3. Purify PCR products using the QIAquick PCR Purification Kit.
4. Perform in vitro transcription using the MEGAshortscript T7 Kit. Add the following to a 0.1 mL plastic tube on ice: 100 ng of purified PCR products, 2 μL of T7 10× reaction buffer, 2 μL of T7 ATP solution, 2 μL of T7 CTP solution, 2 μL of T7 GTP solution, 2 μL of T7 UTP solution, and T7 Enzyme Mix. Adjust volume to 20 μL with nuclease-free water.
5. Incubate the reaction at 37 °C for 2 h.
6. To remove the DNA template, add 1 μL of TURBO DNase, mix well, and incubate for 15 min at 37 °C.
7. Purify RNA using the MEGAclear Kit with RNase-free water.
8. The concentration of RNA can be determined using the absorbance in a spectrophotometer at 260 nm, but we use the Qubit® Fluorometer, which provides a more sensitive means of quantitating RNA in solution. Store RNA at −80 °C until use.

3.1.2 *In Vitro Transcription of Cas9*

1. We use vector pCAG-hCas9 as the template for in vitro transcription. Prepare the following primer set for in vitro transcription of hCas9.

   IVThCas9_F:

   5′-GCGTAATACGACTCACTATAGGGAGAATGGACAAGAAGTACTC-3′.

   IVThCas9_R:

   5′-AGAGTCGCGGCCGCTCAC-3′
2. Amplify the template DNA for in vitro transcription. Add the following to a 0.1 mL plastic tube on ice: 0.2 ng of hCas9 expression vector, 2.5 μL of 10 μM forward primer (final, 0.5

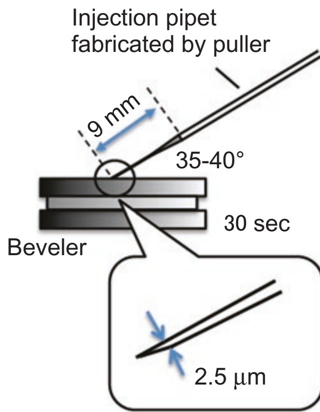
μM), 2.5 μL of 10 μM reverse primer (final, 0.5 μM), 10 μL of 5X Q5 Reaction Buffer, and 0.5 μL of Q5 Hot Start High-Fidelity DNA Polymerase. Adjust volume to 50 μL with nuclease-free water. Perform PCR under the following conditions: 98 °C for 30 s; 20–25 cycles of 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 4.5 min.

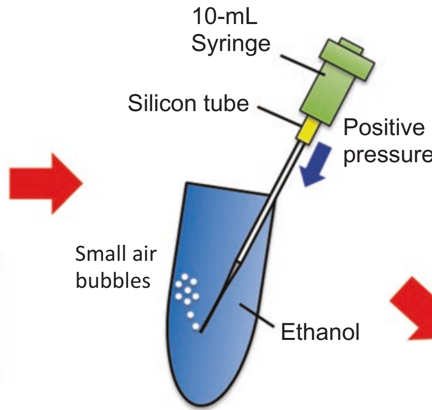
3. Purify PCR products using the QIAquick PCR Purification Kit.
4. Perform in vitro transcription of hCas9 by mMESSAGE mMACHINER T7 ULTRA Kit. Add the following to a 0.1 mL plastic tube on ice: 100–200 ng of purified PCR products, 2 μL of 10X T7 Reaction Buffer, 10 μL of T7 2X NTP/ARCA, and 2 μL of T7 Enzyme Mix. Make up to 20 μL with nuclease-free water.
5. Incubate the reaction at 37 °C for 2 h.
6. To remove the DNA template, add 1 μL of TURBO DNase, mix well, and incubate for 15 min at 37 °C.
7. For poly(A) procedure, add the following reagents to the reaction mixture: 20 μL of 5X *E*-PAP Buffer, 10 μL of 25 mM $MnCl_2$, 10 μL of ATP Solution, and 36 μL of nuclease-free water.
8. Incubate at 37 °C for 30–45 min.
9. Purify RNA using the MEGAclear Kit with RNase-free water.
10. Calculate the concentration of Cas9 RNA and store it at −80 °C until use.

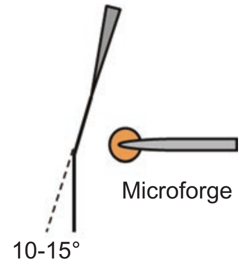
3.2 Embryo Collection and Culture

1. Induce female mice (*see* **Note 2**) to superovulate by the injection of 5 U of PMSG, followed by 5 U of hCG 48 h later (*see* **Note 3**). After injection of hCG, mate females overnight with males of the same strain. The following morning, check for the presence of a vaginal plug to determine whether mating was successful (*see* **Note 4**).
2. At 21 h after hCG injection, sacrifice female mice, dissect out the oviducts.
3. Transfer the oviducts into a 60 mm Petri dish containing M2 medium at room temperature, and remove the blood by shaking the dish.
4. Transfer all oviducts to a new 60 mm Petri dish containing M2-hyaluronidase.
5. Cut the oviduct ampulla using a 27-G hypodermic needle on a 1 mL syringe, and gently squeeze out the cumulus–oocyte complex into M2-hyaluronidase medium.
6. Incubate at 37 °C for several minutes until the cumulus cells fall off.

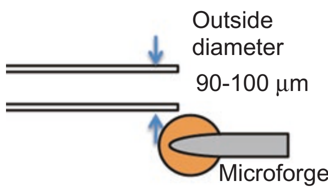
A. Microinjection pipet







B. Holding pipet



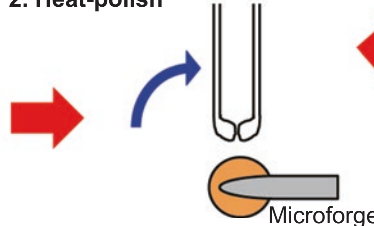


Fig. 1 Preparation of microinjection and holding pipets. (**a**) Microinjection pipet. (**b**) Holding pipet

7. Collect the fertilized eggs using an embryo transfer pipet, and transfer to a fresh dish containing M2 medium. After washing the eggs three times, culture in M16 medium at 37 °C under 5% CO_2 in air until microinjection.

3.3 Cytoplasmic Microinjection

3.3.1 Preparation of Microinjection Pipet

The microinjection pipet for injecting Cas9/gRNA into eggs is produced as shown in Fig. 1a.

1. Parameters such as filament temperature and pulling force should be optimized before the experiment. Pull thin-walled capillary tubes using a pipet puller (*see* **Note 5**).
2. Set a micropipet on the beveler at 35–40°, and grind the tip for 30 s.
3. To remove any residual particles on or in the pipets, wash them in ethanol while maintaining positive pressure using a syringe. Penetration of the micropipet can be confirmed by the appearance of small air bubbles from the end.
4. Bend the tip of the pipet at 10–15° using a microforge.

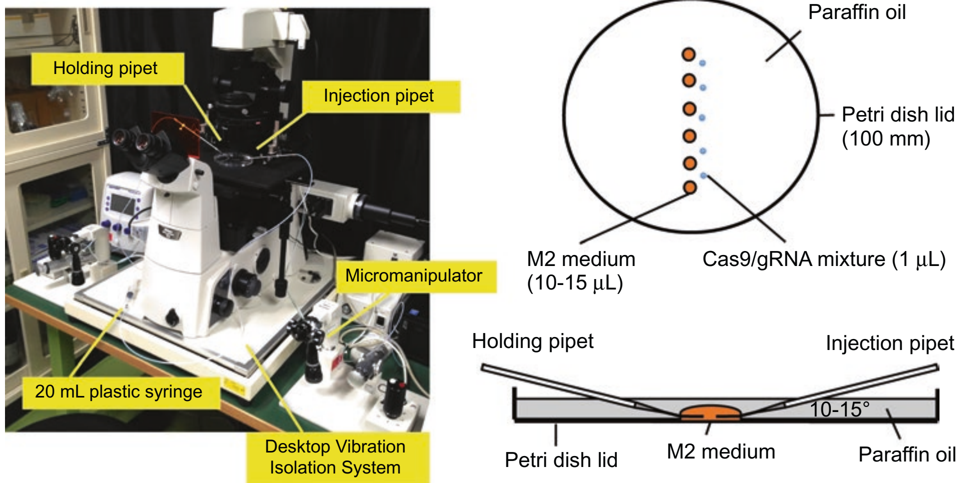


Fig. 2 Arrangement of microscope, micromanipulators, and injection chamber for cytoplasmic injection

3.3.2 Preparation of Holding Pipet

The holding pipet for fixing the egg in position to allow microinjection is produced as shown in Fig. 1b.

1. Pull the non-filament glass tubes as described above for the microinjection pipets.
2. Set the pipet horizontally on the glass ball of the microforge, and cut the end at a width of 90–100 μm wide by flash-heating of the microforge (*see* **Note 6**).
3. Set the pipet vertically on the microforge, and polish the broken end by careful heating.
4. Bend the tip of the pipet at 10–15° on the microforge.

3.3.3 Cytoplasmic Injection

1. Set up the micromanipulation system as shown in Fig. 2 (*see* **Note 7**).
2. In vitro transcribed Cas9 mRNA (50–100 ng/μL) and gRNA (10–50 ng/μL) are generally used for cytoplasmic injection of fertilized eggs. The Cas9/gRNA cocktail should be prepared just before microinjection or stored at −80 °C without repeated freezing and thawing (*see* **Note 8**).
3. We show the setup using the lid of a 100 mm plastic dish, which is used for Hoffman optics (*see* **Note 9**). Make several flat M2 drops (10–15 μL) on the center of the lid, and then place Cas9/gRNA drops (1 μL) to the lower right of the M2 drops (Fig. 2) and cover them with paraffin oil.
4. Set up an injection chamber on the microscope stage. Insert both holding and injection pipets into pipet holders at a slight angle (10–15°) to allow the tips to reach the bottom of the dish (Fig. 2).

5. Move the tip of an injection pipet into a Cas9/gRNA drop, and fill the tip with Cas9/gRNA by maintaining negative pressure on the syringe for a few minutes.
6. Move the tip of the injection pipet into paraffin oil, and firmly push the piston of the syringe to confirm that the injection pipet is not closed (Fig. 3a, arrow).

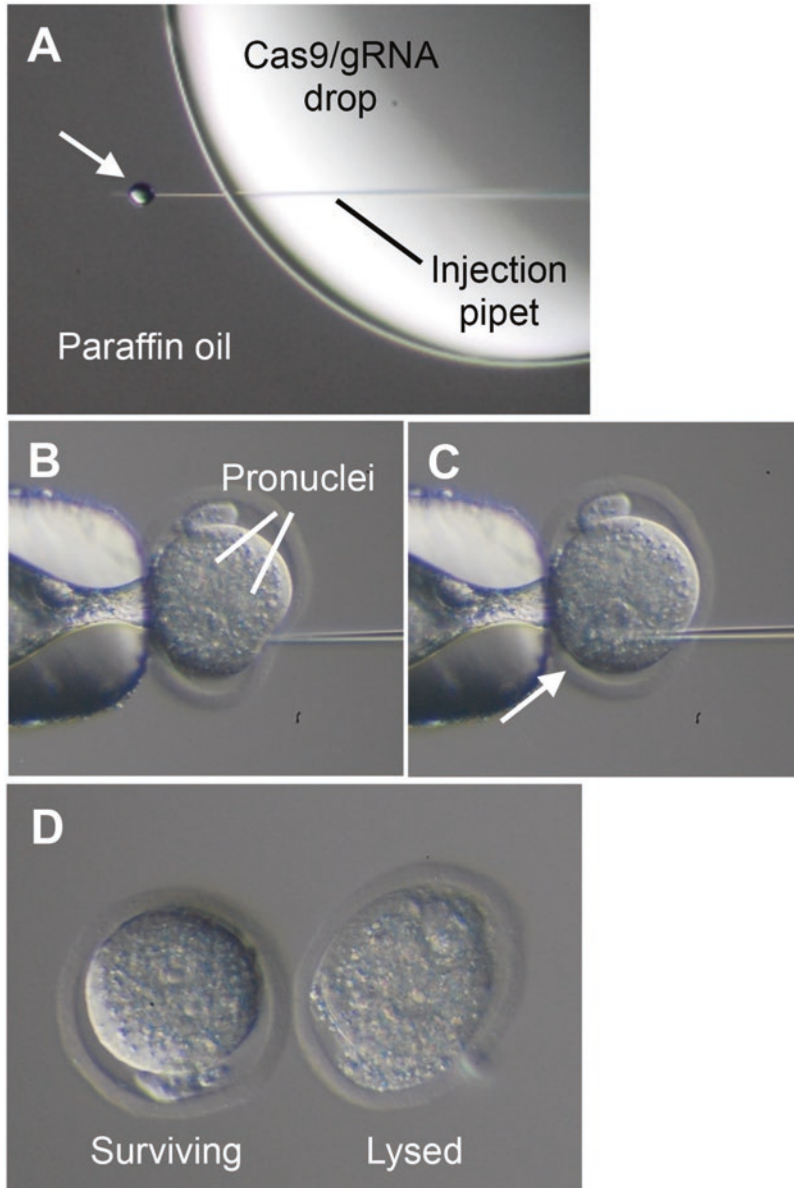


Fig. 3 Procedure for cytoplasmic injection. (**a**) Make sure that the injection pipet is not closed by confirming release of Cas9/gRNA buffer (*arrow*) from the end of the injection pipet. (**b**) Carefully insert the injection pipet into the cytoplasm, taking care not to hurt the two pronuclei. (**c**) If Cas9/gRNA is successfully injected into cytoplasm, cytoplasmic granules around the tip of the pipet will move, and the transparency of the surrounding area will change (*arrow*). (**d**) Surviving eggs exhibit a distinct outline compared with lysed eggs

7. Using an embryo transfer pipet, transfer 20–30 eggs into the M2 drop in the chamber.
8. Place the holding pipet next to an embryo and apply gentle vacuum to affix it to the tip of the pipet.
9. Keeping the holding pipet steady, adjust the focus of the microscope to confirm the position of the pronuclei. Then, focus the microscope on the outline (plasma membrane) of the egg.
10. Squeeze firmly on the syringe just before injection to flush out M2 medium that may have entered the injection pipet, and maintain positive pressure.
11. Carefully insert the injection pipet into the cytoplasm, taking care not to hurt the pronuclei (Fig. 3b) (*see* **Note 10**).
12. After successful penetration of the membrane, Cas9/gRNA will be spontaneously released into the cytoplasm of the egg because the injection pipet is kept at positive pressure. If the cytoplasmic granules around the tip of the pipet move, and the transparency of the surrounding region changes obviously (Fig. 3c), you have successfully injected Cas9/gRNA (*see* **Note 11**).
13. Once Cas9/gRNA is released, quickly pull the injection pipet out of the egg.
14. Release the egg from the holding pipet, and place the injected egg into a separate area of the M2 drop.
15. Pick up a new egg, and perform injection in the same manner. The injection pipet can be used continuously until (1) the injection pipet is unable to penetrate plasma membrane smoothly; (2) two eggs serially lyse immediately after injection; or (3) the injection pipet clogs or breaks.
16. When all the eggs in the drop have been injected, put them back into an M16 drop at 37 °C, and transfer new eggs into the M2 drop in the injection chamber.
17. After injection of all the eggs is completed, incubate them at 37 °C for an additional 30 min.
18. Separate surviving eggs from any that have lysed. Surviving eggs have a distinct outline compared with lysed eggs (Fig. 3d). Culture eggs in M16 medium at 37 °C under 5% CO_2 in air until embryo transfer or other manipulations.

3.4 Embryo Transfer

1. For the production of mutant mice, transfer 1- or 2-cell embryos into the ampulla of the oviduct (10–20 embryos per oviduct) of pseudopregnant foster females at 0.5 days post coitum (dpc) (*see* **Note 12**).

2. Pups will be born at 19.5 dpc.
3. Extract genomic DNA from the tail tips of 3-week-old mice.

3.5 Assay for Genome Modification

1. To detect small genomic modifications, perform PCR using primers flanking the targeted region. For the first screening, PCR products may be digested with restriction enzyme, which cleaves at the Cas9 target site of the nonmodified but not of the modified genomes (*see* **Note 13**). Analyze PCR products by gel electrophoresis.
2. Clone PCR products derived from mutant alleles into a TA-cloning vector and sequence eight or more clones of each mutant allele (*see* **Note 14**).

4 Notes

1. Optimum annealing temperature depends on the target sequence. If PCR products are not well amplified, change the annealing condition.
2. We usually use C57BL/6 J mice for strain establishment and B6D2F1 for the consideration of genome editing conditions. The main advantage of using hybrid mice is the high developmental rate of embryos both in vitro and in vivo.
3. In our lab, we inject ten females with PMSG and hCG at 3:00–4:00 PM (assuming a light period of 8:00 AM–8:00 PM).
4. Vaginal plugs should be checked in the early morning because they are expelled over time.
5. This microinjection pipet may be used without any modifications; however, further modifications such as micropipet beveling increase the survival rate of the eggs.
6. Be sure not to melt the pipet.
7. For holding and injection pipet control, we typically use pneumatic or oil microinjectors constructed with a glass or metal gasket syringe. Electric microinjectors, which make it possible to inject very small volumes of liquid with great precision, are also available for injection pipet control. Alternatively, one can use disposable plastic syringes, the most economical choice. We usually use 10–20 mL plastic syringes for injection pipet control because high pressure can be rapidly delivered to the needle tip to drive DNA/RNA into eggs.
8. RNA solution can be stored at −80 °C for up to a year. Do not repeat freezing and thawing, which promote degradation of RNA. It is also important to use RNase-free water to dilute

the Cas9/gRNA mixture. However, do not use diethylpyrocarbonate (DEPC)-treated water, which is toxic to embryos.

9. Microinjection is generally performed using an inverted microscope with Hoffman modulation contrast optics or Nomarski differential interference contrast optics. A microscope with phase contrast can also be used, but on such instruments it is difficult to identify the location of pronuclei. In general, Nomarski optics are used with a glass injection chamber. However, a recycled chamber is not recommended because it may be contaminated with RNase, which promotes degradation of Cas9 mRNA and gRNA. By contrast, Hoffman optics can be used with disposable plastic dishes, which are free of RNase contamination.
10. In many cases, the injection pipet may be inserted into the boundary of eggs, where pronuclei are not located. Compared with pronuclear injection, cytoplasmic injection is more difficult, because the plasma membrane is more elastic than the nuclear membrane and achieving penetration is more challenging. If you cannot penetrate the membrane, repeat the insertion a few times or change the insertion point. The use of injection pipets with sharp tips improves the success rate.
11. If apparent expansion of the egg is observed, the injection volume is too high.
12. It is better to transfer the manipulated embryos to oviducts as soon as possible after microinjection. Alternatively, embryos can be cultured until the blastocyst stage to determine the developmental rate in vitro or genome editing efficiency.
13. Alternatively, editing efficiency can be assessed using the surveyor nuclease assay, an enzymatic mismatch cleavage assay used to detect single base mismatches or small insertions or deletions.
14. On some occasions, more than two mutant alleles are identified in an individual mouse. This is caused by somatic mosaicism in mice generated by CRISPR/Cas injection [6]. Therefore, sequence analyses of mutant alleles should be performed using eight or more clones. In addition, the mutation profile of the germline in founder (F0) mice is sometimes different from that of the somatic cells used for genotyping; therefore, sequences of the mutant alleles should be checked again in F1 offspring.

Acknowledgment

We thank Dr. Yuji Arai (National Cerebral and Cardiovascular Center) for technical advice on microinjection, and Mika Kimura (Gunma University) for proofreading of the section describing

in vitro transcription of RNA. This work was supported by the Basic Science and Platform Technology Program for Innovative Biological Medicine from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (MEXT) and the Agency for Medical Research and Development (AMED) of Japan.

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Chapter 6

Genome Editing in Mouse Zygotes and Embryonic Stem Cells by Introducing SgRNA/Cas9 Expressing Plasmids

Taichi Noda, Asami Oji, and Masahito Ikawa

Abstract

In mammalian cells, genome editing with the single guide RNA (sgRNA)/Cas9 complex allows for high targeting efficiency within a relatively short time frame with the added benefits of being low cost and easy to design. sgRNA/Cas9-mediated editing in mouse zygotes has accelerated the analysis of gene functions and the generation of mouse models of human diseases. Despite the benefits, this method still suffers from several problems, such as mosaicism in the founder generation which complicates genotyping and phenotypical analyses, and the low efficiency of more complicated genome editing. Thus, we recently established the system for genome editing in embryonic stem (ES) cells and its application for chimeric analysis in mice. In this section, we introduce the procedure for genome editing in mouse zygotes and ES cells.

Key words CRISPR/Cas9, Microinjection, Transfection, Indel, Knockin, Chimeric analysis

1 Introduction

The genetically modified mice has contributed greatly to the study of gene functions [1]. However, the production of mutant mice using the conventional method (*e.g.*, gene targeting) requires several steps, such as constructing a targeting vector, the establishment of ES clones with the desired mutation, the production of chimeric mice, and confirming germline transmission of the desired mutation. The conventional method can take more than 1 year to obtain mutant mice in many cases. Also, specialized, laborious, and costly requirements of the conventional method limited the number of laboratories that were able to take advantage of this method.

The establishment of the CRISPR/Cas9 system opened a new era for mammalian genome editing. It enabled researchers to generate genetically modified mice in a short time, at low cost, and with simplicity in designing single guide RNAs (sgRNA). Insertion/deletion mutations (indel), point mutations, and epitope tagging (*e.g.*, Flag, His) were efficiently induced by the expression of the sgRNA/Cas9 complex in mouse zygotes [2–5]. In fact,

Izuho Hatada (ed.), *Genome Editing in Animals: Methods and Protocols*, Methods in Molecular Biology, vol. 1630,
DOI 10.1007/978-1-4939-7128-2_6, © Springer Science+Business Media LLC 2017

our laboratory has succeeded in creating more than 200 mouse lines within 3 years [6–8]. Meanwhile, the low efficiency of complicated genome editing [*e.g.*, large deletions and knockins (KI)] still requires many mice to be sacrificed [8]. Later experiments of Cas9-mediated genome editing in mouse zygotes revealed the prevalence of mosaicism in the founder generation, which complicated the genotyping and phenotypical analysis [5, 9]. To reduce these problems, we recently established genome editing in ES cells and demonstrated its application in the chimeric analysis in mice [8]. In this section, we describe a procedure for genome editing in mouse zygotes and ES cells by inducing the sgRNA and Cas9 expression from introduced plasmids. We summarize the benefits and drawbacks of our method to allow researchers to determine the most appropriate method for their genome editing experiments (Fig. 1).

2 Materials

2.1 Construction of pX330 and pX459 Plasmids

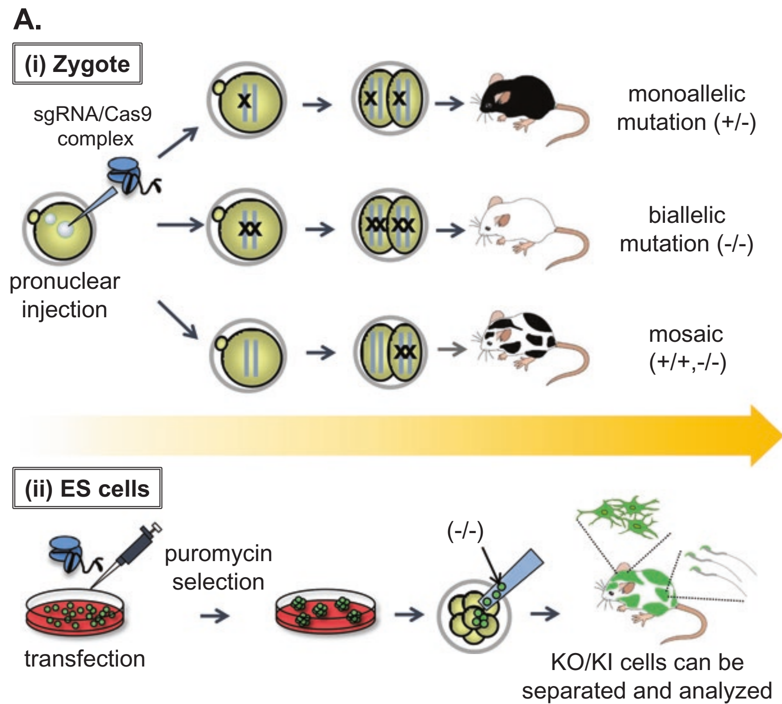
1. pX330 plasmid expressing sgRNA and humanized Cas9 (Addgene #42230, https://www.addgene.org/42230/).
2. pX459 plasmid expressing sgRNA, humanized Cas9, and puromycin resistant gene (Addgene #62988, https://www.addgene.org/62988/).
3. pCAG-EGxxFP reporter plasmid (Addgene #50716, https://www.addgene.org/50716/).
4. Restriction enzymes.
5. A pair of oligonucleotides against target DNA sequence containing a *Bbs*I site (forward: 5′-cacc + 20mer-3′; reverse: 5′-aaac + 20mer-3′).
6. DNA ligation kit.
7. Competent cells (*e.g.*, DH5α).
8. DNA purification kit.

2.2 Construction of the Targeting Vector

1. Cloning plasmid vector [*e.g.*, pBluescript II SK (+)].
2. Other regents required for the cloning were indicated in Subheading 2.1.

2.3 Assessment of sgRNAs

1. Any culture cells of interest (*e.g.*, HEK293T cells).
2. Cell culture medium for HEK293T: FCS [10% (v/v) final conc.] and Penicillin–Streptomycin–Glutamine solution (0.5 mg/mL final conc.) in DMEM medium.
3. Transfection reagents [*e.g.*, 2× BES buffered solution (BBS): 280 mM NaCl, 50 mM BES, and 1.5 mM Na_2HPO_4, pH 6.95, store at 4 °C. 2.5 M $CaCl_2$, store at −20 °C].
4. Gibco distilled water (Thermo Fisher, #15230147).



B.

	Zygotes	ES cells
Gene knockout		
Indel efficiency	53%[†]	91%[¶]
Biallelic mutation	24%[†]	73%[¶]
Genotyping of F0 mice	variegated	predictable
Complicated gene editing		
Large deletion	10%[¶]	27%[¶]
Point mutation/Tag insertion	4%[¶,*]	41%[¶,‡]
KI	2%[¶,§]	8%[¶,§]
Time required	> 1 month	> 2 months

Fig. 1. Experimental designs for genome editing in mouse zygotes and ES cells. Panel (**A**): (*i*) Scheme of genome editing in mouse zygotes. The sgRNA/Cas9 complex was injected into a pronucleus of zygotes. If the monoallelic (+/−) or biallelic (−/−) mutation is induced at the 1-cell stage, all founders will carry the same mutation. However, if the mutation is induced after the 2-cell stage, the founder generation will be mosaic. (*ii*) Scheme of genome editing in ES cells. The sgRNA/Cas9 complex was transfected into GFP-tagged ES cells. After puromycin selection, ES cells with the desired mutation are injected into 8-cell embryos. The mutant cells can be separated and analyzed (*see* Fig. 3C). “X” shows the mutation. (modified from [8]). Panel (**B**): Benefits and drawbacks of genome editing in zygotes and ES cells. [†]Data from the previous paper [13]. [¶]Data from the previous paper [8].[*,‡]ssODN (shown as *) and dsDNA (shown as ‡) were used to induce the point mutation and tag insertion. [§]0.5 kb homology arms were used for the KI

2.4 Pronuclear Injection in Mouse Zygotes

1. Hormones for superovulation (*e.g.*, PMSG, HCG).
2. Embryo culture reagents (nontreated tissue culture dish, medium for embryo culture or handling such as FHM and KSOM, and mineral oil).
3. DNA dilution buffer ($T_{10}E_{0.1}$ buffer: 10 mM Tris–HCl, 0.1 mM EDTA, pH 7.4. Filtered with a hydrophilic 0.2 μm PVDF membrane and store at −30 °C.).
4. Single-stranded oligonucleotides (ssODN).
5. Lysis buffer for tail tip [*e.g.*, 20 mM Tris–HCl (pH 8.0), 5 mM EDTA, 400 mM NaCl, and 0.3% SDS. Store at −20 °C. Add 200 μg/mL Proteinase K solution before using.].
6. Micromanipulator.

2.5 Transfection and Genotyping of ES Cells

1. Gelatin-coated cell culture plate: To make 0.1% gelatin solution, dissolve 0.5 g gelatin from porcine skin in 500 mL sterile distilled water. Mix and autoclave. Add a suitable volume of the gelatin solution to each well of the culture plate [1 mL for 6-well plate; 0.5 mL for 24-well plate; 100 μL for 96-well plate], and incubate for 2 h. Remove the solution, dry overnight, and store at room temperature (RT).
2. Feeder cell medium (FM): Add 5 mL Sodium Pyruvate Solution (1 mM final conc.), 5 mL MEM Non-Essential Amino Acids Solution (0.1 mM final conc.), and 5 mL Penicillin–Streptomycin–Glutamine solution (0.5 mg/mL final conc.) in 435 mL DMEM. Add 50 mL Fetal Bovine Serum (FBS) into the medium for a final concentration of 10%. Store at 4 °C. After the addition of FBS, use within 1 month.
3. KnockOut ES cell medium (KOESM): Add 5 mL Sodium Pyruvate Solution (1 mM final conc.), 5 mL MEM Non-Essential Amino Acids Solution (0.1 mM final conc.), 5 mL Penicillin–Streptomycin–Glutamine solution (0.5 mg/mL final conc.), 0.5 mL 2-Mercaptoethanol (0.1 mM final conc.), and 5 mL 100× Nucleosides solution (3 mM final conc. for Adenosine, 3 mM final conc. for Guanosine, 3 mM final conc. for Uridine, 3 mM final conc. for Cytidine, and 1 mM final conc. for Thymidine) into 480 mL KnockOut™ DMEM. Aliquot 200 mL of the medium to sterilized bottles and add 50 mL FBS (20% final conc.), add 50 μL mouse LIF (1000 U/mL final conc.), and store at 4 °C. After the addition of FBS and LIF, use as soon as possible (~2 weeks).
4. Phosphate-buffered saline (PBS) without calcium and magnesium.
5. Trypsin–EDTA solution: Dilute Trypsin to 0.25% with 1 mM EDTA/PBS. Store at 4 °C.

6. Freezing medium: Mix 5 mL Dimethyl sulfoxide (DMSO) with 20 mL KOESM and store at −20 °C.
7. Mouse embryo fibroblast (MEF) cells [10]: Euthanize a pregnant female (13.5-day postcoitus). Dissect out the uterus with embryos, and place in a 100 mm culture dish with PBS (*see* **Note 1**). Release the embryos and placentas from the uterus. Remove heads and internal organs from all embryos. Mince the fetus with a pair of scissors, dissociate cells with 0.25% trypsine–EDTA solution (about 4 embryos/mL) for 5–10 min at 37 °C. After inactivating the trypsin by adding 10 times volume of FM, pipette well and seed a cell suspension in a dish (about 5 embryos/15 cm dish). Change culture medium after 4–5 h of seeding to remove unattached cells, and let MEF cells grow to ~90% confluence. Cryopreserve the MEF cells (cells from a 100 mm plate/tube) in freezing containers (Thermo Fisher, #5100-0001) and store at −80 °C.
8. Mitomycin C (MMC) treatment of MEF cells [10]: Thaw and expand the MEF cells to confluent state (*see* **Note 2**). Add MMC (10 μg/mL final conc.) and incubate for 2 h at 37 °C. After trypsinization, collect cells, centrifuge, and suspend the pellet with 10% DMSO/FM (1×10^7/tube). Cryopreserve the cells as mentioned above and store at −80 °C for short storage (for several months) or −150 °C for long storage (for years).
9. ES cells: The following ES cell lines are available through Riken Cell Bank (http://cell.brc.riken.jp/en/) [*e.g.*, EGR-G101 (cell# AES0182): C57BL/6NCr-Tg(CAG/Acr-EGFP, EGR-G01 (cell# AES0183): 129S2 x C57BL/6NCr-Tg(CAG/Acr-EGFP), EGR-101 (cell# AES0184): C57BL/6NCr] [11].
10. Transfection reagents [*e.g.*, Lipofectamine® LTX & PLUS™ and Opti-MEM® (Thermo Fisher, #15338-100 and #31985-062, respectively)].
11. Puromycin (P)-KOESM: Dilute 10 mg/mL puromycin to 0.1 μg/mL with KOESM, and store at 4 °C.

2.6 Preparation of ES Cells for 8-Cell Injection

1. Materials required for the preparation of ES cells for 8-cell injection were shown in Subheading 2.5.

2.7 Microinjection of ES Cells

1. PVP medium: Desolve 12% (w/v) polyvinylpyrrolidone (Sigma-Aldrich, #PVP360) in HEPES-buffered CZB at 4 °C overnight. Filter with a hydrophilic 0.2 μm PVDF membrane and store at −30 °C.
2. Medium and regents required for the injection were shown in Subheading 2.4.

3 Method

Carry out all procedures at RT unless otherwise specified.

3.1 Construction of CRISPR/Cas9 and Reporter Plasmids

Details have been reported previously [12].

1. Design sgRNAs using CRISPRdirect (https://crispr.dbcls.jp/). Choose several sgRNA sequences with fewer perfect matches of 12 nucleotides at the 3′ terminus of the sgRNA to suppress off-target effects.
2. Prepare a pair of oligonucleotides including a target DNA (20 nucleotides) with *Bbs*I cohesive ends (*see* Subheading 2.1). Do not include a PAM sequence or polyT tracks (TTTT may terminate sgRNA production) in the sgRNAs. Mix 1 μL of 50 μM forward and reverse oligonucleotides with 48 μL sterile distilled water, and anneal them using a thermal cycler as follows: 95 °C for 5 min; 65 °C for 5 min; RT for more than 60 min.
3. Digest pX330 or pX459 plasmids with *Bbs*I for several hours or overnight, and detect the digested plasmids by an agarose gel electrophoresis. Purify the linearized plasmid from the gel using DNA purification kit.
4. Ligate the annealed product for sgRNA (*see* **step 2** of Sub heading 3.1) with *BbsI* digested pX330/px459 plasmids (*see* **step 3** of Subheading 3.1), transform *E. coli* competent cells, and select ampicillin-resistant clones on a LB plate containing 50 μg/mL ampicillin. Pick up several individual colonies and culture overnight in liquid phase, then purify plasmid using DNA purification kit.
5. To construct the reporter plasmid, amplify 200–600 bp region including the sgRNA target site and place it between EGFP fragments in the pCAG-EGxxFP using restriction enzyme sites (*Bam*HI, *Nhe*I, *Pst*I, *Sal*I, *Eco*RI, and *Eco*RV) [13]. Primers used for this construction can be used for later genotyping assay.
6. Confirm the inserted sequence in each plasmid by Sanger sequencing with a primer (5′-TGGACTATCATATGCT TACC-3′) within the U6 promoter region for pX330 and pX459. For pCAG-EGxxFP reporter plasmid sequencing, the primers to amplify the target region can be used.

3.2 Construction of the Targeting Vector

1. Amplify the 5′- and 3′-homology arms and KI sequence by PCR. Add an appropriate restriction enzyme site at the end of the fragments for easy construction. Place the fragments in the pBluescript II SK (+) plasmid as described in **step 4** of Subheading 3.1. Homology arms greater than 0.5 kb have

higher targeting efficiency (1–2 kb arms are recommended). Recent In-fusion approach also works well if restriction enzyme sites are not available.

2. Transform the targeting vector plasmid into competent cells and plate them onto LB plate with ampicillin as described in **step 4** of Subheading 3.1.
3. Pick up colonies, expand, and purify the targeting vector using DNA purification kit and confirm the inserted sequence in the targeting vector by sequencing.

3.3 Assessment of sgRNAs

1. Design several sgRNAs as the cleavage efficiency largely differs among sgRNAs. To assess sgRNA activity, transfect HEK293T cells with the sgRNA/Cas9 expressing plasmid and pCAG-EGxxFP reporter plasmid containing the sgRNA target region [12, 13]. Before approximately 6 h of transfection, add 2 mL cell culture medium containing 1×10^6 cells in a well of 6-well plates. Mix 90 μL distilled water and 10 μL of 2.5 M $CaCl_2$, and then add 1 μg each of the pX330/pX459-sgRNA and pCAG-EGxxFP-target in the $CaCl_2$ solution. Dilute the DNA mixture with 100 μL 2× BBS and vortex it every 5 min. After 10 min of incubation at RT, add the mixture in each well, joggle the dish to spread the DNA mixture, and incubate HEK293T cells in an incubator at 37 °C, 5% CO_2. Next day, change the cell culture medium. After 48 h of transfection, observe GFP fluorescence under a microscope. Higher GFP fluorescence corresponds to sgRNA with higher cleavage efficiency (*see* **Note 3**).

3.4 Pronuclear Injection in Mouse Zygotes

Details have been reported previously [14].

1. Inject 5 IU of pregnant mare serum gonadotropin (PMSG) in the abdominal cavity of adult B6D2F1 females. After 48 h, inject 5 IU of human chorionic gonadotropin (hCG), and cage with stud males. After 14–16 h of the hCG injection, check the copulatory plug in a vagina indicating the successful mating. At 0.5 embryonic day (ED), perform the euthanasia and collect oviducts from these females. Place the oviducts in the drop of 100 μL KSOM covered with oil, tear the ampulla of the oviduct using tweezers to collect eggs, and remove the cumulus cells using Hyaluronidase (300 μg/mL final conc., Sigma-Aldrich) at 37 °C, 5% CO_2 for 5 min (*see* **Note 4**). Use KSOM for embryo culture and FHM for handling embryos outside the CO_2 incubators.
2. Dilute the pX330 plasmid with $T_{10}E_{0.1}$ buffer to a final concentration of 5 ng/μL (for indel) or 10 ng/μL (for KI). Remove impurities in the solution with a spin column from the DNA purification kit. Use the purified solution for the subsequent

steps. For KI, mix an equal volume of 10 ng/μL pX330 and 20 ng/μL ssODN (5 ng/μL final conc. pX330 and 10 ng/μL final conc. ssODN) (*see* **Note 5**).

3. Perform pronuclear injection as shown in the text book [14]. In brief, load several μL of the DNA solution into an injection pipette, and equip a micromanipulator with the pipette. Inject the DNA solution in a pronucleus using a micromanipulator, and incubate the eggs in KSOM at 37 °C with 5% CO_2 (Fig. 2). Transfer the injected eggs into the ampulla of pseudopregnant females (0.5-day postcoitus). The eggs can be transferred at the day of injection (single cell stage) or the next day (2-cell stage).
4. After 19 days, obtain pups by natural birth or cesarean section and check the genotype of these mice (*see* **Note 6**).

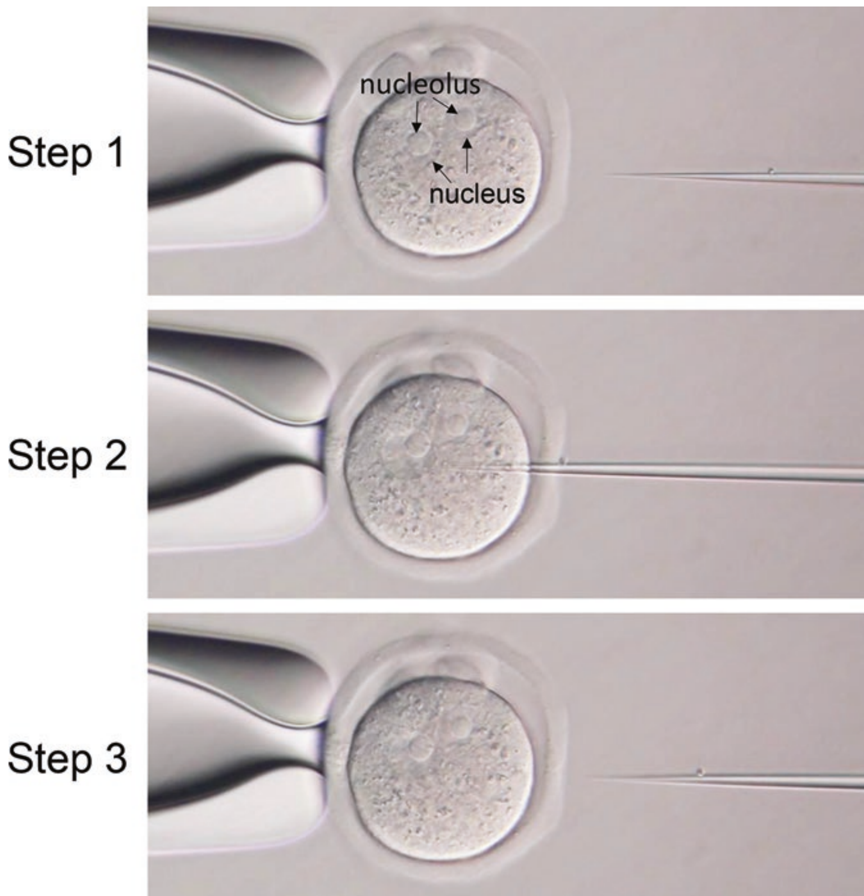


Fig. 2 Injection of the sgRNA/Cas9 complex into a pronucleus. After holding an egg and focusing on the nuclear membrane (*Step 1*), the injection pipette with DNA solution was injected into a pronucleus, and then a small volume of the solution was added (*Step 2*). During the injection, the pronucleus expands (*Step 2*). The injection pipette is then quickly removed (*Step 3*)

3.5 Transfection and Genotyping of ES Cells

Carry out all procedures under sterile conditions, unless otherwise specified.

1. Thaw and seed MMC-MEF cells in a gelatin-coated 6-well plate with FM at least 1 day before seeding ES cells. Thaw a frozen tube containing ES cells quickly, and add 10 times volume of KOESM. Mix well, centrifuge at 500 × *g* for 5 min, and remove the supernatant. After suspending with KOESM, seed 1×10^5 ES cells/well onto the MMC-MEF layer, and incubate at 37 °C with 5% CO_2 (Fig. 3A).
2. Remove KOESM when ES cells reach to ~90% confluency. After washing with PBS, add 200 μL trypsin–EDTA solution, and incubate at 37 °C for 5 min. Add 800 μL KOESM and dissociate ES cells into a single cell suspension by gently pipetting. After 5 min of centrifugation at 500 × *g*, suspend the ES cells and seed into a new 6-well plate (1×10^5 cells/well) with an MMC-MEF layer.
3. For transfection, seed 1×10^5 (B6 background) or 1×10^4 (129 × B6 background) ES cells into a gelatin-coated 6-well plate with an MMC-MEF layer, and incubate at 37 °C, 5% CO_2 for 6–8 h (*see* **Note 7**).
4. Prepare the DNA mixture for transfection. Dissolve 1 μg pX459 (and 1 μg of a targeting vector for homologous recombination) in 10 μL DNase/RNase-free distilled water, and mix with 113 μL Opti-MEM. After mixing well, add 2 μL PLUS reagent (contents of Lipofectamine LTX & PLUS), and gently mix (A solution, *see* **Note 8**). Dilute 5 μL LTX reagent (contents of Lipofectamine LTX & PLUS) with 120 μL Opti-MEM in another tube (B solution). Mix the A and B solution and gently invert. After 5 min at RT, add the mixture into the well of ES cells, and incubate at 37 °C in a 5% CO_2 incubator.
5. Replase the medium with P-KOESM after 14–18 h of transfection. Change P-KOESM every day.
6. To select transiently transfected cells, replace P-KOESM with KOESM after 2–3 days of drug selection (2 days for B6 background and 3 days for 129 × B6 background).
7. Replace KOESM every other day for 3–4 days for recovery.
8. Wash the well with PBS, and trypsinize for 5 min at 37 °C with 200 μL trypsin–EDTA solution. Add 800 μL KOESM and pipette well to dissociate cells into single cells. Seed the ES cell suspension into a freshly prepared gelatin-coated 6-well plate with an MMC-MEF layer in various concentrations (500 and 1000 ES cells/well).
9. After 5–7 days of passage, replace KOESM with PBS, then pick up single colony with 5 μL PBS into 200 uL pipette tips. Place the colonies in PCR tubes with 10 μL trypsin–EDTA solu-

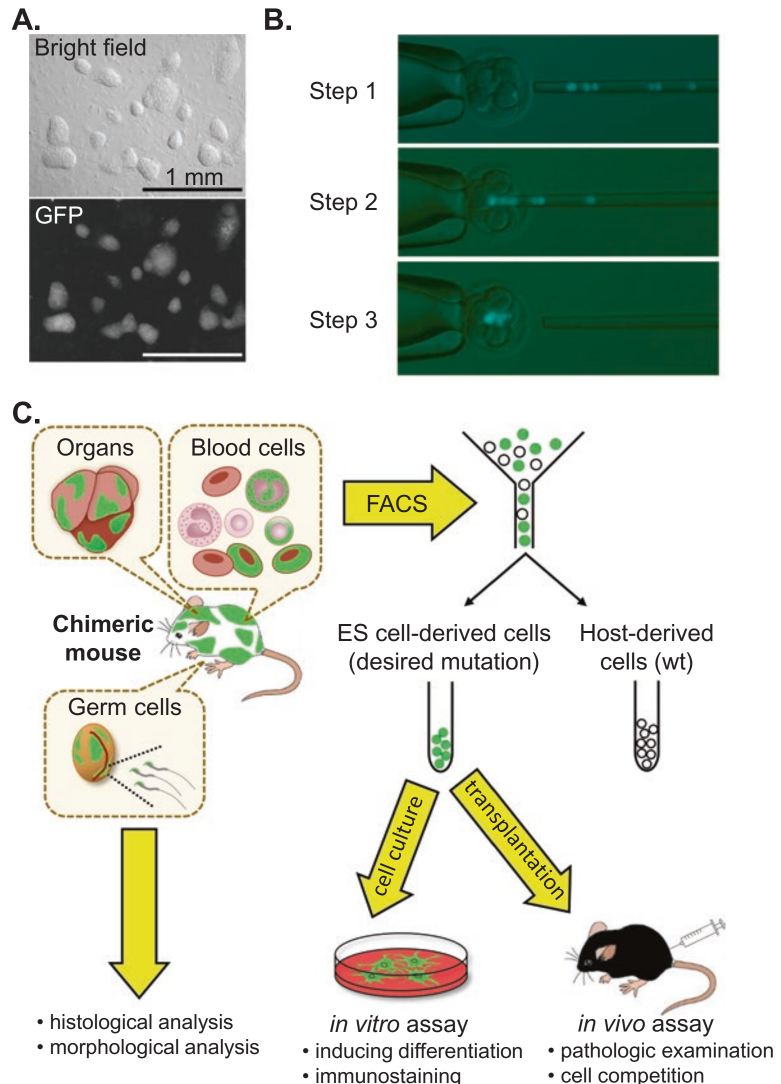


Fig. 3 Injection of GFP-tagged ES cells in an embryo and chimeric analyses. Panel (**A**): GFP-tagged ES cells were observed under a fluorescence microscopy. Panel (**B**): Six GFP-tagged ES cells were aspirated into an injection pipette (*Step 1*). A small opening within the zona pellucida was made with a short piezoelectric pulse. ES cells were injected through the opening (*Step 2*), and then the pipette was quickly removed (*Step 3*). Panel (**C**): Possible experimental applications of chimeric mice are shown. By focusing on the GFP fluorecsence or selecting GFP-positive cells with FACS, it is possible to do the histological and morphological analyses in organs and cells, and in vitro or in vivo assays

tions. Incubate for 5 min at RT (or 37 °C if applicable). Add 90 μL KOESM, and dissociate cells by pipetting. Seed the ES cell suspension from a single colony into a gelatin-coated 96-well plate with an MMC-MEF layer and 100 μL KOESM.

10. Change KOESM every other day and freeze the ES colonies when they grow to ~90% confluence. After washing wells with PBS, add 20 μL trypsin–EDTA solution and incubate for 5 min at 37 °C. Add 80 μL KOESM and mix well. Use 80 and 20 μL of the cell suspension for freezing and genotyping, respectively. For freezing, prepare 80 μL freezing medium (20% DMSO) in a noncoated 96-well plate without MMC-MEFs. Add 80 μL of the ES cell suspension, seal the plate, and transfer into a precooled styrofoam box at 4 °C, then −80 °C freezer as soon as possible. For genotyping, add 80 μL KOESM to the remaining 20 μL cell suspension, and seed all into a gelatin-coated 96-well plate without MMC-MEFs (*see* **Note 9**).
11. Incubate the plate for genotyping at 37 °C, 5% CO_2. After 3 days of incubation, remove KOESM from each well, wash with PBS, add 50 μL lysis buffer, and incubate at 50 °C for 2 h to extract genomic DNA.
12. Purify the genomic DNA if necessary, and perform genomic PCR with primer sets to amplify the target region. Check the sequence and choose the ES clones with the desired mutations for the production of chimeric mice (*see* **Note 10**).
13. To expand the ES cell clones from the frozen stock, warm the cryopreserved plate (*see* **step 10** of Subheading 3.5) at 37 °C in an incubator supplied with 5% CO_2. Prepare 1 mL KOESM in 1.5 mL tubes, and add thawed ES cell suspension. After centrifuging at 500 × *g* for 5 min, suspend the pellet with 500 μL KOESM and seed onto a gelatin-coated 24-well plate with an MMC-MEF layer.
14. Passage ES cells to a 6-well plate for further expansion. Let the ES cells grow to ~90% confluence. Prepare three or four cryotubes for each clone with 0.5 mL of freezing medium and keep at 4 °C. After trypsinization, suspend ES cells with 2 mL KOESM, dispense 500 μL of the suspension per prepared tube, and mix well. Transfer the tubes into the precooled freezing container and place at −80 °C overnight. Store them at −80 °C for short storage (for several months) and −150 °C or liquid nitrogen for long storage (for years).

3.6 Preparation of ES Cells for 8-Cell Injection

1. Thaw and seed ES cells onto a gelatin-coated 6-well plate with an MMC-MEF layer (*see* **step 1** of Subheading 3.5), and incubate it at 37 °C, 5% CO_2.
2. Let ES cells grow to ~90% confluence, and passage one tenth of the cell suspension to another 6-well plate. Incubate for 2 or 3 days.
3. In the morning of the day of injection, trypsinize the 60–70% confluent ES cells and seed them onto a gelatin-coated 60 mm

dish (WITHOUT MMC-MEF cells). Incubate at 37 °C for 30–40 min to let MMC-MEFs settle and attach to the bottom of the dish. Collect the supernatant including ES cells into a 15 mL tube and centrifuge at 500 × *g* for 5 min. Suspend the pellet of ES cells with 0.5–1 mL KOESM. The cells can be kept on ice for several hours.

3.7 Microinjection of ES Cells

1. Inject 5 IU PMSG in the abdominal cavity of adult ICR females. After 48 h, inject 5 IU hCG, and mate the hormone-treated females with adult ICR males. Check the copulatory plug in a vagina. At ED 1.5, collect the ampulla and place them in FHM drops. Recover 2-cell stage embryo by flushing the oviduct with FHM using a flushing needle inserted into the infundibulum and then incubate these embryos in KSOM for 24 h [14]. Transfer the embryos that have developed to 8-cell or morula stage into FHM, and then store them at 4 °C to suspend further development.
2. Prepare glass pipettes for embryo holding and ES cell injection. Load FHM and Hg in holding and ES cell injection pipettes, respectively [14]. Prepare a micromanipulator equipped with these pipettes.
3. Dispense 10 μL drops of FHM and PVP onto a 60 mm dish, and cover the drops with mineral oil. Place embryos in an FHM drop and add 1–3 μL of the ES cell suspension.
4. Wash the inside of an injection pipette with PVP, and pick up 4–6 round-shaped ES cells using the injection pipette (*see* **Note 11**). Hold an embryo and make a hole in the zona pellucida with a piezoelectric pulse. Expel these ES cells in perivitelline space and remove the pipette (Fig. 3A, B).
5. Wash the injected embryos with KSOM, and incubate them in a KSOM drop at 37 °C, 5% CO_2. Select embryos that reach the blastocyst stage, and transfer them into the uterus of pseudopregnant female mice (2.5-day postcoitus).
6. Perform a cesarean section after 17 days of the embryo transfer (ED 19.5). The contribution of ES cells can be determined by GFP fluorescence (*see* **Note 12**).
7. In ES cells where biallelic-mutation was introduced, it is possible to analyze the phenotype in chimeric mice by focusing on the GFP-labeled cells [8]. ES cell-derived cells in chimeric mice can be distinguished and easily utilized for various experiments (Fig. 3C). For example, GFP-positive cells can be visualized within organs using standard cell biology techniques; in addition, these cells can be sorted by FACS and used for further in vitro and in vivo assay.

4 Notes

1. Use dissecting forceps and scissors that have been autoclaved.
2. MEF proliferation is slow and can be passaged 1/10 only for 4–5 times.
3. When high background fluorescence is observed, the inversion of the target sequence or the insertion of longer target sequence into a pCAG-EGxxFP plasmid may reduce background fluorescence.
4. When only a few females are available, we use CARD HyperOva or anti-inhibin methods [15, 16].
5. Due to the low efficiency of KI, it is better to set up ES cell transfection system as plan B [8].
6. The mutation introduced in pups can be determined by direct sequencing of amplified PCR products using genomic DNA extracted from pups. When multiple waves in chromatographs are observed, the mutant allele variant can be predicted using TIDE software (https://tide.nki.nl/) [17].
7. The growth rate of ES cells differs among various backgrounds. It is better to examine growth rates of ES cell lines before starting any experiments.
8. The DNA mixture and Opti-MEM should be mixed well using a vortex prior to adding PLUS reagent.
9. ES cells do not grow well without MMC-MEF cells. However, it is more important to minimize the contamination with MMC-MEF genomic DNA.
10. To analyze the phenotype of a targeted gene in the chimeric mice, ES cells with biallelic mutations need to be selected.
11. Round ES cells should be selected for injection. As incubation time of ES cells at RT increases, the cells become more angular. It is best to inject 20–30 embryos in one injection cycle.
12. There is variation in the ES cell contribution among obtained chimeric mice [8].

Acknowledgment

This work was supported by the MEXT/JSPS KAKENHI grants (JP16J04613 to T.N., JP15J04519 to A.O., and JP25112007 and JP25250014 to M.I.), and the Takeda Science Foundation grant to M.I. The authors would like to thank Dr. Julio Castaneda for critical reading of the manuscript.

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Chapter 7

Genome Editing in Mouse and Rat by Electroporation

Takehito Kaneko

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

Izuho Hatada (ed.), *Genome Editing in Animals: Methods and Protocols*, Methods in Molecular Biology, vol. 1630,
DOI 10.1007/978-1-4939-7128-2_7, © Springer Science+Business Media LLC 2017

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

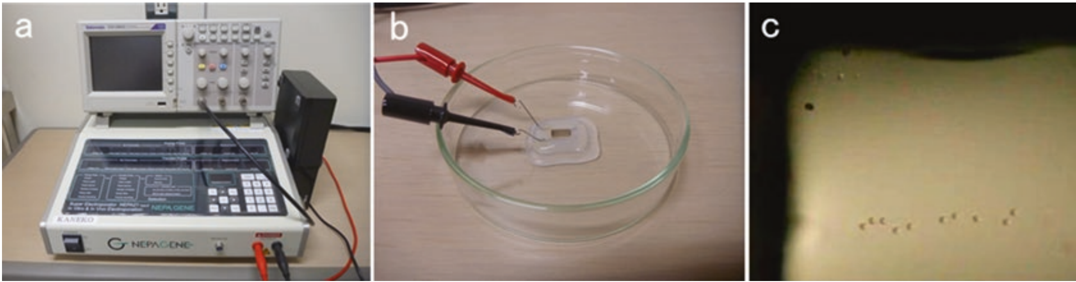

Fig. 1 (**a**) Electroporator NEPA 21. (**b**) Petri dish platinum plate electrodes. (**c**) Embryos are introduced between the electrodes filled with RNA solution

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 GENEX-TRACTOR 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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Chapter 8

Generation of Knock-in Mouse by Genome Editing

Wataru Fujii

Abstract

Knock-in mice are useful for evaluating endogenous gene expressions and functions in vivo. Instead of the conventional gene-targeting method using embryonic stem cells, an exogenous DNA sequence can be inserted into the target locus in the zygote using genome editing technology. In this chapter, I describe the generation of epitope-tagged mice using engineered endonuclease and single-stranded oligodeoxynucleotide through the mouse zygote as an example of how to generate a knock-in mouse by genome editing.

Key words Knock-in mice, Genome editing, Engineered endonuclease, Single-stranded oligodeoxynucleotide (ssODN), Zygote

1 Introduction

Genetically modified mice are useful when performing functional analysis of genes or genome-sequences in vivo. Knock-in technology can be used not only for the accurate deletion of endogenous genome regions but also for mimicking spontaneous mutations such as single-nucleotide polymorphisms (SNPs) by target nucleotide substitution. In addition, knock-in mice incorporating the reporter gene (fluorescein protein, LacZ, synthetic epitope-tag, or other marker genes) can be used for the accurate observation of endogenous gene expressions in vivo. Further, knockin can contribute to the generation of Cre expression mice and floxed mice that can be used for conditional genome modification in vivo.

The conventional knock-in method usually depends on the endogenous homologous recombination reaction using a targeting DNA template, which has flanking DNA of the target locus together with designed modified sequences in vertebrate cells [1–3]. However, other than in some cell lines such as DT40 cells, target modification by homologous recombination in vertebrate cells is known to be inefficient [4, 5]. Therefore, although the conventional knock-in method would allow a targeting DNA tem-

Izuho Hatada (ed.), *Genome Editing in Animals: Methods and Protocols*, Methods in Molecular Biology, vol. 1630,
DOI 10.1007/978-1-4939-7128-2_8, © Springer Science+Business Media LLC 2017

plate to be transfected into a large number of cells, and the modified cells picked up by drug selection, this approach cannot be applied to mammalian zygotes or oocytes because the number of cells that can be simultaneously collected and handled is limited. As an alternative method, pluripotent stem cells (such as embryonic stem cells) or cultural male germline stem cells (known as GS cells) can be used for homologous recombination-mediated generation of knock-in mice [6–8]. However, these multistep experiments (expansion of drug-selected genome-modified cells, generation of chimera/transplanted mouse, and generation of its next generation) are time-consuming, expensive, and laborious.

To overcome these limitations, genome editing technology can be used to generate a knock-in mouse zygote directly. The induction of double-stranded breaks (DSBs) at the target locus by engineered endonuclease can increase DNA repair activity in cells. As a result, homologous recombination with exogenous targeting template DNA is accelerated, and genome-modification efficiency increases [9, 10]. Although sexual maturation for mating is needed for the conventional method, zygote-mediated genome modification can produce a knock-in mouse in one generation. In addition to the zygote-mediated generation, it has been reported that commercially available single-stranded oligodeoxynucleotides (ssODNs), which can be quickly and inexpensively obtained, are available for knockin as a targeting template DNA in cellulo, including mammalian zygotes, using genome editing technology [11–22]. The use of ssODNs could help to simplify the generation of knock-in mice because the construction of the conventional targeting plasmid vectors is complicated and time-consuming. It has been reported that, by the microinjection of ssODN together with engineered endonuclease into the zygotes, various kinds of knock-in mice incorporating exogenous sequences (restriction endonuclease sites [13–15], nucleotide substitution [13, 16, 17], a loxP sequence [18, 19], and a short amino acid epitope-tag sequence [19, 20]) are generated efficiently. On the other hand, if the goal is to introduce fluorescent protein, Cre recombinase, or other longer base length sequences into the knock-in mice, these sequences cannot be covered by commercially available ssODNs because the nucleotide base of the commercially available ssODNs is limited to less than 200 nt among major suppliers. Therefore, it is necessary to prepare a plasmid DNA or amplified double-stranded DNA (dsDNA) as a knock-in template DNA. However, except in a few studies, zygote-mediated knockin has proven inefficient for homologous recombination using a dsDNA as the targeting template DNA [18, 19]. Although novel applications have been studied by many research groups to increase the efficiency of long sequence knockin using a dsDNA template, no highly reproducible method applicable to mammalian zygotes has yet been established. Recently, zygote-mediated long-sequence knockin was reported using self-synthesized ssODNs [21, 22].

In this chapter, I describe a method for generating epitope-tag knock-in mice by the microinjection of ssODN together with engineered endonuclease into mousezygotes, as an example of a knock-in method (Fig. 3). Using this method, I have generated knock-in mice with an efficiency of more than 40% [15, 16]. Almost all of the procedures are the same as those for generating knock-out mice by genome editing. The most important step in the zygote-mediated generation of knock-in mice using genome editing technology is to design the target sequence of the engineered endonuclease and the targeting template DNA (Fig. 1).

2 Materials

Other than for the ssODN preparation, all materials used are the same as the materials used for the generation of knock-out mice by cytoplasmic microinjection described in Chapter 5 (*see* **Note 1**).

2.1 ssODN

1. ssODN: Purchased as a lyophilized product. High-performance liquid chromatography (HPLC) or polyacrylamide gel electrophoresis (PAGE)-purified ssODNs are better, but reverse-phase cartridge purification is usable.
2. RNase- and DNase-free distilled water: Used for the dilution of nucleotides.

3 Methods

3.1 Design of ssODN as Knock-in Targeting DNA

The knock-in template ssODN sequence should be designed so as not to be recognized by the engineered endonuclease. If the DNA sequence of the knock-in allele would be digested by the endonuclease, knockin and indel may be induced simultaneously in the target locus (Fig. 1a). The target sequence of the endonuclease should be designed to overlap the target locus. The length of each homology arm of ssODN is about 45–60 nt (Fig. 1b) (*see* **Notes 2** and **3**).

3.1.1 For Nucleotide Substitution

The ssODN sequence and the target locus of the endonuclease should be designed carefully for the induction of nucleotide substitution by the genome editing, because the substituted nucleotide sequence is not conspicuously different from the wild-type sequence (Fig. 2). In the cases of ZFN and TALEN, the alteration site should be designed on the endonuclease recognition DNA sequence (Fig. 2a). If it is designed at the interval of the recognition sequences, the random indel may be induced together with the precise nucleotide substitution (Fig. 2b). In the case of CRISPR/Cas, even if several mismatched nucleotides exist within the recognition sequence of gRNA, the modified sequence is

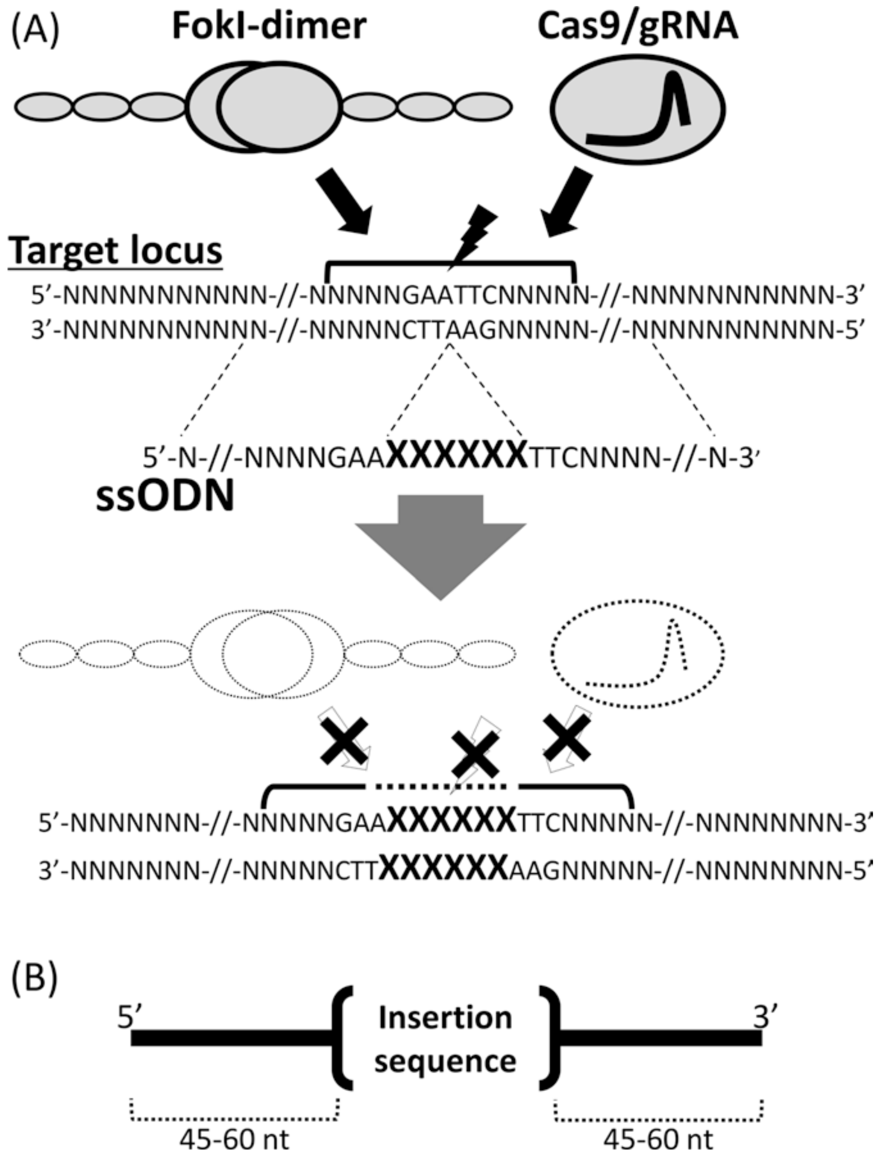

Fig. 1. The knock-in strategy by genome editing technology. (**a**) Schematics of knockin using ssODN and genome editing. Knock-in sequence (indicates as "**XXXXXX**") of ssODN is designed at the target locus of engineered endonucleases (FokI-dimerized ZFN/TALEN pairs or Cas9/gRNA complex). The modified allele should be designed so as not to be recognized by the endonucleases. (**b**) The ssODN used for zygote-mediated knockin together with the endonucleases. Two homology arms (45–60 nt) are added to each side to flank the desired insertion or mutation (indicates as "**Insertion sequence**")

recognizable as a target locus of the Cas9/gRNA complex (Fig. 2d). Therefore, the ideal design for the target nucleotide site is to be on the protospacer adjacent motif (PAM) of gRNA so that the modification disrupts the PAM sequence (Fig. 2c).

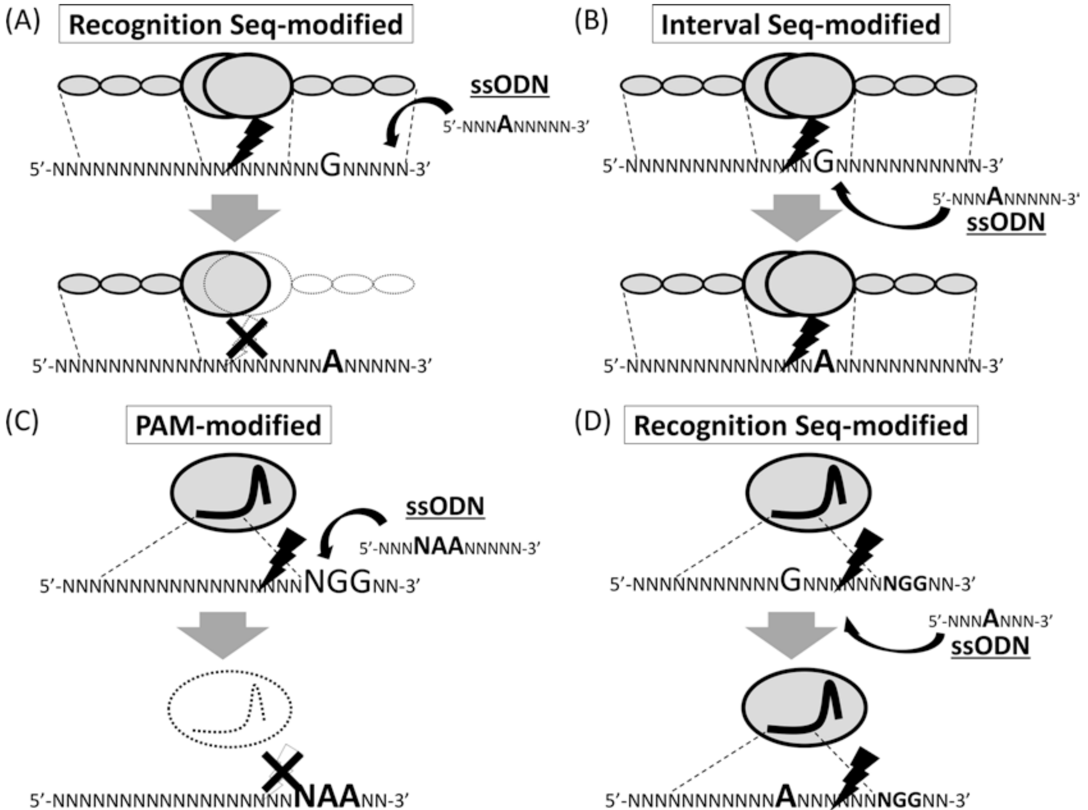

Fig. 2 The design of the nucleotide substitution using ssODN and the engineered endonucleases. (**a**, **b**) The nucleotide substitutions using ZFN or TALEN. When the substitution is introduced to the sequence recognized by nuclease, the modified allele cannot be digested by the same endonucleases again (**a**). In the case of the substitution to the interval of recognition sequence, FokI can digest the modified allele again, and undesired indel can be induced to the locus simultaneously (**b**). (**c**, **d**) The nucleotides substitution using CRISPR/Cas. The modified allele is not recognized by the Cas9-gRNA complex by introducing the substitution on the PAM (**c**). On the other hand, introducing the substitution to the target sequence of the gRNA can cause re-recognition of the same Cas9/gRNA (**d**)

In the case that such a design cannot be applied to the target nucleotide site, it is effective to use ssODNs that have the target substitution together with synonymous substitutions in which the endonucleases are not recognizable [23]. Note that the change of the codon may affect the stability of the RNA and the translation efficiency. In the case of the CRISPR/Cas system, it was reported that orthologous CRISPR-derived Cas9 [16, 24–26] and synthetic mutant Cas9 [27], which recognize the non-conventional sequence as a PAM, are useful for genome editing. In addition, Cas9 nickase [20, 28] and FokI-Cas9 [29, 30] can be used to expand the target locus for knockin.

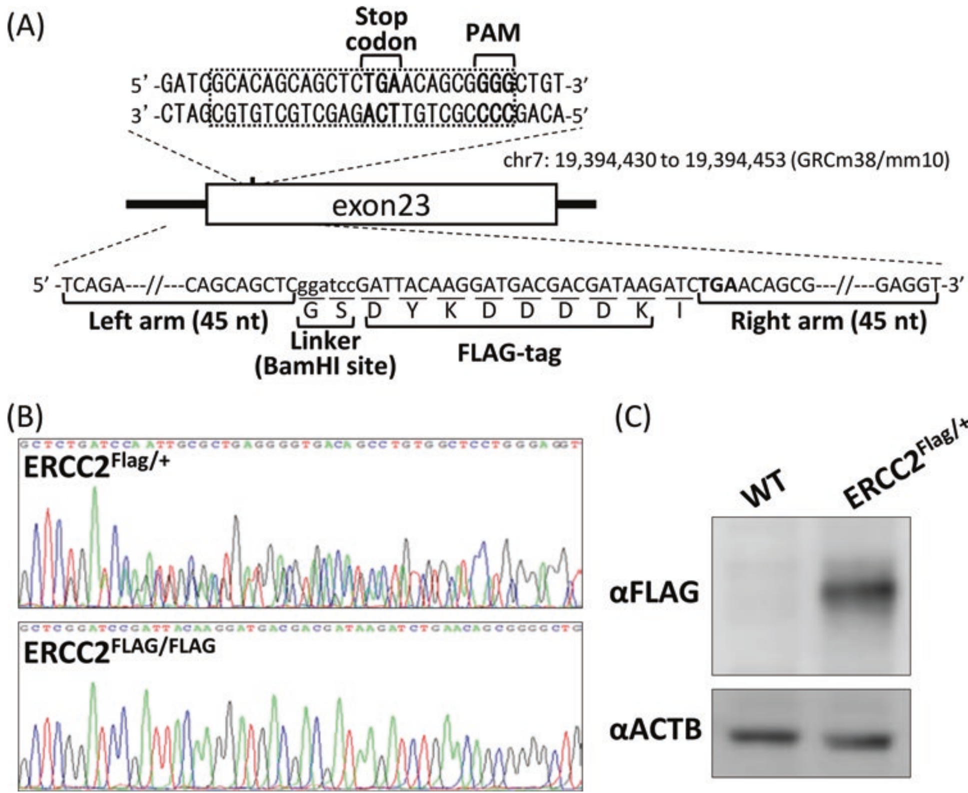

Fig. 3 An example of knock-in mice generation. (**a**) Schematic illustration of the ERCC2 gene structures, sequences around the target locus (upper), and ssODN template (lower). Black-dot box on the sequence around the target locus indicates the PAM and the sequence recognized by the Cas9-gRNA complex. The target sequence contains the stop codon of Ercc2. (**b**) A waveform data of genome sequence of a heterozygous (upper) or a homozygous (lower) knock-in pup. These data were obtained by direct-sequencing of PCR amplicons. (**c**) Immunoblotting of wild-type and tagged mouse. Tail-tip-derived proteins of wild-type (WT) and of heterozygous ($ERCC2^{Flag/+}$) pups were immunoblotted with anti-FLAG antibody and anti-βActin antibody

3.1.2 For Target-Insertion of Short-Epitope Sequence

In the case of the knockin of a short-epitope-tag sequence such as FLAG or HA, the design of endonuclease is easier than the nucleotide substitution, because the DNA sequence of the modified locus becomes much different from that of the unmodified wild-type. As with nucleotide substitution, the target sequence of endonucleases and ssODN should be designed so as not to be recognized by the endonucleases after modification. The tag sequence can be designed at the gap site as well as at the endonuclease recognition sites in the cases of ZFN and TALEN, because the distance between each of the endonuclease pairs is important for FokI endonuclease activity. For CRISPR/Cas-mediated tagging, the recognition sequence of gRNA or PAM should overlap with the tag insertion locus (*see* **Note 4**).

Figure 3 shows an example of a FLAG-tag knockin in the C-terminal region of the mouse Ercc2 gene introduced by using

CRISPR/Cas. Guide-RNA was designed on the stop codon of Ercc2, which is expected not to recognize the target locus after precise modification (Fig. 3a) (*see* **Note 5**).

3.2 Preparation of Injection Nucleotides

The preparations of Cas9 mRNA and gRNA (or other engineered endonuclease RNAs) are the same as described in other chapters.

3.2.1 Dilution of the Purchased ssODN

1. Spin down the dry-storage oligodeoxynucleotides using a desktop centrifuge.
2. Add nuclease-free distilled water to adjust the concentration to 1 mg/mL.
3. Vortex for 10 s.
4. Incubate the ssODN solution at 37 °C for 30 min.
5. Vortex for 10 s.
6. Aliquot for 10 μL and store at −20 °C until use.

3.2.2 Preparation of Injection Solution

1. Cryopreserved ssODN and in vitro transcribed RNA are thawed at room temperature.
2. Mix them with nuclease-free distilled water to adjust the concentrations as follows:
   Cas9 mRNA and gRNA: 10–100 and 10–20 ng/μL
   (or ZFN/TALEN pair: 5–10 ng/μL each)
   ssODN: 100–200 ng/μL
3. Centrifuge for a few minutes at room temperature just before injection.

3.3 Zygote Collection, Microinjection, Embryo Transfer, and Genotyping

These procedures are the same as the methods used for the generation of knock-out mice (*see* Chapter 5) (*see* **Notes 6** and **7**).

4 Notes

1. Although pronuclear microinjection can be used as well as cytoplasmic microinjection for the generation of knock-in mice, cytoplasmic microinjection results in a higher incidence of embryo development after the injection [31]. It has been reported that the electroporation-mediated transduction of ssODN together with engineered endonucleases into mouse zygotes could be used for the generation of knock-in mice as well [32, 33].

2. In a knock-in experiment involving cultured cells, it was reported that the combination of the strand of gRNA and ssODN affected the knock-in efficiency [34]. Either the same combination or a different combination of the strand of gRNA and ssODN was able to generate knockin in the mouse zygote, but this ability may be affected when the efficiency is low.

3. It was previously reported that the most suitable base lengths of the homology arms were 18 and 20 nt in the zebrafish zygote [35], but these lengths were inefficient in the mouse zygote under our experimental conditions. In culture cells, it was reported that a length of 60–90 nt is better than a length of 30 nt [36]. If the knock-in efficiency is low, the extension of each of the homology arms may increase the knock-in efficiency in mouse zygotes.

4. As noted in the introduction, the loxP sequence can be induced efficiently. However, the use of double-knock-in for floxed mice is inefficient. Moreover, the generation of a floxed mouse requires the induction of the loxP sequences of each locus into the same allele or bi-allele. If the target loci are far from each other, the evaluation of the knock-in allele by polymerase chain reaction (PCR) is difficult, and it is necessary to evaluate it in the next generation.

5. Although genomic PCR-direct sequencing is usually used for the detection of genome-modified mice by genome editing, it can be difficult to precisely screen knock-in individuals (Fig. 3b). PCR-restriction fragment length polymorphism (PCR-RFLP) is useful for screening of the knock-in allele. To use PCR-RFLP, the ssODN should be designed so that the restriction enzyme site is created or eliminated after the knockin. For the knockin of an epitope tag-sequence, it is better for the restriction enzyme site to be designed between the open reading frame of the target gene and the epitope sequence. When positioned in this manner, the enzyme site can function not only as a screening marker for PCR-RFLP but also as a linker sequence that does not affect the conformation of the target gene or the interaction of target genes with other molecules. I usually apply the BamHI recognition sequence (5′-GGATCC) because it encodes glycine-serine, which is generally used as an amino acid linker (Fig. 3b).

6. For the efficient generation of knock-in zygotes, it is important to prepare engineered endonuclease(s) that introduce DSB into the target locus efficiently. If the generation efficiency of knock-out mice using genome editing technology in your laboratory is lower than is generally reported, priority should be given to reconsidering the constructs, equipment, or experimental conditions.

   The developmental stage of zygotes used for microinjection and the amount of buffer injected into the cytoplasm may influence the knock-in efficiency. In particular, the amount of the buffer seems to vary among research workers. The condition of

the needle also affects the injection speed of the solution. Because it is more difficult to determine the ideal amount of buffer in a cytoplasmic microinjection than in a pronuclear microinjection, research workers who are already performing this procedure effectively should be observed. Our microinjection condition is shown as a movie file [37].

7. Several studies have reported that non-homologous endojoining (NHEJ) inhibitors such as SCR7 increase the knock-in efficiency when using ssODNs in mouse zygotes [38, 39]. However, the efficiencies of the uninhibited experiments in these reports are lower than the efficiency described in general reports [38, 39]. Therefore, it is not yet clear how effective NHEJ inhibitors are for knockin using ssODN in zygotes. However, the effect of NHEJ inhibitors on the knock-in efficiency using ssODN in zygotes may become clear by applying them to multiple knockins such as flox, because these are much less efficient than a knockin in a single locus.

Acknowledgment

This work was supported by a Grant in-Aid for Scientific Research [26712025 and 15K14884] from the Japan Society for the Promotion of Science.

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Chapter 9

Genome Editing of Rat

Takehito Kaneko

Abstract

Many genetically engineered rat strains have been produced for biomedical research. The simple and quick production of knock-out and knock-in rats is currently possible using genome editing techniques incorporating zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), or clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9. Genome-edited animals have been produced by the introduction of endonucleases into embryos using conventional microinjection and a new electroporation method named Technique for Animal Knockout system by Electroporation (TAKE). This chapter introduces the latest protocols for producing genetically engineered rats using genome editing.

Key words Rat, Gene targeting, ZFN, TALEN, CRISPR-Cas9, mRNA, Cas9 protein, Embryos, Microinjection, Electroporation

1 Introduction

Many genetically engineered rat strains have been produced worldwide for biomedical research. Genome editing techniques that involve zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), or clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 have allowed the simple and quick production of knock-out and knock-in rats [1–4]. Previously, the production of knock-out and knock-in animals required embryos to be injected with embryonic stem (ES) cells or induced pluripotent stem (iPS) cells in which targeted genes had been modified [5]. In rats, however, it was difficult to produce knock-out and knock-in strains because ES and iPS cells had not been established. Genome editing techniques overcame this problem. Target genes can be modified directly by the introduction of ZFN, TALEN, or CRISPR-Cas9 endonucleases into embryos [6– 9]. Genome editing techniques enabled the simple production of knock-out and knock-in strains in animals that did not have established ES and iPS cells. Moreover, a simple electroporation method, Technique for Animal Knockout system by Electroporation

Izuho Hatada (ed.), Genome Editing in Animals: Methods and Protocols, Methods in Molecular Biology, vol. 1630,
DOI 10.1007/978-1-4939-7128-2_9, © Springer Science+Business Media LLC 2017

(TAKE), that can transfect intact embryos with endonucleases was recently established [Chapter 7, [10, 11]]. Genome editing and simple animal production methods offer many advantages for biomedical research using genetically engineered animals. This chapter presents the latest protocols for the production of genetically engineered rats using genome editing.

2 Materials

2.1 Preparation of ZFN/TALEN/Cas9 mRNA and gRNA

1. Custom-designed ZFN, TALEN, Cas9, and gRNA plasmids (*see* **Note 1**).
2. mMESSAGE mMACHINE T7 ULTRA Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA).
3. MEGAshortscript T7 Transcription Kit (Thermo Fisher Scientific).
4. A-Plus Poly (A) polymerase tailing kit (Cellscript).
5. MEGAClear kit (Thermo Fisher Scientific).
6. Distilled water.
7. Phosphate-buffered saline (PBS) buffer without calcium and magnesium.
8. Opti-MEM (Thermo Fisher Scientific).

2.2 Collection of Pronuclear Stage Embryos

1. Mature male and female rats. The F344/Stm rat strain is suitable for gene modification because many embryos can be obtained [12]. This strain is supplied by the National BioResource Project-Rat (Kyoto, Japan, http://www.anim.med.kyoto-u.ac.jp/NBR/Default.aspx).
2. Modified Krebs-Ringer bicarbonate (mKRB) for 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. Sterile mineral oil.
4. 35–60 mm plastic culture dish.
5. CO_2 incubator.
6. Pregnant mare serum gonadotropin (PMSG).
7. Human chorionic gonadotropin (hCG).
8. 1 mL syringe with 30 G steel needle.
9. Pair of small scissors.
10. Fine tipped forceps.
11. Glass capillary pipettes.

Table 1
Components of mKRB medium

Components	mKRB mg/100 mL (mM)
NaCl	553 (94.6)
KCl	36 (4.8)
$CaCl_2$	19 (1.7)
$MgSO_4 \cdot 7H_2O$	29.3 (1.2)
KH_2PO_4	16 (1.2)
$NaHCO_3$	211 (25.1)
Na-lactate (60% syrup)	0.19 mL
Na-pyruvate	6 (0.5)
D-glucose	100 (5.6)
Penicillin G	7
Streptomycin	5
Bovine serum albumin (BSA)	400

2.3 Microinjection of mRNA into Pronuclear Stage Embryos

1. Micromanipulator.
2. Micropipette puller (Sutter Instrument, Novato, CA, USA).
3. Microforge.
4. Glass capillary pipettes.
5. Endonucleases (ZFN mRNA, TALEN mRNA, Cas9 mRNA, gRNA and ssODN).
6. Loading tips.
7. mKRB medium.
8. Sterile mineral oil.
9. 10 cm plastic culture dish.
10. CO_2 incubator.

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

1. *See* Chapter 7 for more detail information.
2. Electroporator (NEPA 21: NEPA GENE Co. Ltd., Chiba, Japan).
3. Petri dish platinum plate electrodes (CUY520P5, NEPA GENE Co. Ltd.).

4. Endonucleases (ZFN mRNA, TALEN mRNA, Cas9 mRNA, gRNA and ssODN).
5. PBS buffer without calcium and magnesium.
6. Opti-MEM medium.
7. Glass capillary pipettes.

2.5 Embryo Transfer

1. Mature female rats.
2. Vasectomized male rats.
3. Isoflurane for anesthesia (2%, 1 L/min).
4. Pair of small scissors.
5. Fine tipped forceps.
6. Glass capillary pipettes.
7. 30 G Steel needle.
8. Wound clips.

2.6 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 ZFN and TALEN mRNA

1. Transcribe in vitro mRNA from custom-designed ZFN and TALEN plasmids using an mMESSAGE mMACHINE T7 ULTRA Transcription Kit.
2. Polyadenylate each mRNA using an A-Plus Poly (A) polymerase tailing kit.
3. Purify each mRNA using a MEGAClear kit.
4. For microinjection, dilute each mRNA to 5–10 μg/mL with distilled water.
5. For electroporation, dilute each mRNA to 40 μg/mL with PBS buffer or Opti-MEM medium.

3.2 Preparation of Cas9 mRNA and gRNA

1. Transcribe in vitro mRNA from Cas9 plasmids using an mMESSAGE mMACHINE T7 ULTRA Transcription Kit.
2. Polyadenylate Cas9 mRNA using an A-Plus™ Poly (A) polymerase tailing kit.
3. Transcribe in vitro mRNA from custom-designed gRNA plasmids using a MEGAshortscript T7 Kit.

4. Purify each mRNA using a MEGAClear™ kit.
5. For microinjection, dilute Cas9 mRNA to 100 μg/mL and gRNA to 50 μg/mL with distilled water.
6. For electroporation, dilute Cas9 mRNA and gRNA to each approximately 500 μg/mL with PBS buffer or Opti-MEM medium.

3.3 Preparation of Pronuclear Stage Embryos

1. Prepare pronuclear stage rat 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 and cervical dislocation.
8. Remove the oviducts using a small pair of scissors (*see* **Note 2**).
9. Flush ampulla with mKRB medium using a 1 mL syringe with a 30 G steel needle.
10. Collect pronuclear stage embryos using a glass capillary pipette and transfer to one of the four drops of mKRB medium.
11. Remove cumulus cells and other debris, and transfer the embryos to another drop of mKRB medium (*see* **Note 3**).
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 microinjection and electroporation.

3.4 Microinjection of mRNA into Pronuclear Stage Embryos

1. Prepare injection and holding pipettes using a micropipette puller and a microforge (*see* **Note 4**).
2. Introduce 2–3 μL of mRNA with distilled water into the tip of the injection pipette using the loading tip (*see* **Notes 5** and **6**).
3. Add appropriate ssODN for the desired knock-in rat to the mRNA solution.
4. Mount the injection pipette in the right pipette holder of the micromanipulator.
5. Mount the holding pipette in the left pipette holder of the micromanipulator.
6. Prepare small 10–20 μL drops of mKRB medium covered with sterile mineral oil in the 10 cm plastic culture dish.
7. Transfer embryos to the drops of mKRB medium.
8. Transfer an embryo to the holding pipette and stabilize.
9. Inject 2–3 pL of mRNA solution into the male pronucleus or cytoplasm.

10. Culture embryos after injection in 100 μL drops of mKRB medium covered with sterile mineral oil at 37 °C under 5% CO_2 and 95% air.

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

1. *See* Chapter 7 for more detail information.
2. Prepare mRNA with PBS buffer or Opti-MEM medium (*see* **Note** 7).
3. Add appropriate ssODN for the desired knock-in rat to the mRNA solution.
4. Introduce 100 μL mRNA solution into the electrode.
5. Place pronuclear stage embryos in a line between electrodes on the petri dish.
6. Connect the electrodes to the electroporator.
7. Set up the poring and transfer electrical pulses on the electroporator.
8. Electroporate the intact embryos (*see* **Note 8**).
9. Place electroporated embryos into fresh drops of mKRB medium and incubate at 37 °C under 5% CO_2 and 95% air until embryo transfer.

3.6 Embryo Transfer

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

3.7 Genotyping of Delivered Pups

1. Collect blood from 3-week-old pups.
2. Blot blood samples onto 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 10**).

4 Notes

1. Custom-designed plasmids can be purchased commercially.
2. Collect oviducts immediately after euthanasia to avoid physiological damage to oocytes.
3. If cumulus cells are attached to embryos, remove using 0.1% hyaluronidase.
4. Products can be purchased commercially.
5. Coinjection of Exonuclease 1 (*Exo 1*) with TALEN mRNA increased the rate of knock-out offspring production [13].
6. Cas9 protein can be introduced into embryos [14].
7. Introduce Cas9 protein into embryos using the same procedure as that used to introduce mRNA.
8. Intact embryos without any chemical treatment, such as zona-thinning using Tyrode's acid solution, can be used for electroporation.
9. Good results are obtained by transfer of embryos at the 2-cell stage.
10. Typical results for the production of embryos by the microinjection of endonucleases are shown in Table 2. The production of embryos by the electroporation of endonucleases is shown in Table 3.

Table 2
Development of rat embryos after microinjection of engineered endonucleases

Targeted genes	Nucleases	Strains	No. embryos injected	No. of embryos survived and transferred	No. (%) of offspring	No. of knock-out offspring	References
Tyr	TALEN	DA	201	86 (43)	20 (23)	0 (0)	[13]
	TALEN + *Exo1*		68	29 (43)	12 (41)	3 (10)	[13]
	CRSPR/Cas9		135	71 (53)	23 (32)	7 (10)	[8]
Il2rg	ZFN	F344	93	41 (44)	9 (22)	3 (7)	[10]
	TALEN		52	20 (39)	6 (30)	6 (30)	[10]
	CRSPR/Cas9		120	100 (83)	41 (41)	21 (21)	[10]

Table 3
Development of rat embryos introduced engineered endonucleases by TAKE method

Animals	mRNA	No. of embryos examined	No. of 2-cell embryos (%)	No. of offspring (%)	No. of knock-out offspring (%)	No. (%) of knock-in offspring	References
Rat	ZFN	63	57 (91)	15 (24)	11 (73)	–	[11]
	TALEN	57	56 (98)	17 (30)	3 (18)	–	[11]
	CRISPR/Cas9	60	45 (75)	24 (53)	21 (88)	8 (33)	[12]
Mouse	CRISPR/Cas9	100	84 (84)	36 (43)	24 (67)	12 (33)	[12]

F344/Stm rat and C57BL/6J mouse was used
Il2rg gene was targeted

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Chapter 10

Gene Targeting in Rabbits: Single-Step Generation of Knock-out Rabbits by Microinjection of CRISPR/Cas9 Plasmids

Yoshihiro Kawano and Arata Honda

Abstract

The development of genome editing technology has allowed gene disruptions to be achieved in various animal species and has been beneficial to many mammals. Gene disruption using pluripotent stem cells is difficult to achieve in rabbits, but thanks to advances in genome editing technology, a number of gene disruptions have been conducted. This paper describes a simple and easy method for carrying out gene disruptions in rabbits using CRISPR/Cas9 in which the gene to be disrupted is marked, the presence or absence of off-target candidates is checked, and a plasmid allowing simultaneous expression of Cas9 and sgRNA is constructed. Next, the cleaving activity of candidate sequences is investigated, and assessments are carried out to determine whether the target sequences can be cut. Female rabbits subjected to superovulation treatment are mated with male rabbits and fertilized eggs are collected, and then pronuclear injection of plasmid DNA is performed. The next day, the two-cell stage embryos are transplanted into pseudopregnant rabbits, and offspring are born within approximately 29–30 days. The genomic DNA of the offspring is then examined to check what types of genetic modifications have occurred. With the advent of CRISPR/Cas9, the accessibility of gene disruptions in rabbits has improved remarkably. This paper summarizes specifically how to carry out gene disruptions in rabbits.

Key words Rabbit, Genome editing, Knockout, Gene targeting, CRISPR/Cas9

1 Introduction

Until the advent of genome editing technology, homologous recombination using mouse embryonic stem (ES) cells and the preparation of chimeric mice were considered mainstream methods for gene disruption in mammals [1]. ES cells from animal species other than mice have an extremely low chimera-forming ability; therefore, performing gene disruption via ES cells was impractical [2]. In addition, if gene disruption is performed via ES cells using species other than mice, ES cells are needed. Furthermore, mice are inexpensive, not subject to certain experimental animal ethics restrictions, and excellent in terms of reproduction.

Izuho Hatada (ed.), *Genome Editing in Animals: Methods and Protocols*, Methods in Molecular Biology, vol. 1630,
DOI 10.1007/978-1-4939-7128-2_10, © Springer Science+Business Media LLC 2017

Moreover, embryonic manipulation and transplantation techniques are already established. Specifically, one sexually mature rabbit costs about 10,000–30,000 JPY, which is five times higher than the cost of mice and rats, but several dozen times lower than the cost of pigs and monkeys; therefore, they can be categorized as relatively inexpensive. Rabbits reach sexual maturity and mate within 4–5 months, have a gestation period of 29–31 days (6–7 pups per birth), and, in experiments aimed at creating chimeric animals and producing transgenic animals, they also allow for easy analysis of next-generation influences because of a fast generation cycle and large number of offspring. In addition, as they only weigh approximately 2.5–4.0 kg, a small facility is sufficient to raise them. Rabbits also have a very gentle temperament, and, as a result, superovulation injections, as well as anesthetic procedures, can be performed with virtually no resistance if done by an experienced operator. If mating occurs after artificial superovulation, an average of 30–50 fertilized eggs can be collected from each animal. Regarding embryonic transplantations, if 15–20 early-stage embryos are transplanted in oviducts, approximately 30–50% will become offspring, which allows embryonic manipulation and transplantation experiments to be carried out very efficiently. Naturally, the option of using rats could also be put into perspective, but when considering the future application of this technique in humans, we started carrying out gene disruptions in rabbits because the latter is a nonrodent species with a body size larger than that of rats and can be used as a reference when spreading the use of this technique to pigs, nonhuman primates, and large mammals. Homologous gene recombination methods using ES cells used to be the most common method for carrying out gene disruption in mammals [3], but unlike common ES cells established from mice whose naive state ES cells allow for the formation of chimera and are capable of differentiating into germ cells, ES cells from humans, and animal species other than mice are known to be primed state cells with a remarkably poor chimera-forming ability [4]. In other words, because rabbit ES cells are primed state cells, their pluripotent stem cells are extremely difficult to use in the creation of chimera and the induction of differentiation into germ cells [5]. Although research on the conversion of primed state rabbit ES cells into naive state cells has progressed, true naive type ES cells that would allow the production of chimeric rabbits have yet to be established [6].

With this background, genome editing technology is very promising, and studies aimed at carrying out gene disruption in rabbits have begun around the world [7–11]. Particularly, the clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated (Cas) (CRISPR/Cas9) system is easy to use and sufficiently simple and efficient to play a leading role in the field of genome editing in rabbits. This paper describes a genome

editing method using CRISPR/Cas9 in rabbits. Particularly, methods aimed at generating gene disruption in rabbits through the pronuclear injection of plasmids are not difficult, even for beginners with little experience in gene recombination. Furthermore, because there is no need to prepare mRNA, this method is highly versatile. The present paper describes both the advantages and disadvantages of the plasmid injection method.

2 Materials

Details pertaining to the construction of plasmids pX330 and pCAG-EGxxFP have been omitted. For more information, please refer to the papers by Mashiko et al. [12] and Honda et al. [10]. In addition, gene transfection of plasmids into HEK293 cells can be achieved by standard transfection methods (chemical transfection or electroporation); therefore, details regarding the transfection method have also been omitted.

2.1 Super Ovulation

1. Sexually mature female rabbits for super ovulation (over 4 months old [<2.0 kg]).
2. Sexually mature male rabbits.
3. Porcine follicle-stimulating hormone (pFSH) suspended in physiological saline at 0.5 IU/mL.
4. Human chorionic gonadotropin suspended in physiological saline at 100 IU/mL.
5. Surgical instruments.

   Surgical scissors (B-1H) (Natsume Seisakusho, Tokyo, Japan) (Fig. 1).
   Biological tweezers (A-4) (Natsume Seisakusho) (Fig. 1).
6. Syringes (5, 50 mL).
7. Injection needles (19- and 26-gauge).
8. 100 mm Petri dish.
9. Pentobarbital sodium.
10. Saline.
11. Retention container for rabbit.

2.2 Fertilized Oocytes Recovery and Embryo Culture

1. RD (HEPES) medium: 50% DMEM, 50% RPMI 1640 (HEPES), 0.5% GlutaMAX.
2. RD medium: 50% DMEM, 50% RPMI 1640, 4 mg/mL bovine serum albumin (*see* **Note 1**).
3. Mineral oil.
4. Glass capillaries.

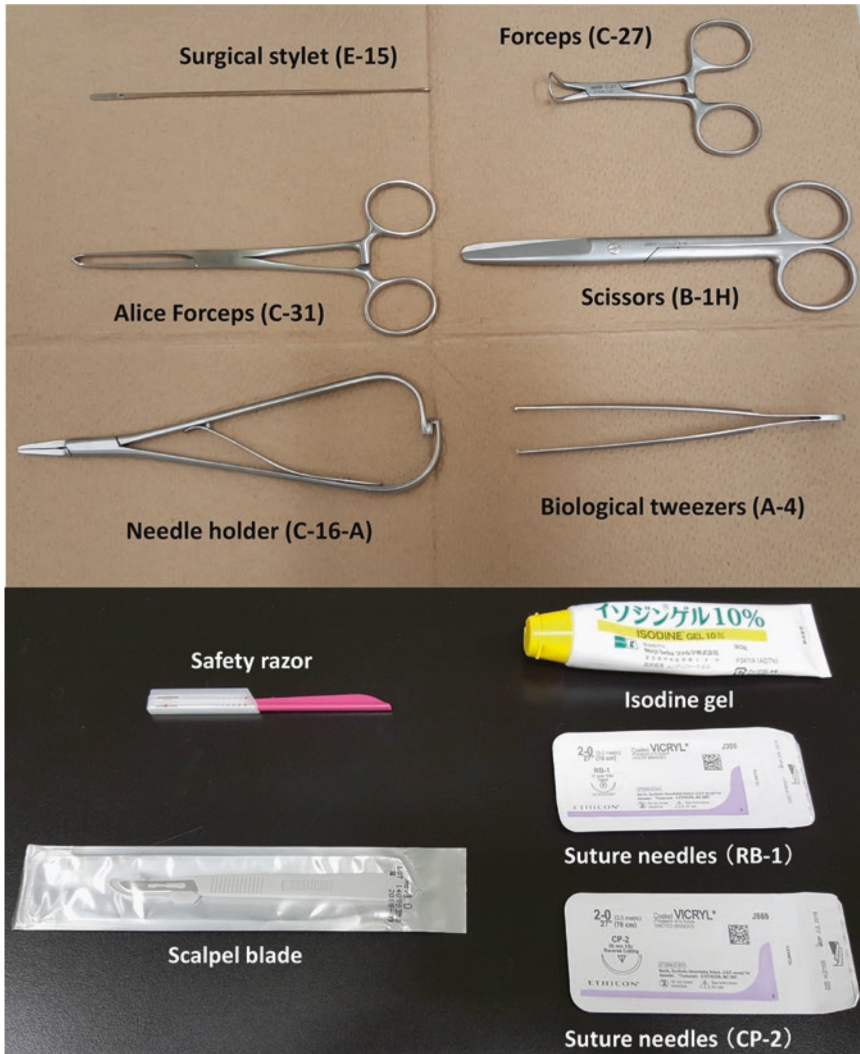

Fig. 1 Surgical instruments. These instruments should be carefully autoclaved and dried to prevent infection during surgery

2.3 Microinjection

1. Phase contrast microscope.
2. Piezo micromanipulator (PMM-150FU) (Prime Tech, Tokyo, Japan).
3. Micropipette puller (P-1000IVF) (Narishige, Tokyo, Japan).
4. Microforge (MF-900) (Narishige).
5. Glass capillary (B100-75-10-PT) (Prime Tech).
6. PVP solution (0.13 g/mL polyvinylpyrrolidone in M2 medium).
7. Plasmids (pX330, pCAG-EGxxFP, pX330-Cetn1, pCAG-EgxxFP-Cetn1) (Addgene, Massachusetts, USA).

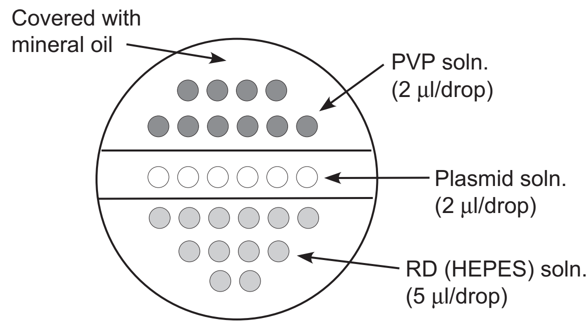

Fig. 2 Injection chamber. The cover of a 100 mm Petri dish filled with appropriate culture medium is used as an injection chamber for injections

8. RD (HEPES) medium.
9. Mineral oil.
10. Cover lid of Petri dish (100 mm) for injection stage (Fig. 2).

2.4 Embryo Transplantation

1. Sexually mature female rabbits for transplantation (foster mothers) (over 4 months old [<2.3 kg]).
2. Surgical instruments (autoclaved) (Natsume Seisakusho).
   Surgical scissors (B-1H) (Fig. 1).
   Biological tweezers (A-4) (Fig. 1).
   Forceps (C27, C31) (Fig. 1).
   Needle holder (C-16A) (Fig. 1).
   Surgical stylet (E-15) (Fig. 1).
3. Glass capillary (handmade from a pasture pipette).
4. RD (HEPES) medium.
5. Insulating container (Cellporter) (Corefront, Tokyo, Japan).
6. Retention container for rabbit.
7. Pentobarbital sodium.
8. Gauze.
9. Surgical cloth.
10. Disinfectant (povidone-iodine, 70% ethanol).
11. 26-gauge needle, 2.5 mL syringe.
12. Safety razor (Fig. 1).
13. Scalpel blade (Fig. 1).
14. Surgical suture needles (Coated VICRYL: RB-1, CP-2 2-0) (Fig. 1).
15. Saline.
16. Surgical gloves.
17. Antibiotics (Cefamezin-α) (Astellas Pharma, Tokyo, Japan).
18. Iodine gel (Meiji Seika Pharma, Tokyo, Japan).
19. Stereomicroscope.

2.5 Parturition and Lactation

1. Nursing cage (W 555 mm × L 605 mm × H 500 mm).
2. Autoclaved floor materials (shredded papers).

3 Methods

3.1 Search for Target Sequences

Investigate the presence or absence of off-target sequences using GGGenome (http://gggenome.dbcls.jp/ja/mm10/2/) and CRISPRdirect (http://crispr.dbcls.jp/). Enter the target sequences (comprising approximately 20 nucleotides), including the PAM (5′-XGG-3′) sequence of the gene that needs to be disrupted (*see* **Note 1**).

3.2 Single Strand Annealing Assay

1. On the basis that the target sequence has as few off-target candidates as possible, complementary oligo-DNA needs to be ordered from a gene synthesis service and then subjected to an annealing reaction. The target sequence needs to be inserted through ligation into the BbsI site of pX330 (For more details, please refer to Honda et al. [10] and Mashiko et al. [12]).
2. A 500–1000 bp gene fragment containing the target sequence near its center needs to be amplified by PCR and inserted into pCAG-EGxxFP.
3. Prepare plasmids by using the ligation reaction described as above (**steps 1** and **2**) for the transformation of *Escherichia coli* cells, and perform restriction enzyme treatment and sequencing to confirm that the target plasmid has been transformed.
4. Transfect the resulting plasmid into HEK293 cells in the same way as the negative control (pCAG-EGxxFP only) and positive controls (pCAG-EGxxFP-Cetn1 and pX330-Cetn1). Confirm EGFP fluorescence under fluorescent microscopy 24–48 h later. In most cases, if four target sequences are selected, one to three targets will exert a cleaving activity equivalent to or stronger than that of Cetn1.

3.3 Superovulation

1. Administer pFSH subcutaneously into the neck of sexually mature female rabbits from 18:00 until 21:00. The pFSH needs to be administered six times in total at intervals of 11–13 h.
2. For rabbits that have completed six administrations of pFSH, approximately 60 h after the beginning of the injections, arrangements need to be made so that rabbits that have been subjected to superovulation treatment are placed in the same cage as sexually mature male rabbits (female rabbits should be placed in male rabbits' cages) between 19:00 and 21:00 (approximately 72 h after administration of pFSH is initiated) for mating to occur (*see* **Note 2**).
3. Once mating is successful, administer hCG to the female rabbits through the ear vein, and when the bleeding has stopped, place them back in their cage.

3.4 Collection of Fertilized Oocytes

1. Euthanize the rabbits with an overdose of pentobarbital sodium (6 mL per rabbit) 19–20 h after mating.
2. Disinfect the abdomen by spraying with 70% ethanol, and open the abdominal cavity with scissors.
3. Extract the right and left ovaries and fallopian tubes, as well as the uterus, and collect them in a 10 cm Petri dish.
4. Using scissors (B-1H), make an incision near the uterotubal junction perpendicular to the angle of the oviduct. Next, take a 10 mL syringe containing RD (HEPES) (10 mL) culture medium pre-warmed at 37 °C and insert it into the oviduct (which looks like a dot). Then, allow approximately 10 mL of the fluid to flow back into the syringe. The fluid is allowed to flow back while the inserted needle is fixed by forceps outside the oviduct. Transfer the fluid to a 100 mm Petri dish and flush fertilized oocytes from the oviduct into the dish (Fig. 3).
5. Using a stereomicroscope, check the fertilized oocytes (check that the cumulus cells have dispersed) and collect them in a mouth capillary. Next, transfer them in a drop of RD culture medium which is covered with mineral oil and equilibrated in an incubator (37 °C, 5% CO_2 in air). Finally, place them in the incubator.

3.5 Injection of plasmid DNA

1. Insert the injection pipette into a drop of PVP, suck and eject the PVP liquid two or three times using the manipulator, and then coat the inner surface of the injection pipette with PVP.

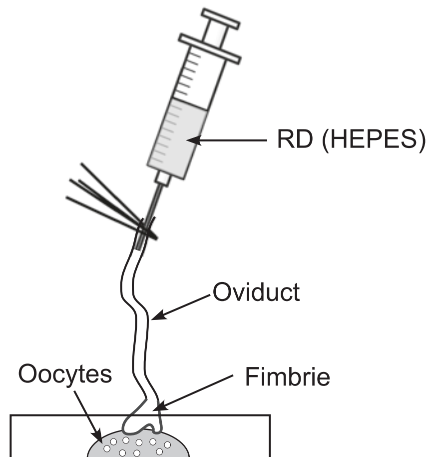

Fig. 3 Oocyte collection. After removing the uterus, ovaries, and most of the fat tissues, insert a round-end needle into the oviduct from the uterus side. The fluid is allowed to flow out while the inserted needle is fixed outside the oviduct using forceps. Fertilized oocytes are then collected in a 100 mm Petri dish

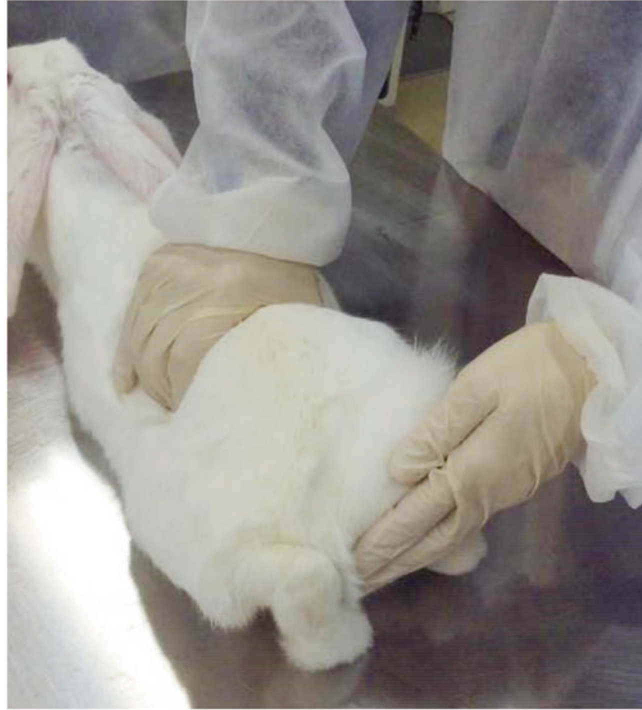

Fig. 4 Preparation of foster mother rabbit for transplantation. Since rabbits ovulate post-copulatory, the foster mother is prepared artificially about 1 day before transplantation

2. Take the injection pipette with a PVP-coated inner surface and insert it into a drop of pX330 solution (5–10 ng/μL). Next, fill it with the solution.
3. Remove the fertilized oocytes with pronuclei out of the incubator and transfer them into a drop of RD (HEPES) medium.
4. Hold the oocyte with a holding pipette, insert an injection pipette into the pronucleus, and inject it with pX330 solution. Confirm that the pronucleus is swollen (*see* **Note 3**).
5. After the injection of oocytes is complete, return them to the incubator and culture overnight.

3.6 Embryo Transplantation

1. Foster rabbits are copulatory stimulated by finger press to the vaginal pore 22–26 h before transplantation (Fig. 4).
2. The next morning, select and pick up embryos that have developed into the two-cell stage and then transfer them into RD (HEPES) medium. Next, move each dish into a thermal insulation container and take them to the transplantation room.
3. Fill a syringe with 2 mL of pentobarbital sodium and apply 70% ethanol to the ear of the rabbit scheduled for use in transplantation. Tap a blood vessel to make sure it is engorged, then

insert a syringe containing pentobarbital sodium into the blood vessel and administer 1.5 mL of the liquid (*see* **Note 4**).

4. Confirm that the anesthesia is working, and then put the animal on the operating table in the supine position.
5. Place the hind limbs in a way that the knee is maintained in extension in a position that does not block the urethra. Place the forelimbs in extension and immobilize the animal obliquely (using duct tape and rope) on the operating table.
6. Apply a disinfectant (iodine scrub) to the hair at the planned location of the incision, and then wash vigorously and make sure it foams.
7. Confirm the position of the nipples and completely shave the hair using safety razor at the planned location of the incision from top to bottom.
8. Disinfect the skin beforehand with gauze soaked in 1% iodine solution.
9. Cover the animal's entire body with a surgical cloth while exposing only the planned location of the incision, and then pinch the cloth and skin using towel forceps (C-27). Finally, fix with a surgical cloth (*see* **Note 5**).
10. Open a vertical incision of approximately 10 cm in the skin of the midline portion of the abdomen (*see* **Note 6**).
11. Pinch the fascia with forceps (A-4) while making sure that the scalpel blade points upward, and then make a partial incision using the tip of the scalpel (*see* **Note** 7).
12. Pinch the portion of the fascia on the incision line using two pairs of Alice forceps (C-31), and then incise above and under the planned location of the incision using a pair of scissors (B-1H).
13. In addition, pinch the fascia at four locations using two pairs of Alice forceps and secure the field of view by placing the former laterally to the Alice forceps.
14. In the case that urine has accumulated in the bladder, deflate by sandwiching between both hands and use towels to absorb the urine.
15. Move the appendix and find the uterus before looking for the ovaries and fallopian tubes (either the ones on the right side or those on the left) (*see* **Note 8**).
16. Once the fimbria of the oviduct is found, confirm the capillary insertion location (at the fimbria of the oviduct) and insertion angle using a surgical stylet.
17. Close the incision temporarily (place the Alice forceps crosswise, cover with gauze, and moisten with physiological saline solution to prevent drying).

18. Take the fertilized oocytes and culture fluid out of the carrier box, place them on a thermo-plate, and check them under a microscope.
19. Move the fertilized eggs into the capillary (transplant 5–10 fertilized eggs into an oviduct on one side).
20. Replace the surgical gloves with new ones, open the incision, reexpose the ovaries and fallopian tubes, and reconfirm the capillary insertion site and insertion angle using a surgical stylet.
21. Insert the capillary into the fimbria of the oviduct and transplant the fertilized egg into the oviduct.
22. Unplug the capillary, return the fallopian tube to its original position, and record the number that has been transplanted.
23. Next, search for the oviduct on the opposite side and move it in the same manner as described above.
24. Return the oviducts to their original location and confirm that no hair is attached to the internal organs (remove any remaining hair with a pair of tweezers).

3.7 Suture: Completion of Surgery

1. Open the bag containing the suture thread (RB-1) and remove with a needle holder.
2. First, suture the center of the surgical wound while gripping the fascia with a pair of tweezers. The suturing has to be performed in 0.5–1 cm intervals using a suturing machine.
3. Open the bag containing CP-2 and take out the suture thread with a needle holder.
4. Let the needle pass through from the edge of the incision to the opposite side of the skin. When performing the suture, take precautions to ensure that the suture thread is not exposed at the surface.
5. Complete the suture by reaching the edges of the wound. Finally, tie a knot using a suturing machine. Make sure that the threads at the stitches and knots are not exposed at the surface (*see* **Note 9**).
6. Inject 1 mL of Cefamezin-α into the thigh muscles.
7. Remove the towel clamps and surgical cloth and apply iodine gel (Fig. 1) to the sutured area.
8. Take the rabbit off the operating table and place it back in its cage.
9. After the rabbit recovers from anesthesia, observe the surgical wound and the animal's general condition closely and confirm that there are no abnormalities.

3.8 Birth of Offspring

1. The morning after the day of surgery, confirm that the wound is closed and observe the rabbit's condition. Inject Cefamezin-α into the thigh muscles.
2. Approximately 20 days after the surgery, if the rabbit exhibits behavior such as pulling its own hair with its mouth, it may be experiencing a pseudopregnancy.
3. Approximately 24 days after surgery, put a floor cover inside the cage (we use autoclaved and dried paper debris or waste-paper discharged from a shredder). Hug and hold the rabbit as gently as possible to achieve communication and get it acquainted to humans.
4. The birth of the offspring will occur 28–32 days after surgery.
5. It takes approximately 1 month until weaning is complete. The floor cover should be replaced once every 2 weeks. Once weaning is complete, the floor cover can be removed.

4 Notes

1. If possible, the off-target search should be performed on the 15 nt containing a PAM sequence, and the results should show as few off-target sequences as possible. Settings allowing for at least four targets per gene are preferable.
2. Ensure that each female mates with two different males, who should be allowed to ejaculate at least twice. Confirm visually that semen is present in the vicinity of the vaginal pore.
3. Plasmid solution needs to be injected correctly into the pronucleus. If the plasmid is accidentally injected into the cytoplasm, the translation of Cas9 proteins will take a long time, and genome editing is likely to occur after the two-cell embryo stage; this is likely to result in the generation of mosaic embryos.
4. The initial 1 mL should be administered in approximately 5–10 s, whereas the injection of the remaining 0.5 mL should take as much time as possible (30 s or longer) for the effects of the anesthesia to last longer. However, the administration of only pentobarbital sodium is obsolete. Henceforth, using ketamine/xylazine or isoflurane is highly recommended.
5. Be careful not to pinch the nipples with the forceps.
6. Make an incision of about 10 cm starting from a point located at 1 cm below the navel all the way down to the lower abdomen.
7. Point the scalpel blade upward to avoid injuring the intestines.

8. Be careful not to pinch the fallopian tubes with your fingers.
9. If the thread is exposed at the surface, a rabbit that is bothered by the surgical wound may remove it with its mouth or teeth, causing the wound to open.

Acknowledgments

We thank Dr. Tadashi Sankai and Atsuo Ogura for their helpful suggestions. This work was supported by the Program to Disseminate Tenure Tracking System from the Ministry of Education, Culture, Sports, Science and Technology, PRESTO of the Japan Science and Technology Agency, the Japan Science and Technology Agency, a Grant-in-Aid for Scientific Research (B) (15H04286), and a Grant-in-Aid for Challenging Exploratory Research (15K14887).

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Chapter 11

Genome Editing of Pig

Masahito Watanabe and Hiroshi Nagashima

Abstract

Pigs are important livestock for food and have been used in various biomedical studies, particularly translational research, as experimental animals because of their anatomical and physiological similarity to humans. The recent development of genome editing techniques, such as ZFN, TALEN, and CRISPR/Cas9, has rapidly expanded the use of genome editing tools in a variety of animals, resulting in the relatively easy and efficient generation of gene knock-out pigs. In the past few years, there has been a sustained increase in reports describing the development of genetically modified pigs. This chapter introduces our workflow for establishing the genetically modified cells (nuclear donor cells) necessary to create gene knock-out pigs using somatic cell nuclear transfer and focuses on the actual generation of gene knock-out pigs using a cytoplasmic injection method.

Key words Pig, Gene knockout, Somatic cell nuclear transfer, Cloning, Cytoplasmic injection

1 Introduction

Pigs have previously been used as large experimental animals for numerous biomedical studies, particularly in the field of translational research [1, 2]. Pigs are suitable for biomedical research because of (1) their similarity to humans in anatomical and physiological characteristics; (2) their high fecundity, which makes it easy to produce offspring; and (3) the applicability of human surgical techniques and instruments without modification.

Since the world's first transgenic pig was established through DNA microinjection into a fertilized egg in 1985 [3], gene modification techniques for pigs have generally involved the introduction of exogenous genes, including methods using sperm as a vector for exogenous genes (Sperm-mediated gene transfer (SMGT) and intracytoplasmic sperm injection (ICSI)-mediated gene transfer) [4, 5] and strategies involving the use of retroviruses and lentiviruses [6, 7]. Homologous recombination using embryonic stem (ES) cells has been used to create gene knock-out mice; however, this method cannot be used for pigs because they lack ES cells capable of germline transmission. The establishment of the

Izuho Hatada (ed.), *Genome Editing in Animals: Methods and Protocols*, Methods in Molecular Biology, vol. 1630,
DOI 10.1007/978-1-4939-7128-2_11, © Springer Science+Business Media LLC 2017

somatic cell nuclear transfer (SCNT) technique was a significant breakthrough in the history of genetic engineering in pigs [8]. SCNT uses a genetically modified (via gene transfer or knockout) nuclear donor cell to produce a genetically modified individual with a desired genotype. However, gene targeting via homologous recombination with somatic cells involves complex operations and exhibits extremely low efficiency. Thus, the generation of gene knock-out pigs was relatively rare until approximately 2010, when genome editing technology was developed. At this time, gene knock-out rats were successfully developed using ZFN [9]. Subsequently, we reported the first study describing the use of ZFN for gene knockout in pigs [10], and thereafter, gene knock-out pigs were produced in 2011 [11, 12]. The second and third genome editing tools TALEN and CRISPR/Cas9 were developed, and a number of groups generated gene knock-out pigs using these tools [13, 14]. Currently, genome editing techniques are used to develop pigs for biomedical research, including model pigs that recapitulate human diseases [15, 16], donor pigs for xenotransplantation [12, 17], and organ-deficient pigs for organ regeneration [18]. Such techniques are also applied to produce genome-edited pigs for agricultural (livestock) use [19, 20].

The following two approaches have been developed to produce gene knock-out pigs using genome editing techniques: SCNT and cytoplasmic injection methods (Fig. 1). Cytoplasmic injection methods are generally used for rodents, but most research groups use SCNT for pigs. Cytoplasmic injection is a simple method, but whether the desired genotype has been successfully obtained only becomes apparent after the individual has been produced. Another practical issue in using gene knock-out animals is the production of mosaic individuals (containing a mixture of cells with multiple different genotypes within a single individual) [21]. Particularly, in

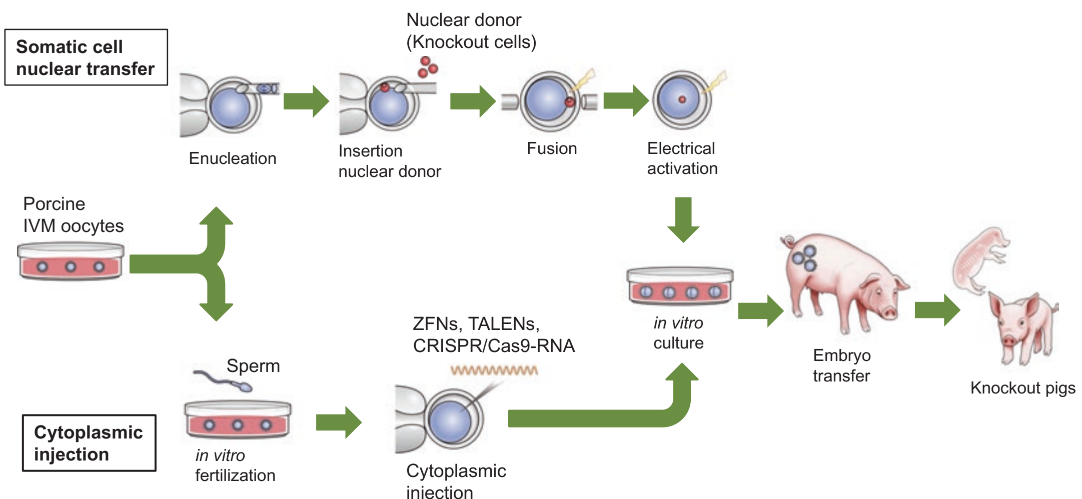

Fig. 1 Schematic representation of two strategies for generating gene knock-out pigs by genome editing

mosaic individuals with a mixture of cells with and without a genetic mutation, an unstable expression of pathology (phenotype) may be observed. Furthermore, mosaic individuals can produce germ cells of multiple mutation types. The selection of individuals in a subsequent generation with the desired genotype requires extensive time, labor, and expense, which is particularly problematic when producing large animals with long gestation periods. In contrast, SCNT methods can reliably produce only individuals with the target genotype by first performing the desired genetic manipulation in nuclear donor cells [22]. Using this method, extraneous individuals are not produced, which is more advantageous from the perspective of animal protection. SCNT techniques also avoid the production of mosaic individuals. Therefore, many groups use SCNT methods to create gene knock-out pigs. The recently developed CRISPR/Cas9 technique may considerably transform the production of gene knock-out pigs. Compared with previous methods using artificial nucleases, CRISPR/Cas9 is not only simpler but also exhibits significantly improved mutation efficiency; thus, the cytoplasmic injection of CRISPR/Cas9 is rapidly increasing in pigs [23, 24].

This chapter introduces the generation of gene knock-out pigs via SCNT and cytoplasmic injection. Here, the focus was on establishing gene knock-out cells (nuclear donors) using genome editing tools. For cytoplasmic injection, we focused on gene knockout using in vitro fertilized eggs.

2 Materials

2.1 Preparation of Genome Editing Tools

2.1.1 ZFNs

1. CompoZr Custom Zinc-finger nuclease (Sigma-Aldrich).

2.1.2 TALENs

1. Custom TALEN synthesis service (ToolGen).
2. Platinum Gate TALEN Kit (Addgene).

2.1.3 CRISPR/Cas9

1. pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene).
2. gRNA Cloning Vector (Addgene).
3. pCAG-hCas9 (Addgene).
4. Guide-it Recombinant Cas9 Nuclease (Takara Bio).

2.1.4 In Vitro Transcription

1. mMESSAGE mMACHINE T7 Ultra Transcription Kit (Thermo Fisher Scientific).
2. MEGAshortscript T7 Transcription Kit (Thermo Fisher Scientific).
3. DNase/RNase-free distilled water.

4. RNase decontamination reagent: for eliminating of RNase contamination.
5. EndoFree Plasmid Maxi Kit (QIAGEN).
6. Isopropanol.
7. Restriction enzyme.
8. 99.5% ethanol.
9. TE saturated phenol: Saturated with 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.
10. Chloroform.
11. 3 M sodium acetate (pH 5.2).

2.1.5 Purification of RNA

1. MEGAclear Transcription Clean-Up Kit (Thermo Fisher Scientific).

2.1.6 Nuclease-Based Mutation Detection Assay

1. DNeasy Blood and Tissue Kit (QIAGEN).
2. Guide-it Mutation Detection Kit (Takara Bio).

2.2 Isolation of Nuclear Donor Cells for SCNT

2.2.1 Preparation for Porcine Fetal Fibroblasts (PFFs) for Gene Knockout

1. Antibiotic-antimycotic solution (Thermo Fischer Scientific).
2. Dulbecco's phosphate-buffered saline (PBS) supplemented with 2% (v/v) antibiotic-antimycotic solution.
3. Iris scissors and forceps.
4. Collagen Type I coated 75 cm^2 culture flask (Asahi Glass).
5. Stainless-steel sieve.
6. Cellbanker (Takara Bio): for cryopreservation of PFFs.
7. 100 mm plastic Petri dish.
8. Cell strainer.
9. Syringe plunger.
10. 0.05% Trypsin-EDTA.
11. Insulin-Transferrin-Selenium-Ethanolamine (ITS-X, Thermo Fischer Scientific).
12. MEM Non-Essential Amino Acid Solution (NEAA, Thermo Fischer Scientific).
13. Culture medium: Minimum Essential Media (MEM)α (Thermo Fisher Scientific) supplemented with 15% (v/v) fetal bovine serum (FBS), 2% (v/v) antibiotic-antimycotic solution, 1% (v/v) ITS-X, 1% (v/v) NEAA.

2.2.2 Electroporation

1. Neon Transfection System (Thermo Fisher Scientific).
2. Dulbecco's Phosphate-Buffered Saline (PBS, DNase-RNase-free grade).
3. Culture medium: MEMα supplemented with heat-inactivated 15% FBS with or without 1% (v/v) antibiotic-antimycotic solution.

2.2.3 Isolation of Gene Knock-out Cells (Nuclear Donor)

1. MEMα (Thermo Fisher Scientific).
2. FBS.
3. Antibiotic-antimycotic solution.
4. 0.05% Trypsin-EDTA.
5. PBS.
6. Collagen Type I coated 60-, 100 mm dish (Asahi Glass).
7. Collagen Type I coated 96-, 48-, 24-, 12-, 6-well plate (Asahi Glass).
8. Countess Automated Cell Counter (Thermo Fisher Scientific).
9. Countess Cell Counting Chamber Slides (Thermo Fisher Scientific).
10. Cryovial tube.
11. Cellbanker.
12. Culture medium: for culture of PFFs. MEMα supplemented with heat-inactivated 15% FBS and 1% (v/v) antibiotic-antimycotic solution.
13. Cell strainer.
14. Multichannel pipette.
15. Pipetting reservoir.
16. MightyAmp DNA polymerase Ver.2 (Takara Bio).
17. PrimeSTAR HS DNA polymerase (Takara Bio).
18. BigDye Terminator Cycle Sequencing Kit (Thermo Fisher Scientific).
19. ABI PRISM 3130xl Genetic Analyzer (Thermo Fisher Scientific).
20. Zero Blunt TOPO PCR Cloning Kit for Sequencing (Thermo Fisher Scientific).
21. LB agar plate including 50 μg/mL of ampicillin or kanamycin.

2.3 Somatic Cell Nuclear Transfer (SCNT)

2.3.1 Preparation of In Vitro Matured (IVM) Oocytes

1. PBS-PVA: PBS containing 75 μg/mL potassium penicillin G, 50 μg/mL streptomycin sulfate, and 0.1% (w/v) polyvinyl alcohol (PVA).
2. Hexadecyltrimethylammonium bromide (CETAB) stock: Dissolve 25 g of CETAB in 125 mL 99.5% ethanol and bring the volume up to 500 mL with water.
3. CETAB solution: Dilute CETAB stock to 0.2% solution with water.
4. Porcine oocyte/embryo collection medium (POE-CM, Research Institute for the Functional Peptides): for oocytes collection.

5. North Carolina State University (NCSU)-23 medium: 108.73 mM NaCl, 4.78 mM KCl, 1.7 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 25.07 mM $NaHCO_3$, 5.55 mM glucose, 7.0 mM taurine, 50 mg/L streptomycin sulfate, 65 mg/L penicillin-G in water.
6. High-performance porcine oocytes medium (HP-POM; Research Institute for the Functional Peptides).
7. Dibutyryl cyclic adenosine monophosphate (dbcAMP, Research Institute for the Functional Peptides).
8. Porcine follicular fluid: collect follicular fluid from ovaries (3–6 mm of follicles in diameter) by aspiration and centrifuged at 1200 × *g* for 60 min at 4 °C.
9. NCSU-based maturation medium (for SCNT): NCSU23 supplemented with 10% (v/v) porcine follicular fluid, 10 IU/mL equine chorionic gonadotropin (eCG) and 10 IU/mL human chorionic gonadotropin (hCG), 0.6 mM cysteine, 10 ng/mL EGF, 1 mM L-glutamine, 5 mM Hypotaurine.
10. POM-based maturation medium (for in vitro fertilization): HP-POM supplemented with 10% (v/v) porcine follicular fluid, 10 IU/mL equine chorionic gonadotropin (eCG), and 10 IU/mL human chorionic gonadotropin (hCG), 1 mM dbcAMP.
11. Paraffin oil.
12. 35-, 60 mm plastic Petri dish.
13. Thermo-container.
14. Needles (20 G).
15. Syringes (10 mL).
16. Warm plate (38.5 °C).

2.3.2 Nuclear Transfer

1. MEMα: for culture of nuclear donor.
2. FBS.
3. HEPES-buffered Tyrode's lactose medium with 0.3% (w/v) PVP (HEPES-TL-PVP): 10 mM HEPES and 0.3% (w/v) polyvinylpyrrolidone in Tyrode lactose medium.
4. Hyaluronidase: 1 mg/mL solution in HEPES-TL-PVP.
5. Demecolcine solution (Sigma-Aldrich): 10 μg/mL in Hank's Balanced Salt Solution (HBSS).
6. Cytochalasin B stock: 5 mg/mL solution in dimethyl sulfoxide (DMSO).
7. Fusion medium: 280 mM mannitol, 0.5 mM HEPES, 0.15 mM $MgSO_4$, 0.01% (w/v) PVA in water.
8. Electrode needles (Nepa Gene).

9. Somatic hybridizer (Nepa Gene).
10. Porcine zygote medium-5 (PZM-5, Research Institute for Functional Peptides): for in vitro culture.
11. PZM-5 (BSA) (Research Institute for the Functional Peptides): PZM-5 without PVA supplemented with 4 mg/mL BSA.
12. Fusion chamber slide (MS Platinum Wire Electrode 1 mm gap, Nepa Gene).
13. Activation solution: 280 mM mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 0.01% (w/v) PVA.
14. Electrical pulsing machine (Multiporator, Eppendorf).
15. Scriptaid stock: 2.5 mM Scriptaid in DMSO.

2.4 Cytoplasmic Injection

2.4.1 In Vitro Fertilization (IVF) of Porcine Oocytes

1. PBS-BSA: PBS supplemented with 0.1% bovine serum albumin (BSA).
2. Porcine fertilization medium (PFM; Research Institute for the Functional Peptides).
3. HEPES-buffered Tyrode's lactose medium with 0.3% (w/v) PVP (HEPES-TL-PVP): 10 mM HEPES and 0.3% (w/v) polyvinylpyrrolidone in Tyrode lactose medium.
4. HEPES-TL-PVP supplemented with 10% FBS: for checking the first polar body.
5. PZM-5.
6. PZM-5 (BSA).
7. Paraffin oil.
8. 35 mm plastic Petri dish.

2.4.2 Cytoplasmic Injection to Porcine Zygotes

1. Micromanipulation system (Narishige).
2. Microinjector (FemtoJet, Eppendolf).
3. Microloader tip (Eppendolf).
4. Injection pipet: the injection pipettes are made with a micropipette puller (Sutter instrument) with the following adjustments: Heat, 675; Velocity, 30; Time, 250.
5. Capillary glass for holding pipet (Harvard Apparatus): diameter is 150 μm.
6. Inverted microscope.
7. DNase/RNase free water: for dilution of RNAs.
8. TE^{-1} buffer: 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0).
9. HEPES-TL-PVP supplemented with 5% FBS: HEPES-TL-PVP containing 5% FBS, adjust osmotic pressure 290 mOsm.
10. PZM-5.

11. Mineral oil.
12. Paraffin oil.
13. 35 mm plastic Petri dish.

2.5 General Equipment and Plastics

1. Humidified incubator at 30 and 37 °C, and 5% CO_2 for cell culture.
2. Humidified incubator at 38.5 °C and 5% CO_2 for embryo culture.
3. Humidified incubator at 38.5 °C and 5% CO_2, 5% O_2, and 90% N_2 for embryo culture.
4. Water bath (37 °C, 55 °C).
5. Freezers (−30 °C, −80 °C) and refrigerators (4 °C).
6. Phase contrast microscope.
7. Stereoscopic microscope.
8. Pipetman (2-, 10-, 20-, 200-, and 1000 μL).
9. Tips for 2-, 10-, 20-, 200-, and 1000 μL pipetman.
10. Plastic tube (1.5 mL).
11. Conical tube (15-, 50 mL).
12. Thermal cycler.
13. Electrophoresis system.
14. 0.2 mL PCR tube and 96-well PCR plate.
15. Spectrophotometer.
16. Liquid nitrogen storage tank.
17. Plastic pipets (1-, 5-, 10-, 25 mL).
18. Centrifuge.
19. Clean bench and biosafety cabinet.
20. 37 °C shaker (at 160–200 rpm).

3 Methods

3.1 Preparation of Genome Editing Tools

Different protocols are available (*see* Chapters 2–4). All steps require DNase/RNase-free reagents and equipment such as microcentrifuge tubes and pipette tips for electroporation or cytoplasmic injection of RNAs.

3.1.1 ZFNs

Basic handling and design/construction for ZFNs are described in Chapter 2 (*see* **Note 1**).

3.1.2 TALENs

The basic handling and design/construction of TALENs are described in Chapter 3 (*see* **Note 2**).

3.1.3 CRISPR/Cas9

The basic handling and design/construction of CRISPR/Cas9 are described in Chapter 4 (*see* **Note 3**).

3.1.4 In Vitro Transcription

Use the mMESSAGE mMACHINE T7 Ultra Transcription Kit for ZFN-, TALEN-, and Cas9-mRNAs. For in vitro transcription of guide RNA (gRNA), use the MEGAshortscript T7 transcription Kit in this step. These protocols essentially correspond to the manufacturer's instructions (*see* Chapters 2–4 and **Note 4**).

3.1.5 Purification of RNA

Use the MEGAClear Kit for this step. This protocol essentially corresponds to the manufacturer's instructions (*see* Chapters 2–4 and **Note 5**).

3.1.6 Nuclease-Based Mutation Detection Assay (Cleavage Detection Assay)

The cleavage detection assay with mismatch-specific nucleases is useful for validating the cleavage efficiency of CRISPR/Cas9 (*see* Chapters 2–4 and **Note 6**). Use the Guide-it Mutation Detection Kit for this step. This protocol essentially corresponds to the manufacturer's instructions.

3.2 Isolation of Nuclear Donor Cells for SCNT

When isolating nuclear donor cells, plasmid DNA is commonly used for ZFNs, TALENs, or CRISPR/Cas9 expression; however, we used RNAs encoding those to prevent foreign genes from being inserted into the individual genomes of animals. In addition, the Cas9 protein can be used instead of Cas9-mRNA in the CRISPR/Cas9 system.

3.2.1 Preparation for PFFs for Gene Knockout

Generally, a primary culture of porcine fetal fibroblasts (PFFs) with high growth performance is suitable as the progenitor line for isolating genetically modified cells (*see* **Note 7**).

1. The uterus containing fetuses is excised surgically from pregnant sow under anesthesia (*see* **Note 8**).
2. Fetuses are recovered from the uterus, sacrificed, and transferred to a plastic dish on ice.
3. In the clean bench, the head and visceral tissue of each fetus are removed using iris scissors and forceps, and then washed three times using PBS supplemented with antibiotic-antimycotic.
4. The remnants are minced with scissors on a 100 mm Petri dish, followed by grinding with a syringe plunger and stainless-steel sieve to eliminate clumps and debris.
5. Rinse the stainless-steel sieve with culture medium; the pass-through sample is collected as dispersed cell suspensions.
6. Seed the cell suspension into 75 cm^2 collagen-coated flasks.
7. When the cells reach the subconfluent stage (70–80% confluency) in the flask, the cells are harvested after treatment with 0.05%

Trypsin-EDTA and passaged to a new flask for the further propagation (*see* **Note 9**).

8. Under subconfluent conditions, the PFFs are harvested and cryopreserved with Cellbanker at a concentration of 5×10^5 cells/tube for later use (*see* **Note 10**).

3.2.2 Electroporation

Use the Neon Transfection System (Thermo Fisher Scientific) for this step. Electroporation is conducted essentially according to manufacturer's instructions.

1. Low passaged PFFs (Passage number 2-4) (Subheading 3.2.1) are seeded to be 70–90% confluent at the time of electroporation on a 100 mm collagen-coated dish.
2. On the day of electroporation, PFFs are washed twice with PBS and harvested after treatment with 0.05% Trypsin-EDTA.
3. After adding the culture medium to stop the trypsin reaction, harvest and centrifuge at $150 \times g$ for 5 min at room temperature.
4. Carefully remove the liquid; the cell pellets are suspended with 1 mL of DNase/RNase-free PBS (*see* **Note 11**).
5. After cell counting, 5×10^5 cells are transferred to a 1.5 mL tube and centrifuged at $150 \times g$ for 5 min at room temperature.
6. Carefully remove the liquid and resuspend the PFFs in 50 μL (1×10^5 cells/10 μL) of R-buffer (included in the Neon Transfection system).
7. Add RNAs encoding ZFNs, TALENs, or CRISPR/Cas9 to the cell suspension and gently mix (*see* **Note 12**).
8. Electroporation is performed using the Neon transfection system with 10 μL Neon tip (*see* **Note 13**).
9. After electroporation is complete, transfer the electroporated cells (5×10^5 cells) to a 100 mm collagen-coated dish containing 10 mL of pre-warmed culture medium without antibiotic-antimycotic (*see* **Note 14**).

3.2.3 Isolation of Gene Knock-out Cells (Nuclear Donor)

An overview of this step is presented in Fig. 2.

1. After electroporation (Subheading 3.2.2), the ZFN- or TALEN-treated cells are cultured at 30 °C for 72 h (*see* **Note 15**). For CRISPR/Cas9, the treated cells are cultured continuously at 37 °C.
2. When the cells reach the subconfluent stage (generally after 48 or 72 h of electroporation), the cells are harvested for limiting dilution to single cell cloning.

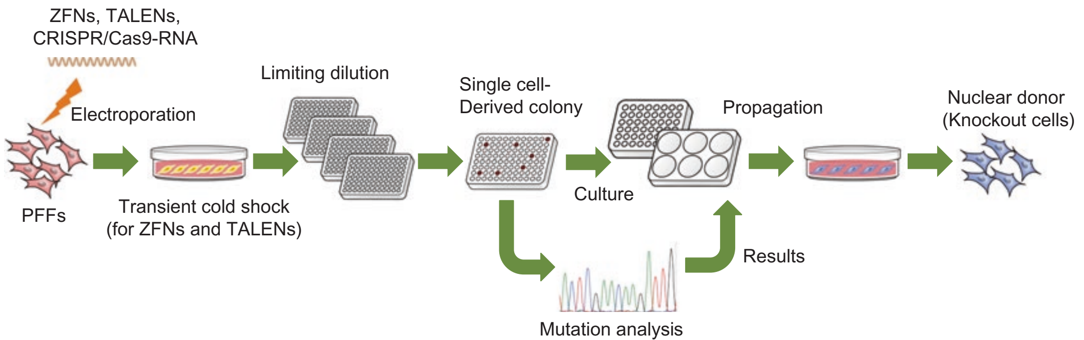

Fig. 2 Schematic representation of isolation for gene knock-out cells (nuclear donor) for SCNT

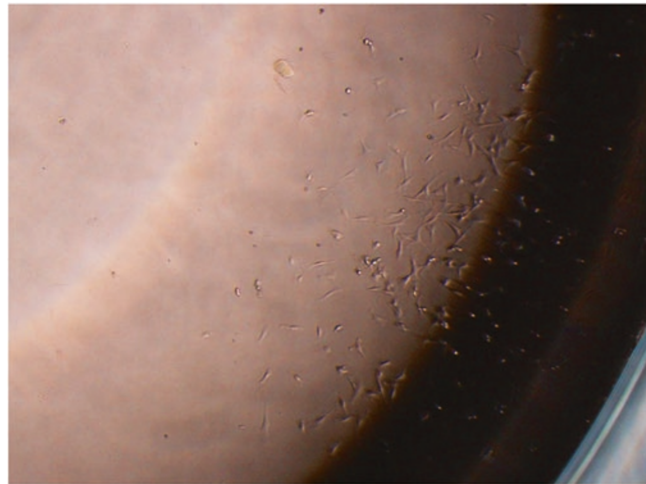

Fig. 3 Single cell-derived colony in the 96-well plate after 7 days of limit dilution

3. Count the number of live cells and dilute them serially with culture medium at a concentration of 5 cells/mL (*see* **Note 16**).
4. Dispense 1 cell/well (200 μL) in 96-well plates using a multichannel pipette and transfer the plates to a humidified incubator at 37 °C.
5. Change the culture medium every 2–3 days (*see* **Note 17**).
6. After 7–8 days of culture, check the colony formation derived from a single cell (*see* **Note 18** and Fig. 3).
7. At 14 days after limiting dilution, cells at relatively high confluency (>50%) with good morphology in each well are selected and divided for further culture (transferred to a 48-well plate) and mutation analysis (*see* **Note 19**).
8. For mutation analysis, direct PCR from the divided cells in the well is performed to amplify the target sequence and confirm

the ZFN-, TALEN-, or CRISPR/Cas9-induced mutations by direct sequencing (*see* **Note 20**).

9. In parallel with mutation analysis (**step 8**), the cell culture is expanded (*see* **Note 21**).
10. Finally, harvest single cell-derived clones with gene knockout, suspend the cells in Cellbanker, and cryopreserve as nuclear donor cells at −80 °C or in liquid nitrogen until SCNT (*see* **Note 22**).

3.3 Somatic Cell Nuclear Transfer (SCNT)

Handling of porcine oocytes is performed on a warm plate at 38.5 °C unless otherwise indicated.

3.3.1 Preparation of In Vitro Matured (IVM) Oocytes

1. Porcine ovaries are collected at a local abattoir and transported to the laboratory in PBS-PVA at 30–35 °C using a thermo-container.
2. The ovaries are washed three times with CETAB solution and rinsed three times with PBS-PVA (*see* **Note 23**).
3. Cumulus-oocyte complexes (COCs) are collected by aspiration using a needle with a syringe from ovarian antral follicles with a diameter of 3.0–6.0 mm.
4. Dilute the collected COCs and follicular fluid with POE-CM, and select COCs which have at least three layers of compacted cumulus cells.
5. Wash selected COCs in three steps into NCSU23-based maturation medium and culture them for 22 h in a humidified atmosphere of 5% CO_2 and 95% air at 38.5 °C. Next, wash COCs with maturation medium and culture for 18–22 h without hormones in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5 °C (*see* **Note 24**).

3.3.2 Nuclear Transfer

An overview of this step is presented in Fig. 1.

1. To prepare the nuclear donor, thaw the stored nuclear donor cells (Subheading 3.2.3) and culture in MEMα supplemented with 15% FBS to sub-confluence followed by culture in MEMα containing 0.5% FBS for starvation to synchronize the cell cycle.
2. IVM oocytes with expanded cumulus cells (Subheading 3.3.1) are treated with hyaluronidase and denuded of cumulus cells by gentle pipetting.
3. Denuded IVM oocytes containing the first polar body are enucleated by gentle aspiration of the polar body and adjacent cytoplasm using a beveled pipette in HEPES-TL-PVP in the presence of 0.1 µg/mL demecolcine, 5 µg/mL cytochalasin B (CB), and 10% FBS.

4. A single donor cell should be inserted into the perivitelline space of an enucleated oocyte (*see* **Note 25**).
5. The donor cell-oocyte complexes are then placed in a fusion medium and held between two electrode needles.
6. Membrane fusion should be induced with a somatic hybridizer by applying a single direct-current (DC) pulse and a pre- and post-pulse alternating current (AC) field of 4 V at 1 MHz for 5 s (*see* **Note 26**).
7. The reconstructed embryos are cultured in PZM-5 (BSA) for 1–1.5 h, followed by electrical activation.
8. To induce electrical activation, the reconstructed embryos are aligned between two wire electrodes (1.0 mm apart) of a fusion chamber slide filled with activation solution. A single DC pulse of 150 V/mm should be applied for 100 μs using an electrical pulsing machine.
9. After activation, the reconstructed embryos are cultured in PZM-5 for 3 h in the presence of 5 μg/mL CB and 500 nM Scriptaid, and then with 500 nM Scriptaid for another 12–14 h (*see* **Note 27**).
10. After these treatments, SCNT embryos are cultured in PZM-5 under a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5 °C until embryo transfer (*see* **Note 28**).

3.4 Cytoplasmic Injection

3.4.1 In Vitro Fertilization (IVF) of Porcine Oocytes

1. A straw containing frozen sperm is thawed by immersion in warm water (37 °C) for 30 s (*see* **Note 29**).
2. The sperms are then suspended in 5 mL PBS-BSA and washed and centrifuged three times at 1000 × *g* for 4 min.
3. After washing, the sperm pellets are resuspended in porcine fertilization medium (PFM) at a concentration of 1.0×10^7 cells/mL.
4. For insemination, 20 IVM oocytes (Subheading 3.3.1) are placed in a 100 μL drop of PFM containing spermatozoa (1.0–5.0×10^6 cells/mL); the oocytes and sperms are incubated for 8 h at 38.5 °C in a humidified atmosphere containing 5% CO_2, 5% O_2, and 90% N_2.
5. After insemination, the embryos are transferred into HEPES-TL-PVP; cumulus cells and excess sperms are removed by gentle pipetting.
6. Embryos showing the release of one or more polar bodies with normal cytoplasmic morphology are cultured (20–30 embryos/20–30 μL drop) in PZM-5 under paraffin oil in a plastic Petri dish maintained in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5 °C.

7. Embryos are cultured in PZM-5 (BSA) until cytoplasmic injection (*see* **Note 30**).

3.4.2 Cytoplasmic injection to porcine zygotes

1. Prepare in vitro fertilized embryos (Subheading 3.4.1) in a drop of HEPES-TL-PVP supplemented with 5% FBS in a Petri dish.
2. Prepare appropriate concentration of ZFN, TALEN-mRNA solution, or CRISPR/Cas9 (gRNA and Cas9-mRNA) solution for cytoplasmic injection (*see* **Note 31**).
3. The RNA solution is injected into the cytoplasm of pronuclear stage embryos using an injection pipet (*see* **Note 32**).
4. Injected embryos are washed twice with HEPES-TL-PVP.
5. Embryos are cultured (20–30 embryos/20–30 μL drop) in PZM-5 under paraffin oil in a plastic Petri dish maintained in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5 °C.
6. To generate knock-out pigs, the injected embryos are transferred into the recipient gilts (*see* **Notes 28** and **33**).

4 Notes

1. The design and construction of ZFNs are complex and difficult; therefore, we purchased them from Sigma-Aldrich if possible. The design, plasmid construction, and validation of ZFNs are performed by Sigma-Aldrich (http://www.sigmaaldrich.com/life-science/zinc-finger-nuclease-technology/custom-zfn.html).
2. We used TALENs purchased from ToolGen (http://toolgen.com); however, this service is no longer available at ToolGen. Alternatively, Platinum TALEN is relatively easy to construct and is capable of high-efficiency knockout in pigs as well as in other animals.
3. When evaluating DNA cleavage activity of CRISPR/Cas9 in vitro, it is convenient to use pX330 plasmid (Addgene) containing a gRNA expression cassette and Cas9 on the same vector. The CRISPR Design (http://crispr.mit.edu/) or CRISPR direct (https://crispr.dbcls.jp/) tools are available for the pig genome. We typically design and construct pX330 vectors targeted to three or four loci in the gene of interest. For off-target site analysis, CRISPR Design tool and COSMID (https://crispr.bme.gatech.edu/) are useful. Recently, Cas9 protein has become commercially available. Cas9 protein is useful for the isolation of gene knock-out cells (nuclear donor) and cytoplasmic injection.

4. Plasmids for templates of in vitro transcription must be linearized with restriction enzymes because circular plasmid templates will generate extremely long, heterogenous RNA transcripts. According to the manufacturer's instructions, ZFN-plasmids (Sigma-Aldrich), TALEN-plasmids (ToolGen), and Platinum TALEN-plasmids (Addgene) are digested with the XbaI, PvuII, and SmaI restriction enzymes, respectively.
5. We adjust the concentration of each ZFN or TALEN-mRNAs to 400 ng/μL using RNase-free water. Equal amounts of the diluted RNAs are mixed to prepare ZFN- or TALEN-mRNA solution (i.e., 200 ng/μL each). For CRISPR/Cas9, we adjust the concentration of gRNA and Cas9-mRNA to 400 ng/μL using RNase-free water, respectively.
6. For electroporation to validate the cleavage efficiency of CRISPR/Cas9, 2 μL of 500 ng/μl pX330 plasmid constructed is added to 2×10^5 cells/20 μL of R-buffer (Subheading 3.2.2). Treated cells are cultured in a 6-well plate at 37 °C for 48 h and then the cleavage detection assay is performed using the Guide-it Mutation detection Kit. To cleave the mismatched-heteroduplex DNA, T7 endonuclease 1 (T7E1) is also available.
7. We have performed efficient knockout of various porcine genes using PFFs. We recommend using PFFs for the isolation of genetically modified cells for SCNT, although fibroblasts from neonatal or adult pigs are also available for the isolation of nuclear donor cells.
8. Generally, we isolate the primary culture of PFFs from fetuses (generally 37–38 days of gestation.
9. This step not only expands the cells but also purifies and selects for PFFs, as well as allows for the removal of cells other than PFFs.
10. We recommend examining the karyotype (38 + XY for male, 38 + XX for female) of the isolated PFFs.
11. From this step, to achieve as much of an RNase-free state as possible during electroporation, DNase/RNase-free reagents and equipment should be used for electroporation.
12. For ZFN or TALEN, 2.5–5 μL (generally 2.5 μL) of 400 ng/μl ZFN or TALEN-mRNA solutions are added to 5×10^5 cells/50 μL of R-buffer. For CRISPR/Cas9, 2.5 μL of 400 ng/μl gRNA and 5 μL of 400 ng/μl Cas9-mRNA are added to 5×10^5 cells/50 μL of R-buffer. When using Cas9 protein instead of Cas9-mRNA, 2.5 μL of 400 ng/μl gRNA and 5 μL of 500 ng/μl recombinant Cas9 nuclease (Takara Bio) are premixed to form a ribonucleoprotein (RNP) complex and then added to 5×10^5 cells/50 μL of R-buffer.

13. After adding RNAs into the cell suspension, electroporation should be started immediately to avoid RNA degradation. Optimal conditions for electroporation should be determined in advance using a reporter plasmid (e.g., EGFP-expressing plasmid etc.). Transfection efficiency should be over 70–80%.
14. After 24 h of electroporation, replace the complete culture medium containing 1% (v/v) antibiotic-antimycotic solution.
15. Transient cold-shock treatment is effective for enhancing the cleavage activity of ZFNs or TALENs [25]. Transient cold-shock treatment at 30 °C may damage electroporated cells. If this occurs, the cells can be cultured under milder conditions (32 °C for 48 or 72 h).
16. Before cell counting, pass the sample through a cell strainer to obtain a single cell suspension and eliminate aggregates for limiting dilution. We typically seed the cell suspension into onto five 96-well plates to obtain 96-192 single cell-derived colonies.
17. The propagation of PFFs from single cells using limiting dilution is difficult. To improve the growth performance of PFFs, adding 2-mercaptoethanol into the culture medium at a concentration of 0.1 mM is effective.
18. At 7–8 days after limiting dilution, small colonies can be observed (Fig. 3). Wells containing only two or three colonies of cells are excluded from further experiments. Moreover, wells containing extremely large colonies compared with other colonies are also excluded from further experiments because such colonies are likely not single cell derived.
19. After stopping the trypsin reaction by adding the culture medium directly into the 96-well plates, the cell suspension is mixed well by gentle pipetting. Subsequently, half of the cells are passaged into 48-well plates, and the remaining cells are allowed to adhere to the plate (generally for 2 or 3 h) to perform direct PCR for the mutation analysis. We typically analyze and passage 96-192 single cell-derived colonies for the mutation analysis.
20. After aspirating the medium, PCR mixtures of MightyAmp DNA polymerase ver.2 (Takara Bio) are directly added to the well in which half of the cells are adherent as described in Subheading 3.2.3, **step** 7. Next, the cells in the well are scratched using a pipet tip, and the liquid PCR sample is transferred to 96-well PCR plates for mutation analysis. Generally, small insertion and deletion (indel) mutations can be identified by direct sequencing in most cell clones analyzed. Subcloning of the PCR products using the TOPO Cloning Kit (Thermo Fisher Scientific) and sequencing may be required to identify the mutations because of the presence of complicated indel

mutations or sequences lacking sequencing primer binding sites as a result of large deletions and insertions. It is important to quickly identify the mutation because culturing many single cell-derived clones continues simultaneously.

21. After passage to a 48-well plate, the cells can typically be passaged every 2–3 days (48-well→24-well→6-well plate→60 mm dish→100 mm dish). The time interval of passage can be used as an indicator to obtain good candidates for nuclear donor cells with potential proliferation abilities.
22. At this step, the passage number of the cell clones will reach approximately P10. Approximately 30–40 days are required to isolate the nuclear donor cells (from electroporation to cryopreservation of nuclear donor cells). At this step, we recommend a re-analysis of the mutations in cryopreserved cells as nuclear donors.
23. Until the aspiration of follicles, keep the washed ovaries in PBS-PVA in a water bath at 38.5 °C.
24. For the preparation of in vitro fertilized embryos (Subheading 3.4.1), wash selected COCs in three steps into POM-based maturation medium and culture them for 20 h, followed by culture for 24 h without dbcAMP and hormones, in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5 °C.
25. Synchronization of the nuclear donor cells at G0/G1 is a key factor for the success for SCNT. Relatively small size cells are suitable as nuclear donors because it is assumed that G0/G1 stage cells are smaller than those in the G2/M stage.
26. Determination of an optimal single direct-current (DC) pulse should be tested depending on the instrument used. We typically induce membrane fusion by applying a single direct-current (DC) pulse (267 V/mm, 20 μs) using a somatic hybridizer (LF201; Nepa Gene).
27. Treatment with histone deacetylase inhibitor, Scriptaid, is effective for improving the in vitro development of SCNT embryos. The morphology of cloned blastocysts treated with Scriptaid is better than that of untreated blastocysts, and the number of constituent cells is high [26].
28. We choose the sites (oviducts or uterine horn) of embryo transfer depending on the stage of embryos. Embryos cultured for 1–2 days are surgically transferred into the oviducts of recipients approximately 10–13 h after the estimated time of ovulation. Embryos cultured for 5–6 days are transferred at the blastocyst stage to the uterine horns of the recipients approximately 105–107 h after the estimated time of ovulation. The genotypes of all cloned offspring from SCNT embryos should contain the same mutation as the nuclear donor cells.

29. We use frozen epididymal sperms from a boar at a concentration of 1.0×10^9 cells/mL as described by Kikuchi et al. [27] prior to use in IVF.
30. We typically use the in vitro fertilized embryos for cytoplasmic injection 8.5–9.0 h after insemination.
31. Generally, we use 2–10 ng/μL of ZFN or TALEN-mRNAs diluted with RNase-free water. For CRISPR/Cas9 system, 2–10 ng/μL of gRNA and 10 ng/μL Cas9-mRNA or Cas9 proteins are used for injection. When using Cas9 protein, Cas9 proteins are diluted with TE^{-1} solution and then premixed with gRNA to form RNP complex just before injection.
32. We typically inject approximately 10 pL of solution into the cytoplasm of the in vitro fertilized embryos.
33. Unlike cloned offspring from SCNT embryos, in some of offspring obtained from embryos by cytoplasmic injection, more than three different allele variants can be found in single individuals because of mosaicism. Therefore, the phenotypes of these mosaic founders should be interpreted with care, particularly for human disease models. Mosaicism in cytoplasmic injection is particularly troublesome for large animals as described in Subheading 1. To date, there are no clear strategies for reducing mosaicism. To reduce mosaicism using the CRISPR/Cas9 system, Cas9 protein rather than RNA may be useful [28].

Acknowledgment

This work was supported by a Grant-in-Aid for Scientific Research (A) from the Japan Society for the Promotion of Science (No. 15H02480 to Hiroshi Nagashima), the Meiji University International Institute for Bio-Resource Research (MUIIBR to Hiroshi Nagashima), the Nakauchi Stem Cell and Organ Regeneration Project, Exploratory Research for Advanced Technology (ERATO), Japan Science and Technology Agency (JST) and the Leading Advanced Projects for medical innovation (LEAP), Japan Agency for Medical Research and Development (AMED). This work was also partially supported by the Practical Research Project for Rare / Intractable Diseases from AMED.

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Chapter 12

Genome Editing of Monkey

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Abstract

Gene-modified monkey models would be particularly valuable in biomedical and neuroscience research. Virus-based transgenic and programmable nucleases-based site-specific gene editing methods (TALEN, CRISPR-cas9) enable the generation of gene-modified monkeys with gain or loss of function of specific genes. Here, we describe the generation of transgenic and knock-out (KO) monkeys with high efficiency by lentivirus and programmable nucleases.

Key words Lentivirus, Programmable nucleases, Gene-modified monkeys, Transgenic, Knockout

1 Introduction

Gene-modified animal models have been widely used in various biological studies. Technologies for generating gene-modified animal models have been developed for several decades. Many methods such as pronucleus microinjection, virus infection, DNA transposon vectors, embryonic stem cell gene targeting, sperm-mediated gene transfer, somatic cell nuclear transfer, semi-clone by haploid stem cell, spermatogonial stem cell-mediated transgene, and recently developed programmable nucleases (ZFN, TALEN, CRISPR-cas9) have been successfully used to generating various and complex animal models in rodents. Taking into consideration feasibility and efficiency, only virus infection and programmable nucleases were successfully applied in monkeys.

The first transgenic monkey was generated by retrovirus in 2001 [1]. Several pioneering works in transgenic monkey such as Huntington's disease model, GFP transgenic marmoset, and Mecp2 transgenic monkey model with germline transmission have been reported by lentivirus infection recently [2–4]. This indicates that it would be a routine method to generate overexpression transgenic monkey models by lentivirus infection. Recently developed programmable nucleases (ZFN, TALEN, CRIPSR-cas9) make it feasible to generate site-specific knock-out monkey models

Izuho Hatada (ed.), *Genome Editing in Animals: Methods and Protocols*, Methods in Molecular Biology, vol. 1630,
DOI 10.1007/978-1-4939-7128-2_12, © Springer Science+Business Media LLC 2017

[5–7]. They were used to generate knock-out various organisms including monkey models rapidly. Obviously, these methods especially the CRISPR-cas9 system will be widely used in generating gene-modified monkey models.

The assisted reproduction technology (ART) of monkey is the fundamental and core procedure in generating gene-modified monkeys. A stable and efficient platform of monkey ART is very necessary. By taking advantage of the high efficient monkey ART established before [8], we have successfully applied lentivirus infection, TALEN, and CRISPR-cas9 in generating transgenic and knock-out monkey (Macaca fascicularis) models.

2 Materials

1. Triptorelin Acetate (Ipsen pharma biotech).
2. Recombinant Human Follitropin (Merck Serono).
3. Chorionic Gonadotrophin Human (Sigma).
4. Zoletil (Virbac).
5. HEPES-buffered TALP medium: add NaCl 6.660 g, KCl 0.239 g, $CaCl_2 \cdot 2H_2O$ 0.294 g, $MgCl_2 \cdot 6H_2O$ 0.102 g, Na_2HPO_4 0.048 g, Glucose 0.900 g, Na lactate 1.87 mL, Phenol red 0.010 g, $NaHCO_3$ 0.168 g, penicillin-G (sodium salt)/streptomycin (100×, 10 mL), HEPES 2.603 g and Na pyruvate 0.060 g to 1 L distilled water and adjust the pH to 7.4.
6. TH3 medium: HEPES-buffered TALP medium, containing 0.3% bovine serum albumin [9].
7. 2.5% Heparin: 1 mg heparin dissolved in 39 mL TH3 medium.
8. Hamster embryo culture medium-9: add NaCl 6.639 g, KCl 0.224 g, $CaCl_2 \cdot 2H_2O$ 0.279 g, $MgCl_2 \cdot 6H_2O$ 0.102 g, Na lactate (60% syrup), 0.632 mL, Polyvinyl alcohol (PVA) 0.1 g and $NaHCO_3$ 2.1 g to 1 L distilled water and adjust the pH to 7.4 [10].
9. 100× Amino acid–pantothenate stock: add Taurine 6.250 g, Asparagine 0.132 g, Cysteine 0.121 g, Histidine 0.155 g, Lysine 0.146 g, Proline 0.115 g, Serine 0.105 g, Aspartic acid 0.133 g, Glycine 0.075 g, Glutamic acid 0.147 g, Glutamine 2.92 g and Pantothenic acid 0.07 g to 1 L distilled water, filter and split it.
10. HECM-9aa medium: Add amino acid–pantothenate stock to HECM-9 medium at a ratio of 1:100 before use.
11. HECM-9aa medium + 5% FBS: used for in vitro culture of monkey embryos to blastocyst stage.
12. 10% PVP: 1 g Polyvinylpyrrolidone dissolved in 9 mL TH3.

13. 2 mg/mL Hyaluronidase: 20 mg Hyaluronidase dissolved in 10 mL TH3.
14. mMESSAGE mMACHINE T7 ULTRA kit (Life Technologies).
15. MEGAshortscript T7 kit (Life Technologies).
16. MEGAclear kit (Life Technologies).
17. Mineral oil).
18. Cell strainers (BD).
19. Capillary glass (Sutter Instrument, B100-75-10, BF100-75-10).
20. P97 micropipet puller (Sutter Instrument).
21. Laparoscope operating system (endoscope, display screen, vacuum aspiration system, veress needle, trocar, grasping forceps).
22. Tube incubator (Cook).
23. Shadowless lamp.
24. Ultrasound instrument.
25. Restraint chair.
26. Electric stimulator (Shanghai Jialong instrument, JL-C4).
27. Inverted microscope (Olympus IX-83).
28. Micromanipulators (Narishige).
29. Syringe (IM9B, Narishige; CellTram vario, Eppendorf).
30. IM300 microinjector (Narishige).
31. Microscope heating stages (Tokai Hit).
32. Table centrifuge.
33. Piezo impact drive system (Prime Tech).

3 Methods

3.1 Monkey Superovulation

1. Monitor monkey menstruation cycle by directly observing or gently inserting cotton swab into the vagina of adult female monkey (*see* **Note 1**).
2. Administer sustained-release triptorelin at a dose of 0.5 mg/kg subcutaneously on day 2 of menstrual cycle to prevent spontaneous LH surges.
3. From day 3 of menstrual cycle, intramuscular injections of 15–25 IU (adjust doses according to weight) recombinant human follitropin twice daily for 7–8 days.
4. Monitor the ovarian morphology and follicle size by ultrasonography from day 10 of menstrual cycle. If the ovary diameter is smaller than 5 mm with no obvious responsive follicles, the superovulation should be canceled.

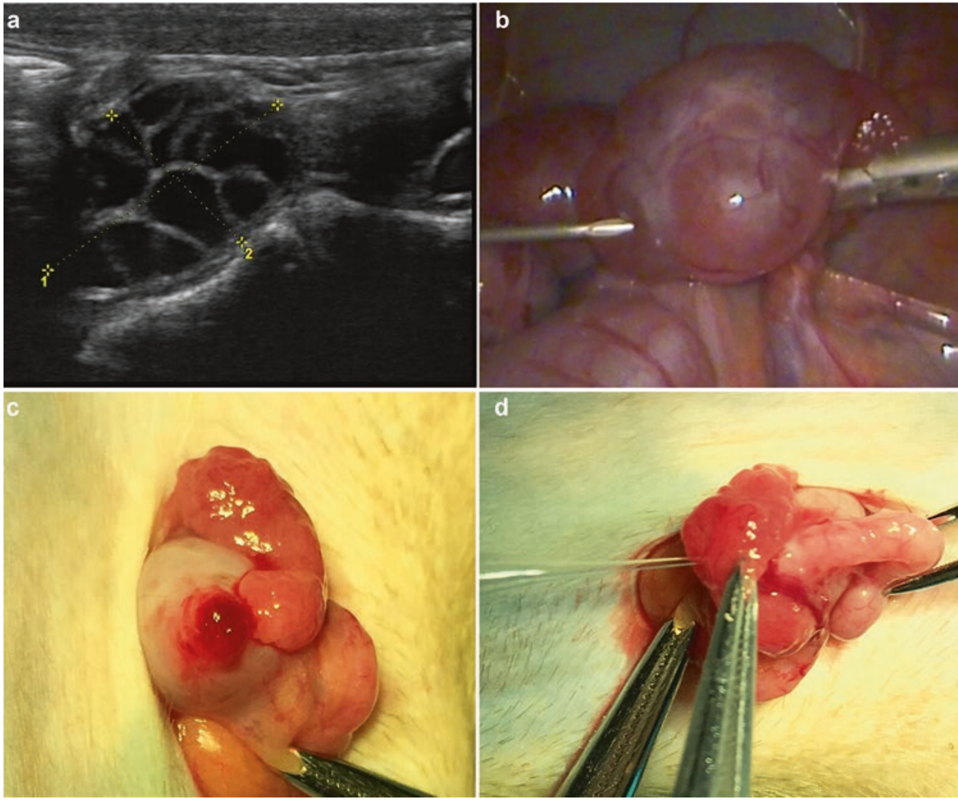

Fig. 1 Monkey surgery for oocytes collection and embryo transfer. (**a**) Ovary and follicles morphology by ultrasonography of the stimulated monkey on day 11 before hCG injection (1D = 19 mm, 2D = 12 mm). (**b**) Follicles aspiration by laparoscope operating system. (**c**) Stigma of the surrogate. (**d**) Monkey embryo transferred to ovarian duct

5. Intramuscular injections of Chorionic Gonadotrophin Human (hCG) 1000–1500 IU to the monkeys on day 11 of menstrual cycle (Fig. 1a). Usually, we inject 1000 IU per monkey and inject 1500 IU if the monkey is heavier than 5 kg. Postpone the hCG injection for 1 day if the follicle diameter is smaller than 2 mm).

3.2 Monkey Oocytes Collection

1. Anesthetize the female with zoletil 36 h after hCG injection.
2. Shave the abdominal fur and sterilize with iodine tincture and 75% medical ethanol.
3. Place the monkey on operating table in Trendelenburg and fix it by binding arms and legs on the table legs.
4. Cover the monkey by aseptic hole-towel with the sterile abdomen exposed.
5. Insert Veress needle into abdominal cavity placed above the navel. Inflate the abdominal cavity with N_2 at 1 L/min to 15 mmHg pressure and keep it. Pull out Veress needle and

insert trocar into abdominal cavity through the same position. Pull out obturator, leave the cannula connected with the gas tube.

6. Insert the endoscope into cannula, view the position and size of two ovaries. Position two accessory ports placed in the paralumbar region which is used for ovary stabilization and follicles aspiration.
7. As described in **step 5**, insert trocar into abdominal through positioned paralumbar region with the exception of gas linked. Pull out obturator and insert grasping forceps into abdominal cavity to stabilize ovary.
8. Insert a 18 g follicle aspirated needle which is connected with a negative pressure aspirator and a 15 mL centrifuge tube into abdominal cavity through opposite position of which grasping forceps is inserted. Aspirate all follicles of the two ovaries into a 15 mL centrifuge tube that contains 2–3 mL TH3 medium with heparin (Fig. 1b).
9. Stitch the incisions of the skin and muscle separately by using absorbable suture and silk sutures. Sterilize the incision with iodine tincture.
10. Transfer the tube to laboratory immediately and add 1 mL hyaluronidase (2 mg/mL) to the tube. Remove the cumulus cells by gently pipetting the follicular aspirates. Pour the follicular aspirates in cell strainer to remove blood and cumulus cells. Oocytes are still on the mesh of the cell strainer. Wash the strainer with 3–5 mL TH3 medium to remove remaining hyaluronidase.
11. Transfer the cell strainer to a 6 cm culture dish and turn it over. Wash the strainer with TH3 medium and collect the medium (*see* **Note 2**).
12. Transfer the medium to the stereomicroscope. Collect the oocytes in all stages and transfer the oocytes to pre-equilibrated HECM9aa medium quickly by using a ~300 μm diameter oral suction glass pipet. Distinguish the developmental stage of oocytes into GV, MI, and MII by checking the germinal vesicle and first polar body and transfer them into different drops. Transfer the oocytes to an)incubator at 37 °C in 5% CO_2.

3.3 Monkey Sperm Preparation

1. Fix the adult male monkey on the chair by binding arms and legs on the table legs, shave the fur around the testicular and inner thighs. Wash the penis by using warm saline and wipe it with dry medical cotton ball.
2. Two 5 mm wide aluminum strips covered with wet cotton are wrapped in the base of the penis and coronary sulcus. Two electrodes are connected with the two aluminum strips separately.

3. Turn on the power button of the electric stimulator. Adjust the prime period to 4 s and the frequency to 30 Hz. Set the volt range to 80 V.
4. Turn on the start button of the electric stimulator. Increase the volt from 0 V until the animal response to the volt. Do not increase the volt beyond 36 V. Stimulate the male monkey until the semen ejaculation. A 1.5 mL sterile EP tube is used to collect the semen (*see* **Note 3**).
5. Transfer the ejaculate to a tube incubator at 37 °C for liquefying at least 15 min.
6. Aspirate the liquid from the ejaculate tube and transfer it to a 15 mL centrifuge tube. Wash it twice by adding 5 mL TH3 medium followed by centrifugation 5 min at 200 × *g*.
7. Remove supernate and resuspend the sperm again by adding 1.5 mL TH3 medium. Centrifuge it at 200 × *g* for 5 min.
8. Transfer the tube to tube holder carefully without shaking the tube. Wait 15–30 min for the vigorous sperm swimming from bottom to the supernate.
9. Aspirate 30 μl supernate with vigorous sperm to the 3.5 cm dish and cover it with mineral oil. Transfer the dish to incubator at 37 °C in 5% CO_2 for use.

3.4 Lentivirus Injection in MII Stage Oocytes

For generation of transgenic monkey models with gene over expression, lentivirus infection method is the most common method now. Lentivirus injection is performed before ICSI. Usually, the lentivirus is packaged by company (*see* **Note 4**).

1. Perform the lentivirus injection by using an inverted microscope equipped with micromanipulators and an IM300 microinjector. The left micromanipulator controls the micropipet holder that is connected with a holding pipet. The right micromanipulator controls the micropipet holder that is connected with an injection pipet. The holding pipet is controlled by an oil-filled IM9B syringe and the injection pipet is controlled by the IM300 microinjector).
2. Prepare the holding pipet and injection pipet by using capillary glass and P97 micropipet puller. The holding pipet pulled by thin-wall borosilicate tubing without filaments (B100-75-10) was processed to 120–150 μm outer and 30 μm inner diameter. The injection pipet pulled by thin-wall borosilicate tubing with filaments (BF100-75-10) can be used directly with a thin and sharp tip.
3. Thaw one tube of lentivirus and add polybrene to a final concentration of 6 μg/mL in the tube. Position one injection pipet into the tube inversely and immerse the bottom of the pipet into lentivirus. The tip of the pipet will be full of lentivirus due to capillarity phenomena.

4. Turn on IM300 microinjector that is connected with N_2 and open up the control valve of N_2 to a pressure of 30 psi which indicates that the pressure of the injection pipet can adjust from 0 to 30 psi through knob "Injection." Adjust the IM300 to "action" model. Adjust the injection pressure to ~20 psi through knob "Injection." Check the "balance" and adjust the "balance" value to 0.3–0.5.
5. Prepare a dish with several drops of TH3 medium (15–20 μL/drop) which is covered by mineral oil. Set up the holding pipet and injection pipet well in the microscope. Break the tip of the injection pipet by bumping the holding pipet gently and generate a 1–2 μm fracture in the tip. At this time, the lentivirus is flowing out from the injection pipet due to the positive pressure.
6. Immobilize MII stage oocytes by holding pipet. Penetrate the zona pellucida gently by using the injection pipet and inject lentivirus into perivitelline space by IM300 until the oocyte cytoplasm become at most 1/2 primary volume of the oocyte (Fig. 2a *see* **Note 5**).
7. Finish the lentivirus injection of all MII oocytes and transfer the oocytes to pre-equilibrated HECM9aa medium and culture at 37 °C in 5% CO_2 for at least 2 h before ICSI (*see* **Note 6**).

3.5 Intracytoplasmic Sperm Injection)

1. In the ICSI procedure, the injection is performed by using an inverted microscope equipped with Piezo impact drive system. The left micromanipulator controls the micropipet holder that is connected with a holding pipet. The right micromanipulator controls the micropipet holder that is connected with a Piezo-drived injection pipet. Both the holding pipet and sperm injection pipet are controlled by oil-filled IM9B and CellTram vario syringes.
2. Prepare the sperm injection pipet by using capillary glass and P97 micropipet puller. The sperm injection pipet pulled by thin-wall borosilicate tubing without filaments (B100-75-10) was processed to 6–8 μm outer and 5–6 μm inner diameter. Mercury (~2 mm) should be back loaded into injection pipet by using a 1 mL syringe.
3. Prepare a dish with several drops of TH3 medium (15–20 μL/drop) and one drop of TH3 + PVP (10%) which is covered by mineral oil. Set up the holding pipet and injection pipet well in the microscope. Wash the injection pipet in the TH3 + PVP medium by using piezo unit that is applied with high power (>5) and high speed (>5).
4. Transfer 3–5 oocytes to one drop of TH3 medium, and dilute 3–5 μL sperm into another TH3 drop. Immobilize sperm by gently striking tip with injection pipet. Aspirate one sperm into the injection pipet tail first).

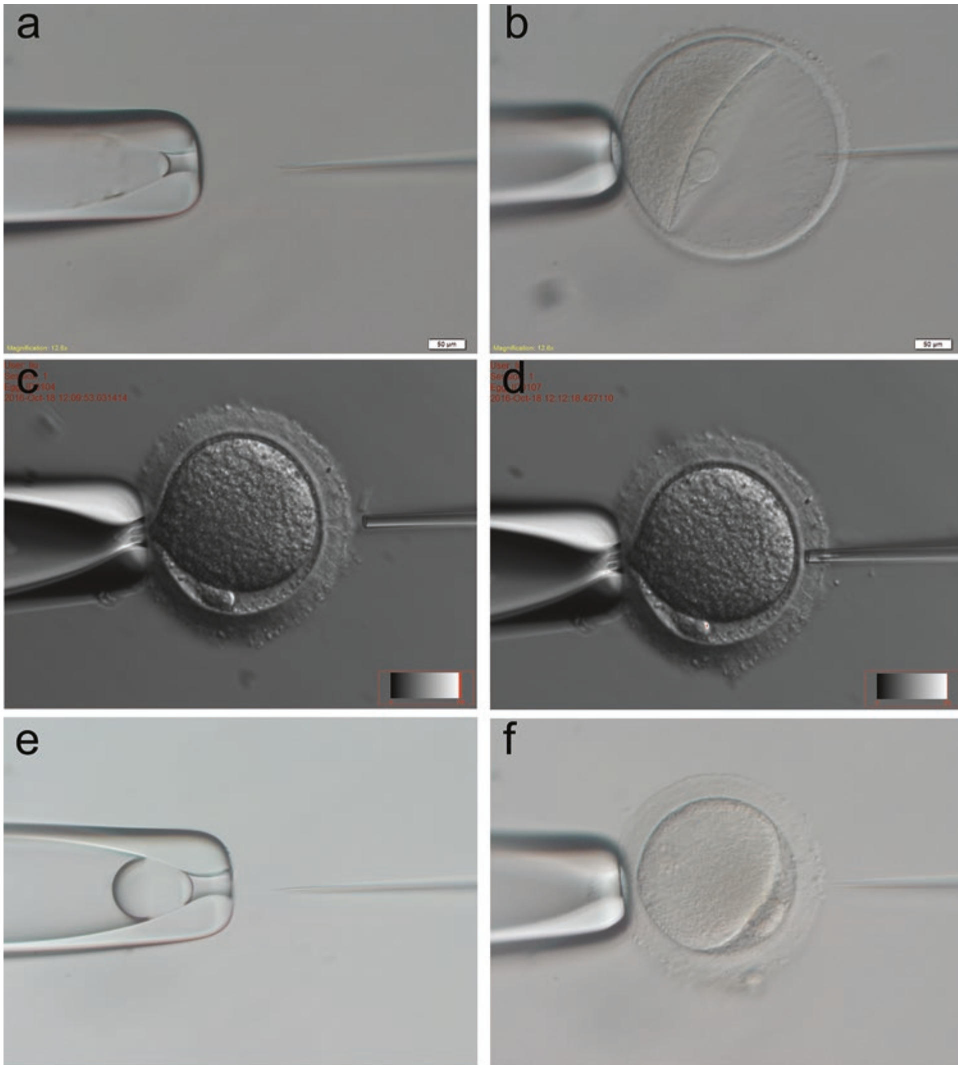

Fig. 2 Monkey embryo manipulation. (**a**, **b**) Lentivirus injection in monkey MII stage oocyte. (**c**, **d**) Intracytoplasmic sperm injection of monkey oocyte. (**e**, **f**) SgRNA and cas9 mRNA injection into monkey zygote

5. Immobilize MII stage oocytes by holding pipet with first polarbody positioned at either 6 or 12 O'clock. Penetrate the zona pellucida gently by using the Piezo-drived injection pipet which is applied with low power (2–3) and low speed (2–3). Push one sperm to the tip of the injection pipet and insert the pipet into the oocyte nearly to the opposite. Break the oocyte membrane by aspirating the cytoplasm into the pipet gently or using the Piezo-drived injection pipet that is applied with low power (1) and low speed (1). Inject the sperm to the oocyte cytoplasm with least TH3 medium if possible (Fig. 2c, d).
6. Finish the sperm injection of all MII oocytes and transfer the oocytes to HECM9aa medium and culture at 37 °C in 5% CO_2.

3.6 SgRNA and cas9 mRNA Injection

This procedure is performed to generate knock-out monkey models. ICSI is performed directly after oocytes collection without lentivirus injection and the resultant zygotes were used for RNA injection (*see* **Notes 7** and **8**).

1. Prepare sgRNA and cas9 mRNA as previously reported [11]. For sgRNA preparation, a T7 promoter contained specific F primer and a common R primer are used to amplify the sgRNA template by PCR and the resulted PCR product is used for in vitro transcription by using MEGAshortscript T7 kit. For cas9 mRNA, a T7 promoter contained specific F primer and a common R primer are used to amplify the Cas9 coding region and the resulted PCR product is used for in vitro transcription by using mMESSAGE mMACHINE T7 ULTRA kit. MEGAclear kit is used for Cas9 mRNA and sgRNA purification. In our experience, 50 ng/μl sgRNA and 100 ng/μl cas9 mRNA are suggested. Mix the sgRNA and cas9 mRNA and split each PCR tube 5–10 μl and store at −80 °C.
2. Check the fertilization of oocytes after ICSI for 6 h and pick the zygotes with 2 pronucleus. Generally, >80% oocytes should be fertilized by ICSI.
3. Prepare holding pipet, injection pipet and perform the injection as a lentivirus injection procedure).
4. Thaw one tube of mixed RNA in the tube which is positioned in 4 °C. Position one injection pipet into the tube inversely and immerse the bottom of the pipet into RNA. The tip of the pipet will be full of RNA due to capillarity phenomena.
5. Turn on IM300 microinjector that is connected with N_2 and open up the control valve of N_2 to a pressure of 30 psi which indicates that the pressure of the injection pipet can adjust from 0 to 30 psi through knob “Injection.” Adjust the IM300 to “action” model. Adjust the injection pressure to ~20 psi through knob “Injection.” Check the “balance” and adjust the “balance” value to 0.1–0.3.
6. Prepare a dish with several drops of TH3 medium (15–20 μL/drop) which is covered by mineral oil. Set up the holding pipet and injection pipet well in the microscope. At this time, the RNA mix in the pipet should be injected by stepping on the pedal.
7. Immobilize a zygote by holding pipet. Penetrate the zona pellucida and membrane gently by using the sharp and thin injection pipet and inject 5–10 pL RNA mix into cytoplasm by the IM300 (Fig. 2e, f).
8. Finish the RNA injection of all zygotes and transfer the zygotes to HECM9aa medium and culture at 37 °C in 5% CO_2.

3.7 Monkey Embryo Transfer

For transgenic monkey generation, check the fertilization of lentivirus injected-oocytes after ICSI for 6 h and zygotes with two pronucleus are transferred to the surrogate monkey mothers.

For knock-out monkey generation, zygotes were transferred to the surrogate monkey mothers after RNA injection for at least 2 h).

1. Monitor the menstrunos cycle of adult female monkeys. Adult female monkeys whose menstrunos cycles are synchronous to that of the oocytes donor are chosen as surrogate candidates (Fig. 1c).
2. Check the ovaries of surrogate candidate which is on day 14–18 after menstruation onset by laparoscopic surgery as described in oocytes collection. The synchronous females whose ovaries have a stigma or fresh corpus luteum are used as surrogates for embryos transfer in next step.
3. Insert trocar into abdominal through positioned paralumbar region which is near to the ovary. Pull out the trocar and obturator and expand the incision to 1–2 cm by cutting the skin and muscles using a sterile scissor. Pull out the ovary and ovarian duct by using a clamp to seize the ovary connected adipose. Turn on the shadowless lamp.
4. Transfer 2–3 embryos from HECM9aa medium to TH3 medium and wash for at least three times in different TH3 medium to remove the residual HECM9aa medium by using ~300 μm diameter oral suction glass pipet with a blunt section. Aspirate the embryos to the pipet with less TH3 medium and inset the pipet to the ovarian duct. Transfer the embryos to ovarian duct by blowing the pipet (Fig. 1d).
5. Check the pipet in the TH3 medium immediately after the transfer to ensure all of the embryos are transferred.
6. Stitch the incisions of the skin and muscle separately by using absorbable suture and silk sutures. Sterilize the incision with iodine tincture).

3.8 Pregnancy Test and Monitoring

Perform ultrasound test of the surrogate uterus to confirm the pregnancy 28–30 days after embryo transfer. Monitor the pregnant surrogate once a month. Cesarean section was suggested for single fetus beyond 160 day or twin fetus beyond 145 day.

3.9 Genotype Analysis

Generally, DNA from skin and blood are used for genotype analysis.

1. For transgenic monkey models, the transgene is detected by PCR first. The copy number and insert position of the transgene were further analyzed by AccuCopy assay and deep-sequencing-based method in the PCR positive founders.
2. For knock-out monkey models, PCR was performed using targeted gene-specific primers and PCR products are then subcloned to pMD19-T vector for sequencing and verifying the mutations.

4 Notes

1. For monkey menstruation cycle monitoring, it is necessary to train the monkeys to follow the instruction from the breeder. This will save lot of time and reduce the stress reaction to monkeys.
2. After oocytes collection, the collected medium is poured in the cell strainer again for a second round wash. Usually, this procedure will add several oocytes in total because some oocytes will be left in the first round wash especially for the blood-rich follicle aspirate.
3. For monkey sperm collection, the monkey should not be stimulated too frequency. Usually, we collected sperm from the same monkey at most twice 1 week. Ejaculation failure is rarely seen in our lab. Change the monkey if the monkey does not ejaculated for up to 10 min.
4. The titer and purity of the lentivirus is the key point for generating transgenic monkeys with a high efficiency. The titer is highly associated with the positive rate and at least 5×10^8 is suggested. The purity is associated with the pregnancy rate and birth rate.
5. In lentivirus injection, the tip of the injection pipet should be broken by bumping the holding pipet gently as the volume of the injected lentivirus is relative large. The flow velocity of the injected lentivirus could be controlled by the injection pressure.
6. For the transgenic monkey generation, 2 h gap is needed and enough for oocytes to restoration between lentivirus injection and ICSI. Shorter gap may increase the oocytes mortality during the ICSI procedure and longer gap may reduce the fertilization rate and development potential of oocytes.
7. For sgRNA preparation, we usually design at least three sgRNAs at different regions of the exon or different exons for one targeting gene. We test the targeting efficiency of the sgRNAs in monkey embryos by collecting sample at morula/blastocyst stage. The sgRNAs with high efficiency are used for injection into embryos which will be transferred to surrogates.
8. For sgRNA injection, the concentration and injection volume is critical for knock-out efficiency and embryo development. The suggested parameter is based on our experience and operation habit.

Acknowledgment

This work was supported by grant "National Key Technology R&D Program of China 2014BAI03B00629(Q.S.)" and "Shanghai City Committee of science and technology project 14140900100 630 (Q.S.)."

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Chapter 13

Genome Editing Mediated by Primordial Germ Cell in Chicken

Jae Yong Han and Hong Jo Lee

Abstract

Rapid development of genome editing technology has facilitated the studies on exploring specific gene functions and establishment of model animals. In livestock, the technology has contributed to create high value in industry fields, e.g., enhancing productivity or acquiring the resistance against disease. Meanwhile, genome editing in avian species has been emphasized because of their applicable possibilities in terms of highly productive chickens, disease-controlled avian lines, and development of novel biological models. Induction of exogenous gene using virus system or transposition in chicken primordial germ cells (PGCs) has been widely used for producing transgenic chicken, and recently developed programmable genome editing (PGE) technologies such as tale transcription activator-like effector nuclease (TALEN) and clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated (Cas9) are expected to maximize the applicable potentials of avian species. In this regard, this chapter will cover the methods for producing genome-edited chicken by *piggyBac* transposition and gene targeting technology, TALEN, and CRISPR/Cas9.

Key words Genome editing, Chicken, Primordial germ cell, *piggyBac* transposition, TALEN, CRISPR/Cas9

1 Introduction

Genome editing in livestock provides enormous benefits to industry fields as well as research fields. Regulation of specific gene expression that is related to growth rate and virus susceptibility could contribute to enhancing productivity and viral disease resistance [1]. In avian species, genome editing technology has been widely attempted because of its unique biological characteristics [2]. Poultry meat itself is a qualified protein resource, and egg from chicken is considered a valuable reservoir for functional proteins. Therefore, adopting the genome editing technologies on avian genome is expected to open new era of agriculture [3].

Izuho Hatada (ed.), *Genome Editing in Animals: Methods and Protocols*, Methods in Molecular Biology, vol. 1630,
DOI 10.1007/978-1-4939-7128-2_13, © Springer Science+Business Media LLC 2017

Genome editing technology in avian species has been largely focused on chicken. After the production of the first transgenic chicken mediated virus vector system, diverse approaches to produce genome-edited chicken have been tried [4–13]. Especially, primordial germ cells (PGCs)-mediated germline transmission system in avian species has been considered as most efficient methods for transmitting genetic information to next generation [14–16]. This germline transmission system was adapted to produce transgenic chickens that express functional proteins in their eggs [17]. Furthermore, recently developed chicken genome editing systems adopting programmable genome editing (PGE) technology including TALEN and CRISPR/Cas9 to chicken PGC is expecting to contribute to the establishment of novel chicken lines that are valuable in poultry industry as well as in research area [18–20].

In this regard, we describe methods to produce genome-edited chicken mediated by chicken PGCs as well as in vitro culture of chicken PGCs in detail.

2 Materials

2.1 Isolation and In Vitro Culture of PGCs

1. Chickenembryos at Hamburger and Hamilton (HH) stages 26–28 [21].
2. Sharpened forcep.
3. Trypsin/ethylenediaminetetraacetic acid.
4. PGC culture medium: Knockout Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% (vol/vol) FBS, 2% (vol/vol) chicken serum, 1× nucleosides, 2 mM L-glutamine, 1× nonessential amino acids (NEAA), β-mercaptoethanol, 10 mM sodium pyruvate, and 1× antibiotic–antimycotic (ABAM), 10 ng/mL Human basic fibroblast growth factor (bFGF).
5. 1 × Hank's balanced salt solution (HBSS) without $CaCl_2$ or $MgCl_2$.
6. Mouse embryonic fibroblast (MEF, ICR strain mouse) (*see* **Note 1**).
7. Mitomycin C.
8. 0.1% gelatin.

2.2 PGC Transfection and Selection

1. Lipofectamine 2000 (Invitrogen).
2. Opti-MEM medium (Invitrogen).
3. PBS, without Ca^{2+} or Mg^{2+}.
4. HBSS without Ca^{2+} or Mg^{2+}.
5. CAGG-PBase (pCyL43B) plasmid donated by the Sanger Institute [22], concentrated at 1 μg/μL.

6. *piggyBac* transposon (pCyL50) plasmid donated by the Sanger Institute [22], concentrated at 1 μg/μL.
7. The plasmids for TALEN pair expression from Feng Zhang (Addgene kit # 1000000019) [23], concentrated at 1 μg/μL.
8. hCas9 plasmid from George Church (Addgene plasmid # 41815) [24], concentrated at 1 μg/μL.
9. Guide RNA (gRNA) expression plasmid (Bioneer, Daejeon, Korea), concentrated at 1 μg/μL.
10. Hemocytometer (Marienfeld).
11. Fluorescent microscope.
12. Geneticin® Selective Antibiotic (G418).
13. FACSAria III cell sorter (Becton, Dickinson and Company, Franklin Lakes, NJ).

2.3 Validation of Genome Edited PGC

1. DNeasy blood & tissue kit (QIAGEN).
2. Fluorescent microscope.
3. T7E1 endonuclease (New England Biolabs, Ipswich, MA).
4. Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI).
5. pGEM-T easy vector (Promega, Madison, WI).
6. ABI 3739XL DNA Analyzer (Applied Biosystems, Foster City, CA).
7. Donor PGC-specific primer set.
8. Transgene-specific primer set.
9. PCR instrument.
10. PCR reagents: PCR buffer, dNTP, Taq polymerase.
11. Agarose.
12. 50× TAE buffer: 242 g of Tris-base, 57.1 mL of Acetate, 18.61 g of disodium EDTA in 1 L of distilled water.
13. Electrophoresis equipment.

2.4 PGC Transplantation

1. Small-diameter (25 μm) glass micropipette (made with micro-electrode pipette puller Shutter Instrument Co.) and a micro grinder (NARISHIGE).
2. Mouth-controlled pipette.
3. HBSS without $CaCl_2$ or $MgCl_2$.
4. Recipient chicken embryos at HH stages 14–17.
5. PBS, without Ca^{2+} or Mg^{2+}.
6. Pipette washing solution. 0.1% hydrogen peroxide in autoclaved distilled water.
7. Forceps.

8. Stereomicroscope with illuminator.
9. Hot-melt glue sticks and glue gun.
10. Parafilm.
11. Donor PGC prepared at 3×10^3 cells/μL in HBSS.
12. Confocal laser scanning microscope (LSM 700, Carl Zeiss).

2.5 Testcross for Germline Transmission

1. Wild-type (WT) mature chicken.
2. 1 mL syringe.
3. PBS, without Ca^{2+} or Mg^{2+}.

2.6 Validation of Genome Edited Chicken and Line Establishment

1. DNeasy Blood & Tissue kit (QIAGEN).
2. Donor PGC-specific primer set.
3. Transgene-specific primer set.
4. PCR instrument.
5. PCR reagents: PCR buffer, dNTP, Taq polymerase.
6. Agarose.
7. 50× TAE buffer: 242 g of Tris-base, 57.1 mL of Acetate, 18.61 g of disodium EDTA in 1 L of distilled water.
8. Electrophoresis equipment.
9. Fluorescent excitation lamp with detection filters (BLS Ltd., Budapest, Hungary).
10. Transgene-specific restriction enzyme.
11. Nylon membrane (Hybond-N+, Amersham Biosciences, Piscataway, NJ, USA).
12. Random Primer DNA Labeling Kit (Takara Bio Inc., Shiga, Japan).
13. α-^{32}P-dCTP.
14. DNA Walking SpeedUp Premix Kit-II (Seegene, Seoul, Korea).
15. Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI).

3 Methods

Here, we describe the methods to produce genome-edited chicken mediated by PGC transplantation that was modified by *piggyBac* transposition, TALEN, and CRISPR/Cas9 (*see* Fig. 1) [20, 25].

3.1 Isolation and In Vitro Culture of PGCs

1. Incubate fresh chicken eggs (Eyal-Giladi and Kochav (EGK) stage X [26]) at 37 °C for 5.5 days (HH stage 26–28) for gonadal PGC isolation.

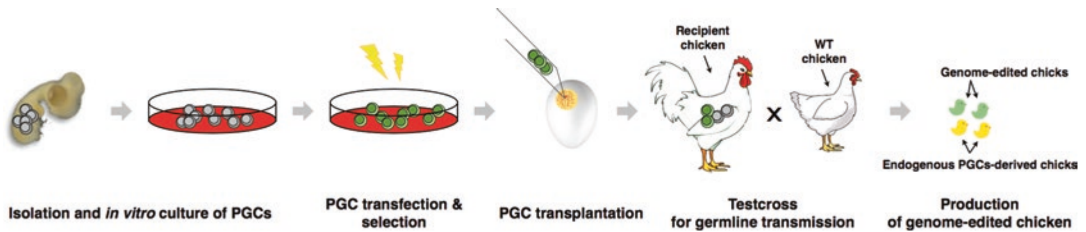


Fig. 1 Schematic representation of genome editing in chicken. Genome-edited PGCs are transplanted to recipient chicken embryo. After sexual maturation of the recipient that has donor PGCs, genome-edited chicks are produced by testcross with wild-type (WT) chicken

2. Extract embryonic gonads from embryos at HH stages 26–28 with sharpened forceps, and incubate with 500 μL of 0.05% trypsin/EDTA at 37 °C incubator for 5 min.
3. Add 50 μL FBS for inactivation, and centrifuge (200 × *g*, 5 min) and remove the supernatant.
4. Resuspend dissociated gonadal cells and maintain with PGC media.
5. The cultured PGCs are subcultured onto mitomycin C-inactivated MEFs in 5- to 6-d intervals by gentle pipetting without any enzyme treatment.

3.2 PGC Transfection and Selection

1. For the production of transgenic chicken, mix 4 μg helper plasmid containing CAGG-PBase (pCyL43B) and 6 μg the *piggyBac* transposon (pCyL50) containing a reporter gene (e.g., Green fluorescent protein, GFP) or selectable marker (e.g., neomycin resistance gene) with 100 μL Opti-MEM and incubate for 5 min at room temperature (RT). For TALEN or CRISPR/Cas9 transfection, mix 2.5 μg CMV GFP expression plasmid vector, 2.5 μg each TALEN pairs or hCas9 plasmid and guide RNA expression plasmid with 100 μL Opti-MEM and incubate for 5 min at RT.
2. Mix 10 μL Lipofectamine 2000 reagent with 100 μL Opti-MEM and incubate for 5 min at RT.
3. Mix the plasmids with Opti-MEM and Lipofectamine 2000 reagent with Opti-MEM and incubate for 20 min at RT.
4. During incubation, harvest cultured PGCs and centrifuge (200 × *g*, 5 min). Discard the supernatants.
5. Add 1 mL HBSS to the harvested PGCs and gently pipette.
6. Determine the number of PGCs using a hemocytometer and seed 5×10^5 PGCs in a 12-well plate with 1 mL PGC culture medium without antibiotics.
7. Apply DNA-Lipofectamine complex to PGCs and incubate for one day at 37 °C in a CO_2 incubator.

Table 1
Information of the primer sets used for PCR analysis

Gene	Primers sequence	Product size (bp)
OV	**F:** CTGGGACAGTTTGCTACCCA **R:** CTGGTGCTGTCTTTTGCACC	427

8. Harvest the transfected PGCs and centrifuge (200 × *g*, 5 min). Remove the supernatants.
9. Wash the PGCs with 1 mL PBS three times and suspend them with PGC culture medium with antibiotics.
10. One day after lipofection, reporter protein (e.g., GFP)-expressing cells were sorted using a FACSAria III cell sorter. If the plasmids contain drug selectable marker (e.g., neomycin resistance gene), add 300 μg/mL G418 to the culture media for selection (*see* **Note 2**).

3.3 Validation of Genome Edited PGC

1. After FACS enrichment or drug-selection, monitor reporter gene expression of PGCs using fluorescent microscope and analyze reporter gene expression by flow cytometry.
2. Extract genomic DNA of transfected PGCs using DNeasy Blood & Tissue kit according to manufacturer's protocol.
3. Perform PCR of the genomic DNA using donor PGC-specific primer set or transgene-specific primer set, and identify genome-edited PGC specific band by electrophoresis.
4. Perform the T7E1 assay to identify gene mutation in TALEN or CRISPR/Cas9 treated PGCs.
5. Amplify the genomic region encompassing the TALEN or CRISPR/Cas9 targeting site with a specific primer set by PCR (Table 1).
6. Re-anneal the PCR amplicons to form heteroduplex DNA structure.
7. Treat T7E1 endonuclease to re-annealed heteroduplex amplicons at 37 °C for 15 min, and identify cleaved amplicons by electrophoresis (*see* **Note 3**).
8. To confirm target locus mutation, PCR amplicons were cloned into a pGEM-T easy vector and sequenced with an ABI 3730XL DNA Analyzer.

3.4 PGC Transplantation

1. Incubate recipient eggs up to HH stages 14–17 at 37 °C in air with 60–70% relative humidity.
2. Make a small window on the pointed end of the recipient egg and a 2 μL aliquot containing more than 3000 PGCs was

microinjected with a micropipette into the dorsal aorta of the recipient embryo.

3. Seal the egg window of the recipient embryo with paraffin film and the egg was incubated with the pointed end down until further screening and hatching at 37 °C in air with 60–70% relative humidity.
4. To detect GFP-expressing PGCs in embryonic gonads, the gonads or testes were dissected at different stages and live images of GFP-expressing transplanted PGCs were observed using a confocal laser scanning microscope.

3.5 Testcross for Germline Transmission

1. After sexual maturation of recipient, collect the semen from recipient rooster twice in 1 week.
2. Introduce 50 μL semen from mature male recipients to WT laying hens.
3. Collect eggs from WT laying hens the day after artificial insemination, and incubate the egg with the pointed end down until hatching at 37 °C in air with 60–70% relative humidity.
4. Donor PGCs-derived progeny can be distinguished from the recipient endogenous PGCs-derived progeny by feather color (*see* Fig. 2a) (*see* **Note 4**).

3.6 Validation of Genome-Edited Chicken and Line Establishment

3.6.1 Validation of Transgenic Chicken

1. To validate transgenic chicken that expresses fluorescent reporter protein, donor PGCs-derived chickens are monitored by a fluorescent excitation lamp with detection filters (*see* Fig. 2b).
2. To confirm the transgene of transgenic chicken, perform PCR of the chicken's genomic DNA using donor PGC-specific primer set or transgene-specific primer set, and identify genome-edited chicken-specific band by electrophoresis.

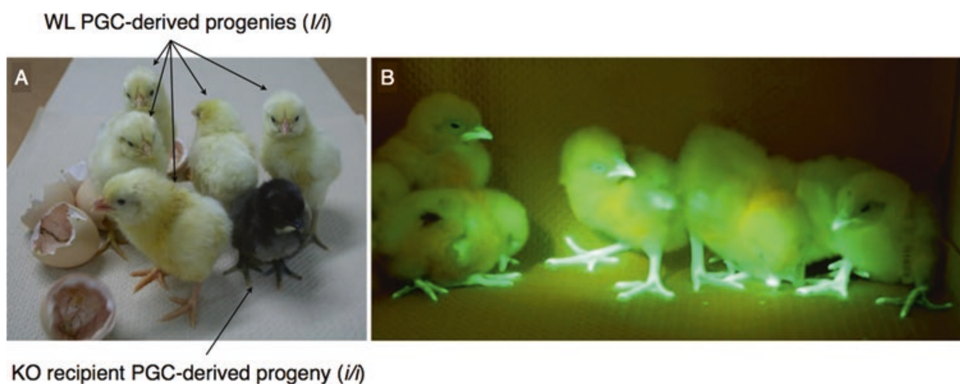


Fig. 2 Validation of donor PGCs-derived progenies and transgenic chickens. (**a**) Validation of donor PGCs-derived hybrid progenies (*I/i*) compared to endogenous PGCs-derived progenies (*i/i*) by feather color. (**b**) Validation of transgenic chicken by a fluorescent excitation lamp with detection filters. (Reproduced from ref. 21 with permission from National Academy of Sciences)

3. To verify copy number of transgene in transgenic chicken genome, perform southern blot analysis. For southern blotting, 10 μg of genomic DNA from transgenic chickens is digested with transgene-specific restriction enzyme. The digested DNA is electrophoresed in agarose gel and then transferred to a nylon membrane. Blots were hybridized with α-^{32}P-labeled transgene-specific probe prepared using a Random Primer DNA Labeling Kit. Hybridization signals were detected by autoradiography.
4. To identify the integration sites of *piggyBac* transposon, perform DNA walking analysis by using the DNA Walking SpeedUp Premix Kit-II. The products of the third round of DNA walking PCR were cut out of the agarose gel and purified with the Wizard SV Gel and PCR Clean-Up System and then cloned directly into the pGEM-T Easy. The cloned plasmids were sequenced using an ABI Prism 3730 XL DNA Analyzer. The sequences of the transgene-flanking region were analyzed using the BLAST Assembled Genome Database (http://blast.ncbi.nlm.nih.gov).

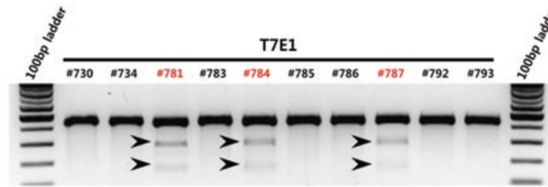
3.6.2 Validation of Gene-Targeted Chicken

1. To validate gene targeting in chicken, perform PCR, T7E1 assay, and sequencing analysis.
2. Amplify the genomic region encompassing the TALEN or CRISPR/Cas9 targeting site with a specific primer set by PCR.
3. Re-anneal the PCR amplicons to form heteroduplex DNA structure.
4. Treat T7E1 endonuclease to re-annealed heteroduplex amplicons at 37 °C for 15 min, and identify cleaved amplicons by electrophoresis (*see* Fig. 3a).
5. To confirm target locus mutation, PCR amplicons were cloned into a pGEM-T easy vector and sequenced with an ABI 3730XL DNA Analyzer (*see* Fig. 3b).
6. Potential off-target effects of TALEN or CRISPR/Cas9 are predicted using the web-based software program, TAL Effector Nucleotide Targeter 2.0 or CRISPR Design [27, 28] (*see* **Note 5**).

4 Notes

1. MEFs are isolated at 12.5–13.5 day post coitum (dpc) ICR strain pregnant mice and cultured to P3 at culture dish. And the MEFs are treated with mitomycin C at 37 °C for 3 h and frozen in liquid nitrogen. The day before subculture of PGCs, the mitomycin C-treated MEFs were seed onto 0.1% gelatin-coated culture dish with DMEM supplemented with 10% (vol/vol) FBS and 1× ABAM. Optimal confluency of the MEFs is 30–50%.

A



B

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                                             M   G   S   I   G   A   A   S    amino acids
ID#648 TGCTGTTTGCTCTAGACAACTCAGAGTTCACC ATG GGC TCC ATC GGT GCA GCA AGC A  wild
       -------------------------GTTCACC ATG GGC TCC ATC GGT GCA GCA AGC A  29nt del

ID#704 TGCTGTTTGCTCTAGACAACTCAGAGTTCACC ATG GGC TCC ATC GGT GCA GCA AGC A  wild
       TGCTGTTTGCTCTAGACAACTCAGAGT----- --- --- --- --- --- -CA GCA AGC A  21nt del  frameshift

ID#736 TGCTGTTTGCTCTAGACAACTCAGAGTTCACC ATG GGC TCC ATC GGT GCA GCA AGC A  wild
       TGCTGTTTGCTCTAGACAACTCAGAGTT---- --G GGC TCC ATC GGT GCA GCA AGC A   6nt del  frameshift

ID#781 TGCTGTTTGCTCTAGACAACTCAGAGTTCACC ATG GGC TCC ATC GGT GCA GCA AGC A  wild
       TGCTGTTTGCTCTAGACAACTCAGAGTT---- --G GGC TCC ATC GGT GCA GCA AGC A   6nt del  frameshift

ID#784 TGCTGTTTGCTCTAGACAACTCAGAGTTCACC ATG GGC TCC ATC GGT GCA GCA AGC A  wild
       TGCTGTTTGCTCTAGACAACTCAGAGTT---- --G GGC TCC ATC GGT GCA GCA AGC A   6nt del  frameshift

ID#787 TGCTGTTTGCTCTAGACAACTCAGAGTTCACC ATG GGC TCC ATC GGT GCA GCA AGC A  wild
       TGCTGTTTGCTCTAGACAACTCAGAGTT---- --G GGC TCC ATC GGT GCA GCA AGC A   6nt del  frameshift

ID#803 TGCTGTTTGCTCTAGACAACTCAGAGTTCACC ATG GGC TCC ATC GGT GCA GCA AGC A  wild
       TGCTGTTTGCTCTAGACA-------------- -TG GGC TCC ATC GGT GCA GCA AGC A  15nt del  frameshift
```

Fig. 3 Validation of donor PGCs-derived progenies and genome-edited chickens. (**a**) The hatched chicks produced from the founder through testcross are identified by T7E1 assay. (**b**) Targeted DNA sequences in the OV gene were analyzed in the OV mutant donor PGCs-derived offspring. A dash in the DNA sequences denotes deleted nucleotides, and bold ATG indicates the translational initiation codon in the second exon of the OV gene. (Reproduced from ref. 20 with permission from National Academy of Sciences)

2. To select transfected PGCs, diverse fluorescent reporter gene could be used. In case of drug selection, apply optimal amount of drug after analyzing the kill curve of PGCs with drug-resistant MEF.
3. T7E1 assay is widely used in detection of genetic mutations. To identify gene mutation using T7E1 assay, apply optimal amount of T7E1 endonuclease and incubate proper times with control. Prolonged incubation or applying excess amount of enzyme could result in over-digestion.
4. Donor PGCs-derived progenies could be distinguishable for feather color. Because WL feather color (*I/I*) is dominant and KO feather color (*i/i*) is recessive. Therefore, recipient KO chickens that have donor WL PGCs (*I/I*) and their own PGCs (*i/i*) could produce WL phenotype progenies (*I/i* or *I/I*) through testcross with wild-type KO chicken (*i/i*) or WL chicken (*I/I*). And also donor PGCs could be KO breed. Recipient WL that have donor KO PGCs (*I/I* and *i/i*) could produce donor KO PGCs-derived progenies (*i/i*) through testcross with wild-type KO chickens (*i/i*).

5. Off-target effect is an unavoidable issue on gene targeting. To assess further nonspecific mutation in the gene-targeted chicken, not only web-based off-target screening but also whole genome sequencing (WGS) of the chicken genomic DNA needs to be performed. Although many software tools provide a list of potential off-target effects for gene-targeting, the predictions with the tools can cause false positives and negatives [29].

Acknowledgment

This work was supported by a National Research Foundation of Korea (NRF) grant, funded by the Korean government (MSIP; No. 2015R1A3A2033826), and the Next-Generation BioGreen 21 Program Grant PJ008142, Rural Development Administration, Republic of Korea.

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Chapter 14

CRISPR/Cas9-Mediated Targeted Knockin of Exogenous Reporter Genes in Zebrafish

Atsuo Kawahara

Abstract

Genome editing technologies such as ZFN, TALEN, and CRISPR/Cas9 efficiently induce DNA double-stranded breaks (DSBs) at a targeted genomic locus, often resulting in a frameshift-mediated target gene disruption. It remains difficult to perform targeted integration of exogenous genes by genome editing technologies. DSBs can be restored through DNA repair mechanisms, such as non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), and homologous recombination (HR). It is well known that HR facilitates homology-dependent integration of donor DNA template into a targeted locus. Recently, both NHEJ-mediated and MMEJ-mediated targeted integrations of exogenous genes have been developed in zebrafish. This chapter summarizes the application of CRISPR/Cas9-mediated knock-in technology in zebrafish.

Key words Zebrafish, CRISPR/Cas9, NHEJ, MMEJ, HR, Knockout, Knockin

1 Introduction

The zebrafish is an ideal model vertebrates that can be easily modified through forward and reverse genetics [1]. A number of zebrafish mutants defective in organogenesis have been identified from chemical mutagenesis screening [2, 3]. Through unbiased forward genetics, the genes responsible for the phenotypes of these mutants have been identified as key regulators of early zebrafish embryogenesis, including hematopoietic and cardiovascular development [4, 5]. Recent advances in genome editing technologies (ZFN, TALEN, and CRISPR/Cas9), which are powerful reverse genetic tools in various model organisms, enable the introduction of locus-specific DNA double-stranded breaks (DSBs) that are restored by DNA repair mechanisms, such as non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), and homologous recombination (HR) [6]. DSBs can be repaired by HR using long homologous fragments from the targeted locus. In the absence of donor DNA templates, NHEJ connects the end of

Izuho Hatada (ed.), *Genome Editing in Animals: Methods and Protocols*, Methods in Molecular Biology, vol. 1630,
DOI 10.1007/978-1-4939-7128-2_14, © Springer Science+Business Media LLC 2017

broken strands and often leads to insertion and/or deletion (indel) mutations. Recently, an alternative DNA repair mechanism has been revealed in which the exposed ends with microhomology sequences (3–30 bases) at the target site are mutually annealed and repaired by MMEJ, thereby resulting in the production of predictable small deletions. Thus, frameshift-mediated targeted gene disruption can be easily generated through the processes of NHEJ and MMEJ.

In zebrafish, the targeted integration of exogenous genes (knockin) remains more technically challenging than targeted gene disruption (knockout). Because the frequency of HR is enhanced by the generation of site-specific DSBs, the genome editing technologies TALEN and CRISPR/Cas9 have been used as a tool for the targeted integration of exogenous genes. Zu Y et al. have reported the TALEN-mediated knockin of a donor vector containing homology arms (approximately 900 bp) into the *tyrosine hydroxylase* locus [7]. The authors obtained four founders with knockin alleles from a F0 screen of 275 potential founders (germline transmission frequency: ~1.5%). This relatively low frequency limits the practical use of zebrafish genome editing. Shin J et al. have improved the configuration of the targeting construct and have adjusted the length of the homology arm, thus resulting in more efficient TALEN-mediated knockin of a reporter into the *sox2* locus: 29 of 363 F0 founders produced knock-in alleles (germline transmission frequency: ~8%) [8]. More recently, Irion U et al. have reported the HR-mediated knockin of a donor vector into the *albino* (*alb*) locus by using the CRISPR/Cas9 system [9]. The authors observed the HR-directed repair of the *alb* mutation by using a circular donor vector containing homology arms (approximately 900 bp) and CRISPR target sites, and 3 of 28 F0 founders produced repaired alleles (germline transmission frequency: ~11%). Although the construction of donor vectors is complicated, HR-mediated knockin of donor vectors is very useful for precise genome editing in zebrafish.

Auer et al. have reported the NHEJ-mediated knockin of a driver gene [10]. The authors observed the CRISPR/Cas9-mediated targeted insertion of a donor vector, when the vector and the targeted genomic locus were simultaneously cleaved via the CRISPR/Cas9 system. Kimura Y et al. have reported a vector containing a CRISPR target site, the hsp70 promoter, and reporter/driver genes [11]. The authors have successfully performed NHEJ-mediated locus-specific insertion of a reporter, thus allowing real-time visualization of target gene expression. We have recently demonstrated that NHEJ-mediated integration of the eGFP reporter into both targeted *pax2a* alleles causes loss-of-function phenotypes identical to that of the *no isthmus/pax2a* mutant [12] (Fig. 1). Thus, NHEJ-mediated reporter gene integration at a targeted locus is a powerful genetic tool allowing

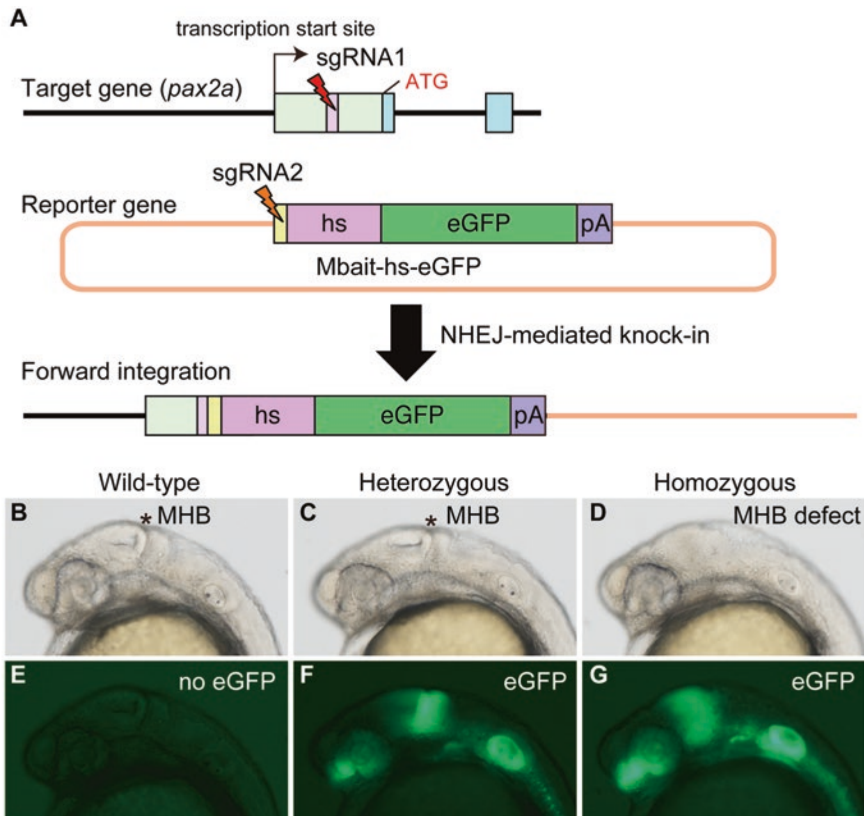

Fig. 1 Strategy of NHEJ-mediated knockin of an exogenous reporter gene. (**a**) Genomic locus of the *pax2a* gene and the reporter gene structure. Two sgRNAs (sgRNA1; for the targeted genome digestion, sgRNA2; for the reporter gene digestion) and the eGFP reporter gene together with Cas9 mRNA were injected into 1-cell stage embryos, and a transgenic line containing the eGFP gene in the *pax2a* locus was established. ATG; initiation start codon, hs; hsp70 promoter, eGFP; enhanced GFP, pA; polyA signal. (**b–d**) Morphology of the midbrain-hindbrain boundary (MHB). The MHB formed normally in wild-type and heterozygous Tg[pax2a-hs:eGFP] embryos, but not homozygous Tg[pax2a-hs:eGFP] embryos. The MHB defect in the homozygous embryo was identical to the *no isthmus*/*pax2a* mutant. (E-G) The eGFP expression at 30 hpf embryo. As described previously [12], eGFP expression in heterozygous Tg[pax2a-hs:eGFP] embryo was detected in the optic stalk, the MHB, the otic vesicles and the hindbrain where the endogenous *pax2a* gene is expressed. The eGFP expression around the midbrain of homozygous Tg[pax2a-hs:eGFP] embryo was anteriorly expanded, whereas the eGFP expression in the optic stalk, the otic vesicles, and hindbrain was normally detected

investigation of the real-time expression of a reporter in the heterozygous embryos and loss-of-function phenotypes of target gene in homozygous embryos.

Recently, we have developed a method allowing MMEJ-mediated knockin of exogenous reporter genes [13](Fig. 2). An eGFP reporter construct containing bait sequences targeted by sgRNA/Cas9 and short homologous sequences (40 bases) flanking the targeted genomic locus is used. We have found that the reporter gene is precisely integrated into the targeted genomic locus by MMEJ, thus suggesting that the MMEJ-mediated knockin method

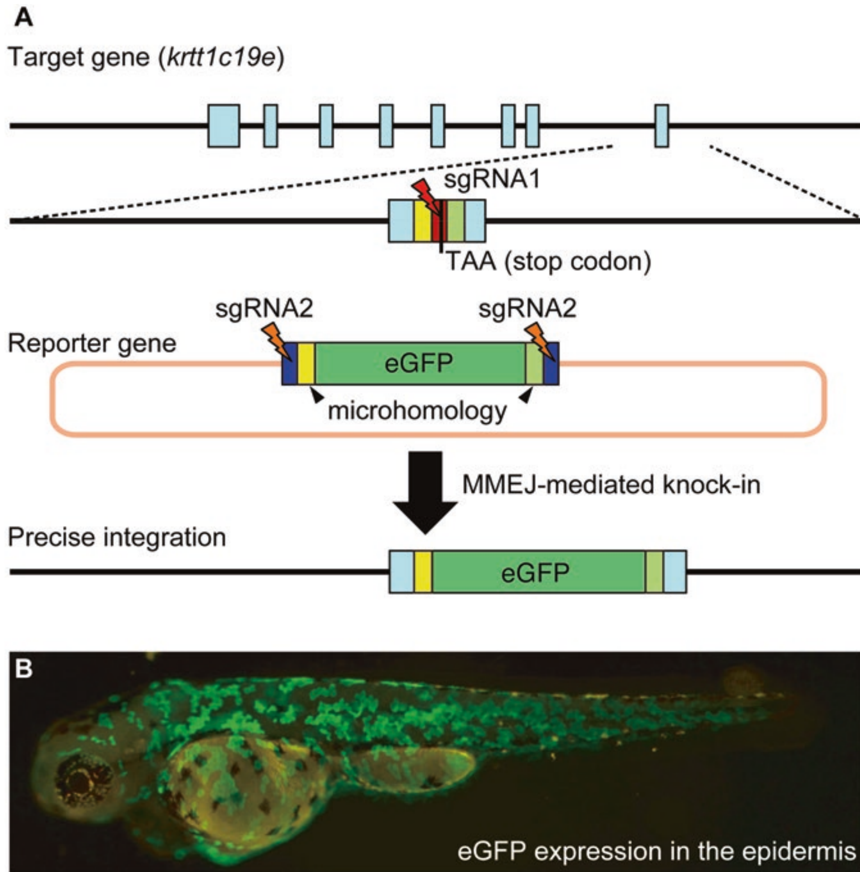

Fig. 2 Strategy of MMEJ-mediated knockin of an exogenous reporter gene. (**a**) Genomic locus of the *krtt1c19e* gene (a *keratin* gene) and the eGFP reporter gene construct. The reporter gene contains two homologous sequences (*yellow* and *light green boxes*; 40 bases each) around the stop codon of the *krtt1c19e* gene. Two sgRNAs (sgRNA1; for the targeted genome digestion, sgRNA2; for the reporter gene digestion) and the reporter gene together with Cas9 mRNA were injected into 1-cell stage zebrafish embryos, and a transgenic line containing the eGFP gene in the *krtt1c19e* locus was established. (**b**) The eGFP expression in the epidermis at 2 dpf. As described previously [13], eGFP expression was detected in the epidermis of Tg[krtt1c19e:eGFP] embryos

is useful for precise genome modifications in various model organisms. Indeed, the MMEJ-mediated integration of the eGFP donor vector by using TALENs has been founded to be functional in frogs and silkworms [14]. This chapter describes the procedures of NHEJ- and MMEJ-mediated knockin of exogenous reporter genes into genomic loci targeted by CRISPR/Cas9.

2 Materials

2.1 Preparation of sgRNA and Cas9

1. pDR274 (Addgene plasmid number: 42250) [15].
2. pCS2 + hSpCas9 (Addgene plasmid number: 51815) [16] (*see* **Note 1**).

3. *Bsa*I-HF (NEB).
4. *Dra*I (NEB).
5. *Not*I-HF (NEB).
6. 20 mg/mL Proteinase K.
7. phenol/chloroform/isoamyl alcohol (25:24:1).
8. 3 M sodium acetate.
9. 5 M ammonium acetate.
10. 100% ethanol.
11. 70% ethanol.
12. QIAprep Spin Miniprep Kit (Qiagen).
13. MAXIscript T7 Transcription Kit (Thermo Fisher Scientific).
14. mMESSAGE mMACHINE SP6 Transcription Kit (Thermo Fisher Scientific).
15. PD SpinTrap™ G-25 (GE Healthcare Life Sciences).
16. Primer: M13 reverse primer; 5′-CAGGAAACAGCTATGAC-3′.
17. LB medium: 1% polypeptone, 0.5% yeast extract, 1% NaCl.
18. Antibiotics: kanamycin.

2.2 Microinjection

1. Injection buffer: 40 mM HEPES [pH 7.4], 240 mM KCl, 0.5% phenol red.
2. E3 medium: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$.
3. Puller PC-10 (Narishige).
4. Manipulator MMN-8 (Narishige).
5. Microinjector IM 300 (Narishige).

2.3 Genomic PCR and Sequencing Analysis

1. pGEM-T Easy (Promega).
2. M13 forward primer; 5′-GTAAAACGACGGCCAGT-3′.
3. MinElute PCR Purification Kit (Qiagen).
4. Thermal Cycler 2720 (Applied Biosystems).

3 Methods

3.1 Construction of sgRNA Expression Vectors

1. Search sgRNA target sequences for individual target locus: the NGG sequence is required for the recognition of sgRNA/Cas9 complex. Choose 20-nucleotide sequences upstream of the NGG as an sgRNA target site. Prepare oligonucleotides encoding the sgRNA target sequences (sense oligonucleotide; 5′-TAGGN$_{18}$-3′, and antisense oligonucleotide; 5′-AAACN$_{18}$-3′) (*see* **Note 2**).

2. Mix sense and antisense oligonucleotides (1 μM each), heat the solution at 98 °C for 3 min, incubate at 65 °C for 10 min, and leave the solution at room temperature.
3. Mix 1 μL of the annealed oligonucleotides, 1 μL of *Bsa*I-digested pDR274 vector (10 ng/μL), and 2 μL of ligase (ligation high ver. 2: TOYOBO) and incubate at 16 °C for 30 min.
4. Transform the ligation solution (2 μL) into competent *E. coli* (DH5α: 20 μL).
5. Select the bacterial colonies containing sgRNA sequences and grow the bacteria in LB medium with kanamycin.
6. Purify the sgRNA expression vector from bacterial cultures by using the QIAprep Spin Miniprep Kit according to the manufacturer's instructions.
7. Read the nucleotide sequences of the sgRNA expression vector by using the M13 reverse primer.

3.2 Preparation of sgRNA

1. Linearize 5 μg of the sgRNA expression vector by *Dra*I digestion.
2. Incubate the linearized DNA with 200 μg/mL proteinase K at 50 °C for 30 min (*see* **Note 3**).
3. Extract the linearized DNA by using phenol/chloroform/isoamyl alcohol (25:24:1).
4. Transfer the supernatant into a 1.5 mL tube, add a one-tenth volume of 3 M sodium acetate and two volumes of 100% ethanol, and centrifuge at 15,000 × *g* for 10 min.
5. Rinse the pellet with 70% ethanol and dry the pellet.
6. Dissolve the pellet in sterilized water.
7. Transcribe sgRNA from the linearized DNA (1 μg) by using the MAXIscript T7 kit according to the manufacturer's instructions.
8. Add 1 μL of DNase I, and incubate at 37 °C for 15 min (*see* **Note 4**).
9. Extract the RNA with phenol/chloroform/isoamyl alcohol (25:24:1).
10. Remove unincorporated nucleotides by gel filtration using the PD SpinTrap™ G-25 column.
11. Precipitate the sgRNA with a one-tenth volume of 5 M ammonium acetate and two volumes of 100% ethanol.
12. Dissolve the pellet in sterilized water.

3.3 Preparation of Cas9 mRNA

1. Linearize 5 μg of pCS2 + hSpCas9 by *Not*I digestion.
2. Incubate the linearized DNA with 200 μg/mL proteinase K at 50 °C for 30 min to abolish RNase activity.

3. Extract the linearized DNA with phenol/chloroform/isoamyl alcohol (25:24:1).
4. Transfer the supernatant into a 1.5 mL tube, add a one-tenth volume of 3 M sodium acetate and two volumes of 100% ethanol, and centrifuge at 15,000 × *g*.
5. Transcribe mRNA from the linearized DNA (1 μg) by using the mMESSAGE mMACHINE SP6 kit according to the manufacturer's instructions.
6. Follow the same processes (**steps 8–12**) in Subheading 3.2.

3.4 Microinjection

1. Prepare the injection needle (glass capillary: GD-1) with a Puller PC-10.
2. Dilute sgRNAs, donor vector and Cas9 mRNA solutions with injection buffer (sgRNA: 25 ng/μL, donor vector: 25 ng/μL, Cas9 mRNA: 250 ng/μL) (*see* **Note 5**).
3. Fill the needle with the solution.
4. Inject approximately 1 nL of the solution into the 1-cell stage zebrafish embryos.
5. Incubate the injected embryos in E3 medium at 28.5 °C (*see* **Note 6**).

3.5 Preparation of Genomic DNA

1. Put zebrafish embryos (1 day post-fertilization, dpf) into a 1.5 mL tube and remove as much liquid as possible.
2. Add 108 μL of 50 mM NaOH and incubate at 98 °C for 10 min.
3. Add 12 μL of 1 M Tris–HCl [pH 8.0], mix the solution and store at −30 °C.

3.6 Genomic PCR

1. Amplify the DNA fragment containing the junction by genomic PCR using the locus-specific primer and the reporter-specific primer (*see* **Note 7**).
2. Add 2 μL of 6× loading dye to the reaction solution and separate PCR products on a 1% agarose gel.
3. Take an image of the gel with a transilluminator.
4. Determine the potential founders containing the reporter.

3.7 Determination of the Integration Site by Sequence Analysis)

1. Amplify the junction region from genomic DNA of the potential F0 founders by PCR using the locus-specific primer and the reporter-specific primer.
2. Purify the PCR products using the MinElute PCR Purification Kit according to the manufacturer's instructions.
3. Insert the purified PCR products into the pGEM-T Easy vector.

4. Transform the ligation solution (2 μL) into competent *E. coli* (DH5α: 20 μL).
5. Select the bacterial colonies containing the reporter sequences by PCR using the M13 forward and reverse primers.
6. Purify the PCR products by using the MinElute PCR Purification Kit according to the manufacturer's instructions.
7. Sequence the purified PCR products and identify the F0 founders containing a knock-in allele.
8. Maintain F1 embryos derived from the F0 founders to adult fish.

4 Notes

1. Various types of sgRNA expression vector and Cas9 expression vector are commercially available. It is important to check the genome editing activity of your vector system.
2. The CRISPR/Cas9target site has the sequences, 5′- N_{20}-NGG-3′. To make an sgRNA expression vector, NN at the 5′ end of the target sequence can be replaced with a GG sequence because the 5′ end sequence is not essential for sgRNA/Cas9 recognition.
3. Proteinase K is used to abolish RNase activity.
4. DNase I is used to digest the template DNA.
5. Two sgRNAs are used: one is for the targeted genomic sequence digestion, and the other is for the reporter plasmid digestion.
6. The genome editing activity of sgRNA/Cas9 is determined with a heteroduplex mobility assay (HMA) using genomic DNA derived from the injected embryo.
7. The locus-specific primers (approximately 20 bp) are selected by using a primer design tool "Primer3" (http://bioinfo.ut.ee/primer3-0.4.0/).

Acknowledgment

This work was supported by the Japan Society for the Promotion of Science (JSPS, 26640064), Japan Agency for Medical Research and Development (AMED, 16km0210077j0001), the Takeda Science Foundation, and the SENSHIN Medical Research Foundation.

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Chapter 15

Genome Editing of Medaka

Satoshi Ansai and Masato Kinoshita

Abstract

Medaka (*Oryzias latipes*), along with zebrafish (*Danio rerio*), is a useful experimental model fish. Here, we describe a simple method for generating medaka gene knock-out strains using an automated microchip electrophoresis system. We also describe a method for targeted gene knock-in using a plasmid carrying a sequence that does not cause off-target effects in medaka.

Key words Medaka, Genome editing, CRISPR/Cas, Targeted mutagenesis, Heteroduplex mobility assay, Targeted integration

1 Introduction

Medaka (*Oryzias latipes* species complex) is a small freshwater teleost fish native to East Asia and has been used as an experimental vertebrate model since the early twentieth century [1]. In recent years, owing to availability of a number of genetic resources, including highly polymorphic inbred strains, mutants with unique phenotypes, and complete genome sequence, medaka has become an attractive model system for genetic analysis in many areas of biological research [1]. In particular, the availability of genetic resources has contributed to advances in forward genetics studies, such as identification of the sex-determining gene [2] and a novel gene responsible for a human disease [3].

Reverse genetics is another approach used to determine gene function and understand biological processes. Although gene targeting in mouse embryonic stem cells is a powerful tool for reverse genetics, this technique has not been a feasible option yet in medaka because of the difficulty in establishing germline-competent stem cell lines. Genome editing has become a robust and versatile technology for carrying out reverse genetics studies in a wide range of species, including medaka and other fish. In this technology, site-specific DNA double-stranded breaks (DSBs) induced by targetable nuclease systems result in targeted genome

Izuho Hatada (ed.), *Genome Editing in Animals: Methods and Protocols*, Methods in Molecular Biology, vol. 1630,
DOI 10.1007/978-1-4939-7128-2_15, © Springer Science+Business Media LLC 2017

manipulations, such as targeted mutagenesis with a small insertion and/or deletion (indels) or targeted gene integration, during the subsequent repair processes. RNA-guided nuclease (RGEN) based on the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system is one of the simplest nuclease systems to implement because target specificity of the Cas9 nuclease can be easily tailored by customizing the single guide RNA (sgRNA) or CRISPR RNA (crRNA) sequence.

In this chapter, we describe protocols for targeted mutagenesis and targeted integration in medaka using the CRISPR/Cas system. First, we describe methods to prepare genome editing tools for microinjection, which is commonly used to introduce genome editing tools into fish eggs. Then, we describe heteroduplex mobility assay (HMA) performed with an automated microchip electrophoresis system (MultiNA; HMA–MultiNA), a simple method to detect small indels induced by the RGEN. This method is convenient to evaluate DSB-inducing activity of the sgRNA or crRNA and to identify mutated individuals for targeted mutagenesis experiments. Finally, we describe the procedure for targeted integration using a plasmid that contains a unique target sequence (bait sequence).

2 Materials

2.1 Preparation of Single Guide RNA (sgRNA)

1. DR274 (Addgene Plasmid #42250; https://www.addgene.org/).
2. Pairs of oligonucleotide DNA containing 18 bp of the targeted sequence (*see* Subheading 3.1, **steps 1–3**).
3. NucleoSpin Plasmid QuickPure Kit (Macherey-Nagel, Düren, Germany).
4. 20 mg/mL Proteinase K solution.
5. 10% SDS solution.
6. AmpliScribe T7-*Flash* Transcription Kit (Epicentre, Madison, WI).
7. RNeasy Plus Mini Kit (Qiagen, Hilden, Germany).

2.2 Production of Cas9 RNA

1. pCS2 + hSpCas9 (Addgene Plasmid #51815).
2. QIAGEN Plasmid Midi Kit (Qiagen).
3. 20 mg/mL Proteinase K solution.
4. 10% SDS solution.
5. mMessage mMachine SP6 Transcription Kit (Thermo Fisher Scientific, Waltham, MA).
6. RNeasy Plus Mini Kit (Qiagen).

2.3 Detection of Mutations in Injected Embryos and Fin Clips Using Heteroduplex Mobility Assay with an Automated Microchip Electrophoresis System (HMA–MultiNA)

1. Alkaline Lysis Buffer: 25 mM NaOH and 0.2 mM EDTA.
   Prepare fresh by diluting 1 M NaOH (40× stock solution) and 10 mM EDTA (50× stock solution).
2. Neutralization Buffer: 40 mM Tris–HCl (pH 8.0).
3. High success-rate DNA polymerase KOD-FX (Toyobo, Osaka, Japan).
4. Gene-specific primers: a primer pair that can amplify an 80–250 bp genomic DNA fragment containing the target site (*see* **Note 1**).
5. Thermal cycler.
6. MCE-202 MultiNA microchip electrophoresis system for DNA/RNA analysis with DNA-500 reagent kit for MultiNA (Shimadzu, Kyoto, Japan).

2.4 Microinjection into Fertilized Eggs

1. Medaka eggs within 20 min post-fertilization (*see* **Note 2**).
2. Plastic dishes (30 mm diameter).
3. Ice or a refrigerated chamber.
4. Micromanipulator.
5. Injector.
6. Stereomicroscope.
7. Egg holder.

2.5 Evaluation of Guide RNA (gRNA) Activity

1. 25 ~ 50 ng/μL sgRNAs (*see* Subheading 2.1) or 10–25 ng/μL crRNA and 40 ng/μL tracrRNA (*see* **Note 3**).
2. 100–200 ng/μL Cas9 RNA (*see* Subheading 2.2).
3. Medaka eggs <20 min post-fertilization.
4. Microinjection apparatus (*see* Subheading 2.4).
5. Materials for HMA–MultiNA (*see* Subheading 2.3).

2.6 Establishment of Gene Knock-out (KO) Strains

1. Incubator (26–28 °C).
2. Plastic cups (200 mL).
3. 25 ng/μL sgRNAs (selected in Subheading 2.5).
4. 100–200 ng/μL Cas9 RNA (*see* Subheading 2.2).
5. Microinjection apparatus (*see* Subheading 2.4).
6. Materials for HMA–MultiNA (*see* Subheading 2.3).

2.7 Preparation of Donor Plasmids and Single Guide RNAs for Targeted Integration

1. RSB bait plasmid: this plasmid contains two RSB bait (bRSB) sequences (5′-GGAGCTGCTTCACGATGTCCCGG-3′) and a multiple cloning site (MCS) (Fig. 1a) (*see* **Note 4**).
2. Two pairs of gene-specific primers: primer pairs that amplify about 500 bp of upstream (left homology arm; HA-L) and downstream (right homology arm; HA-R) regions of the

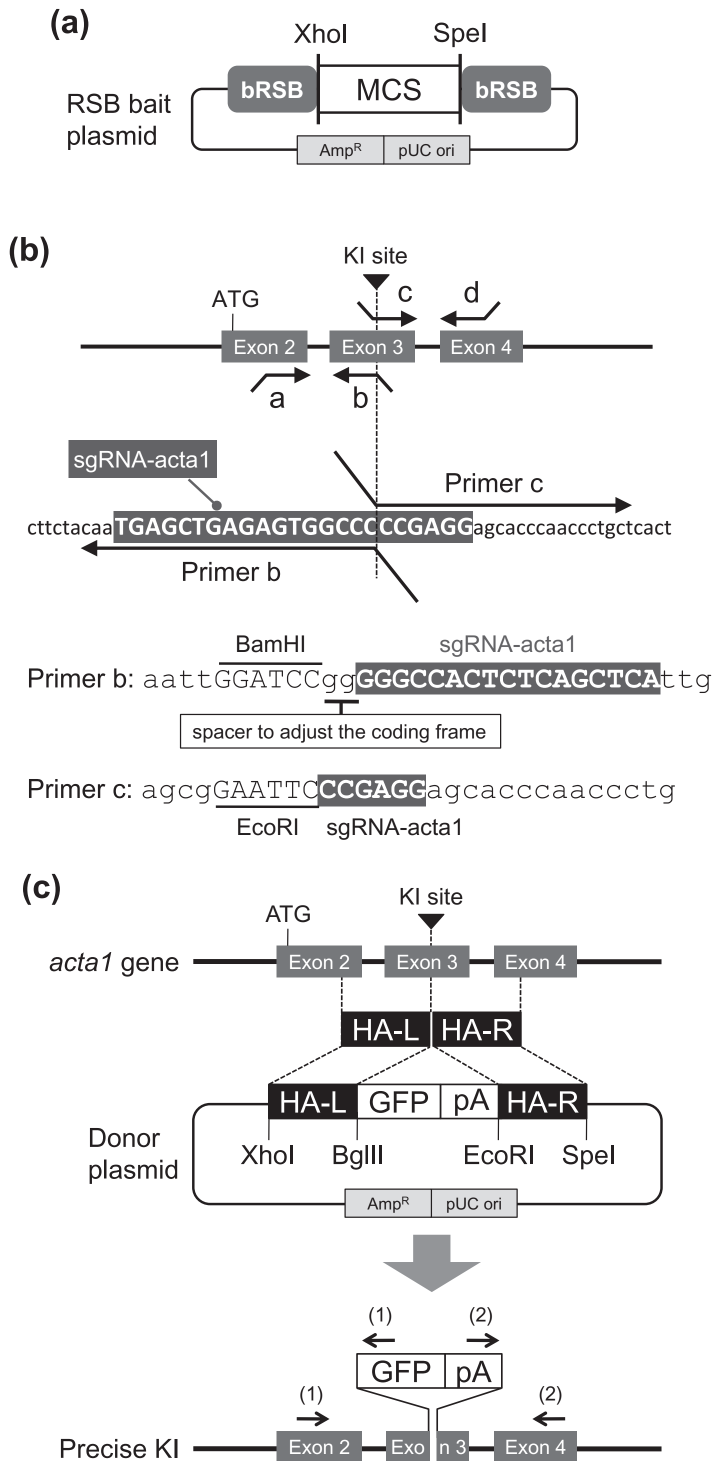

Fig. 1 Schematic illustration of a gene knock-in (KI) experiment. (**a**) Schematic design of the RSB bait plasmid. The multiple cloning site (MCS) contains XhoI and SpeI recognition sequences. (**b**) Design of primers to construct homology arms of a donor vector. Primer pairs a/b and c/d can be used to amplify the left homology arm (HA-L) and the right homology arm (HA-R), respectively. A target site of sgRNA for the *acta1* gene (sgRNA-acta1) is indicated in upper case characters within a *gray box*. Oligonucleotide sequences of primers b and c are shown below the panel. Two nucleotides, "GG," are incorporated in primer b to adjust the coding frame. (**c**) Schematic design of a donor vector for precise integration of *GFP* gene in the muscle actin gene (*acta1*) locus. *Arrows* labeled (1) and (2) indicate primer pairs for PCR genotyping to detect precise integration

targeted integration site with the appropriate restriction enzyme sites to construct a donor plasmid (*see* Fig. 1b, c for restriction enzyme sites) (*see* **Note 5**).

3. PCR fragment containing GFP coding sequence and polyadenylation site with BamHI and EcoRI recognition sites (*see* **Note 5**).
4. Restriction enzymes XhoI.
5. Restriction enzyme BamHI.
6. Restriction enzyme EcoRI.
7. Restriction enzyme SpeI.
8. NucleoSpin Plasmid QuickPure Kit (Macherey-Nagel, Düren, Germany).
9. 20 mg/mL Proteinase K solution.
10. 10% SDS solution.
11. NucleoSpin Gel and PCR Clean-Up Kit with Buffer NTB (Macherey-Nagel).
12. DR274 (Addgene Plasmid #42250; https://www.addgene.org/).
13. Pairs of oligonucleotide DNA containing 18 bp of the targeted sequence (*see* Subheading 3.1, **steps 1–3**).
14. AmpliScribe T7-*Flash* Transcription Kit (Epicentre, Madison, WI).
15. RNeasy Plus Mini Kit (Qiagen, Hilden, Germany).

2.8 Establishment of Gene Knock-in (KI) Strains

1. Incubator (26–28 °C).
2. Plastic cups (200 mL).
3. 50 ng/μL sgRNA for bRSB.
4. 25 ng/μL sgRNA for the muscle actin (*acta1*) gene.
5. 100 ng/μL Cas9 RNA (*see* Subheading 2.2).
6. 2.5 ng/μL Donor plasmid and sgRNAs (*see* Subheading 2.7).
7. Microinjection apparatus (*see* Subheading 2.4).

3 Methods

3.1 Preparation of Single Guide (sgRNA)

1. Select “5′-N_{21}GG-3′” or “5′-CCN_{21}-3′” sequences in loci that are to be targeted. Choose two or more target sites in the target locus with reference to (a) potential off-target sequences and/or (b) microhomology sequences.

   (a) Some genome sequences containing up to several base pairs of mismatches adjacent to a NGG protospacer adjacent motif (PAM) can be disrupted in the injected fish [4].

Target candidates with less potential off-target sites in the medaka genome can be predicted using the CRISPR/Cas9 target online predictor (CCTop, http://crispr.cos.uni-heidelberg.de) [5] or CRISPRscan (http://www.crisprscan.org/) [6]. Potential off-target sites in the medaka genome can also be found using the NBRP medaka pattern match tool for CRISPR/Cas (http://viewer.shigen.info/medakavw/crisprtool/).

(b) We previously demonstrated that microhomologous sequences that stride across the DSB point frequently induce specific patterns of deletions between two homologous sequences [7]. Candidates that will frequently produce specific patterns of deletions in the RNA-injected fish can be identified using the program hosted at the NBRP medaka web site (http://viewer.shigen.info/cgi-bin/crispr/crispr.cgi) to search for CRISPR target sites with microhomology sequences.

2. Enter the selected target sequences as "5′-N_{21}GG-3′" in the Microsoft Excel file "sgRNA_design.xls" (available at http://satoshi-ansai.github.io/en/misc.html). Pairs of 22-mer oligonucleotide sequences for subcloning into the DR274 vector are created in the file.
3. Order and obtain the designed oligonucleotides from a supplier of choice.
4. Clone each pair of annealed oligonucleotides into the DR274 vector digested with the restriction enzyme BsaI.
5. Each modified DR274 vector is purified from cultured *E. coli* using NucleoSpin Plasmid QuickPure Kit. Linearize 5 μg of purified plasmids by DraI digestion in reaction volume of 100 μL.
6. Add 5 μL of 10% SDS and 1 μL of proteinase K solution to the DraI-digested plasmids and incubate at 55 °C for 30 min to eliminate residual RNase activity. Purify each digested plasmid using phenol/chloroform extraction followed by ethanol precipitation. Dissolve the pellet in 5–10 μL of RNase-free water. This solution can be stored at −20 °C (*see* **Note 6**).
7. Assemble 9 μL of reaction solution for in vitro transcription by the AmpliScribe T7-*Flash* Transcription Kit according to manufacturer's instruction in a 0.2 mL PCR tube. Add 1 μL of DraI-digested template DNA in each tube. Incubate at 37 °C for 3–4 h in a thermal cycler.
8. Purify the transcribed sgRNA using RNeasy Plus Mini Kit. Elute the RNA with 30 μL of RNase-free water. Use 1 μL of each eluted RNA solution to estimate concentration using a spectrophotometer and for carrying out electrophoresis to assess quality of the RNA. Store sgRNA at −80 °C (*see* **Note 7**).

3.2 Production of Cas9 RNA

1. Purify pCS2 + hSpCas9 vector from cultured *E. coli* using Qiagen Midi Prep Kit. Linearize 10 μg of the purified plasmid by NotI digestion in reaction volume of 100 μL.
2. Add 5 μL of 10% SDS and 1 μL of proteinase K solution to the NotI-digested plasmid and incubate at 55 °C for 30 min. Purify the digested plasmid using phenol/chloroform extraction followed by ethanol precipitation (*see* **Note 6**). Dissolve the pellet in 10–20 μL of RNase-free water. This solution can be stored at −20 °C.
3. Assemble 9 μL of reaction solution for in vitro transcription by mMessage mMachine SP6 Transcription Kit according to manufacturer's instruction in a 0.2 mL PCR tube. Add 1 μL of NotI-digested template DNA and incubate at 37 °C for 3–4 h in a thermal cycler.
4. Purify the transcribed RNA solution using RNeasy Plus Mini Kit. Elutethe RNA with 30 μL of RNase-free water (*see* **Note 7**). Use 1 μL of the eluted RNA solution to estimate concentration using a spectrophotometer and for carrying out electrophoresis to assess quality of the RNA. Store the transcribed RNA at −80 °C.

3.3 Detection of Mutations in Injected Embryos and fin Clips Using Heteroduplex Mobility Assay with an Automated Microchip Electrophoresis System (HMA–MultiNA)

1. Extract crude genomic DNA (gDNA) from embryos or fin clips as follows. Place each embryo or fin clip in a 0.2 mL PCR tube and add 25 μL of alkaline lysis buffer. In case of embryos, the egg envelope should be broken using a forceps or a truncated 2–200 μL micropipette tip (*see* **Note 8** and Fig. 2). Incubate at 95 °C with frequent vortexing until the embryo or fin clip is dissolved (*see* **Note 9**). Now, add 25 μL of neutralization buffer. These crude DNA samples can be stored at −20 °C. The fish whose tail fin had been clipped can be bred for more than 2 weeks in a plastic cup (Fig. 2).
2. Assemble 10 μL of KOD-FX reaction mixture using primer pairs designed for each target site. Amplify the genomic region containing the target site of the injected guide RNA using standard PCR conditions.
3. Analyze the PCR product using automatic electrophoresis system MultiNA along with DNA-500 reagent kit.

3.4 Microinjection into Fertilized Eggs

1. Collect eggs within 20 min post-fertilization in a plastic dish (*see* ref. 8).
2. Remove attaching filaments of the eggs using forceps and place the plastic dish containing the eggs on a sheet of paper placed on ice or in a refrigerated chamber (*see* **Note 10**).
3. Inject the mixture of genome editing tools into the cytoplasm of 1- or 2-cell stage eggs (*see* **Note 11**). It is recommended

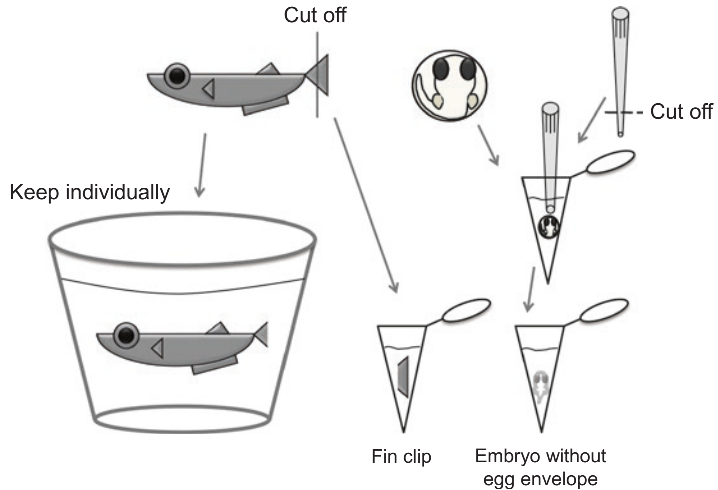

Fig. 2 Fin clip and egg treatment for genomic DNA extraction. Cut off less than each half of tail fin (fin clip) and place it in a 0.2 mL PCR tube containing 25 μL of lysis buffer. The fish is bred in a plastic cup. To break the egg envelope, put an embryo into a 0.2 mL PCR tube containing lysis buffer and squash it with a truncated 2–200 μL micropipette tip

that the injection be performed with earlier stage (1-cell stage) eggs. Do not inject into the yolk sac. The volume of injected mixture should be less than 4 nL, as a larger volume will kill the embryos.

3.5 Evaluation of Guide RNA (gRNA) Activity

1. Prepare the mixture containing Cas9 RNA and each designed gRNA. The final concentration of each RNA solution should be as follows: 25–50 ng/μL for sgRNA, 10–25 ng/μL for crRNA, 40 ng/μL for tracrRNA, and 100–200 ng/μL for Cas9 RNA.
2. Inject each sgRNA or crRNA and tracrRNA with Cas9 RNA mixture into the fertilized eggs (*see* Subheading 3.4).
3. Incubate injected eggs for 3–5 days at 26–28 °C.
4. Analyze the target region using HMA–MultiNA (*see* Subheading 3.3).
5. Evaluate in vivo activity of gRNAs. Embryos injected with active gRNAs show a number of bands with different mobilities, which are derived from many types of homo- and heteroduplexes containing the wild type and/or various types of indel sequences. As shown in Fig. 3, gRNA with high DSB-inducing activity leads to weak PCR amplification of wild-type sequence (arrowhead).
6. Select the gRNA(s) for the following experiments.

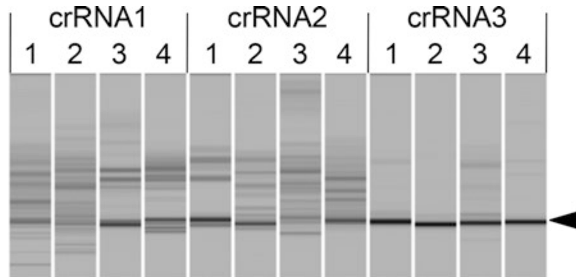

Fig. 3 DNA double-stranded breaks (DSBs)-inducing activity of each CRISPR RNA (crRNA). Three crRNAs are designed for a target gene. The in vivo DSB-inducing activity of each crRNA was evaluated using a heteroduplex mobility assay (HMA-MultiNA). crRNA1 and crRNA2 showed high activity, while crRNA3 showed low activity. *Black arrowhead* indicates the PCR product obtained using wild-type sequence as template. Activity of sgRNAs is evaluated using the same method

3.6 Establishment of Gene Knock-out (KO) Strains

1. Prepare mixture containing 100–200 ng/μL Cas9 RNA and 25 ng/μL selected gRNA(s) (*see* Subheading 3.5).
2. Inject the mixture into fertilized eggs (*see* Subheading 3.4) (*see* **Note 12**).
3. Incubate the injected embryos at 26–28 °C and rear them until they reach sexual maturity (G_0 adults).
4. Mate the G_0 adult fish with their wild-type counterparts.
5. Collect 8–16 F_1 embryos. Extract gDNA from each embryo and analyze the mutation in gRNA target region using HMA-MultiNA (*see* Subheading 3.3).
6. Directly sequence the PCR fragments showing multiple band patterns in HMA.
7. Select G_0 individuals that produce desired mutant sequences in their progenies (F_1) and obtain their F_1 fish by mating with wild-type fish (Fig. 4a) (*see* **Note 13**).
8. After the F_1 have attained body length > 1 cm, cut off the tail fin from each F_1 fish (Fig. 2).
9. Extract gDNA from each clipped fin and carry out HMA–MultiNA (*see* Subheading 3.3). Determine genotypes by direct sequencing.
10. Select both male and female fish harboring the same desired mutation (Fig. 4b).
11. Mate selected F_1 fish to obtain F_2 fish, and rear them until they have attained body length > 1 cm (*see* **Note 14**).
12. Cut off the tail fin from each F_2 fish. Perform HMA–MultiNA. Heterozygotes show multiple banding patterns in HMA. Though both wild-type and homozygous mutant (KO) fish show single band in HMA, the bands derived from wild-type or KO fish can generally be distinguished according to their size using gel-electrophoresis (Fig. 4c).

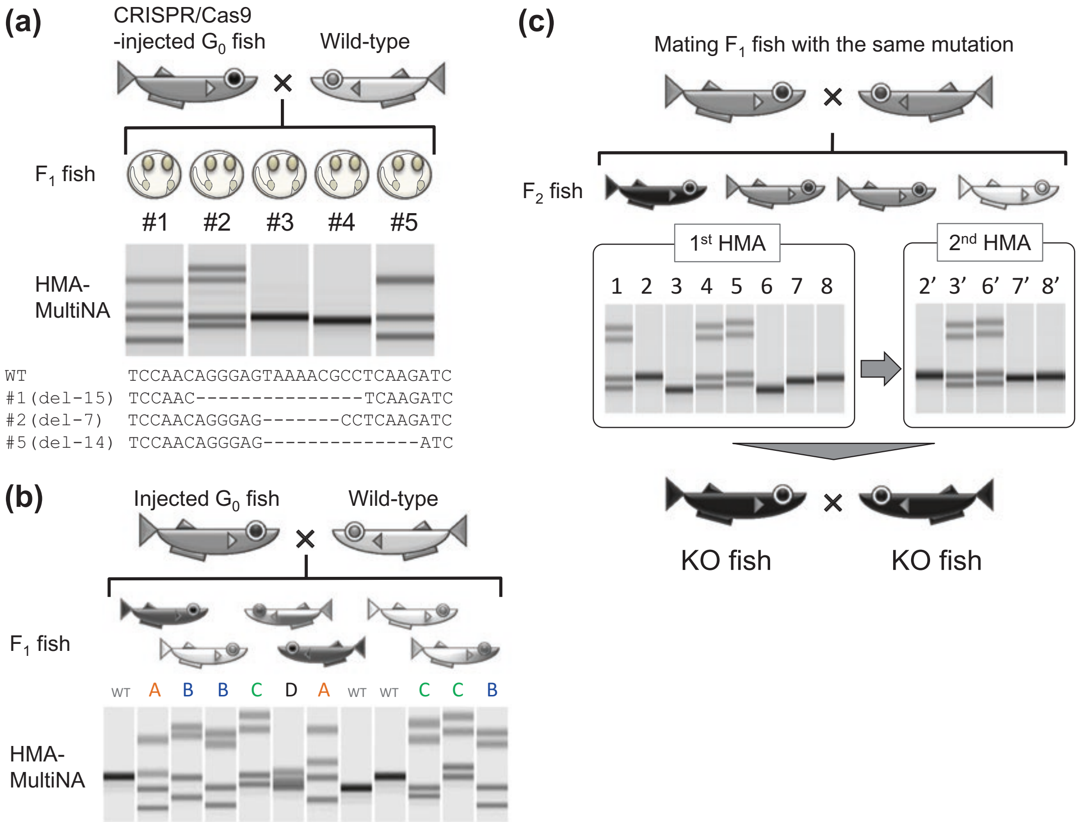

Fig. 4 Heteroduplex mobility assay with an automated microchip electrophoresis system (HMA-MultiNA) to establish gene knock-out (KO) strains. (**a**) Selection of G_0 founder fish. Based on HMA-MultiNA and sequencing data of F_1 embryos, select the founder fish that can generate the F_1 generation with desired mutations. In order to establish gene KO fish, frameshift mutations (#2 and #5) are required. (**b**) Selection of F_1 founder fish to produce F_2 generation. HMA–MultiNA genotyping is performed using genomic DNA extracted from a fin clip of each F_1 fish. The HMA profiles indicate the pattern of mutated sequences. A selected fish is then mated with a fish harboring the same mutation pattern to produce the F_2 generation. (**c**) Identification of gene KO fish in a F_2 family obtained by mating F_1 fish harboring the same mutation with each other. Wild-type (2, 7, and 8), heterozygous mutant (1, 4, and 5), and homozygous mutant (3 and 6) fish are distinguished by HMA-MultiNA genotyping using genomic DNA extracted from each fin clip of F_2 fish (1st HMA). To clearly discriminate homozygous mutants from wild-type fish, each PCR product from the 1st HMA is mixed with a wild-type PCR product, and the mixture is reannealed. The PCR products from homozygous mutant fish (3′ and 6′) show a heterozygous pattern (2nd HMA)

13. (Optional: for reliable distinction between wild-type and KO fish) To a PCR product showing a single band pattern in HMA, add a PCR product separately prepared from wild-type template. Reanneal the mixture by heating at 95 °C for 5 min and gently cooling to room temperature. Perform HMA-MultiNA (2nd HMA). The wild-type will show only one band, while KO fish will show a multiple banding pattern similar to heterozygotes (Fig. 4c).

3.7 Preparation of Donor Plasmids and Single Guide RNAs for Targeted Integration

1. Clone PCR fragments containing homologous genomic sequences on either sides of a KI target site (HA-L and HA-R, *see* Fig. 1c) and *GFP* gene (including polyadenylation site) in the RSB bait plasmid using restriction enzymes. This plasmid is designated as the donor plasmid (Fig. 1c).
2. Transform *E. coli* with the donor plasmid and culture the *E. coli*.
3. Purify the plasmid from the *E. coli* culture using a plasmid preparation kit. Adjust the volume of plasmid solution to 100 μL using sterile water.
4. Add 5 μL of 10% SDS and 1 μL of proteinase K solution in the plasmid solution and incubate at 55 °C for 30 min (*see* **Note 6**).
5. Purify the SDS/proteinase K-treated plasmid using NucleoSpin Gel and PCR Clean-Up Kit with Buffer NTB (Macherey-Nagel) according to manufacturer's instructions.
6. Prepare the gRNA for bRSB (gRNA-bRSB) as described above (*see* Subheading 3.1) (target 23 bp sequence: 5′- GGAGCTGCTTCACGATGTCCCGG-3′).
7. Prepare the gRNA for a target site (in this study, the *acta1* gene; gRNA-acta1) (*acta1*; Ensembl gene number ENSORLG00000010881) (target 23 bp sequence: 5′-TGAGCTGAGAGTGGCCCCCGAGG-3′) (*see* Subheading 3.1).

3.8 Establishment of Gene Knock-in (KI) Strains

1. Prepare injection mixture containing the following components: 100–200 ng/μL Cas9 RNA, 25 ng/μL sgRNA-acta1, 50 ng/μL sgRNA-bRSB, and 2.5 ng/μL donor plasmid.
2. Inject the mixture into fertilized eggs (*see* Subheading 3.4 and **Note 15**).
3. Incubate the injected embryos at 26–28 °C and rear them to sexual maturity (2–3 months). Figure 5 shows a G_0 embryo in which a precise integration event had occurred in a part of the skeletal muscle.
4. Mate the G_0 fish with their wild-type counterparts.
5. Collect 8–16 F_1 embryos and extract gDNA from each embryo (*see* Subheading 3.3).
6. Confirm the precise KI event by PCR genotyping. The primer pairs for this analysis are indicated in Fig. 1c.
7. Select G_0 individuals that produce embryos with the precise KI fragment in their progenies (F_1 generation) and obtain the F_1 fish by mating with their wild-type counterparts.
8. After the F_1 fish attain body length > 1 cm, cut off the tail fin from each F_1 fish and select KI fish to establish KI strains.

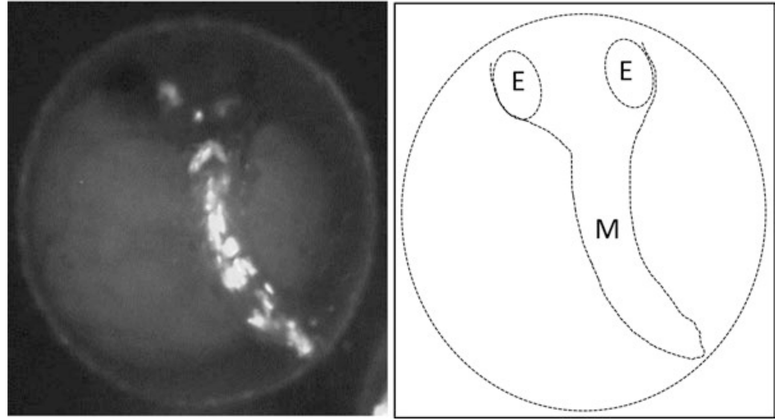

Fig. 5 Precise targeted integration of *GFP* gene into the third exon of the muscle actin (*acta1*) gene. Green fluorescence is observed in the skeletal muscle of an injected embryo 5 d post-fertilization. *E* eye, *M* skeletal muscle

4 Notes

1. Fragments >250 bp lead to decreased resolution of mobility difference among homoduplexes and heterodupulexes.
2. One-cell stage eggs are recommended. Two-cell stage eggs can also be used, but eggs later than the 4-cell stage are not recommended because the ratio of mutated cells in an embryo decreases.
3. Usually, crRNA and tracrRNA are chemically synthesized. Order and obtain them from a supplier of choice.
4. RSB bait (bRSB) is a target sequence of sgRNA-bRSB (*see* Fig. 1b). The bRSB located in the *myostatin* gene of red sea bream (*Pagrus major*) is recognized by the sgRNA-bRSB, and cleaved by the sgRNA-Cas9 complex (unpublished data). No off-target candidates for this gRNA can be detected by interrogating the medaka genome sequence. Therefore, a plasmid containing two bRSB (Fig. 1a) is available as a donor plasmid for targeted integration at any target site in the medaka genome (*see* Fig. 1c).
5. To produce a fusion protein by targeted integration, the primers sequences should be designed such that the integrated gene fragment (e.g., *GFP* gene) is in-frame with the target native gene.
6. RNase contamination drastically reduces the yield of RNA and success rate of genome editing. To remove RNase activity, the SDS/proteinase K treatment described here is strongly recommended. Alternatively, plasmid preparation using a RNase-free plasmid preparation kit is recommended.

7. RNeasy Plus Mini Kit is recommended to remove template plasmid DNA without DNase. Removal of the template plasmid DNA by standard DNase treatment can also be carried out.
8. The front ends of commercially available 2–200-μL tips are too narrow to break the egg envelope of medaka. Therefore, cut off the front ends to obtain ~1 mm diameter orifices.
9. Usually, it takes ~5 min to dissolve the fin clip or the embryo.
10. By cooling the fertilized eggs, the 1-cell stage period can be extended. However, too low a temperature kills the eggs. Therefore, a 6 °C temperature is recommended. Placing the plastic dish directly on ice decreases the temperature below the recommended one; to avoid this, put a sheet of paper between the plastic dish and ice.
11. It is recommended that the mixture be injected into the cytoplasm of earlier stage (1-cell stage) eggs. Do not inject into the yolk sac. The volume of injected mixture should be less than 4 nL, as a larger volume will kill the embryos.
12. Injection into 30–50 eggs is enough to establish a gene KO strain.
13. To disrupt the gene of interest, G_0 founder fish that generate frameshift mutants in the F_1 generation should be selected.
14. The F_2 generation consists of wild-type, heterozygotes, and homozygotes for the mutated sequence in the ratio 1:2:1.
15. Injection into >200 eggs is recommended to establish a gene KI strain.

Acknowledgments

This work was supported by a Grant-in-Aid for JSPS Research Fellows (Grant Number 13J01682)(SA).

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Chapter 16

A Simple Protocol for Loss-of-Function Analysis in *Xenopus tropicalis* Founders Using the CRISPR-Cas System

Yuto Sakane, Ken-ich T. Suzuki, and Takashi Yamamoto

Abstract

Xenopus tropicalis is a versatile model organism for studying basic biology such as developmental biology and cell biology, and for biomedical research on human diseases. Current genome editing techniques enable researchers to easily perform gene targeting in various animals. Among them, gene knockout using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR-associated (Cas) (CRISPR-Cas) system has recently become an indispensable strategy for loss-of-function analysis in vivo. Because of its ease of use, time, and cost efficiencies, CRISPR-Cas has also been applied to *X. tropicalis* where the gene disruption is highly efficient. In this chapter, we introduce a simple CRISPR-Cas system protocol for gene disruption in *X. tropicalis.* Based on our protocol, researchers can generate knock-out phenotypes within the shortest of timeframes, a week, and analyze genes of interest in founder generation.

Key words *Xenopus tropicalis*, CRISPR-Cas, Gene knockout, Loss-of-function analysis

1 Introduction

The Western clawed frog (*Xenopus tropicalis*) has been used in various research fields from cell and developmental biology to drug screening over the past two decades [1, 2]. Compared with other animals, this species offers many experimental advantages: a diploid genome, a relatively short life cycle, and low rearing costs. In addition, the genome sequence of this tetrapod has been analyzed and found to have highly conserved synteny and orthologs for about 1700 human disease-related genes [3]. In the post-genomic era, researchers can easily retrieve information from whole genome data for various organisms and, accordingly, effective use of reverse genetics tools has become a major strategy for understanding gene function in vivo. However, knock-out genes of interest (GOI) in *X. tropicalis* was difficult. Instead, antisense oligonucleotide-mediated knockdown is a popular method used for performing loss-of-function (LOF) analyses.

Izuho Hatada (ed.), *Genome Editing in Animals: Methods and Protocols*, Methods in Molecular Biology, vol. 1630, DOI 10.1007/978-1-4939-7128-2_16, © Springer Science+Business Media LLC 2017

Although antisense oligonucleotides can inhibit splicing or translation of target mRNAs in the injected embryos, its effect gradually attenuates during the embryo stage, and its effect is finally lost in the tadpole and adult stages. Also, this technique possibly causes experimental artifacts leading to misinterpretation of its results. Consequently, *Xenopus* researchers have longed for more robust gene knock-out techniques to be developed.

Recent progress in genome editing enables us to perform gene knock-out and knock-in experiments in various organisms [4]. Genome editing tools such as Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR-associated (Cas) (CRISPR-Cas) system can recognize a target DNA sequence and induce DNA double strand breaks (DSBs) in a site. Most of the DSB sites are repaired by two endogenous mechanisms: non-homologous end joining (NHEJ) and microhomology-mediated end joining (MMEJ) [5]. NHEJ repair often induces small insertions or deletions (indels) into the target site. MMEJ repair also produces deletions of more than 2 bp via microhomologies in the target sequence [6]. Accordingly, knock-out GOI is done by frameshift (out-of-frame) or nonsense mutations.

CRISPR-Cas, an RNA-guided endonuclease (RGEN) system, has been applied to genome editing in a number of organisms including *Xenopus* [7–11]. This technique is responsible for a dramatic paradigm shift in all life science fields [12]. In this chapter, we present a simple and efficient protocol to generate knock-out founders using the CRISPR-Cas system in *X. tropicalis*, from single guide RNA (sgRNA) design to genotyping. This CIRSPR-Cas-based protocol can be accomplished within the shortest of timeframes, a week, and produce almost complete knock-out founder embryos for rapid and efficient functional analysis of GOI [10] (Fig. 1).

2 Materials

Note that this protocol is based on *Streptococcus pyogenes* (*Sp*) Cas9 and T7 RNA polymerase in this chapter.

2.1 sgRNA and Cas9 mRNA Synthesis

1. Vectors of Cas9 cDNA for in vitro transcription (e.g., pCS2 + – T7/h*Sp*Cas9, *see* **Note 1**). We use human codon-optimized *Sp*Cas9, which contains two nuclear localization signals.
2. An appropriate restriction enzyme for plasmid linearization (e.g., *Not*I in the case of pCS2+, TaKaRa).
3. Forward oligonucleotide

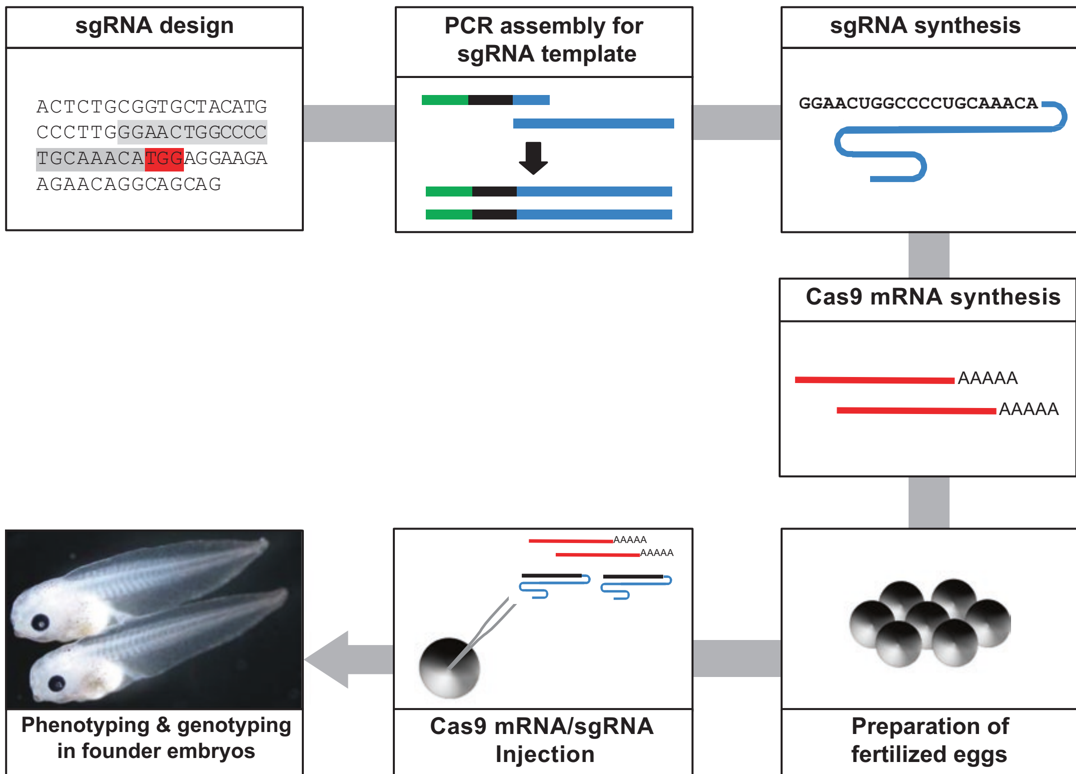


Fig. 1 CRISPR-Cas-mediated genome editing workflow for *Xenopus tropicalis*. Researchers select the gene of interest, and then design and search for sgRNA on- and off-target sites. The DNA template for sgRNA is constructed by a PCR-based assembly using commercially ordered oligonucleotides. Cas9 mRNA and sgRNA are synthesized by in vitro transcription, and are injected into fertilized eggs at the one-cell stage. Reared founder embryos are subjected to phenotyping and genotyping. All procedures using this protocol are accomplished within a week

(5′-TAATACGACTCACTATAGG$(N)_{18}$GTTTTAGAGCTAGAAATAGCAAG-3′). $(N)_{18}$ is 18 nucleotides upstream of the protospacer adjacent motif (PAM) in each GOI. GG are the first and second nucleotides for T7 RNA polymerase based in vitro transcription (*see* **Notes 2** and **3**). The forward oligonucleotide is prepared for each target site.

4. Reverse oligonucleotide (5′-AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC-3′, *see* **Note 2**). The reverse oligonucleotide contains the sgRNA scaffold sequence and is commonly used in all PCR assemblies.
5. PCR enzyme (e.g., KOD FX Neo, TOYOBO).
6. PCR product purification kit (e.g., QIAquick PCR Purification Kit, Qiagen: 28104).

7. In vitro transcription kit for sgRNA (e.g., MEGAshortscript™ T7 Transcription Kit, Thermo Fisher Scientific: AM1354).
8. Purification kit for sgRNA (e.g., MEGAclear™ Transcription Clean-Up Kit, Thermo Fisher Scientific: AM1908).
9. In vitro transcription kit for mRNA (e.g., mMESSAGE mMACHINE® T7 ULTRA Transcription Kit, Thermo Fisher Scientific: AM1345).
10. mRNA purification kit (e.g., RNeasy mini kit, Qiagen: 74104).

2.2 Preparation of In Vitro Fertilized Eggs

1. 10× Marc's Modified Ringers (MMR) stock solution: 1 M NaCl, 20 mM KCl, 10 mM $MgCl_2$, 20 mM $CaCl_2$, 50 mM HEPES, pH to 7.4. Sterilize with 0.2 μm filter or autoclave.
2. 0.1× MMR with 50 μg/ml gentamicin.
3. 1× Modified Barth's Saline (MBS): 88 mM NaCl, 1 mM KCl, 1 mM $MgSO_4$, 5 mM HEPES, 2.5 mM $NaHCO_3$, 1 mM $CaCl_2$, pH to 7.8. Sterilize with 0.2 μm filter or autoclave.
4. 2% (w/v) L-cysteine (in 0.1× MMR), adjust to pH 7.8 with 10 N NaOH just before use.
5. 15 U/100 μl and 100 U/100 μl human chorionic gonadotropin (hCG). Dissolve in 0.6% NaCl solution and store at −20 °C before use. Human and ovine luteinizing hormone can be used instead of hCG [13].
6. 70% Leibovitz L15 medium diluted with sterilized water. Sterilize with 0.2 μm filter.
7. 1% (w/v) MS-222.
8. 100 mm petri dish coated with 1.5% agarose dissolved in 0.3× MMR.

2.3 Injection of Cas9 mRNA and sgRNA

1. Puller for microneedles (e.g., PN-31, NARISHIGE).
2. Microinjector (e.g., NANOJECT II, Drummond Scientific).
3. Stereo microscope.
4. Micromanipulator.
5. Microneedle (e.g., 3 1/2 in. glass capillaries for Nanoject II, Drummond Scientific: 3-000-203-G/X).
6. Incubator (22–26 °C).
7. 60 mm petri dish coated with 1.5% agarose dissolved in 0.3× MMR.
8. Injection buffer: 5% (w/v) Ficoll 400 dissolved in 0.33× MMR. Sterilize with 0.2 μm filter.
9. Nuclease-free water. (Do not use DEPC-treated water; e.g., Thermo Fisher Scientific: AM9938.)

2.4 Genotyping

1. Genomic DNA extraction kit (e.g., DNeasy Blood & Tissue Kit, Qiagen: 69504).
2. Oligonucleotide sets for on- and off-target PCR amplicons and colony PCR.
3. PCR enzymes (e.g., KOD FX Neo, TOYOBO, LA Taq, TaKaRa, or general products).
4. Electrophoresis equipment. Agarose gel electrophoresis is good for routine work, but using a microcapillary electrophoresis system is better (e.g., MCE®-202 MultiNA, Simadzu).
5. Cloning vector for Sanger DNA sequencing (e.g., TOPO® TA Cloning Kit for Sequencing, Thermo Fisher Scientific; 450071).

3 Methods

3.1 Design and In Vitro Synthesis of sgRNA

1. Find the 5′-$(N)_{20}$-NGG-3′ sequences of GOI on both sense and antisense strands using sgRNA design and prediction tools based on *X. tropicalis* genome data (e.g., CRISPRdirect [14]). Underline denotes the PAM sequence. We usually select and enlist several sgRNA candidates targeting each GOI according to the criteria and scores from these bioinformatics tools, focusing on specificity and fewer off-target candidate sites (*see* **Notes 3–5**).
2. Order forward oligonucleotides designed for each target sequence.
3. Mix 200 pmol of each forward and reverse oligonucleotide in a total 100 μl PCR reaction with PCR enzyme. Perform PCR assembly using the cycling conditions as follows: 94 °C for 5 min, 10–15 cycles of 98 °C for 20 s, 56 °C for 20 s, 68 °C for 20 s, and 68 °C for 1 min.
4. Purify the PCR product with a silica column-based kit. PCR product is eluted with 35–50 μl nuclease-free water. After purification, check its concentration and specific amplification by agarose electrophoresis. The expected PCR product size is 117 bp on agarose gel electrophoresis.
5. Perform in vitro transcription using MEGAshortscript™ T7 Transcription Kit, following the manufacturer's user guide. Add 0.5–2 μg of the purified PCR product as template DNA in a 20 μl reaction. Incubate at 37 °C for over 4 h.
6. Add 1 μl of TURBO DNase and incubate at 37 °C for 15 min to remove the template DNA.
7. Purify sgRNA using a MEGAclear™ Transcription Clean-Up Kit. Finally, the sgRNA is eluted with 35 μl of the elution solution included in this kit.

8. Quantitate the concentration of the sgRNA synthesized. Check its quality by agarose electrophoresis. The concentration should be 0.5–1.5 μg/μl or more. Dispense the sgRNA into nuclease-free tubes and store at −70 °C.

3.2 In Vitro Synthesis of Cas9 mRNA

1. Linearize 4 μg of Cas9 plasmid with an appropriate restriction enzyme in a 50 μl reaction leaving blunt or 5′ overhanging ends. Incubate to completely digest the plasmid at 37 °C overnight.
2. Purify the linearized plasmid with a silica column-based kit. DNA is eluted with 30 μl of nuclease-free water. After purification, check the concentration and linearization by agarose electrophoresis.
3. Synthesize mRNA using the mMESSAGE mMACHINE® T7 ULTRA Transcription Kit, following the manufacturer's user guide. In brief, mix T7 2× NTP/ARCA, 10× T7 reaction buffer, enzyme mix, and linearized DNA template (~1 μg) in a 20 μl reaction. Incubate at 37 °C for 2 h.
4. Add 1 μl of TURBO DNase and incubate at 37 °C for 15 min to remove the DNA template.
5. Add the polyA tailing reagent included in the kit to the reaction. Before adding the polyA tailing enzyme (E-PAP), keep a 1 μl aliquot of the reaction mixture. Incubate at 37 °C for 45 min.
6. Clean up the transcribed Cas9 mRNA using an RNA purification kit. Finally, the mRNA is eluted with 30–50 μl of nuclease-free water.
7. Check the successful polyA tailing by comparing the before and after aliquots using agarose electrophoresis. Quantitate the concentration of mRNA. Finally, 10–20 μg of mRNA can be obtained in a 20 μl reaction volume using this kit. Dispense Cas9 mRNA to nuclease-free tubes and store at −70 °C. Do not freeze-thaw and the dispensed mRNA should be for single use.

3.3 Preparation of In Vitro Fertilized Eggs

Good quality eggs are the most important factor for successful genome editing experiments in *X. tropicalis*. To reduce mosaicism in founder embryos, Cas9 mRNA and sgRNA should be injected during the early period of the one-cell stage, as quickly as possible. For more details on handling *X. tropicalis* embryos, refer to del Viso and Khokha (2012) [15]. We prefer to use the Golden strain provided by the National BioResource Project "*Xenopus tropicalis*" at Hiroshima University, because this strain is microinjection robust and always yields good results from our experimental system. Researchers should gain approval from their affiliated institutes and perform experiments according to the ethics guidelines for animal studies.

1. Inject males with 15 U of hCG (15 U/100 μl) 3–7 days pre-experimentation. Frogs are kept at 25–26 °C.
2. Inject two females and two males with 15 U of hCG (15 U/100 μl) for priming about 20 h before the day of the experiment.
3. Inject females and males with 100 U of hCG (100 U/100 μl) for boosting on the morning of the experiment.
4. After 3–4 h, the females will start to lay eggs. Then, euthanize the males with 1% MS-222 and isolate their testes. Each testis is stored in 70% Leibovitz L15 medium at 14–25 °C until just before use (*see* **Note 6**).
5. Gently squeeze the eggs from the females into an agarose gel-coated 100 mm petri dish. Wipe any water away from the dish.
6. Transfer the testis to a 1.5 ml Eppendorf tube and add 500 μl of 1× MBS. Gently grind the testes with pestles and sprinkle the suspension on the collected eggs. Gently spread them to form a layer and leave them for 5 min at 22–26 °C.
7. Soak the dish with 0.1× MMR.
8. After 15 min, de-jelly the embryos with 2% L-cysteine.
9. Discard the L-cysteine, and then wash the embryos several times with enough 0.1× MMR.
10. Soak the eggs in the dish containing the injection buffer.

3.4 Injection of Cas9 mRNA and sgRNA

1. Dilute and mix an appropriate concentration of Cas9 mRNA and sgRNA with nuclease-free water for injection (*see* **Note 7**).
2. Set up stereo microscope and microinjector (we used NanoJect II in this experiment). Load Cas9 mRNA and sgRNA mixture into the pulled microneedles.
3. Place the embryos in the agarose gel-coated 60 mm dish filled with the injection buffer.
4. Inject 2.3 nl of the Cas9 mRNA and sgRNA mixture into the one-cell stage embryos. To reduce mosaicism, the injection should be finished within 45 min post-fertilization.
5. Keep the injected embryos at 22–26 °C for several hours.
6. Transfer the embryos to 0.1× MMR at the blastula stage (3 to ~4 h post-injection). Keep embryos at 24–26 °C. The correct incubation temperature is critical for normal development of *X. tropicalis.*
7. Check the phenotypes at the time point when the GOI should be functioning. When knock-out the melanin synthesis related genes (e.g., *tyrosinase* and *slc45a2*), the phenotypes can be evaluated by the black pigmentation grade in the retinal pigment epithelium and melanophore at 3 days post-injection

([10]; weak, moderate, severe; Fig. 2a). Severe phenotypes targeting *tyrosinase* show almost complete albinism in the founder (Fig. 2b). Based on our protocol, 20–30% severe phenotypes can be obtained [10].

3.5 Genotyping Using Heteroduplex Mobility Assay

Based on our protocol, although the majority of the injected embryos will have severe phenotypes, we occasionally observe different severity levels in the F0 founders caused by mosaicisms and individual differences as shown in Fig. 2a. Therefore, evaluating the knock-out (gene disruption) efficiency in founders is required with every experiment. There are many methods to detect somatic mutations introduced by genome editing tools. For example, SURVEYOR nuclease [16] and restriction fragment length polymorphism (RFLP) [10, 17] are convenient and commonly used assays for estimating target disruption efficiency. Also, RGEN-RFLP analysis can accurately estimate the somatic mutation rate [10, 18] (*see* **Note 8**). Amplicon-seq and fluorescence PCR-based fragment analysis [10, 19] are also excellent methods, but they

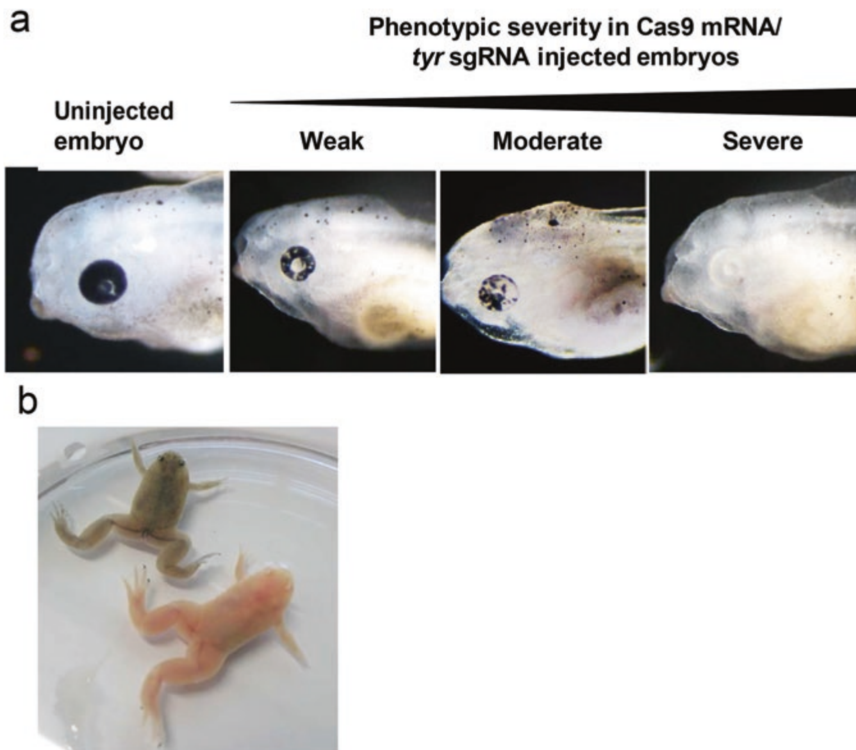


Fig. 2 Representative phenotypes in the Cas9 and sgRNA injected embryos (founder generation). (**a**) Phenotypic severity in the founder generation. In the case of *tyrosinase* (*tyr*) phenotypes, severities of pigmentation loss in the retinal pigment epithelium and melanophore can be classified into four groups: normal, weak, moderate, and severe. (**b**) An example of an F0 *tyrosinase* disrupted froglet (*lower*) shows almost completely loss of pigmentation in the whole body compared with the wild-type (*upper*)

require the availability of expensive equipment such as a next-generation sequencer and capillary sequencer. Therefore, first, we describe an easy and cost-effective analysis, the heteroduplex mobility assay (HMA), for routine work [10, 20, 21]. Because both wild-type and mutant alleles are intermingled in founder individuals, genome PCR products containing sgRNA target regions greatly promote the formation of heteroduplexes between wild-type and mutant alleles over the 30 cycles when the PCR has reached its plateau [10]. Heteroduplex mobility is slower than homoduplex mobility, accordingly, indel mutations can be easily visualized as up-shifted bands on agarose or polyacrylamide gels. Previously, we have reported that HMA can be employed to check indel mutations for not only on-target but also off-target sites [10] (*see* **Note 9**).

1. Rear Cas9 and sgRNA injected embryos to a stage where you can evaluate the knock-out phenotypes of the GOI and collect samples (whole embryos, tail fin clips, or tips of digit) into 1.5 ml tubes, individually. We usually collect at least five individual samples in every experiment.
2. Extract genomic DNA from each sample using a silica column-based DNA purification kit. Elute 50 μl of elution buffer and determine the concentration of each sample (*see* **Note 10**).
3. Perform genome PCR on the target region to produce 100–300 bp amplicons containing the sgRNA target site. Typical PCR conditions for this are shown in Table 1. To obtain enough heteroduplex products, 35 cycles PCR should be performed (*see* **Note 11**).
4. Diluted PCR products are electrophoresed using agarose or polyacrylamide gels or by microchip (microcapillary) electrophoresis (*see* **Note 12**). Figure 3 shows a comparison of agarose gel and microchip electrophoresis images of HMA targeting of the *tyrosinase* locus. The PCR product from the uninjected embryo is a single band (homoduplex form of the wild-type allele; closed arrowhead), whereas up-shifted bands (heteroduplex form of the wild-type and mutant alleles; parentheses) are only detected for amplicons from the Cas9 and sgRNA injected embryos.

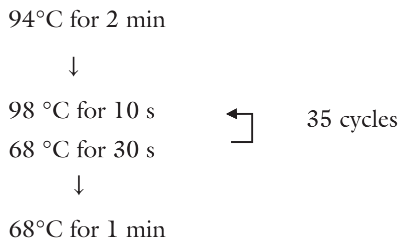
3.6 Genotyping Using DNA Sequencing

Because multiples of 3 bp indels result in in-frame mutations and will not disrupt the function of target genes, we also confirm the frequency and types of mutations in the sgRNA target sites in the injected embryos by DNA sequence analysis. Although amplicon-seq using a next-generation sequencer is the best way to do this because it provides an absolutely quantitative profile of the mutant allele [10] (*see* **Note 13**), it is not cost-effective and suitable for routine work. Therefore, here, we describe conventional Sanger DNA sequencing for routine work.

Table 1
PCR conditions for the heteroduplex mobility assay

Reagents	Volume (μl)
2 × PCR buffer for KOD FX Neo	5
2 mM dNTPs	2
10 pmol/μl forward primer	0.2
10 pmol/μl reverse primer	0.2
Genomic DNA	~50 ng
KOD FX Neo (1 U/μl)	0.15
Distilled water	Up to 10 μl

The PCR cycle condition



1. PCR amplify a genomic region containing the sgRNA target site using the same primer pair used for HMA. We recommend that PCR product sizes are from 100 to 300 bp as in the case of HMA. Heteroduplex products often present some problems in this analysis (e.g., sequencing errors). Therefore, to reduce heteroduplex formation, no more than 30 PCR cycles should be conducted. According to our experience, 24–28 cycles seems to be appropriate for subcloning and downstream Sanger DNA sequencing.
2. Check PCR products using agarose gel electrophoresis. Some extra bands may be detected if large insertions and deletions are present.
3. Subclone PCR products into the cloning sites of cloning vectors. Mix 3 μl of the purified PCR products, 0.5 μl of salt solution, and 0.5 μl of the pCR4.0 TOPO TA vector. Incubate at room temperature for 5–60 min, followed by transformation. If the PCR products carry blunt ends, adenine attachment is required for TA cloning before the subcloning step.
4. Perform colony PCR with the appropriate forward and reverse primers to identify inserted clones. If you observe many doublet bands in the colony PCR, these clones are most likely to have heteroduplex inserts. This is probably caused by excessive

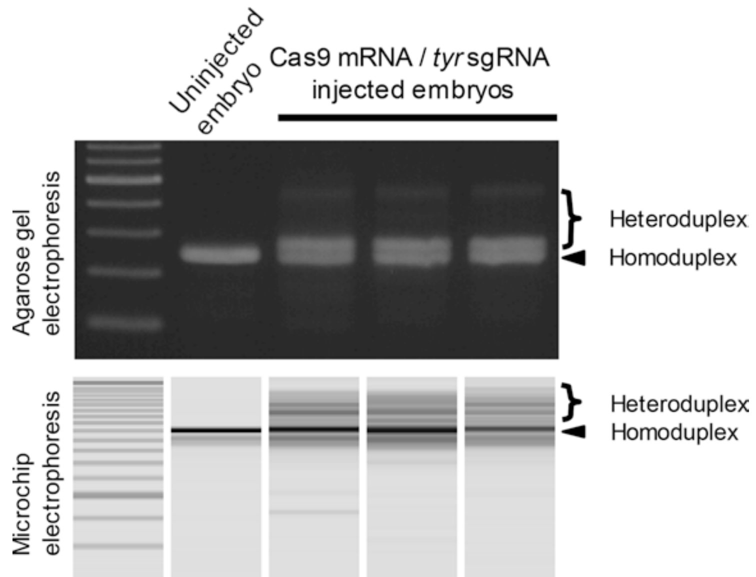


Fig. 3 Detection of CRISPR-Cas-mediated mutagenesis using heteroduplex mobility assay. The PCR products containing *tyrosinase* (*tyr*) sgRNA target site from the uninjected control and Cas9 and sgRNA injected embryos were analyzed using agarose gel (*upper*) and microchip (*lower*) electrophoresis. The single band from the uninjected embryo represents the homoduplex form of the wild-type allele (*closed arrowheads*). Up-shifted bands (heteroduplex form of the wild-type and mutant alleles) are only detected in the injected embryos (*parentheses*)

PCR cycles on the genomic DNA, so reducing the number of cycles to 28 or fewer is recommended.

5. Perform Sanger DNA sequencing on the plasmids or colony PCR products from the clones using inside primers such as T7 or SP6 primers. We usually analyze 10–20 clones from each embryo to estimate the somatic mutation rate (*see* **Note 13**).
6. Align the wild-type and mutant allele sequences. Representative data are shown in Fig. 4. Cas9 cleaves the target site 3 bp upstream from PAM; consequently, DSB and NHEJ or MMEJ repairs are introduced there. There is evidence of repair by MMEJ (the microhomologous sequences are underlined in Fig. 4).

4 Notes

1. We use the pCS2 + −T7/h*Sp*Cas9 plasmid [10], which contains a human codon-optimized *S. pyogenes* Cas9 open reading frame derived from pX330 [22] and a new T7 promoter site for in vitro mRNA synthesis using T7 RNA polymerase. We strongly recommend using the mMESSAGE mMACHINE®

***tyr* sgRNA : 13/13 (mutants/ total clones)**

```
GCCTGTTCTTCTTCCTCCATGTTTGCAGGGGCCAGTTCCCAAGGGCATGT     wild-type
GCCTGTTCTTCTTCCTCCATG-TTGCAGGGGCCAGTTCCCAAGGGCATGT     Δ1 X2
GCCTGTTCTTCTTCCTCCATGT----AGGGGCCAGTTCCCAAGGGCATGT     Δ4 X3
GCCTGTTCTTCTTCCTCCATG----CAGGGGCCAGTTCCCAAGGGCATGT     Δ4 X4
GCCTGTTCTTCTTC-------------------AGTTCCCAAGGGCATGT     Δ19
GCCTGTTCTTCCTCCTCCATGTGGGGGCAGGGGCCAGTTCCCAAGGGCATGT   Δ2 +4
GCCTGTTCTTCTTC---TGTTC-TGCAGGGGCCAGTTCCCAAGGGCATGT     Δ9 +5
GCCTGTTCTTCTT----TTCCA-TGCAGGGGCCAGTTCCCAAGGGCATGT     Δ10 +5
```

Fig. 4 Sequence analysis of a *tyrosinase* disrupted embryo. Representative example of the sequence alignment of the *tyrosinase* target site from the Cas9 mRNA and sgRNA injected embryo. All sequenced clones contained indels near the target site in this severe phenotype embryo. The sgRNA target protospacer and PAM sequences are highlighted in *gray* and *red*, respectively. Deletions and insertions are shown by *dashes* and *blue* letters. Putative microhomologous sequences are underlined. Types and frequencies of each indel mutation are shown at the right panel

T7 ULTRA Transcription Kit (Thermo Fisher Scientific) for in vitro transcription. mRNA synthesized using this kit possesses a higher translational efficiency than provided by conventional mRNA synthesis kits because it incorporates a nucleotide cap analog and polyA tailing reagents. This should result in high genome editing efficiency in the injected embryos.

2. Forward oligonucleotide for the sgRNA template contains a T7 promoter and protospacer targeting GOI, and a region of crRNA sequence. The reverse oligonucleotide contains a region of crRNA, a linker loop, and tracrRNA sequences (called sgRNA scaffold sequences), and is common to all sgRNA templates. The scaffold sequence from the pDR274 plasmid has been described previously [23]. Because the 3′ terminal regions are partially complementary in each oligonucleotide, the DNA template for sgRNA can be produced by annealing and extension in the PCR assembly step.
3. To increase the in vitro transcript yield with T7 RNA polymerase, the +1 and +2 nucleotides from the transcription start site must be guanines (G); consequently, the 5′ terminal of the transcribed sgRNA becomes GG. The proximal region of the protospacer sequence (12 nucleotides adjacent to PAM) known as the seed region is critical for target specificity, whereas the distal region known as the nonseed region (8 nucleotides) seems to be somewhat tolerant of mismatches [24, 25]. The target genome sequences do not necessarily need to contain the 5′-GG; therefore, at least N_{18} should be specific for them.
4. For the LOF analysis, the sgRNAs should be designed to effectively perform GOI knock-out as per the following criteria: just downstream of the start codon, and the exon-intron junction and coding regions of the crucial domains involved in protein

function. When researchers analyze genes of unknown function, we recommend trying two or three sgRNAs against each GOI to confirm the phenotypes obtained are phenocopies.

5. As representative examples of online tools for sgRNA design, CRISPRdirect (https://crispr.dbcls.jp/) [14], Cas-Designer (http://www.rgenome.net/cas-designer/) [26], CRISPRscan (http://www.crisprscan.org/) [27], and Cas-OFFinder (http://www.rgenome.net/cas-offinder/) [28] can search the on- and off-target sequences based on the *X. tropicalis* genome. The Microhomology-Predictor (http://www.rgenome.net/mich-calculator/) [6] also predicts the deletion patterns mediated by MMEJ repair that can be applied to perform effective gene knock-out by out-of-frame mutations by design.
6. Isolated *X. tropicalis* testis cannot be stored at 4 °C. We recommend using all prepared testes on the same day because sperm motility decreases gradually over time.
7. Although some Cas9 constructs are reported to produce high efficiency knock-outs in *X. tropicalis* [7–10], the doses of Cas9 mRNA and sgRNA vary among these reports. Additionally, CRISPR-Cas activity seems to depend on the targeted sequence and the chromatin status; therefore, researchers need to optimize the doses and ratios of both RNAs for every target site. In our experimental system using pCS2 + –T7/h*Sp*Cas9-based Cas9, we generally use 2.2 ng and 200 pg of the Cas9 mRNA and sgRNA per embryo, respectively. Although we have tested over 30 sgRNAs, no significant toxic effects on embryos were observed according to our results.
8. In principle, RGEN-RFLP analysis is the same as conventional RFLP analysis except for use of the ribonucleoprotein (RNP) complex of recombinant Cas9 protein and sgRNA [18]. Genomic PCR products from the wild-type target allele are completely digested in vitro with RNP. Conversely, PCR products from the mutant allele can no longer be cut because the sgRNA target sites are altered. Thus, we can calculate the somatic mutation frequency for the injected embryos from the ratio of cut and uncut PCR products (bands) in the electrophoresis images. We previously reported a high correlation between RGEN-RFLP analysis and amplicon-seq in *X. tropicalis* embryos [10]; therefore, this method is also recommended.
9. The mutation detection limit for HMA is considered to be approximately 0.5–2.5% in human cultured cells, mouse, and *X. tropicalis* [10, 21].
10. To perform genomic DNA extraction cost-effectively, we use the easy method that follows, which is a modification from the Nakayama et al. study [29]. Briefly, DNA samples are lysed in lysis buffer (50 mM Tris, 1 mM EDTA, 0.5% Tween 20) with

a final concentration 200 μg/ml proteinase K. After incubation at 56 °C for >2 h, phenol/chloroform extraction and ethanol precipitation is performed with linear polyacrylamide and sodium acetate. This purification step improves the success rate of target amplification. Finally, resuspend the genome DNA in 20–40 μl of TE buffer (pH 8.0). Add 0.5 μl of the DNA solution to a 10 μl PCR.

11. It is hard to distinguish between heteroduplex and homoduplex bands by agarose electrophoresis when the PCR product sizes are over 300 bp. PCR products sizes should be less than 300 bp. Also, PCR cycles should be at least 30 cycles to detect heteroduplexes as up-shifted bands on electrophoresis gels.
12. To visualize up-shifted bands clearly by gel electrophoresis, the agarose concentration should be at least 3%. Alternatively, polyacrylamide or microcapillary electrophoresis methods are the best ways to clearly separate heteroduplexes (e.g., MultiNA system, Shimadzu or TAPEStation, Agilent) [10, 21].
13. More than 90% of mutations are observed within ±11 bp around the DSB site [10]. In some severe phenotypes, the somatic mutation rates approach almost 100%, as shown by amplicon sequencing, suggesting that an almost complete knock-out in *X. tropicalis* can be generated in the founder generation based on our protocol [10].

Acknowledgments

We are grateful to Drs. Akihiko Kashiwagi and Keiko Kashiwagi (Hiroshima University) for technical advice on animal husbandry and to Ms. Mitsuki Shigeta and Miyuki Suzuki for experimental support. *X. tropicalis* (Golden strain) was provided by the Institute for Amphibian Biology (Hiroshima University) through the National Bio-Resource Project, Japan Agency for Medical Research and Development, Japan. This work was supported by the Japan Society for the promotion of Science KAKENHI Grant Number 15K06802 [Grant-in-Aid for Scientific Research (C)], The Narishige Zoological Science Award and The Naito Foundation to K.T.S. and JSPS KAKENHI Grant Number 15J05833 [Grant-in-Aid for JSPS Fellows] to Y.S.

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Chapter 17

Genome Editing of Silkworms

Takuya Tsubota and Hideki Sezutsu

Abstract

Silkworm is a lepidopteran insect that has been used as a model for a wide variety of biological studies. The microinjection technique is available and it is possible to cause transgenesis as well as target gene disruption via the genome editing technique. TALEN-mediated knock-out is especially effective in this species. We also succeeded in the precise and efficient integration of a donor vector using the Precise Integration into Target Chromosome (PITCh) method. Here, we describe protocols for ZFN, TALEN, and CRISPR/Cas9-mediated genome editing as well as the PITCh technique in the silkworm. We consider that all of these techniques can contribute to the further promotion of various biological studies in the silkworm and other insect species.

Key words Silkworm, ZFN, TALEN, CRISPR/Cas9, Knock-in, PITCh

1 Introduction

Silkworm, *Bombyx mori*, is a lepidopteran insect that has been used as a model in various biological studies including physiology, biochemistry, pathology, and genetics. In 2000, the transgenic technique was established [1] and this greatly contributed to the facilitation of a wide variety of silkworm studies including gene functional analysis as well as practical research. The availability of the genome sequence [2] is also valuable not only for silkworm studies but also for other lepidopteran species. It is obvious that this is the most advanced model insect in this order.

The genome editing of the silkworm was first carried out using ZFN technology [3]. In this study, the knock-out of the *biogenesis of lysosome-related organelles complex 1, subunit 2* (*BLOS2*) gene was attempted and this resulted in the successful disruption of this gene. *BLOS2* is an excellent model for genome editing assay for the following reasons; first, its mutant shows a translucent skin phenotype that can be easily detected [3, 4] (Fig. 1), and second, *BLOS2* is located on the Z chromosome that permits the germline knock-out detection in the hemizygous females in G1. After the success

Izuho Hatada (ed.), *Genome Editing in Animals: Methods and Protocols*, Methods in Molecular Biology, vol. 1630,
DOI 10.1007/978-1-4939-7128-2_17, © Springer Science+Business Media LLC 2017

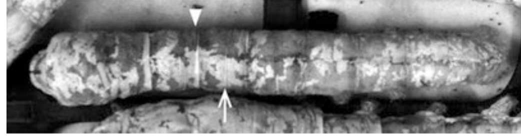

Fig. 1 Oily mosaic G0 larva caused by the *BLOS2* knock-out. The *arrow* indicates the normal epidermis region and the *arrowhead* depicts the oily skin

of the ZFN-mediated knock-out, another programmable nuclease TALEN was developed and the effectiveness of this tool was evaluated in the silkworm using *BLOS2* as a model. Initially, the efficiency of TALEN was comparable to that of ZFN [5]; however, the TALEN vector was improved so that the synthesized RNA can be compatible with the silkworm translation machinery or the TALEN activity can be expressed at a higher level. The utilization of this vector increased knock-out efficiency by more than one order of magnitude [6]. Due to this result, a number of knock-out studies are ongoing using this improved TALEN [7–11]. CRISPR/Cas9, another genome editing tool mediated by RNA, is also functional although its effectiveness appears to be lower compared to TALENs [12].

Targeted integration of the donor vector is another important application of genome editing. Conventionally, this was approached via the homology-directed repair (HDR) pathway, but the efficiency was very low in the silkworm and other species presumably due to the variable frequencies of homologous recombination in cell types and organisms [12]. To overcome this, the donor vector was designed so that it can be linearized by TALENs and the microinjection of this arranged vector in mixture with TALENs could successfully cause site-specific integration with comparable efficiency to transposon-mediated transgenesis in the silkworm [13]. In contrast to this method, we recently developed another knock-in system termed PITCh [14]. This method is based on a microhomology-mediated end-joining (MMEJ) pathway, a recently identified DSB repair mechanism that uses microhomologous sequences (5–25 bp) for error-prone end-joining [15]. We demonstrated that the TALEN-based PITCh system (TAL-PITCh) could efficiently integrate a donor vector harboring the *hsp90* promoter and GFP into the *BLOS2* genomic locus in a very precise manner [14]. The PITCh system was also effective in mammalian cell culture and in the frogs [14, 16]. It is possible that this technique can be utilized in a wide variety of organisms.

Here, we describe protocols of ZFN, TALEN, and CRISPR/Cas9-mediated genome editing in the silkworm. In addition, we also describe the method of the TAL-PITCh system using *BLOS2* as a model. We consider that all of these techniques will be promising tools for the further promotion of silkworm and other insect studies.

2 Materials

2.1 ZFN-Mediated Knock-out

1. Silkworms and related resources.

   Silkworms are available from the public bioresource center in Japan (National BioResource Project, http://silkworm.nbrp.jp/index_en.html; Genebank Project NARO, https://www.gene.affrc.go.jp/about_en.php), India (India Central Sericultural Germplasm Resources Centrehttp://csgrc.res.in/indent-germplasm.htm), and other countries. The silkworm gene information is available in the Japanese silkworm genome research program (http://sgp2010.dna.affrc.go.jp/index.html) and the Chinese silkworm genome database (http://silkworm.genomics.org.cn/). ESTs can be searched and requested in SilkBase (http://silkbase.ab.a.u-tokyo.ac.jp/cgi-bin/index.cgi). The gene expression profile is available in the microarray database [17].

   Silkworms can be reared using an artificial diet (Nihon Nosan Kogyo, Yokohama, Japan) or mulberry leaf. We usually rear on the artificial diet because we need to constantly have non-diapausing strains for the microinjection experiments (see below).
2. Equipment required for the molecular biological experiments.
3. DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) or other genome extraction reagents.
4. PCR enzymes.
5. Sequencing reagents.
6. pENTR-NLS-ZFN (available from Carroll lab; www.biochem.utah.edu/carroll).
7. pDEST14 or other Clonase reaction destination vectors with T7 sequence.
8. T4 DNA ligase.
9. *E. coli* competent cells.
10. Plasmid midiprep kit.
11. mMESSAGE mMACHINE kit with T7 polymerase (Thermo Fisher Scientific, Waltham, USA).
12. Injection buffer: 0.5 mM potassium phosphate buffer (pH 7.0), 5 mM KCl.
13. A micromanipulator and microinjection system.

2.2 TALEN-Mediated Knock-out

1. Silkworms and related resources (*see* Subheading 2.1, **item 1**).
2. Equipment required for the molecular biological experiments.
3. DNeasy Blood & Tissue Kit (QIAGEN) or other genome extraction reagents.
4. PCR enzymes.

5. Sequencing reagents.
6. Plasmids from Golden Gate TALEN and TAL Effector Kit 2.0 (Addgene, Cambridge, USA).
7. pBlue-TAL vector (Addgene).
8. T4 DNA ligase.
9. *E. coli* competent cells.
10. Plasmid midiprep kit.
11. mMESSAGE mMACHINE kit with T7 polymerase (Thermo Fisher Scientific).
12. Injection buffer: 0.5 mM potassium phosphate buffer (pH 7.0), 5 mM KCl.
13. A micromanipulator and microinjection system.

2.3 CRISPR/Cas9-Mediated Knock-out

1. Silkworms and related resources (*see* Subheading 2.1, **item 1**).
2. Equipment required for the molecular biological experiments.
3. DNeasy Blood & Tissue Kit (QIAGEN) or other genome extraction reagents.
4. PCR enzymes.
5. Sequencing reagents.
6. pDR274 (Addgene).
7. T4 DNA ligase.
8. *E. coli* competent cells.
9. Plasmid midiprep kit.
10. MAXIscript T7 Kit (Thermo Fisher Scientific).
11. pMLM3613 (Addgene).
12. mMESSAGE mMACHINE kit with T7 polymerase.
13. Injection buffer: 0.5 mM potassium phosphate buffer (pH 7.0), 5 mM KCl.
14. A micromanipulator and microinjection system.

2.4 TAL-PITCh-Mediated Knock-in

1. Silkworms and related resources (*see* Subheading 2.1, **item 1**).
2. Equipment required for the molecular biological experiments.
3. The template plasmid for PITCh vector construction. Here, we utilize pBachsp90GFP-3xP3DsRed, a plasmid with an *hsp90* promoter and GFP [14, 18] (*see* **Note 1**).
4. The PCR primers for inverse PCR from the template plasmid. Here, we utilize BLTS6DsRedF1 (5′-CCGATCCTAGCCGC GAACCTGAACAATAGTCACCACCTGTTCCTGTAG-3′) and BLTS6pBacLR1 (5′-CGGCTCCTTATACTTGGTGATCG CGCTCGAA TTAGATCTTTGG-3′) (see below).
5. PCR enzymes.

6. T4 DNA ligase and kinase.
7. Competent *E. coli* cells.
8. Plasmid miniprep kit.
9. Plasmid midiprep kit.
10. Phenol/chloroform.
11. 100% ethanol.
12. 70% ethanol.
13. Injection buffer: 0.5 mM potassium phosphate buffer (pH 7.0), 5 mM KCl.
14. A micromanipulator and microinjection system.
15. A stereo fluorescence microscope.
16. PCR primers for the detection of the knock-in. BLOS2F1 (5′-AGCAAATTGTTGCAAGGGAC-3′) and g2900_120R (5′-ATTTGTTGGCAGCACTGCTT-3′) are utilized here for the detection of the 5′ junction, and pBacL3490 (5′-ATAAC GACCGCGTGAGTCAA -3′) and BLOS2R1 (5′-AGCTAGC CACAGTGTTTATC-3′) are used for the 3′ junction.
17. Agarose.

3 Methods

The outline of the knock-out experiment (Subheadings 3.1, 3.2 and 3.3) is shown in Fig. 2.

3.1 ZFN-Mediated Knock-out

1. Select the target site using the "Zinc Finger Tools" program (http://www.scripps.edu/mb/barbas/zfdesign/zfdesign-home.php) [19].
2. Extract the genome from the *w1 pnd* strain (*see* **Note 2**) using a genome extraction kit. Amplify the target genome region by PCR. Analyze its sequence and determine the presence or absence of the polymorphic nucleotide in the ZFN target site.
3. Construct the ZFN expression vector following the method described previously [3, 20].
4. Carry out the in vitro transcription using a message machine kit with a T7 polymerase or equivalent. Dissolve 100 ng/μl of each RNA in injection buffer.
5. Collect eggs from the *w1 pnd* strain by mating newly eclosed adult moths.
6. Inject the ZFN RNA using a microinjector (*see* **Note 3**). Seal the eggs using bonds.
7. Incubate the eggs keeping the humidity until hatched.

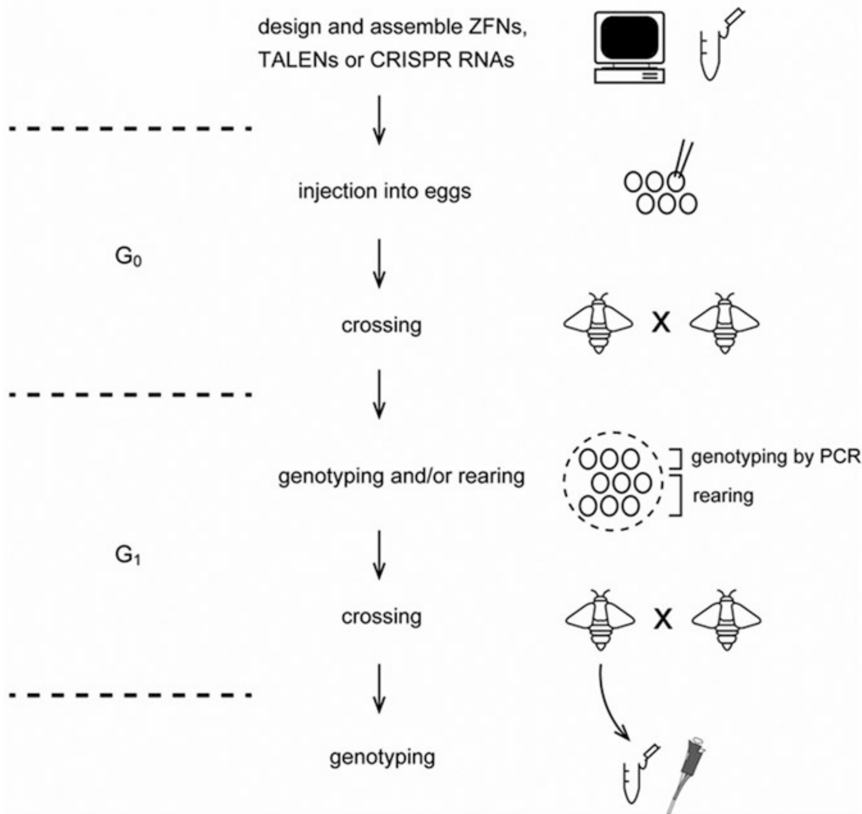

Fig. 2 Diagram of the genome editing experiment for the generation of *B. mori* knock-out individuals

8. Rear the G0 individuals until adulthood and obtain fertile eggs by sib-mating or mating with noninjected individuals.
9. Incubate the eggs at 25 °C and collect 25–50 eggs from each brood for genomic DNA extraction.
10. Amplify the target region by PCR. Analyze the sequence of the PCR product and examine whether the mutation is present. For broods in which the mutation could be detected, rear the remaining individuals.
11. Cross the G1 adults with *w-c* or other diapausing strains to keep stocks. After crossing, extract the genome from each G0 adult and check the genotype by PCR and sequencing.

3.2 TALEN-Mediated Knock-out

The detailed protocol for a TALEN-mediated knock-out was previously published [21, 22].

1. Select the TALEN target site. In comparison to ZFN, the TALEN target selection is flexible and the target site can be selected at almost any region in the gene of interest [21, 22]. It is preferable that a natural restriction enzyme site exists within the target.

2. Assemble TALEN using the Golden Gate TALEN kit. Insert the assembled arrays into pBlue-TAL [6] which is an expression vector optimized for the silkworm translation system. Utilization of this vector is critical for a highly efficient knock-out.
3. Carry out the in vitro transcription using a message machine kit with a T7 polymerase. Dissolve 125 ng/μl of each RNA in injection buffer.
4. Microinject the RNA into the *w1 pnd* eggs and generate the knock-out strain following the method described in Subheading 3.1 (*see* **Note 4**).

3.3 CRISPR/Cas9-Mediated Knock-out

1. Select the target site using CRISPRdirect (https://crispr.dbcls.jp/) [23] or other target selection tools.
2. Synthesize the sgRNA. Insert the oligonucleotides bearing the sgRNA into the pDR274 vector [24] and transcribe using a T7 polymerase with the MAXIscript T7 Kit (or equivalent).
3. Synthesize the Cas9 mRNA from the Cas9 expression vector pMLM3613 using the mMESSAGE mMACHINE T7 ULTRA kit [24].
4. Mix the sgRNA and Cas9 mRNA at a concentration of 50 ng/μl of sgRNA and 300 ng/μl of Cas9 mRNA.
5. Follow the protocol described in Subheading 3.1 (*see* **Note 5**).

3.4 Knock-in Using TAL-PITCh

Here, we describe a protocol of integrating a plasmid with the *hsp90* promoter-GFP (*see* Subheading 2.3, **item 4**) into the *BLOS2* locus. The flowchart of the knock-in experiment is shown in Fig. 3.

1. Select the TALEN target site as described in Subheading 3.2, **step 1**. Here, we select the exon 3 of the *BLOS2* gene as the target (Fig. 4).
2. Design the donor (PITCh) vector. In the TAL-PITCh system, the PITCh vector needs to be designed so that TALENs targeting the genomic site can also cleave this vector [14, 25]. The TALEN recognition site should therefore be introduced into the PITCh vector (Fig. 5). At this time the anterior and posterior halves of the spacer sequence should be switched compared to that of the genomic target [14, 25] (Fig. 4). Following these rules we can design the TALEN target sequence in the vector (Fig. 4).
3. Construct the PITCh vector. Design primers including the vector sequence and TALEN target (BLTS6DsRedF1 and BLTS6pBacLR1, Fig. 6). Perform PCR from pBachsp90GFP-3xP3DsRed (*see* **Note 6**) and self-ligate the PCR product. Transform into the *E. coli* competent cells.
4. Pick 4 ~ 6 colonies. Purify the plasmid using a plasmid mini-prep kit and verify the sequence around the TALEN target site.

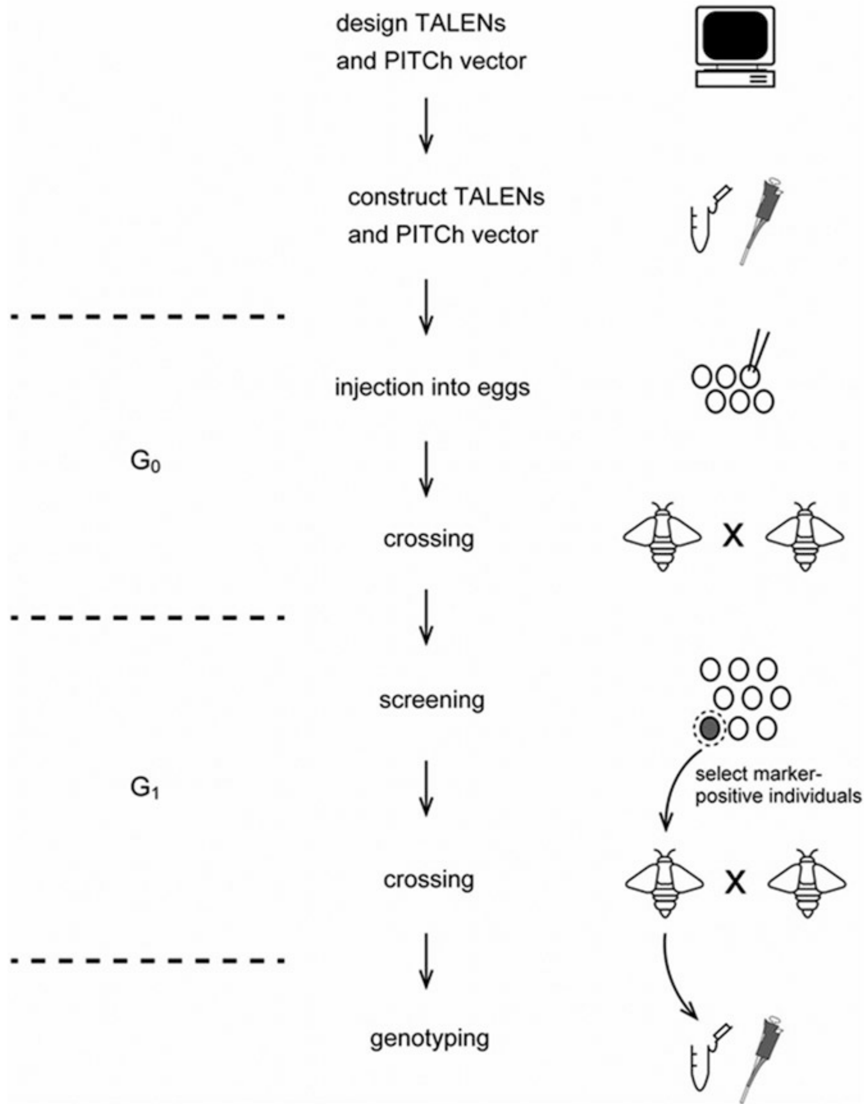

Fig. 3 Diagram of the genome editing experiment for the generation of *B. mori* knock-in individuals

Occasionally, mutations can exist within the primer sequence utilized for the inverse PCR.

5. For colonies with the appropriate plasmid, purify again using a midiprep kit.
6. Extract the plasmid with phenol/chloroform, precipitate with 100% ethanol, wash with 70% ethanol three times, and dry. Suspend in injection buffer.
7. Mix TALENs and the PITCh vector at 125 ng/μl for each TALEN and 500 ng/μl for the PITCh vector.

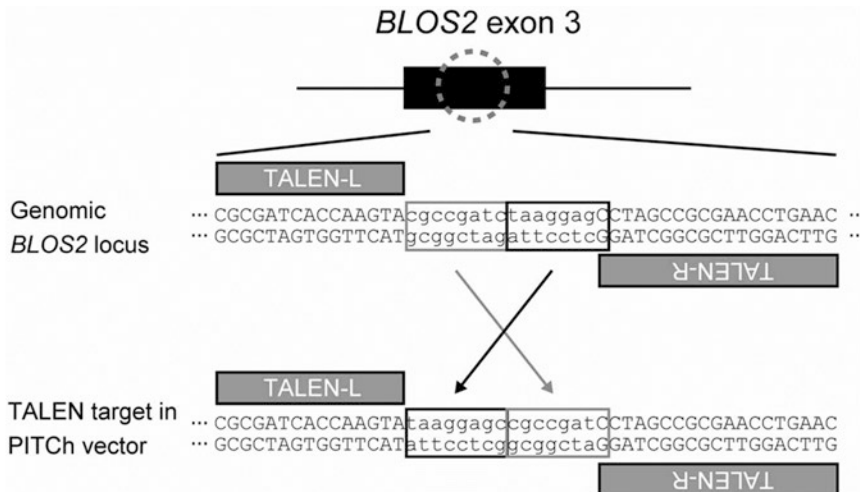

Fig. 4 Design of the microhomology sequence

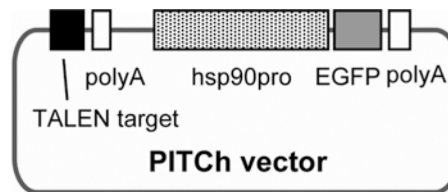

Fig. 5 Architecture of the PITCh vector

8. Microinject the TALEN and PITCh vector into the *w1 pnd* eggs (*see* **Note** 7) and obtain the G1 broods following the method described in Subheadings 3.1, **steps 6–8**.
9. Observe the fluorescence of the G1 embryos. According to the activity of the *hsp90* promoter, the GFP expression can be detected as early as 2–3 days after egg laying [18]. The GFP-positive individuals are possible candidates for knock-in (Fig. 7). Select the GFP-positive individuals and keep rearing (*see* **Note 8**).
10. Cross the GFP-positive individuals with a diapausing strain such as *w-c* and obtain fertile eggs. After crossing extract the genome from each adult.
11. Perform PCR using primers designed against the vector and genome sequence (Fig. 8). Analyze the PCR product using gel electrophoresis (Fig. 9).
12. Verify the sequence of the PCR product and evaluate whether the knock-in has occurred as planned. In the case of *BLOS2*, knock-in into the targeted locus was detected for six individuals (4, 8, 16, 17, 34, and 35; Fig. 9) [14]. Among these individuals, precise knock-in occurred for both the 5′ and 3′ junctions

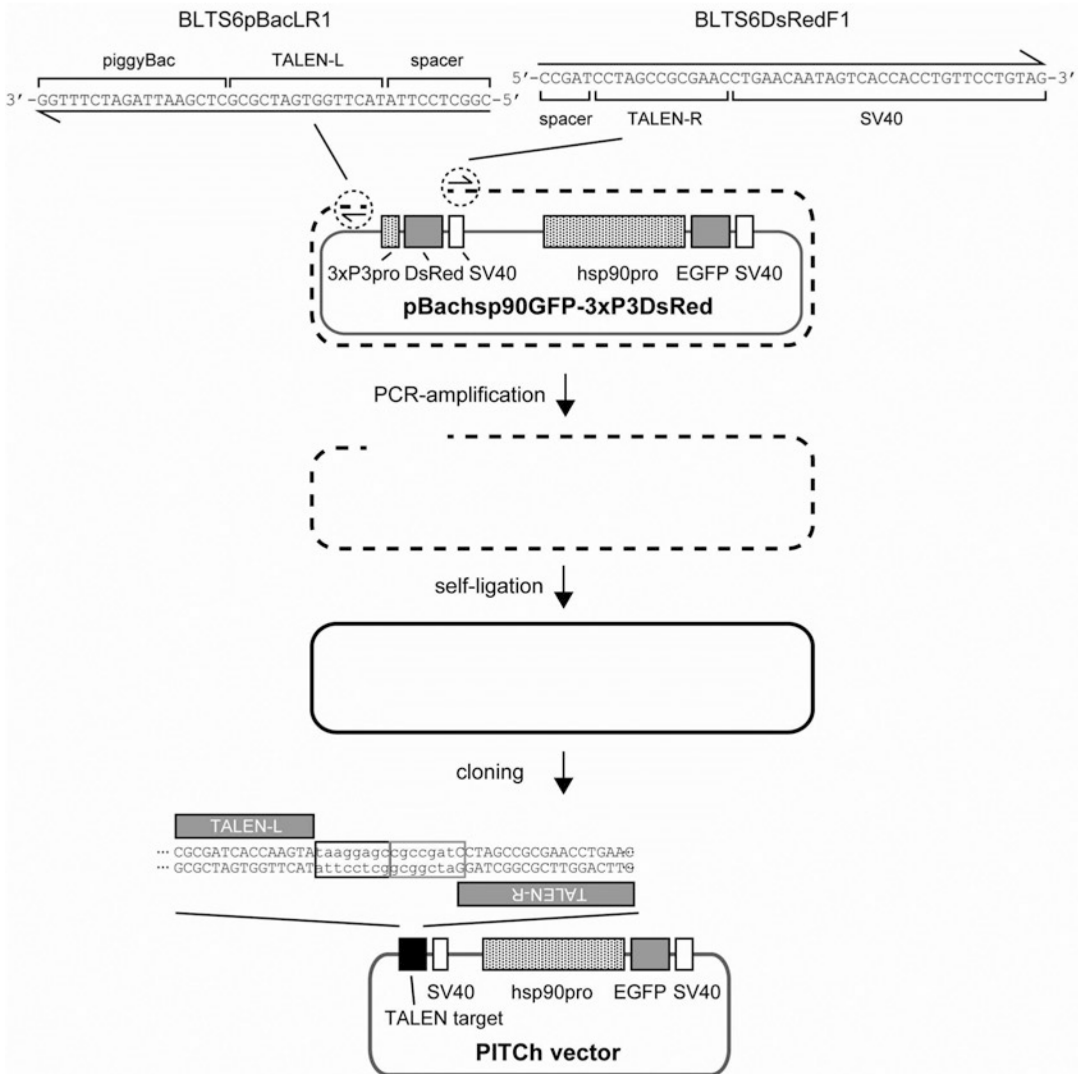

Fig. 6 Diagram of PITCh vector construction via inverse-PCR. The TALEN target site can be inserted into the pBachsp90GFP-3×P3DsRed plasmid using primers with the shown sequences

for 4, 8, 16, and 17, whereas for 34 and 35, insertion of extra nucleotides (around 1.7 kb) was present only in the 3′ junction (Fig. 9) [14].

4 Notes

1. The silkworm *hsp90* promoter exhibits strong and ubiquitous transcriptional activation activity in various developmental stages [18] and thus utilization of this promoter permits easy knock-in detection. Besides this promoter, the 3×P3 [26], *ie1*

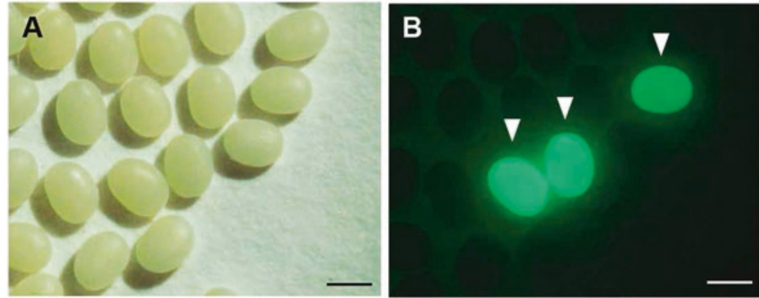

Fig. 7 Microscopy images of G1 embryos for the knock-in experiment. (**a**) Bright field. (**b**) Fluorescence. The *arrowheads* indicate GFP-positive individuals. *Scale bar*, 1 mm

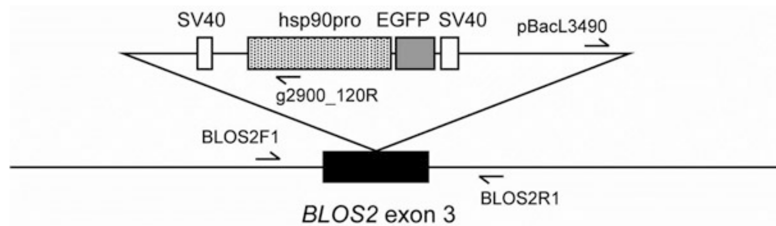

Fig. 8 Primer position for knock-in detection

[27], *A3* [1], and *fibroin light chain* promoters [28] are available in the silkworm. These promoters are connected with marker genes such as the fluorescent protein genes EGFP, DsRed and CFP, or genes related to larval coloration such as kynurenine 3-mono oxygenase (KMO) [29] and arylalkylamine-*N*-acetyl transferase (aaNAT1) [30] for use as knock-in reporters.

2. We recommend the use of a non-diapausing strain for injection, although the silkworm genome sequence has been determined for the diapausing strain *daizo* [2]. The silkworm is a polymorphic organism and it is critically important to determine the target site sequence in the strain actually being utilized for the microinjection (not only for the ZFN experiments but also for TALEN and CRISPR/Cas9). In our laboratory we usually use *w1 pnd*, a non-diapausing strain with a white egg color mutation. This permits the easy detection of transgenic marker gene expression and is therefore suitable for the knock-in experiment.
3. We could obtain the *BLOS2* knock-out strains from at least 5 broods by injecting 480 embryos [3].
4. TALEN efficiency is very high and in most cases injection into ~100 embryos is sufficient for knock-out strain generation [6].

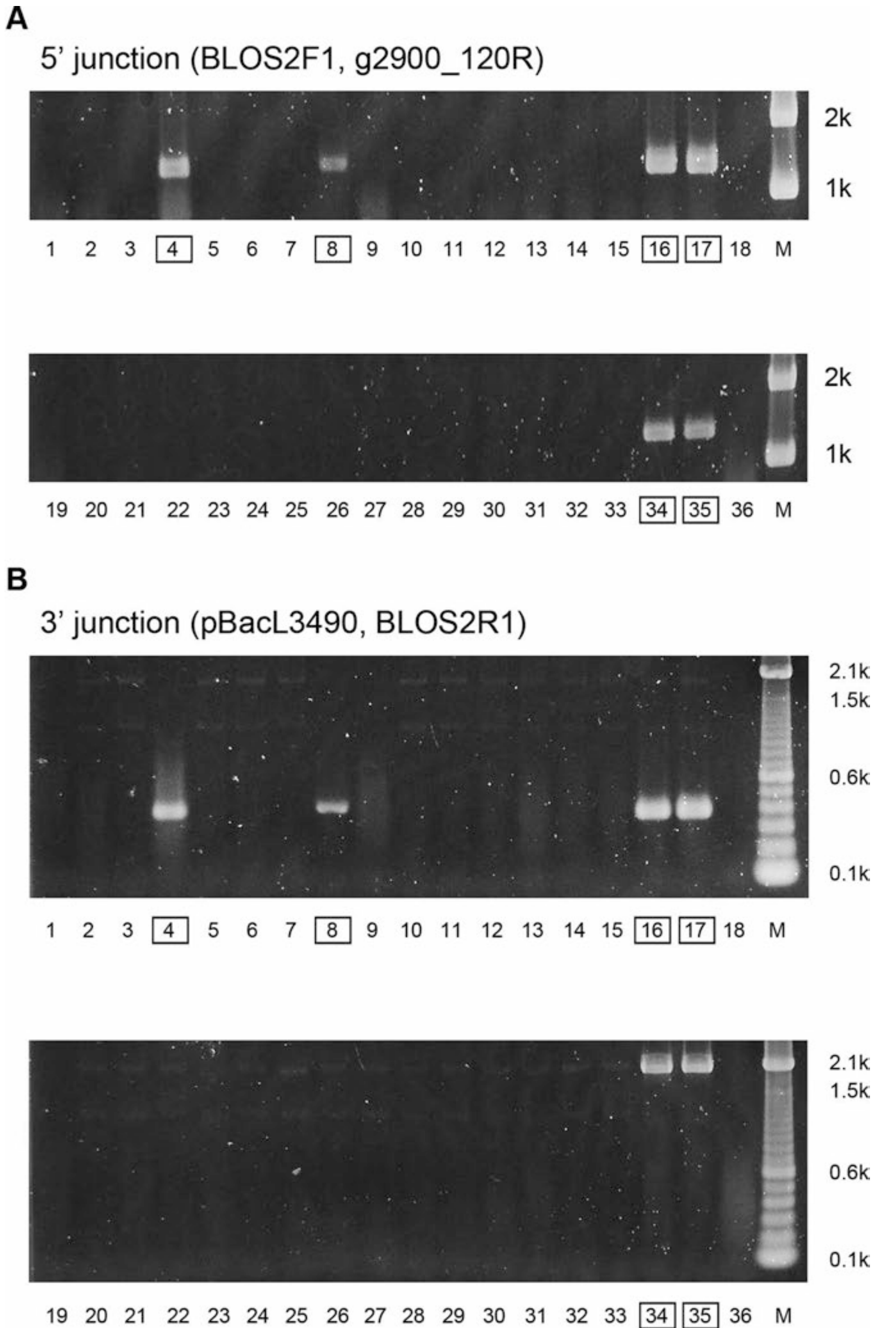

Fig. 9 Genotyping of *BLOS2* knock-in individuals. The PCR was carried out using the primers shown in Fig. 8, and the product was analyzed by gel electrophoresis. (**a**) is the 5′ junction and (**b**) is the 3′ junction. A total of 36 investigated individuals are shown here

5. In the case of CRISPR/Cas9, microinjection into ~200 embryos is sufficient for knock-out strain generation if the synthesized sgRNA is functional [12].
6. Preferably polymerases with high fidelity should be used.
7. We could obtain four individuals that showed precise integration for both the 5′ and 3′ junctions by injecting ~200 embryos [14].
8. All the GFP-positive individuals should be reared because in many cases the donor vector is not integrated into the targeted genomic locus [14].

Acknowledgment

This work was supported by grants-in-aid from the Ministry of Agriculture, Forestry, and Fisheries.

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Chapter 18

Genome Editing in the Cricket, *Gryllus bimaculatus*

Takahito Watanabe, Sumihare Noji, and Taro Mito

Abstract

Hemimetabolous, or incompletely metamorphosing, insects are phylogenetically basal and include many beneficial and deleterious species. The cricket, *Gryllus bimaculatus*, is an emerging model for hemimetabolous insects, based on the success of RNA interference (RNAi)-based gene-functional analyses and transgenic technology. Taking advantage of genome editing technologies in this species would greatly promote functional genomics studies. Genome editing has proven to be an effective method for site-specific genome manipulation in various species. Here, we describe a protocol for genome editing including gene knockout and gene knockin in *G. bimaculatus* for functional genomics studies.

Key words Cricket, Knockout, NHEJ-mediated knockin

1 Introduction

The two-spotted cricket, *Gryllus bimaculatus* (Orthoptera: Gryllidae), is one of the most abundant cricket species. It can be easily bred in the laboratory and has been widely used to study insect physiology and neurobiology [1]. This species was recently established as a model system for studies on molecular mechanisms of development and regeneration. To analyze gene functions during embryogenesis and regeneration in *G. bimaculatus*, the RNA interference (RNAi) technique can be used [2, 3]. At any developmental stages, RNAi were effectively in few weeks after injecting double-stranded RNA. Furthermore, a technique to generate transgeniccrickets with the *piggyBac* transposase has recently been established [4, 5]. *eGFP*-expressing transgenic cricket lines allowed us to perform live imaging of fluorescently labeled embryonic cells and nuclei. The generation of transgenic crickets would also be useful for analyzing the functions of genes and *cis*-regulatory elements.

Although the above RNAi and transgenic systems are effective for analyzing gene function, each system has some shortcomings. For example, inhibition of gene function by RNAi is easily and

Izuho Hatada (ed.), *Genome Editing in Animals: Methods and Protocols*, Methods in Molecular Biology, vol. 1630,
DOI 10.1007/978-1-4939-7128-2_18, © Springer Science+Business Media LLC 2017

efficiently but not completely, because some RNA remains intact. In the case of *piggyBac*-based transgenesis, transgenes are randomly integrated into the genome, meaning that one cannot control either the copy number or the genomic locus of a transgene. Consequently, for more sophisticated functional analyses of individual genomic region, a technique for modifying the cricket genome at a specific site is needed.

Genome editing tools such as ZFNs, TALENs, and CRISPR/Cas9 system can be used to induce targeted DNA double-strand breaks (DSBs) into specific regions of the genome [6–8]. DSBs induced by any of these genome editing tools activate the DNA damage response [9]. DSBs can be repaired by nonhomologous end joining (NHEJ), in which short random insertions or deletions are generated at the cleavage site [10], or by homology directed repair (HDR) in which gene knockin results in a sequence replacement, if a modified template is used [11]. HDR can be much more precise, since DSBs are repaired through a specific homologous template. Although gene knockin is indispensable for further detailed analysis of genome function, establishment of the knock-in method through an HDR pathway is far more difficult than gene knockout, because of the general low efficiency of HDR in eukaryotes [12].

Recently, efficient methods for gene knockin through NHEJ have been developed in zebrafish [13]. In this method, both genome and donor vectors are cleaved in vivo. Then some percentage of the time, the cleaved donor vector is ligated to the genomic cut site through NHEJ. This method has advantages in that the knock-in efficiency is greater compared to HDR-mediated knockin.

In the cricket, we described examples for generating knock-out animals using ZFNs and TALENs [14–16]. We have also demonstrated gene knockouts using the CRISPR/Cas9 system and explored the role of dopamine in learning and memory in the cricket [17]. In addition, performing functional analysis of developmental genes was succeeded by generating knock-out lines using the CRISPR/Cas9 system. Recently, we also succeeded in generating knock-in crickets by utilizing a homology-independent gene knock-in method [18]. In this chapter, we provide the protocols for using ZFNs, TALENs and CRISPR/Cas9 system to generate knock-out and knock-in cricket lines.

1.1 Generating Knock-Out Cricket Lines

To facilitate the production of homozygous knock-out animals via genome editing, we examined an effective strategy for selecting animals with a mutant allele, independent of their genetic tractability or their phenotypic characteristics. We reported the first research in hemimetabolous insects to show the effectiveness of ZFNs and TALENs for generating knock-out animals [14]. ZFNs and TALENs were designed for targeting to the *G. bimaculatus laccase2* (*Gb'lac2*) locus. This gene was selected based on

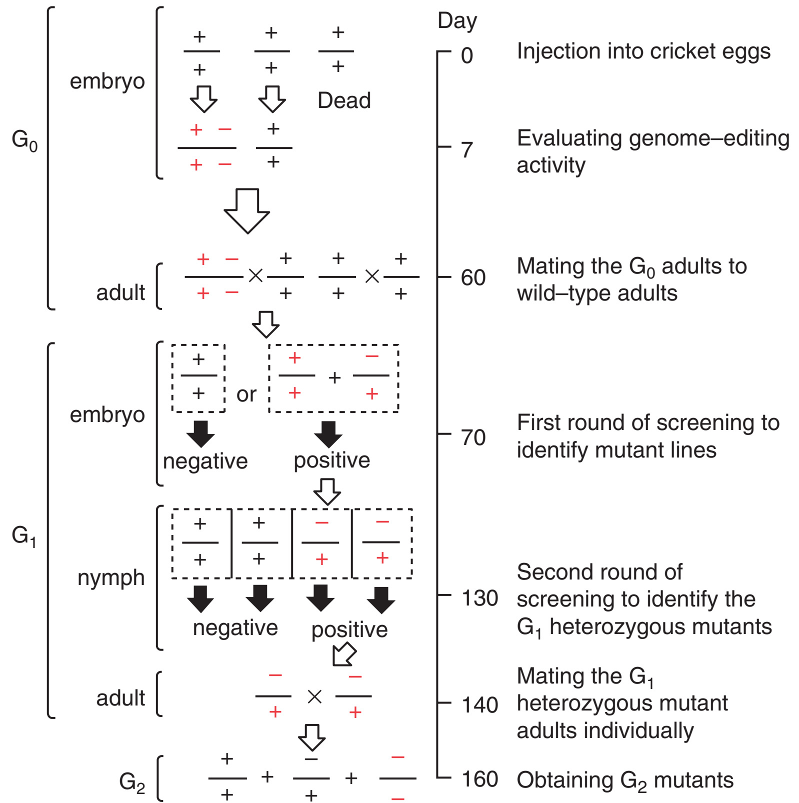

Fig. 1 Summary of the scheme to isolate homozygous mutants in the cricket. Evaluating genome editing activity by the SURVEYOR nuclease assay in 10 mutagenized G_0 embryos (day 7). Mutagenized G_0 adults are crossed to wild-type adults (day 60). G_1 embryos are checked for heterozygous mutations in a first round of screening (day 70). Positive G_1 individuals are developed into final instar nymphs and subjected to a second round of screening (day 130). Positive G_1 adults are crossed by strain to obtain homozygous mutants in the G_2 generation (day 140). Adapted with modification from ref. 15

previous studies, in which RNAi targeting of *Gb'lac2* in the nymphal stages of the cricket resulted in loss of cuticle tanning after molting.

After in vitro transcription, ZFN or TALEN mRNAs were injected into cricket eggs just after fertilization. To generate knock-out crickets, we designed a two-step screening method based on mutant detection assays using SURVEYOR nuclease (Fig. 1). Mutant lines transmitted from the founder crickets were detected in several batches of G1 eggs in the first round of screening. Heterozygous offspring were selected with mutation detection assays in the second round of screening. Subsequent sibling crosses created homozygous knock-out crickets exhibiting the cuticle tanning defect phenotype. This selection strategy independent of mutant phenotypic characteristics can be used to generate knock-out crickets using the CRISPR/Cas9 system [17].

1.2 Generating Knock-In Cricket Lines

For NHEJ-mediated gene knockin, the CRISPR/Cas9 system was used because the activity of DNA digesting was remarkably higher than ZFNs and TALENs. This knock-in method is consisted of four components, a donor vector contained gRNA target sequence upstream of gene of interest, two gRNAs cleaved target region of genomic DNA and donor vector, and Cas9 mRNA. We generated a donor vector containing gRNA target sequence from the DsRed gene, which does not exist in the cricket genome, and targeted this sequence upstream of autonomous GFP expression cassette (Fig. 2) [18]. Cas9 mRNA, gRNAs targeted against the genome sequence and a donor vector were co-injected into fertilized eggs. To check for the presence of the knock-in event, inside-out PCR was performed at 7 days post-injection using specific primers for each 5′ and 3′ junction point (Fig. 2). For some genomic loci, we were able to isolate knock-in lines and observe GFP expression in embryos (unpublished).

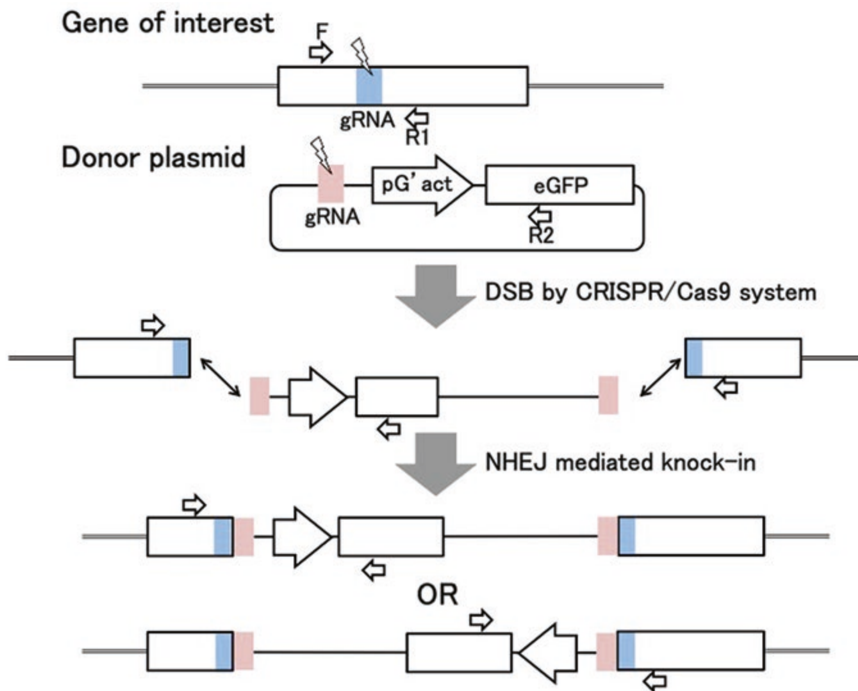

Fig. 2 Outline of NHEJ-mediated knock-in method**.** The donor plasmid contains target site of gRNA (sequence from DsRed) and expression cassette (eGFP derived *Gryllus* actin promoter). After introducing DSBs for target genome and donor plasmid, break points are ligated between genome and donor plasmid via NHEJ repair pathway. For the detection of a knock-in event, inside-out PCR are performed using three primers, two primers for genome (F and R1), and one primer for donor plasmid (R2) because of forward and reverse directions

2 Materials

2.1 Maintenance of Wild-Type Crickets

1. Artificial fish food (e.g., TetraFin and TetraGold (Tetra)).
2. Paper towels for cover of dishes to collect eggs and for water tubes.
3. Insect cages.

2.2 Preparation of Plasmid for ZFNs/TALENs/Cas9 mRNA and gRNAs In Vitro Transcription

1. Plasmid purification kit.
2. TE buffer: 10 mM Tris–HCl, 1 mM EDTA.
3. Phenol:chloroform:isoamyl alcohol (25:24:1, v/v).
4. Restriction enzyme.
5. T4 DNA ligase.

2.3 In Vitro Transcription of ZFNs/TALENs/Cas9 mRNA and gRNA

1. mMessage mMachine T7 Ultra Kit (AM1345, Thermo Fisher Scientific).
2. MEGAshortscript T7 Transcription Kit (AM1354, Thermo Fisher Scientific).
3. Injection solution: 1.4 mM NaCl, 0.07 mM Na_2HPO_4, 0.03 mM KH_2PO_4, 4 mM KCl.

2.4 Injection of ZFNs, TALENs, or CRISPR/Cas9 System Cocktail into Cricket Eggs

1. Glass capillary with filament: ID, 0.6 mm; OD, 1 mm.
2. Micropipette puller (P-1000IVF, Sutter Instruments).
3. Micropipette grinder (EG-44, Narishige).
4. Pneumatic microinjector (IM-300, Narishige, Fig. 3c).

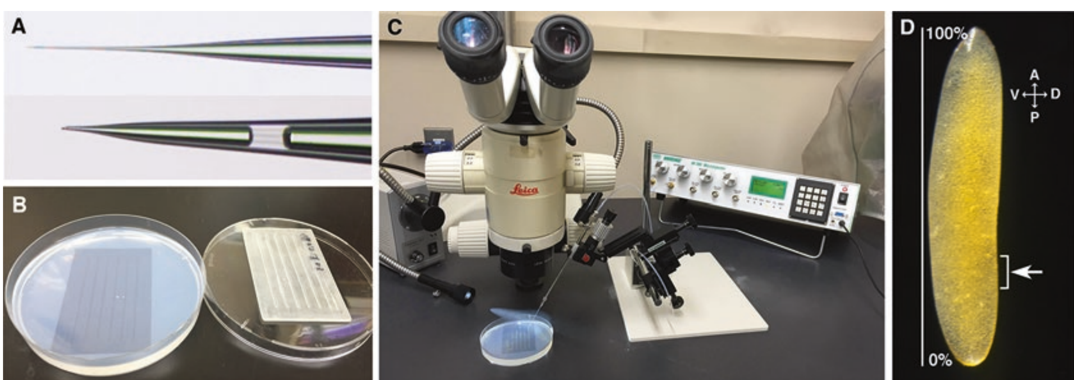

Fig. 3 Injection system for cricket eggs. (**a**) Needle for egg injection. *Upper panel* indicates needle before grind, and lower indicates after grind. (**b**) The egg trough and mold. (**c**) Injection apparatus for cricket eggs. This apparatus consists of the microscope and microinjector and compressor (NARISHIGE IM300 MICROINJECTOR). (**d**) Picture of a cricket egg. Anterior end is up, dorsal side is right. The injection needle is inserted from the dorsal side of the egg near the posterior end (20–25% of the egg length from the posterior end; *arrow*). Adapted with modification from ref. 18

5. Stereoscopic microscope.
6. Plastic dish.
7. Plastic mold: 0.7 mm wide × 0.5 mm deep.
8. Agar for injection.

2.5 Generation of Homozygous Knock-Out Cricket Lines via Two Selection Stages

1. Genomic DNA purification kit.
2. Taq DNA polymerase.
3. Primer pairs.
4. Thermal cycler.
5. SURVEYOR Mutation Detection Kit (706020, Integrated DNA Technologies).
6. Agar for electrophoresis.

2.6 Construction of the Donor Vector for Knock-In Experiment and Checking the Knock-In Event

1. Plasmid purification kit.
2. TE buffer: 10 mM Tris–HCl, 1 mM EDTA.
3. Phenol:chloroform:isoamyl alcohol (25:24:1, v/v).
4. Restriction enzyme.
5. T4 DNA ligase.
6. mMessage mMachine T7 Ultra Kit (AM1345, Thermo Fisher Scientific).
7. MEGAshortscript T7 Transcription Kit (AM1354, Thermo Fisher Scientific).
8. Injection solution: 1.4 mM NaCl, 0.07 mM Na_2HPO_4, 0.03 mM KH_2PO_4, 4 mM KCl.
9. Glass capillary with filament: ID, 0.6 mm; OD, 1 mm.
10. Micropipette puller (P-1000IVF, Sutter Instruments).
11. Micropipette grinder (EG-44, Narishige).
12. Pneumatic microinjector (IM-300, Narishige).
13. Stereoscopic microscope.
14. Plastic dish.
15. Plastic mold: 0.7 mm wide × 0.5 mm deep.
16. Agar for injection.
17. Genomic DNA purification kit.
18. Taq DNA polymerase.
19. Primer pairs.
20. Thermal cycler.
21. Agar for electrophoresis.

3 Methods

3.1 Maintenance of Wild-Type Crickets

Rear nymphs and adults at 29 °C and 50% humidity under a 10-h light: 14-h dark photoperiod. Feed nymphs and adults with artificial fish food (*see* **Note 1**). Under these conditions, the generation time of the cricket is about 2 months.

Maintain first to third instar nymphs in plastic insect cases (*see* **Note 2**), and fourth instar nymphs to adults in plastic containers (e.g., cloth cases) (*see* **Note 3**). Each plastic insect case should contain one 50 ml plastic tube plugged with a paper towel for supplying water and several crumpled papers as a shelter. Each plastic container for rearing nymphs and adults should contain a pile of moist and folded paper towels (~1.5 cm in height) in a plastic dish for supplying water (*see* **Note 4**) and for adult females to lay eggs during the dark period.

Renew the paper towels for collecting eggs each day, and place the collected eggs under the same conditions as used for rearing adult crickets with closing the lid of plastic dish. After the first instar nymphs hatch from the eggs (~13th day of incubation), transfer the nymphs to the rearing cases.

3.2 Preparation of Plasmid for ZFNs/TALENs/Cas9 mRNA and gRNAs In Vitro Transcription

For obtaining plasmids for ZFNs/TALENs/Cas9 mRNA and gRNAs in vitro transcription, *see* Chapters 2, 3, and 4. We previously used self-constructed ZFNs and the Custom TALEN™ Service offered by Cellectis Bioresearch for these plasmids [14]. In *Gryllus*, TALEN pairs constructed by Transposagen Biopharmaceuticals and Platinum Gate Assembly also induced mutations into the target genomic locus with high efficiency [15, 16].

For the CRISPR/Cas9 system, we use the pMLM3613 plasmid containing wild-type *Streptococcus pyrogenes* Cas9 (*SpCas9*) under the T7 promoter and without codon optimizing [19] (*see* **Note 5**). For in vitro transcription of designed gRNAs, we usually use the pDR274 plasmid, obtained from Addgene, containing an empty gRNA scaffold under control of the T7 promoter [19].

To design efficient gRNAs, we used the UI/UX of the "focas" website (http://focas.ayanel.com/) created by Ayanel & Co (Tokyo) [20]. This website was developed using the standalone softwares, CasOT [21], and the slight modification of on_target_score_calculator.py [22]. In *Gryllus*, CasOT for avoiding gRNA with high off-target activity could not be used because *Gryllus* genome project is uncompleted and privately (*see* **Note 6**). To clone designed target sequences into pDR274, each target must have a GG at the 5′ end, because transcription is started from the GG of the T7 promoter 3′ end. Protocol of cloning designed target sequences into pDR274 is described below:

1. For cloning designed target sequence into pDR274, prepare two oligo DNAs.
   Forward: TA-GGNNNNNNNNNNNNNNNNNN
   Reverse: AAAC-NNNNNNNNNNNNNNNNNN
2. Mix 1 μl of each 100 μM oligo DNAs and 8 μl water, and anneal using decreasing temperature steps as follows:

   95 °C, 1 min, (−2.0 °C/s); 85 °C, 10 s, (−0.3 °C/s); 25 °C, Hold.
3. Dilute annealed oligo DNA to 1 μM.
4. Ligate pDR274 and annealed oligo DNA using the Golden Gate Cloning method with *Bsa*I and T4 DNA Ligase.

10× T4 DNA Ligase buffer	2 μl
100 ng/μl pDR274 plasmid	1 μl
Annealed oligo DNA	3 μl
*Bsa*I	1 μl
T4 DNA Ligase	1 μl
ddH_2O	up to 20 μl

   [37 °C, 5 min/16 °C, 10 min] × 10 cycles; 12 °C, Hold.
5. Add 0.5 μl BsaI to the Golden Gate Cloning mix for complete digestion of pDR274.

Golden Gate cloning mix	20 μl
*Bsa*I	0.5 μl

   50 °C, 60 min; 80 °C, 15 min; 12 °C, Hold.
6. Transform competent cells using 10 μl of the Golden Gate Cloning mix.
7. After plasmid preparation, check the cloned oligo DNA sequence.

3.3 In Vitro Transcription of ZFNs/TALENs/Cas9 mRNA and gRNA

1. Purify plasmids for the Cas9 mRNA and gRNA using a plasmid purification kit.
2. Suspend plasmids in 50 μl of TE buffer.
3. Linearize the plasmids for mRNA synthesis with a restriction enzyme (*Pme*I for pMLM3613, *Dra*I for pDR274).
4. Extract linearized DNAs using phenol:chloroform:isoamyl alcohol.
5. Perform ethanol precipitation, and resuspend DNA in TE buffer at 0.5 μg/μl.

6. Synthesize ZFNs/TALENs/Cas9 mRNA with the mMessage mMachine T7 Ultra Kit and gRNA with MEGAshortscript T7 Transcription Kit, according to the manufacturer's protocol.
7. Resuspend RNA pellets in the injection solution (*see* **Note 7**), and adjust to a final concentration of 2 μg/μl.
8. Aliquot the solution into a 1.5 ml tube, and store at −80 °C.

3.4 Injection of ZFNs, TALENs, or CRISPR/Cas9 System Cocktail into Cricket Eggs

1. Needle preparation;
   (a) Needles are pulled in the micropipette puller using the following program:
      - HEAT 816, PULL 0, SPEED 15, TIME 250, PRESSURE 500.
      - HEAT 816, PULL 0, SPEED 15, TIME 250, PRESSURE 500.
      - HEAT 816, PULL 0, SPEED 15, TIME 250, PRESSURE 500.
      - HEAT 816, PULL 80, SPEED 15, TIME 200, PRESSURE 0.

   (b) Bevel the tip to 20 degrees using a micropipette grinder with dropping water (Fig. 3a) (*see* **Note 8**).
2. Preparation of egg trough.
   (a) Pour approximately 20 ml of hot 1.0% agarose into a 9 cm Petri dish.
   (b) Set the mold groove side down, tapping to eliminate bubbles. Grooves on mold are 0.7 mm wide × 0.5 mm deep (Fig. 3b).
   (c) When solidified, remove the mold and wrap dish in paraflim, and store at 4 °C.
3. Make injection solution; Mix ZFNs, TALENs, or CRISPR/Cas9 system for the injection solution (final conc. 1 μg/μl for ZFNs/TALENs/Cas9 mRNA and 0.5 μg/μl for gRNA, basically) (*see* **Note 9**).
4. Make and place an egg dish to collect eggs in the cage with the crickets. Moisten the towels generously with water (*see* **Note 10**).
5. Allow females to oviposit eggs for 1 h (*see* **Note 11**).
6. Remove the egg dish and place in a 28 °C incubator for 1 h (*see* **Note 12**).
7. Transfer eggs into troughs filled with 20 ml of 1× PBS with 50 U/ml penicillin and 50 μg/ml streptomycin. Each trough can hold ~35 embryos.
8. Transfer Petri dish to a dissecting microscope equipped with fluorescence.

9. Fill the injection needle from the blunt end with the injection solution.
10. Mount needle onto a micromanipulator.
11. Connect to a pneumatic microinjector.
12. Set injection pressure to 5–10 psi and injection time to 0.1 s.
13. Inject the posterior end of the egg (20–25% of egg length from the posterior end; Fig. 3d) with approximately 4 nl of solution.
14. Use forceps to slide the egg off the needle.
15. Using a P1000 micropipette, transfer injected eggs to a 6 cm plastic dish filled with PBS$^+$ buffer.

3.5 Generation of Homozygous Knock-Out Cricket Lines via Two Selection Stages (Fig. 1)

1. On day 0, inject RNAs into the posterior end of ~150 eggs.
2. On day 7, evaluate the ZFNs, TALENs, or CRISPR/Cas9 system activity using ten eggs (Fig. 1, Day 7).
   (a) Select ten injected eggs, and extract genomic DNA from each egg individually, using a genomic DNA purification kit.
   (b) Amplify the 150- to 300-bp fragment, including the gRNA target site, using a 20 μl PCR reaction (Fig. 4a) (*see* **Note 13**). Divide the PCR products into halves for SURVEYOR nuclease treatment or nontreatment.
   (c) To allow complementary but mismatched strands to anneal, incubate 10 μl of the PCR products at 95 °C for 5

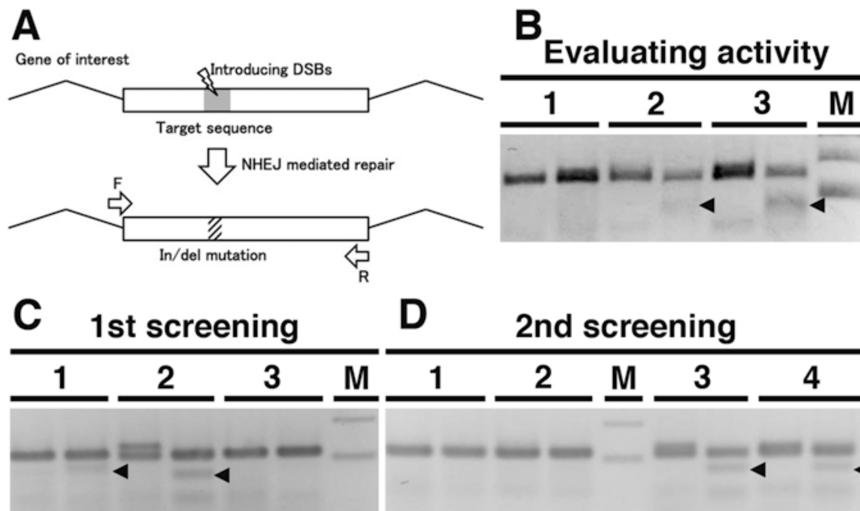

Fig. 4 Isolation of homozygous mutants with two-step screenings. (**a**) Illustration of introducing in/dels after repair of DSBs. Primers "F" and "R" are designed to bind genomic DNA for amplifying 150–300 bp of the PCR product. (**b–d**) Agarose gel pictures of the SURVEYOR nuclease assays. Left lane of each sample is untreated DNA, and right lane is DNA treated with SURVEYOR nuclease. The expected band cleaved by SURVEYOR nuclease is indicated by *arrowhead*. (**b**) Evaluating activity on day 7. (**c**) First round of screening on day 70. (**d**) Second round of screening on day 130. M; marker DNA. (**b–d**) Adapted from ref. 15

min, ramp the temperature from 95 to 85 °C at −2 °C/s, and ramp the temperature from 85 to 25 °C at −0.1 °C/s.

(d) Add 0.5 μl Nuclease S and 0.5 μl Enhancer S to the reannealed PCR products.

(e) Incubate the mixture at 42 °C for 45 min (*see* **Note 14**).

(f) Visualize the SURVEYOR nuclease-treated and -untreated products by agarose gel electrophoresis immediately after incubation (Fig. 4b) (*see* **Note 15**).

(g) Calculate the percentage of eggs for which mutations have been introduced into a gRNA target site (*see* **Note 16**).

3. Approximately 70–80% of nymphs will hatch on day 13. This hatching rate will be lower if genes related to embryonic development have been targeted. If the knock-out phenotype is visible (as is the case in *laccase2*), then nymphs will exhibit a partial knock-out phenotype because G_0 nymphs are chimeric insects consisting of genome-edited mutant cells and wild-type cells.
4. From days 14–50, rear the G_0 nymphs until the eighth instar (*see* **Note 17**).
5. 3–7 days after the nymphs become adults (about day 60), mate the G_0 adults to wild-type adults, and collect 200–300 G_1 eggs (Fig. 1).
6. One week after the eggs have been laid (approximately 70 days from the original injection), perform the first round of screening with nuclease, to identify cricket lines containing genome-edited mutants (Fig. 1, Day 70).

(a) Combine 20–25 embryos from each line and extract genomic DNA, using a genomic DNA purification kit (*see* **Note 18**).

(b) Perform SURVEYOR nuclease treatment (Fig. 4c) (*see* Subheading 3.5, **step 2** b–f).

(c) Isolate SURVEYOR nuclease-sensitive (positive) lines, which contain the mutants.

7. Keep the G_1 eggs from the positive lines of the first screening, rearing the hatched nymphs until the eighth instar stage (day 70–120).
8. A few days after the nymphs becoming eighth instar nymphs (about day 120–130), perform a second round of screening with SURVEYOR nuclease to identify the G_1 heterozygous mutants (Fig. 1, day 130).

(a) Separately extract genomic DNA from the T3 leg tips of 24 nymphs (12 males and 12 females) in each line, using a genomic DNA purification kit.

(b) Perform SURVEYOR nuclease treatment for each genomic DNA (Fig. 4d) (*see* Subheading 3.5, **step 2** b–f).

(c) Isolate the SURVEYOR nuclease-sensitive (positive) crickets, which correspond to the G_1 heterozygous mutants.

9. Rear the G_1 heterozygous mutants until the adult stage.
10. At 3–7 days after the G_1 heterozygous mutants become adults (about day 140), mate the G_1 heterozygous mutant adults individually, and collect the G_2 eggs.
11. Based on Mendelian inheritance, G_2 eggs will include homozygous mutants, heterozygous mutants, and wild-types.
12. Genotype the eggs using the SUVEYOR nuclease system or by direct sequencing of the target region.

3.6 Construction of the Donor Vector for Knock-In Experiment and Checking the Knock-In Event

1. Construction of the donor vector: The summary of NHEJ-mediated knockin is shown in Fig. 2. The donor vector includes a gRNA target sequence, which does not exist in the cricket genome (we usually use partial sequence of *DsRed* gene). We constructed the donor vector containing an *eGFP* expression cassette driven by *Gryllus* actin (G'act) promoter.
2. Construct some gRNAs for targeting genomic region and select a gRNA with high activity (*see* **Note 19**).
3. Injection of knock-in cocktail into cricket eggs: Microinject as described in Subheading 3.4. The injection solution contains: 100 ng/μl Cas9 mRNA, 100 ng/μl the donor vector, 50 ng/μl gRNA for the genome target, and 50 ng/μl gRNA for the donor target (*see* **Note 20**).
4. Check the knock-in event with inside-out PCR (Fig. 2).
   (a) Extract genomic DNA from G_0 eggs 7 days after injection.
   (b) Amplify and determine the junction sequence between the integrated donor vector and flanking genome region using donor vector-specific and target genomic region-specific primers.
5. Check for the presence of marker fluorescence in the embryonic body from 5 days after injection onward (*see* **Note 21**).
6. Obtaining knock-in lines.
   (a) Obtain G_1 eggs by crossing G_0 adults and wild type as described in Subheading 3.5, **steps 3–5**.
   (b) Perform inside-out PCR using genomic DNA extracted from batches of 25 G_1 eggs at 7 days after egg-laying for each cross (*see* **Note 22**).
   (c) To identify knock-in individuals, perform an additional round of inside-out PCR using genomic DNA separately extracted from the T3 leg tips of 25 nymphs.

4 Notes

1. Provide flesh feed every three or 4 days because crickets prefer to eat flesh feed. For the first instar nymphs, crushed feed was provided using a coffee mill.
2. The maximum population per one insect case ($15 \times 20 \times 15$ cm^3) was approximately 300 nymphs. Too dense of an insect population promotes cannibalization.
3. First to third instar nymphs are separated by hatching day, and fourth to eighth instar nymphs by week. Maintain adults in plastic containers, separated by the week in which the cricket reaches adulthood.
4. Fresh water in a plastic dish should be provided daily, and the paper towels should be replaced every 3 days.
5. Currently, although plasmids containing Cas9 variants from other bacteria species (e.g., *Staphylococcus aureus*, *Neisseria meningitidis*) are available, cleavage activities of these variants have not been evaluated in the cricket. Cas9 protein is also available from PNA Bio and Thermo Fisher Scientific, and they work well in cricket eggs.
6. Although designed gRNA occasionally exhibit the off-target activity, off-target mutants were eliminated from on-target mutant lines after several crossings focusing on on-target site only.
7. Use the typical Triboleum injection solution. 10× recipe is: 14 mM NaCl, 0.7 mM Na_2HPO_4, 0.3 mM KH_2PO_4, 40 mM KCl. Rhodamine dextran can be added to the injection solution in order to visualize the injection.
8. The opening should be 9–13 μm in diameter. Sharpness and fineness of capillary tip is quite important for viability after injection.
9. To obtain maximal efficiency for each of target, one may need to adjust the concentration of ZFNs/TALENs and the ratio of Cas9 mRNA and gRNA conc.
10. Crickets will produce few eggs in the paper towels if there is too much water. To reduce the amount of water, drop the extra water by flipping the plastic water dish upside down.
11. In order to collect eggs for injections, adult cricket should be deprived of the egg dish for at least 8–10 h.
12. Although this step is not necessary, the mortality rate of the eggs can be reduced experimentally.
13. Although 500- to 1000-bp fragment is generally amplifying for SURVEYOR nuclease assay, we strongly recommend amplifying short fragment, in *Gryllus*. Because many SNPs

exist in *Gryllus* genome as a corollary of an unestablished pure strain.

14. If the digested bands appear smeared during electrophoresis, reduce the reaction time to 15 or 30 min.
15. The digested bands are often broad because the size of the insertions/deletions can vary among the mutagenized sequences. By using gels that have 6 or 7 mm wells, a clearer band pattern may be achieved.
16. To obtain G_1 mutants effectively, we recommend the use of gRNAs which introduce mutations into over 50% eggs.
17. To avoid unintentional mating, divide the G_0 nymphs into males and females once they become eighth instar nymphs, and continue to rear separately.
18. By using a larger number of embryos, the sensitivity of mutation detection may be reduced.
19. The efficiency of knock-in event is depending on activity of gRNA for genomic target. Therefore, we strongly recommend that a gRNA is selected cautiously. For checking gRNAs activity, SURVEYOR nuclease assay is used as described in Subheading 3.5, **step 2**.
20. We recommend the number of injected egg is about 200–300, because the efficiency of knock-in event is significantly low compared to knock-out event. To obtain maximal knock-in efficiency for each of the targets, one may need to adjust the ratio of the donor vector and Cas9 mRNA and gRNAs conc.
21. Because activity of our G'act promoter would depend on integrated loci, the marker expression may not be detectable as fluorescence.
22. If fluorescence is detectable in embryos, a positive line can be identified without inside-out PCR.

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Chapter 19

Genome Editing of the Ascidian *Ciona intestinalis* with TALE Nuclease

Yasunori Sasakura, Keita Yoshida, and Nicholas Treen

Abstract

The ascidian *Ciona intestinalis* is an important model animal for studying developmental mechanisms for constructing the chordate body. Although molecular and embryological techniques for manipulating *Ciona* genes were developed a long time ago, recent achievements of genome editing in this animal have innovated functional analyses of genes in *Ciona*. Particularly, knockout of genes in the G0 generation coupled with tissue-specific expression of TALENs enables us to rapidly address gene functions that were difficult using previous methods.

Key words Ascidian, *Ciona intestinalis*, Genome editing, TALEN, Knockout, Electroporation

1 Introduction

The ascidians are a major group of the subphylum Tunicata of the phylum Chordata (*see* **Note 1**) [1]. The phylogenetic position suggests that ascidians share a basic body plan with vertebrates. Indeed, ascidians possess chordate-specific characteristics such as the notochord, dorsal neural tube, pharyngeal gills, the endostyle/thyroid gland, and so on. The similarity between vertebrates and ascidians is best represented by the tadpole shape of ascidian larvae (Fig. 1). In addition to that, ascidians generally have very simple bodies compared to vertebrates. The tadpole larvae of ascidians consist of countable numbers of cells, and their cell lineages during embryogenesis have been described in detail [2]. These features enable us to observe cellular and molecular mechanisms for constructing chordate-specific structures [3]. Among ascidians, *Ciona intestinalis* is the representative species by several reasons. First, the genome sequence of this ascidian became public more than a decade ago [4]. Accompanying it, information of genes encoded in the genome were described in detail, including exon-intron structures, expressed sequence tags, and full-length cDNA collections [5, 6]. This information facilitates functional analyses of genes by skipping laborious

Izuho Hatada (ed.), *Genome Editing in Animals: Methods and Protocols*, Methods in Molecular Biology, vol. 1630, DOI 10.1007/978-1-4939-7128-2_19, © Springer Science+Business Media LLC 2017

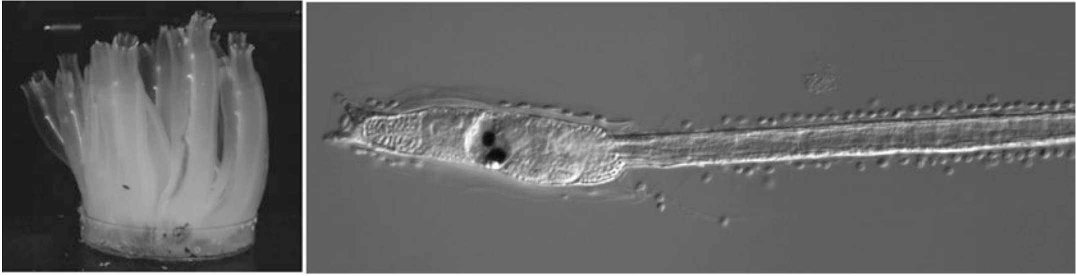

Fig. 1 The ascidian *Ciona intestinalis*. *Left* is adults cultivated by the National BioResource Project Japan. Several animals are shown. The diameter of the petri dish at the bottom is 9 cm. *Right* is a tadpole larva of *Ciona intestinalis*. A posterior portion of the tail is omitted from the panel. The body length of a larva is approximately 1 mm long

cloning processes. In *Ciona*, molecular techniques for examining gene functions have been well established. Microinjections of exogenous nucleic acids into cells enable us to knock down genes by introducing antisense morpholino oligonucleotides that specifically disrupt translation of target genes [7, 8]. Electroporation into fertilized eggs of plasmid DNAs that contain the fusion of a *cis* element of a tissue-specific gene and a reporter gene can label tissues, cells, and subcellular components in living embryos to visualize their behaviors during development [9, 10]. Electroporation is a time-saving method compared to microinjection and is easy to carry out. The ease of this technique and the compact genome of *Ciona* make it possible to analyze transcriptional regulation of genes in a short time period [11]. *Ciona intestinalis* has relatively short generation time and they can be cultured in laboratories [12], indicating that genetic approaches are possible in *Ciona*. Indeed, transgenic and mutant lines of *Ciona* have been created with transposon-based transgenesis [13–15]. The availability of genetics for *Ciona* is exceptional in marine invertebrates, because inland culturing of marine invertebrates is usually difficult. Advances in our understandings about developmental mechanisms of *Ciona* have been provided with these developmental and genetic techniques.

Although *Ciona intestinalis* is a special marine invertebrate in which sophisticated techniques of molecular and genetic biology are available, knockout of genes, a best method for addressing gene functions, was not established for a long time. Recently, the so called genome editing techniques brought revolution in biology since the techniques have provided easy knock-out methods in many animals (e.g., [16–18]. In *Ciona*, targeted knockouts of genes in its genome were reported with zinc finger nuclease [19], TALEN [20], and CRISPR/Cas9 [21, 22]. Among them, we favor TALEN-based knockouts because of their reproducibly high mutagenesis rates. In this chapter, we summarize TALEN-based gene knockouts in *Ciona* and its application for conditional knockouts.

2 Materials

2.1 Animals

Wild-type *Ciona intestinalis* can be collected from quiet seaside locations such as fishery harbors. In Japan, the delivery system of wild-type animals is conducted by the National BioResource Project (NBRP). The project cultivates wild-type animals almost throughout the year (Fig. 1), and researchers can order animals by its web-based ordering system (the URL is http://w-ciona.lab.nig.ac.jp/cgi-bin/ghost_order_top.cgi).

2.2 TALENs

The ways to assemble TALENs are described in other chapters. We recommend using Platinum TALENs for knockouts in *Ciona* [23], because TALENs generated with this have a high probability of producing a good mutation rate. Our rough estimation suggests that about 70% of constructed TALENs have mutation rates sufficient for functional analyses of genes in the G0 generation and germline mutagenesis. The TALEN repeat arrays can be assembled in vectors that are compatible for expression systems in *Ciona* (Fig. 2) [20]. Vector information is available in our website at http://marinebio.nbrp.jp/ciona/forwardToKnockOutAction.do.

2.3 Reagents

1. Dechorionation solution: 1% sodium thioglycolate and 0.05–0.1% actinase E (a kind of protease) in seawater. Store at 4 °C. Long-term storage is not recommended.
2. 2 M NaOH.
3. Mannitol-seawater solution: 1:9 dilution of seawater and 0.77 M mannitol.
4. Wizard genomic DNA purification kit (Promega).
5. Surveyor nucleases such as Cel-I: We use Surveyor nuclease kit (706025 of Transgenomics).
6. 15% polyacrylamide gel in 1× TAE.
7. In-Fusion cloning kit (Clontech).
8. *Xho*I.
9. MegaScript T3 kit (Ambion) .
10. Cap structure analog (S1404 of New England Biolabs) .
11. Poly A tailing kit (Ambion).
12. Chloroform: 49:1 mixture of chloroform and isoamyl alcohol.
13. Phenol/chloroform: 1:1 mixture of TE-saturated Phenol and Chloroform/isoamyl alcohol.
14. 70% ethanol. Prepare with RNase-free H_2O for use with RNA.
15. RNase-free H_2O.
16. 100% isopropanol.

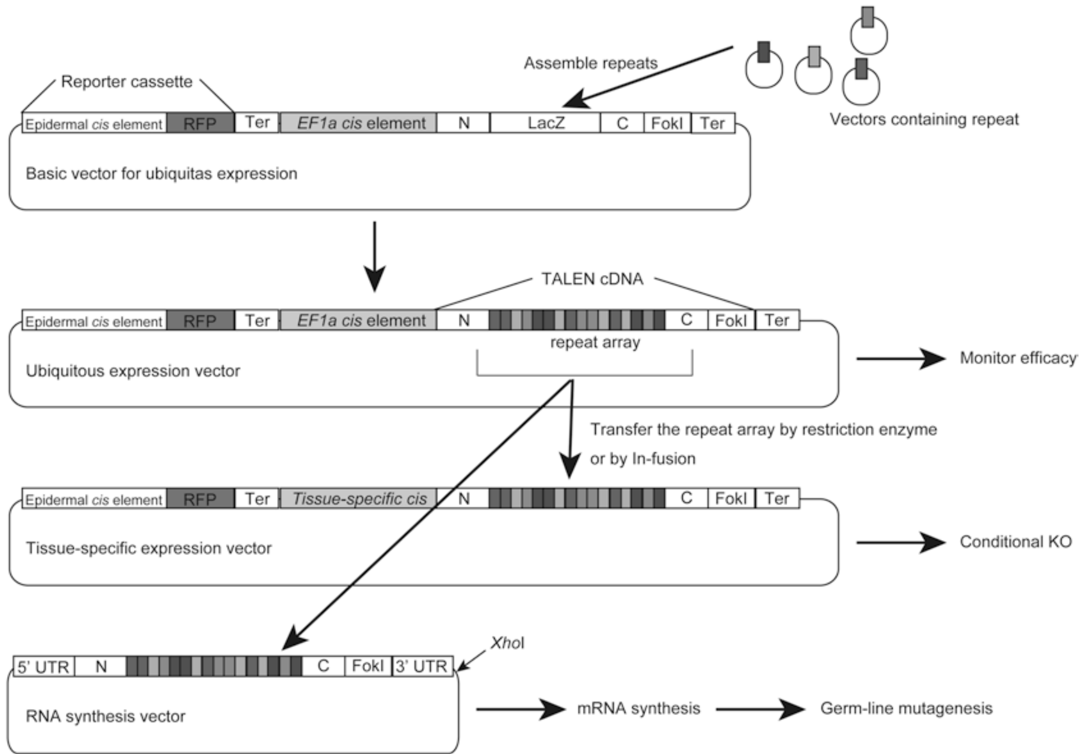

Fig. 2 Our TALEN vector systems for gene knockouts in *Ciona intestinalis*. *Ter* transcription termination sequence, *RFP* cDNA encoding red fluorescent protein, *LacZ* a portion of *LacZ* cDNA, *N* cDNA encoding amino terminal region of TALEN, *C* cDNA encoding carboxyl terminal region of TALEN, *FokI* cDNA encoding the nuclease domain of *Fok*I. 5′ UTR and 3′ UTR, untranslated regions from cDNA encoding beta tubulin of *Halocynthia roretzi*. *XhoI Xho*I restriction site

2.4 Apparatus

1. GenePulser Xcell™ CE system (1652661 of Bio-rad).

3 Methods

3.1 Electroporation of Vectors

Constructed TALENs can be introduced into *Ciona* embryos by means of electroporation [9, 20]. There are several methods of electroporation and the following one is an example.

1. Unfertilized eggs collected from wild types are dechorionated with the dechorionation solution. Add 200 μl of 2 M NaOH to 10 ml dechorionation solution before use. Gently pipette to dissolve a white cloudy precipitate that forms. Enter eggs into the prepared dechorionation solution, and mix them by occasional pipetting. Removal of chorion should be checked with a microscope.
2. After dechorionation (*see* **Note 2**), wash naked eggs several times by exchanging seawater (*see* **Note 3**), followed by insem-

ination. The occurrence of fertilization can be seen by the shape change of eggs.

3. After fertilization, eggs are washed once with the 1:9 dilution of seawater and 0.77 M mannitol (mannitol-seawater solution) .
4. 30 μg each of plasmid DNAs harboring paired left and right TALEN cDNAs are mixed in 500 μg of mannitol-seawater solution in a cuvette (4 mm width) with a glass pipette. Then enter 300 μg mannitol-seawater solution containing fertilized eggs (prepared in **step 3**) into the cuvette. Mix by gentle pipetting. Usually, we do not adjust the number of eggs for an electroporation.
5. The cuvette is set in the machine (we use GenePulser II), and then a pulse is given with the condition of 50–52 V for 20 ms. Usually, we provide a pulse 20–25 min after fertilization at 18 °C.
6. Eggs are washed several times with seawater and they are cultured at 16–18 °C.

3.2 Estimation of Mutation Rates

Before functional analysis, the efficacy of constructed TALENs should be analyzed because constructed TALENs are not always active enough to mutate genes efficiently. For this purpose, the TALENs are first assembled in a vector harboring the *cis* element of *EF1α* that can express downstream genes in the ubiquitous manner (Fig. 2) [20, 24]. Our TALEN vector expresses a reporter gene along with TALENs, and the fluorescence derived from the reporter gene can be used to check the efficiency of electroporation. A brief procedure of estimating mutation rates is as follows:

1. When electroporated animals reach late tailbud to larval stages, pick up about 50–100 animals that emit fluorescence in the almost entire region of their body with a fluorescent microscope. Embryos generally show abnormal morphology due to a side effect of TALENs expressed too much by the ubiquitous promoter, but it is not necessary to avoid collecting these embryos when estimating mutation rates. Collect embryos in a 1.5 ml tube. Centrifuge briefly and discard excess seawater.
2. Extract genomic DNA from the gathered embryos in bulk. We use Wizard genomic DNA purification kit for isolating genomic DNA. Dissolve collected embryos in 600 μl of Nuclei lysis solution. Incubate for several hours at 50 °C. Then precipitate proteins with 200 μl protein precipitation solution according to the manufacturer's instruction. After isopropanol precipitation, dissolve DNA in 10–20 μl water.
3. A 200–300 base pair amplicon containing the target site of a TALEN pair is amplified by PCR. The PCR fragment is analyzed by means of surveyor nucleases such as Cel-I [19, 25],

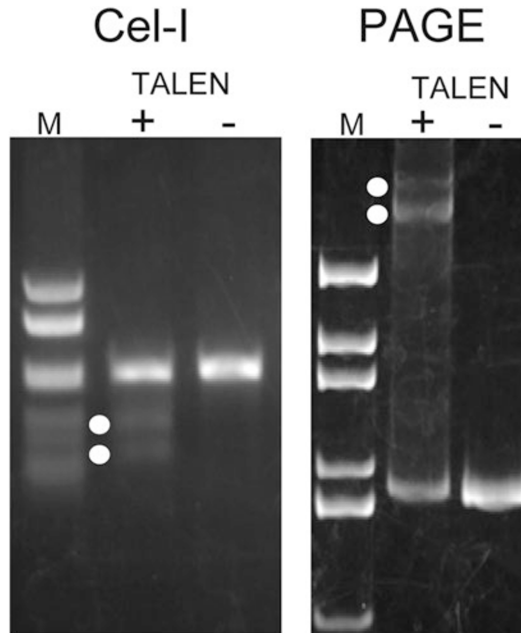

Fig. 3 Gel electrophoreses for monitoring mutations induced by TALENs. The *left panel* is a Cel-I restriction enzyme-mediated method, and the *right panel* is a polyacrylamide gel-mediated method. In both gels, mutations in the same gene induced by the same TALENs are detected. *M* marker. + *lanes* exhibit PCR products derived from animals in which the target gene is knocked out with TALENs, and – *lanes* exhibit PCR products from animals into which control TALENs (the TALENs targeting a gene different from the detected gene) were introduced. The bands suggesting mutations are shown with *white dots*

polyacrylamide gel electrophoresis [26], and/or sequencing after subcloning, for estimating mutation rates (Fig. 3). We recommend the polyacrylamide gel electrophoresis because no post-PCR treatment is necessary other than electrophoresis with 15% polyacrylamide gel (*see* **Note 4**). We suppose that TALEN pairs that have mutation rates higher than 60% by sequencing can be used for subsequent analyses.

3.3 Conditional Knockouts in the G0 Generation

When a TALEN pair with a good mutation rate is constructed, the TALENs are transferred to vectors with appropriate expression systems for functional analyses of genes (Fig. 2). A recommended functional analysis is expressing TALENs with tissue-specific *cis* elements (Fig. 4) to destroy the target gene in the conditional manner. The advantages of this method are: (1) reducing expressing tissues of TALENs can reduce the damage given to embryos; (2) limiting the tissue where a target gene is knocked out gives us a strong cue to identify the tissue where the gene majorly functions; (3) the method can distinguish different gene functions in different tissues or developmental timings, particularly this is

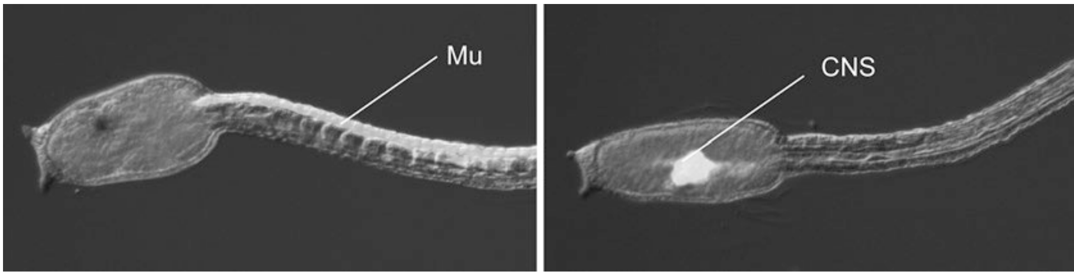

Fig. 4 Tissue-specific expression of fluorescent protein in *Ciona* larvae. The left larva express mCherry in the muscle (Mu), and the right larva express mCherry in the central nervous system (CNS)

essential when destroying a function of the gene in a tissue or developmental time can hide its another function. We already reported TALEN expression vectors for the epidermis, neural tissue, endoderm, and differentiated neurons [20, 27]. In *Ciona*, many more tissue-specific *cis* elements have been reported and they can also be used. In our initial reports [20, 27], the tissue-specific expression vectors contain a cassette expressing GFP or RFP in the epidermis, which can be used for monitoring electroporation efficiencies. This is not the ideal way because expression of the fluorescent proteins cannot label the tissue where TALENs are actually expressed, and further improvements are now in progress. The transfer of TALEN repeats can be easily done with restriction enzymes or in vitro recombination kits such as In-Fusion cloning kit (Fig. 2). The expression vectors can be introduced by means of electroporation as mentioned above.

When we use TALENs with good mutation rates, we can observe phenotypes at the G0 generation (the generation at which TALEN vectors are introduced). This indicates that we can investigate gene functions without establishing mutant lines. If a gene-of-interest has a function at an embryonic stage, the phenotype could be seen within 24 h after fertilization, because *Ciona* need less than 24 h from fertilization until hatching. We need to take care that we could not address gene functions with this method when the target genes have functions at very early embryonic stages when zygotic gene expression has not started (*see* **Note 5**) or it is not yet active, because TALENs require a certain time for accumulating translated proteins for introducing mutations. We have successfully monitored a gene function at early tailbud stage (~8 h after fertilization) [20], but we have not tested at earlier stages and more experience will be necessary to see the temporal limit of this approach.

3.4 Germline Mutagenesis

As mentioned in the previous subchapter, a limitation of G0-knockouts is incompatibility for addressing gene functions when target genes have a function at very early embryonic stages. In this case, we need to establish mutant lines for observing

functions. Additionally, mutant lines are useful for functional analyses of genes because it is the only way to generate mutant animals without any worry of mosaicism of mutations among cells constituting the animal. Although G0-based knockout is a time-saving method, we always need to take mosaicisms of mutations into account that might affect phenotypes from two major aspects (*see* **Note 6**). First, mosaicism could weaken phenotypes because some cells have functional copies of target gene. Second, some mutations could generate unexpected effects on gene functions that would possibly oppose gene functions, such as constitutive active forms. For avoiding these disadvantages, establishing mutant lines is ultimately the best way to describe gene functions.

In *Ciona*, germ cell mutagenesis is achieved by introducing TALEN mRNAs into eggs [28]. The injected mRNAs diffuse in the egg cytoplasm to reach the region where primordial germ cells (PGCs) emerge (*see* **Note 7**), and translated TALENs mutate their target genes in the germ cell genome. Knockout lines can be established by generating progeny derived from mutated germ cells. We reported that the efficiencies of mutating germ cell genomes in the G0 generation tend to be lower and more variable among individuals than those in somatic cells [28]. This phenomenon was explained because *Ciona* PGCs are small and a smaller amount of TALEN mRNAs is uptaken by PGCs. Therefore, a larger number of G0 animals should be screened to obtain G1 families with a high rate of mutant carriers. Electroporation has not been adopted for germ cell mutagenesis in *Ciona* because a *cis* element capable of expressing a gene in the germ line has not been isolated.

Because a detailed method of microinjections will be described in another chapter of an ongoing book from Springer, here we describe how to prepare for TALEN mRNAs:

1. We need to introduce TALEN repeats into a vector for in vitro synthesis of mRNAs. The vector contains partial cDNA encoding the N and C terminals of TALENs that are flanked by untranslated regions of a tubulin gene from the ascidian *Halocynthia roretzi* (Fig. 2) [29]. The untranslated regions facilitate translation in *Ciona* embryos [24]. Without such translated regions, in vitro synthesized mRNAs are not translated efficiently in *Ciona*.
2. One microgram of the constructed vectors is linearized with *Xho*I. Check the vectors by electrophoresis.
3. Phenol-chloroform extraction and chloroform extraction followed by ethanol precipitation to remove RNase and the restriction enzyme.
4. mRNA is synthesized with MegaScript T3 kit and Cap structure analog according to the manufacturer's protocols. The cap structure analog (Capped GTP) is necessary for translation

in *Ciona* embryos. The ratio of GTP and capped GTP in the solution is recommended to be 1:4.

5. After mRNA synthesis, digest template DNA with DNase I provided in the kit. Template DNA needs to be removed from mRNA because a high amount of DNA is toxic for *Ciona* embryos.
6. Elongate the poly A tail with the Poly A tailing kit. A long poly A tail will increase the efficiency of translation. This step is optional.
7. Precipitate mRNA with the LiCl solution provided in kit. This step removes excess nucleotides, short RNAs, and digested DNA. Rinse pellet well with 70% ethanol to remove LiCl.
8. Dissolve mRNA in RNase-free H_2O and adjust concentration. The recommended final concentration of TALEN mRNAs in the injection solution is 100 ng/μl each of the left and right TALENs [28].

3.5 Database and Website

The history of genome editing in *Ciona* is not long now, and many *Ciona* researchers have not been accustomed to the techniques. To help researchers use these techniques, we developed a website in which information related to genome editing in *Ciona* is accumulated. The URL of the website is http://marinebio.nbrp.jp/ciona/forwardToKnockOutAction.do. The website deals with vector sequences of TALEN and CRISPR/Cas9 vectors, the genes that are so far targeted by genome editing, target sites of the genes, primers for detecting mutations, gel images detecting mutations, sequences of mutated genes and references. Expression vectors can be requested from the website.

4 Notes

1. A paper [30] asserts that Tunicata, Vertebrata, and Cephalochordata can be regarded as phylum in the branch of deuterostomes.
2. Usually, it takes a few minutes for dechorionation at 18 °C. The time varies by subtle change in the room temperature and seasonal differences of *Ciona* eggs.
3. There are two ways to perform this step. One is that gently swirling a petri dish causes dechorionated eggs to accumulate in the center. Using a glass pipette collect the eggs and transfer them to another petri dish filled with seawater. The other way is using a manual centrifuge. Centrifuging too vigorously decreases the quality of the eggs.
4. Our condition of electrophoresis is 100 V for 2–2.5 h at room temperature. Higher voltages may disturb DNA bands.

5. Zygotic gene expression starts at the 8-cell stage during *Ciona* embryogenesis [31].
6. Mosaicism is thought to be caused by the unequal distribution of expression vectors among blastomeres.
7. PGCs of *Ciona* are located at the posterior end of cleavage stage embryos. At the larval stage, they are located at the ventral side of the tail [32].

Acknowledgments

We would like to thank Drs. Hiroshi Ochiai, Tetsushi Sakuma, and Takashi Yamamoto for their help to support our researches. This study was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) and Ministry of Education, Culture, Sports, Science and Technology (MEXT) to Y.S. Y.S. was supported by the Toray Science and Technology Grant. Further support was provided by grants from the National Bioresource Project.

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Chapter 20

Genome Editing of *C. elegans*

Takuma Sugi

Abstract

Caenorhabditis elegans, a 1 mm long free-living nematode, is a traditional model animal for genetic investigations of various biological processes. Characteristic features that make *C. elegans* a powerful model of choice for eukaryotic genetic studies include its rapid life cycle, well-annotated genome, simple morphology, and transparency. Recently, genome editing technologies have been increasingly used in *C. elegans*, thereby facilitating their genetic analyses. Here, I introduce a protocol frequently used in *C. elegans* genome editing.

Key words *C. elegans*, CRISPR/Cas9, Homology-dependent repair

1 Introduction

The nematode *Caenorhabditis elegans* became a powerful model organism just over 50 years ago [1]. This tiny animal has greatly contributed to investigations of the functions of genes in development and cellular biology. One advantage of using *C. elegans* as a model animal is its simple morphology; the adult hermaphrodite is composed of only 959 somatic cells, including 302 neurons, and cell number does not vary among individuals. Researchers have tracked the fate of every cell, from fertilization to adulthood, in living animals and have generated a complete cell lineage [2]. Features that make the nematode highly amenable to laboratory research also include its small size, short life cycle, and ability to survive long-term freezing for storage. These advantageous features have allowed *C. elegans* researchers to uncover novel biological mechanisms, such as apoptosis, and develop novel techniques, such as green fluorescent protein (GFP) tagging, in the life sciences research field.

C. elegans is a genetically tractable model system, for which many genetic resources and tools have been developed. There are approximately 20,359 protein-coding genes in *C. elegans* (WormBase referential freeze WS250, November 2015). In

Izuho Hatada (ed.), *Genome Editing in Animals: Methods and Protocols*, Methods in Molecular Biology, vol. 1630,
DOI 10.1007/978-1-4939-7128-2_20, © Springer Science+Business Media LLC 2017

OrthoList, a compendium of *C. elegans* genes with human orthologs, 7663 protein-coding genes are predicted to have human orthologs, corresponding to approximately 38% of the *C. elegans* genome [3].

Microinjection of a gene of interest has been an indispensable genetic technique to study gene functions in *C. elegans*. The microinjection of genes including fluorescent protein-coding genes into the gonad of the adult hermaphrodite results in the formation of semi-stable extrachromosomal arrays that are comprised of many copies of the injected DNA [4]. The transgenes, which are typically overexpressed in somatic tissues and silenced in the germline and early embryo, can be integrated into the genome by UV irradiation or microparticle bombardment [5, 6]. These integration methods have allowed the generation of low-copy transgenes, which are expressed at closer to endogenous levels [7]. However, this experiment is less efficient, time-consuming, and not easily able to control genomic location for gene insertion. Thus, a strategy for inducing a stable gene knockin at a desired genomic location has been required in the nematode research field.

Due to these requirements, *C. elegans* researchers have recently utilized targeted genome editing technologies. A large number of protocols for genome editing technologies have been established since Wood et al. reported a landmark paper on genome editing in *C. elegans* [8–10]. These protocols include several strategies for homology-dependent repair (HDR)-mediated gene knockin of *C. elegans* genome. In particular, Dickinson et al. have established a versatile and simple strategy for precise gene insertion without an exogenous sequence at the targeted locus or marker mutations [11, 12]. They have designed a plasmid that includes a fluorescent tag gene and the self-excising cassette (SEC) carrying a drug resistance gene, a visible phenotypic marker, and a heat-shock-inducible Cre recombinase gene. Incorporation of the SEC from the plasmid into genomic loci permits easy excision of unwanted sequences upon heat-shock treatment after the identification of the desired mutant using drug selection and screening for a visible phenotype.

Here, I introduce this protocol by applying it to insert the fluorescent protein TagRFP-T into the genomic region downstream of the promoter of *glr-1* gene [13, 14] (a *C. elegans* AMPA-type receptor ortholog) (Fig. 1).

2 Materials

2.1 Plasmids

1. Cas9 and sgRNA co-expression plasmid: Purchase the pDD162 vector (Addgene, #47549) or pJW1219 vector (Addgene, #61250) to coexpress Cas9 and sgRNA under the control of R07E5.16 *U6* promoter from Addgene. pJW1219 vector is a pDD162 derivative with the higher efficiency.

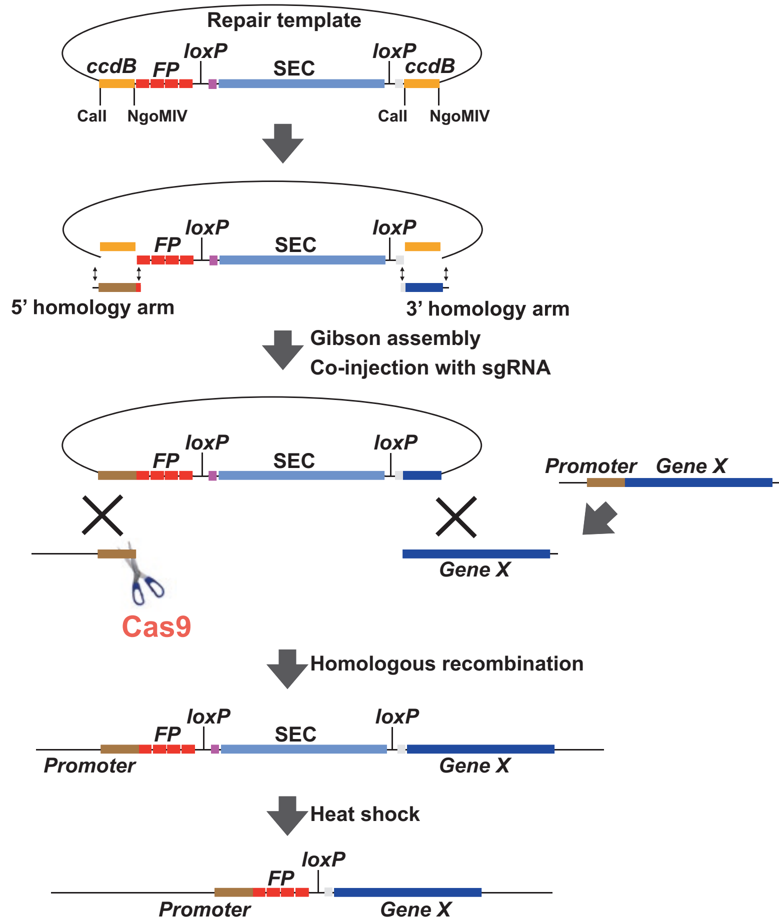

Fig. 1 Schematic of homologous recombination-mediated *FP* insertion using CRISPR/Cas9 system. The self-excising cassette is composed of a drug resistance gene, a visible phenotypic marker, and a heat-shock-inducible Cre recombinase gene. The SEC selection marker in the resulting *FP*-inserted worms can be excised by heat-shock treatment of worms

2. Q5 Site-Directed Mutagenesis Kit (New England Biolabs, #E0554S).
3. Forward primer 1: 5′-N$_{20}$GTTTTAGAGCTAGAAATAGCAAGT-3′.

   Reverse primer 1: 5′-CAAGACATCTCGCAATAGG-3′.

   Forward primer 2: 5′-ATCGACCGTAAGCGTTACACCTCCACCAAGG-3′.

   Sequencing primer 1: 5′-GGTGTGAAATACCGCACAGA-3′.

   M13 forward primer: 5′-TGTAAAACGACGGCCAGT-3′.

   M13 reverse primer: 5′-CAGGAAACAGCTATGACCATG-3′.

4. Homologous repair template plasmid: Several homologous repair templates are available for knockin of a gene-encoding fluorescent protein. pDD286 plasmid for knockin of TagRFP-T was chosen here and purchased from Addgene as transformed bacteria. Streak bacteria onto an ampicillin plate. Pick a single colony and culture it at 30 °C for 16 h. Purify the plasmid DNA using miniprep kit (e.g., QIAGEN, #27104).
5. Restriction enzymes: ClaI (e.g., New England Biolabs, #R0197S) and NgoMIV (e.g., New England Biolabs, #R0564S).
6. Purification of PCR products: QIAquick PCR Purification Kit (e.g., QIAGEN, #28104).
7. Gibson assembly: NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs, #E5520S).
8. Injection marker: Several plasmids for germ transformation in *C. elegans* are available. pCFJ104 (Addgene, #19328) for expressing mCherry in body wall muscles under the control of the *myo-3* promoter were used here.

2.2 C. elegans Preparation

1. *C. elegans*: Obtain the wild-type N2 strain from *Caenorhabditis Genetic Center*. Maintain and handle the wild-type N2 strain using standard methods [1].
2. Nematode Growth Medium (NGM) plate: Weigh 6 g NaCl, 40 g agar, 5 g peptone and transfer to the 3 L flask. Add 1950 mL of water and autoclave at 120 °C for 20 min. Cool to approximately 60 °C and add 2 mL of 1 M $CaCl_2$, 2 mL of 1 M $MgSO_4$, and 50 mL of 1 M KPO_4 solution (108.3 g KH_2PO_4 and 35.6 g K_2HPO_4 in water). Pour 10 ml of autoclaved solution into a 60 mm Petri plate. Streak *Escherichia coli* OP50 propagated at 37 °C for 12 h.

3 Methods

3.1 DNA Construction for Cas9-sgRNA Plasmid

1. Choose the target site and submit the genomic sequence to the CRISPR guide RNA selection tool website (http://genome.sfu.ca/crispr/about.html).
2. The tool returns target sites of the form 5′ N_{20}-NGG-3′, where N is any base. The GG at the 3′ end of each guide are known to have higher efficiency for NGG PAM [15]. Insert the N_{20} sequence into the pDD162 or pJW1219. I used pDD162 here.
3. Transform the fragment created by PCR using the forward primer 1, the reverse primer 2 (*see* **Note 1**), and Q5 Site-Directed Mutagenesis Kit to suitable competent cells and culture it on an ampicillin plate for 14 h.

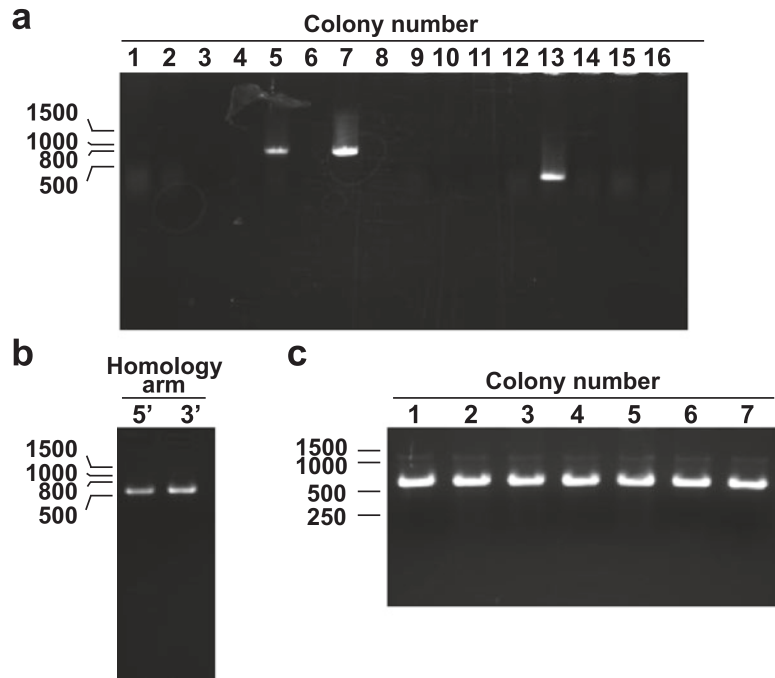

Fig. 2 Electrophoresis check of the DNA construction process. (**a**) Identification of an *E. coli* colony carrying the appropriate Cas9-sgRNA plasmid by colony PCR and agarose electrophoresis analysis. This analysis was performed using forward primer that anneals to the sequence of the original pDD286 vector and reverse primer that anneals to the inside of the forward primer used in the original PCR. (**b**) Agarose electrophoresis of PCR products for amplification of the 5′ and 3′ homology arms. (**c**) Identification of a colony carrying the appropriately assembled plasmid by colony PCR. The M13 forward primer and the second primer used for amplifying a homology arm were used for the analysis and agarose electrophoresis analysis

4. Identify the colony carrying the appropriate Cas9-sgRNA plasmid by colony PCR using forward primer 2 that anneals to the sequence of the original pDD286 vector and a reverse primer 2 that anneals to the inside of the forward primer used in the above PCR (Fig. 2a).
5. Sequence the resultant plasmid using the sequencing primer 1.

3.2 DNA Construction for Homologous Recombination Template

1. Digest the FP-SEC vector using one of two pairs of restriction enzymes recommended by Dickinson et al. [12] (*see* **Note 2**). ClaI and NgoMIV were used here, because the 5′ homology arm could be fused directly to the fluorescent protein TagRFP-T, without additional sequence.
2. Choose the genomic regions suitable for homology arms (500–700 bp long) and design the primers to assemble homol-

ogy arms into the FP-SEC vector (pDD286; Fig. 1). The homology arms are inserted in place of the *ccdB* negative selection markers, which are flanked by restriction sites. Because *E. coli* DH5α harboring the original plasmid expression cannot grow on the usual ampicillin plate due to the *ccdB* marker, successful insertion should be verified by picking a single colony that grows on the plate.

3. Using the two sets of the primers, generate two PCR products by amplifying homology arms with sequences overlapping with sequences of the FP-SEC vector (Fig. 2b) (*see* **Note 3**).
4. Mix the PCR products and purify them using QIAquick PCR Purification Kit. Elute the purified PCR products with 35 μL of autoclaved water.
5. Assemble the purified PCR products with FP-SEC vector using NEBuilder HiFi DNA Assembly Cloning Kit. Mix 2 μL of PCR products, 0.5 μL of vector, and 2.5 μL of NEBuilder HiFi DNA Assembly Master Mix. Incubate this mixture at 50 °C for 1 h. Transform 2 μL of the reaction to NEB 5-α competent cells.
6. Perform colony PCR using the M13 forward primer and the second primer used for amplifying a homology arm to identify a colony expressing appropriately assembled plasmid (Fig. 2c).
7. Sequence the resultant plasmid using the M13 forward and reverse primers.

3.3 Injection

1. Prepare the injection solution:
   - 10 ng/μL homologous repair template.
   - 50 ng/μL Cas9-sgRNA plasmid.
   - Suitable co-injection marker (5 ng/μL pCFJ104 for expressing mCherry in body wall muscles under the control of *myo-3* promoter was used here).
2. Inject the solution into the gonads of a few young adult worms using standard methods [4].
3. Cultivate the injected worms on the NGM plate at 25 °C for 2.5 days.

3.4 Screening

1. Dissolve hygromycin in water to prepare a 5 mg/mL hygromycin solution. Pour 500 μL of this solution onto the surface of each NGM plate in which worms were cultivated for 2.5 days. Swirl so that the solution covers the entire surface of the plate and let it dry.
2. Identify worms that survive the antibiotic screening and exhibit roller phenotype. Among them, transfer worms that do not carry co-injection marker (e.g., *myo-3p::mCherry*) as an extrachromosomal array individually to new NGM plates. The NGM plate does not need to contain antibiotic such as hygromycin. Cultivate worms at 20 °C for a few days.

3. Find an NGM plate on which all adult worms have roller phenotype. These worms should be homozygous knock-in animals. Transfer 5–10 L1/L2 worms from this plate to another new NGM plate.
4. To remove SEC, induce expression of *heat-shock promoter::cre* by heat-shock treatment of worms. Place the NGM plate at 32 °C for 20 min and allow them to recover at 20 °C.
5. Pick a few knockin worms that do not exhibit roller phenotype and maintain them for each experiment.

4 Notes

1. The primers used for creating the Cas9-sgRNA plasmid should not include the PAM sequence.
2. The pair of restriction enzymes used for FP-SEC vector construction should be chosen according to each FP-SEC vector and application. For example, if AvrII and NgoMIV are used with pDD286, the repair template includes a flexible linker between the 5′ homology arm and TagRFP-T. This combination is suitable for generating C-terminal tags. On the other hand, if ClaI and NgoMIV are used, the 5′ homology arm will be fused directly to the TagRFP-T, without additional sequence. This is useful for creating N-terminal tags without flexible linker.
3. Design a primer for creating FP-SEC vector so that FP-SEC insertion disrupts the sgRNA target site. Ideally, the insertion site is within 10 bp of the PAM sequence. Otherwise, use a primer that introduces silent mutations to prevent Cas9 from cutting the repair template.

Acknowledgment

I thank *Caenorhabditis* Genetic Center for sharing strains and *C. elegans* knockout facility at University of British Columbia. The writing of this review was made possible in part by a grant from the Japan Society for the Promotion of Science, and Japan Science and Technology Agency under Precursory Research for Embryonic Science and Technology (PRESTO).

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