

Production of knock-in mice in a single generation from embryonic stem cells

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The system-level identification and analysis of molecular networks in mammals can be accelerated by ‘next-generation’ genetics, defined as genetics that does not require crossing of multiple generations of animals in order to achieve the desired genetic makeup. We have established a highly efficient procedure for producing knock-in (KI) mice within a single generation, by optimizing the genome-editing protocol for KI embryonic stem (ES) cells and the protocol for the generation of fully ES-cell-derived mice (ES mice). Using this protocol, the production of chimeric mice is eliminated, and, therefore, there is no requirement for the crossing of chimeric mice to produce mice that carry the KI gene in all cells of the body. Our procedure thus shortens the time required to produce KI ES mice from about a year to ~3 months. Various kinds of KI ES mice can be produced with a minimized amount of work, facilitating the elucidation of organism-level phenomena using a systems biology approach. In this report, we describe the basic technologies and protocols for this procedure, and discuss the current challenges for next-generation mammalian genetics in organism-level systems biology studies.

INTRODUCTION

To identify and analyze molecular networks and/or cellular circuits in organisms, gene knockout (KO) or KI technologies are powerful tools and are often applied in mammalian reverse genetics studies. However, to produce KO or KI mice using classic genetics is demanding with respect to time, space, and effort. In conventional schemes, after target mutations are introduced into embryonic stem cells (ESCs) by homologous recombination, the mutant ESCs are injected into wild type blastocysts to produce chimeric mice. To produce mice in which all the cells of the body carry KO or KI mutations, a requirement for reliable phenotype analysis, further crossings of the offspring are required. These crosses take several months to years to carry out, as a period of at least 3 months is required per generation. Therefore, to comprehensively identify and quantitatively analyze the molecular networks and/or cellular circuits in an organism with minimal effort requires next-generation genetics, i.e., highly efficient production technologies for obtaining mice that carry KO or KI mutations throughout all the cells of the body without crossing.

Methods developed to accelerate KO and KI mouse production.

Recent advances in genome-editing techniques have accelerated KO studies^{1–7}. We recently established an efficient method for the generation of mice that carry biallelic KO mutations in all cells of the body in one stage^{8,9}. After identifying a molecule of interest, it is also important to create KI animals that have modified proteins. Ideally, these KI animals will have a null phenotype that can be rescued with the expression of a mutant form of the deleted gene. This enables the evaluation of the organism-level effect of modifying the protein. However, in contrast to the improved KO rates obtained with one-stage production schemes, zygotic KI remained inefficient, particularly long-fragment insertion by CRISPR-mediated genome editing (initially a KI rate of only ~20%¹⁰). Several studies have sought to improve the KI rate, for example, by inhibitor treatments^{11,12}, by using Cas9 protein rather than Cas9 mRNA^{13–15}, and by using two single-stranded

oligodeoxynucleotides (ssODNs) as a bridge between the large fragment and the genome¹⁶. Although one of these studies showed an increased genome-editing (KI) rate, an up to ~45% KI efficiency for live-born pups, when injecting a complex of Cas9 protein/dual crRNA/tracrRNA into pronuclei¹⁴, some of the results were based on a small number of experiments, and thus the reproducibility of these recent studies must be further evaluated. In addition, usually not all cells of produced KI mice carry the KI mutation, so further crossing of the offspring is required to produce mice that carry KI mutations throughout the body. In summary, further improvements to overcome editing inefficiency (particularly with a large-fragment KI) in the zygote were needed to achieve one-step generation of KI mice.

An attractive alternative method for producing mice that carry KI mutations throughout the body without crossing is the two-step production of KI mice as almost completely ESC-derived mice (ES mice). The advantages of using ESCs are the ease of and flexibility for more complex genome editing (e.g., multi-gene KI) in *in vitro* culture, the ability to select for sex, the ease of storage, and the ability to introduce a loss-of-function mutation in an embryonic lethal gene.

ES mouse production within a single generation was first tried using the tetraploid complementation method^{17–24}. However, this method has several potential drawbacks, such as developmental abnormalities caused by substantial contamination by host cells^{24,25}, and the difficulty of using inbred ESCs^{21,23,26}. Another method for the generation of ES mice is to inject ESCs into, or to allow ESCs to aggregate with 8-cell embryos^{27–29}. All three germ layers (ectoderm, endoderm, and mesoderm) of the adult mice are developed from the inner-cell mass (ICM) of the blastocyst-stage embryo. After injection or aggregation, the ESCs show a strong bias toward integration into the ICM of blastocyst-stage embryos, and the ICM is occupied by ESCs. As the ESCs are derived from ICM, the cell–cell adhesion properties of the ESCs might enhance the commitment of ESCs into ICM^{30,31}.

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Although the developmental mechanism of ES mice remains unclear, 8-cell injection and aggregation methods can generate ES mice with ~0.1% or less contamination by host-embryo cells. To minimize such contamination, ESCs are established and maintained in serum-free three-inhibitor/two-inhibitor (3i/2i) + leukemia inhibitory factor (LIF) medium. ESCs of various mouse strains, even from inbred strains such as C57BL/6 (B6), can be produced with this method and can stably and efficiently be used to generate ES mice via 8-cell injection, even after many passages and gene targeting³². An ES mouse method combining 3i/2i + LIF-cultured ESCs with 8-cell injection/aggregation has already been used to make mice that carry KI mutations throughout the body without crossing^{33–35}, indicating that this ES mouse method is feasible and robust. The combination of the genetic background of the ESCs and host embryos highly influences the efficiency of the somatic and germline contribution of the ESCs³⁶. In the case of ICR mouse embryos and B6 ES cells, a high production efficiency is achieved for chimeric mice. Moreover, it is also possible to easily determine the contribution rate of ES cells in chimeric mice by using the difference in coat color between ICR mice and B6 mice³⁶. Thus, it is preferable to use a combination of ICR mouse embryos and B6 ES cells.

Development of a system for the efficient parallel establishment of KI ESCs that are competent for ES mice production

To improve the throughput of KI ESC production, we focused on developing the following three methods: (i) a feeder-free culture method to eliminate the preparation of the mouse-embryonic fibroblast (MEF) feeder; (ii) a highly efficient and small-scale KI protocol to enable parallel gene targeting, and (iii) a simple colony-pickup method to reduce the work involved in handling a large number of samples in parallel (Fig. 1).

We carefully optimized the feeder-free culture conditions under which ESCs retain their ability to efficiently contribute to embryos. We found that good chimerism could be obtained using amine-coated dishes pretreated with 6-bromoindirubin-3'-oxime (BIO)-containing medium (BIO medium; Fig. 2a,b)^{37,38}. Without the BIO medium pretreatment, ESCs tend to differentiate over several passages. Although the mechanism underlying this observation is not fully understood, we speculate that the BIO medium influences the distribution of the three inhibitors in the 3i culture medium by changing the surface charge of the positively charged amine-coated dish. This idea was based on our finding that ESCs could be cultured without impairing quality through several passages in 3i medium on type-B gelatin, which is negatively charged in a neutral culture medium.

Gene-transfer techniques can be roughly classified into two types: electroporation and chemical-reagent methods. In this step, we optimized the chemical-reagent method, as it has several features that make it suitable for a small-scale protocol. In particular, it is scalable and has higher viability than electroporation. Although the conditions that result in high gene-transfer efficiency usually decrease cell viability, we found that the viability could be improved by treating the cells with BSA after transfection (Fig. 2c,d)³⁷. As a genome-editing tool introduced by chemical transfection to increase the KI efficiency, we used the transcription activator-like effector nuclease (TALEN)^{5,37,39}. The DNA cleavage by TALEN requires dimerization of its *FokI* endonuclease catalytic domain⁴⁰.

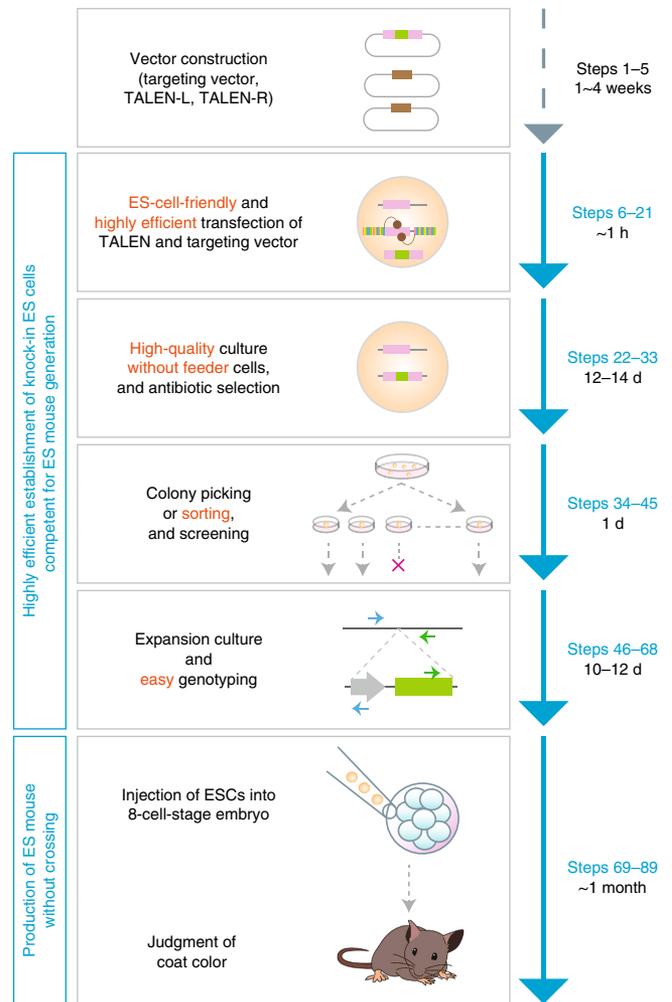


Figure 1 | Overview of advanced ES-mouse protocol. Workflow for the high-efficiency production of a mouse that carries a knock-in (KI) mutation throughout the body within a single generation. Our workflow is classified into two parts: high-efficiency establishment of KI ES cells (ESCs) and the production of ES mice from KI ESCs without crossing. For the high-efficiency establishment of KI ESCs, there are four main stages: (i) a high-efficiency KI protocol using a genome-editing tool introduced by ESC-friendly and highly efficient transfection (Steps 1–21), (ii) a high-quality-culture protocol using 3i conditions without the time-consuming preparation of feeder cells (Steps 22–33), (iii) an easy single-colony-isolation protocol using a cell sorter for high-throughput (parallel) applications (Steps 34–36), and (iv) an easy genotyping protocol that avoids laborious procedures such as Southern blotting (Steps 37–68). To produce a mouse that carries a KI mutation throughout the body without crossing, ESCs cultured in 3i medium are injected into 8-cell-stage embryos (Steps 69–89).

Thus, two TALEN molecules must bind to both the right and left sides of the target site with the appropriate orientation and spacing. As a result, the dimer recognizes a target-site sequence that is twice the length recognized by single TALEN molecules. This molecular property also results in higher specificity and fewer off-target effects. We tested several homology arm lengths for the targeting vector and concluded that the optimal length was 4–8 kbp³⁷. We also confirmed that there was less random insertion with a circular vector than with a linear one³⁷. Using our optimized TALEN-based targeting protocol with a circular vector, the efficiency of correct genome editing is almost 90%³⁷.

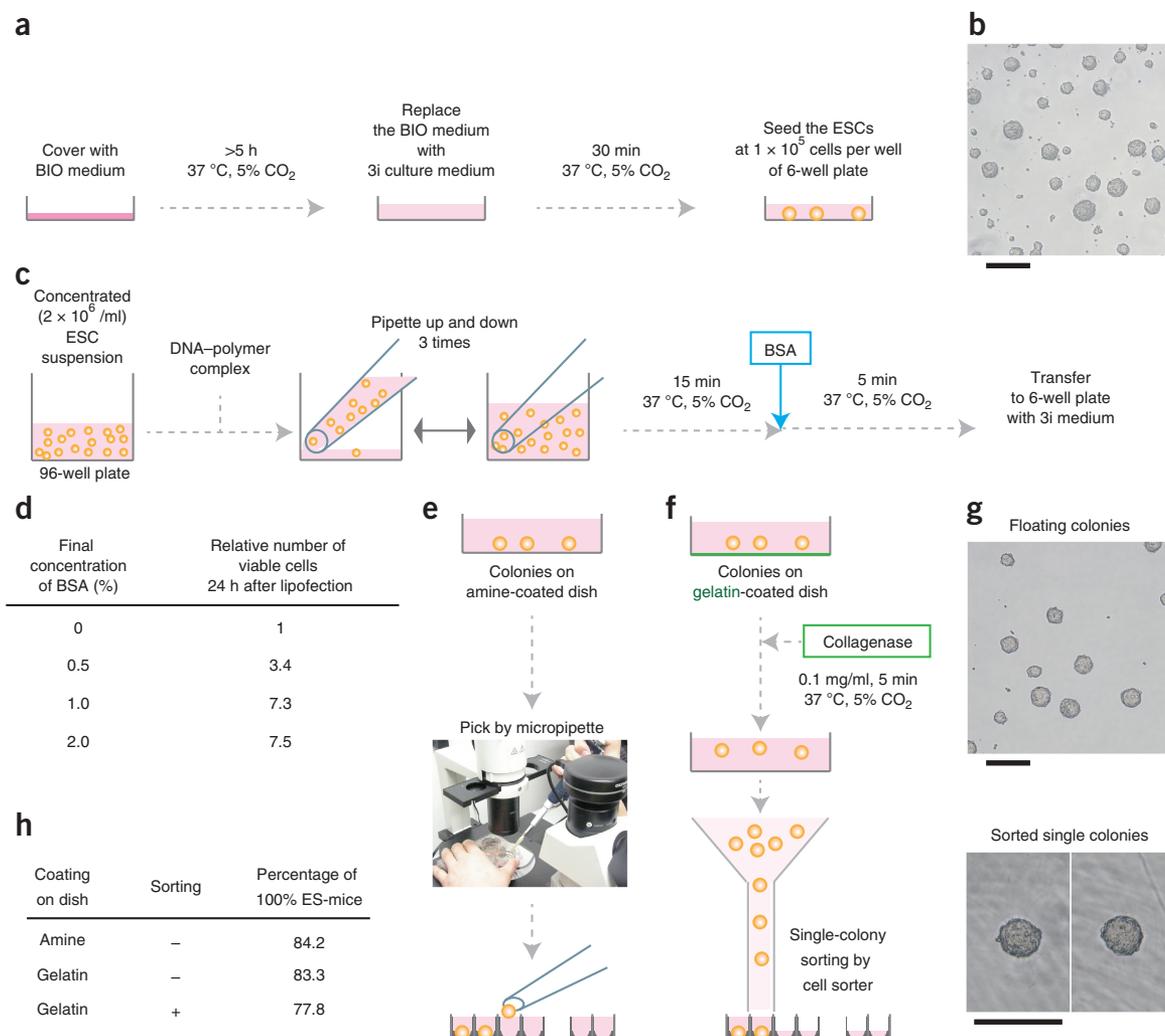


Figure 2 | Simple and efficient protocols for establishing KI ESCs under feeder-free conditions. **(a)** Illustration of the dish pretreatment for feeder-free culture. To maintain ESC's pluripotency, it is essential to pretreat the surface of amine-coated dishes (or wells) with BIO medium for more than 5 h at 37 °C in 5% CO₂. Replace the BIO medium with 3i culture medium 30 min before starting the ESC culture, and then incubate the plates at 37 °C in 5% CO₂. Seed the ESCs at 1 × 10⁵ cells per well of an amine-coated 6-well plate, and start the culture at 37 °C in 5% CO₂. **(b)** Photograph of representative ES colonies cultured under the feeder-free conditions. Undifferentiated ESCs form round colonies under feeder-free conditions. Scale bar, 200 μm. **(c)** Illustration of the high-efficiency-transfection protocol. A concentrated ESC suspension (2 × 10⁶/ml) is mixed with the DNA-polymer complex. ESC viability after transfection is improved by treating the cells for 5 min with BSA before seeding them into the dish. With this protocol, highly efficient gene transfer is achieved without exposing the ESCs to anything but 3i-culture medium for long periods. **(d)** BSA concentration-dependent change in the viability of transfected ESCs. The transfected ESCs were treated with various concentrations (%) of BSA for 5 min. Relative values are shown with viability defined as 1 when no BSA was added. **(e)** Illustration of the conventional procedure for picking colonies. ES colonies are picked up using a micropipette under a microscope, and dispensed one by one into the wells of a 96-well plate. When using the highly efficient KI protocol, the number of colonies picked per construct may be small. Therefore, when only a few kinds of KI constructs are used, a conventional manual method can be used. **(f)** Illustration of the procedure for automated-colony dispensing for high-throughput applications. Colonies cultured on gelatin-coated dishes are suspended by collagenase treatment (final concentration = 0.1 mg/ml, 5 min). Floating colonies are dispensed one by one into each well of a 96-well plate using a cell sorter. **(g)** Photographs of representative floating ES colonies before and after sorting. Scale bars, 200 μm. **(h)** Effect of sorting and gelatin coating on the chimerism of ESCs. ES colonies were cultured on amine-coated or gelatin-coated dishes, and then separated into 96-well plates manually using a cell sorter. Colonies after sorting were dispersed into single cells by trypsin treatment and used for 8-cell injection. The number of 100% ES-derived chimeras relative to the total number of pups was calculated. All animal experiments depicted here were approved by the Institutional Animal Care and Use Committee of RIKEN Kobe Branch, and all the animals were cared for and treated humanely in accordance with the institutional guidelines for experiments using animals.

Single-colony manipulation, i.e., the picking up of a colony from a culture dish hinders the parallel handling of multiple KI ESC lines. Therefore, we examined protocols for floating colonies into the culture medium and manipulating them using a cell sorter. First, we found that ESCs can be cultured on type-B gelatin without impairing their ability to contribute to chimeric embryos. We then used collagenase to degrade the type-B gelatin

and detach the ESCs, and confirmed that this had little effect on the adhesion between ESCs. Moreover, unlike trypsin, collagenase is not inhibited by divalent ions and retains its activity in the 3i medium that is preferable for ESC culture. Therefore, the addition of collagenase to 3i medium causes the ESC colonies to detach from the type-B gelatin and to float into the culture medium while maintaining their colony form. These floating colonies can

be automatically dispensed into the small wells of a 96-well plate one by one with a cell sorter (Fig. 2f–h). By combining these three procedures, we created an efficient protocol for establishing KI ESCs.

Overview of the procedure

In this report, we describe the entire procedure for the efficient production of mice that carry KI mutations throughout the body. The workflow of this procedure is presented in Figure 1. It includes the TALEN-mediated efficient KI of a gene cassette including a puromycin-resistance gene into the ROSA26 locus in 3i/2i + LIF-cultured B6-ESCs (Steps 1–68), followed by 8-cell injection (Steps 69–89).

Construction of the targeting (donor) vector and TALEN (Steps 1–5). Targeting vectors are constructed by conventional molecular-biological methods. When arms of an optimal length (4–8 kb) are used, the targeting efficiency is usually >90%³⁷. However, when arms of ~1 kb are used, the targeting efficiency decreases to ~50%³⁷. The linearization of targeting vectors increases the frequency of random integration. Thus, we recommend using circular targeting vectors³⁷. Our KI protocol was optimized using C-terminal-truncated (+63) TALENs^{41,42} that are expressed depending on the CAG promoter⁴³. Target sequences of TALENs can be designed using TALEN-NT⁴⁴. TALEN expression vector construction kits can be obtained from Addgene (<https://www.addgene.org/TALEN/>). We follow Addgene’s detailed protocol for constructing the TALENs.

Genome-editing tools ideally are easy to use; they have strong activity as well as sequence specificity, and they permit flexibility of target-sequence design. The CRISPR/Cas9 system may be suitable as an alternative genome-editing tool². However, as the target-sequence length of CRISPR/Cas9 is about half that of TALEN⁴⁵, CRISPR/Cas9 has lower sequence specificity than TALEN. Moreover, flexibility of its target-sequence selection is not higher than that of TALEN because of restriction by the PAM sequence⁴⁶. In addition, as for the strength of activity, i.e., genome-editing efficiency, TALEN is not inferior to CRISPR/Cas9 in genome editing of ESCs, in which antibiotic selection can be used³⁷. Thus, although its specificity and flexibility have been improved by using Cas9 nickase^{47,48} or Cas9 derived from different organism species^{49,50}, using TALEN might have more advantages at the present time.

Although we used a targeting vector with long homology arms for homologous recombination (HR)-mediated KI, using vectors with extremely short microhomology sequences (10–40 bp) for microhomology-mediated end joining (MMEJ)-mediated editing is an attractive alternative^{51,52}. For example, the TAL-PITCH (precise integration into target chromosome) system^{53–55} that combines TALEN and MMEJ-assisted KI was reported. However, as this system’s KI efficiency is <67%⁵⁵, further improvement is necessary.

Establishment of KI ESCs (Steps 6–33). We use a commercially available 3i medium (iSTEM, Takara Bio) for ESC culture. By culturing ESCs on amine-coated dishes that have been pretreated with BIO medium³⁷ for more than 5 h at 37 °C in 5% CO₂ (Fig. 2a), the cells can undergo >10 passages without any impairment in their ability to contribute to chimeric embryos. Under these culture conditions, the ESC colonies maintain a clear spherical shape (Fig. 2b).

We use a commercially available transfection reagent (Xfect Transfection Reagent, Takara Bio) to transfect the targeting vector and TALEN-expression vectors into ESCs, using a protocol modified from that provided by the manufacturer. In our protocol, we treat ESCs similar to nonadherent cells. The DNA–Xfect complex is mixed with a single-cell suspension of ESCs by pipetting. The mixture is incubated for 15 min at 37 °C in 5% CO₂, and then BSA is added to a final concentration of 2%³⁷ (Fig. 2c). The addition of BSA with a final concentration of 1% or more suppresses the transfection-induced decrease in cell viability (Fig. 2d). The transfected ESCs are dispersed on BIO-treated amine-coated or gelatin-coated dishes as a single-cell suspension, and then they are selected twice by puromycin with a final concentration of 1.2 ng/μl. Puromycin-resistant ESC colonies are isolated into the wells of a 96-well plate one by one with a micropipette or a cell sorter (Fig. 2e,f). The culture on gelatin and sorting with a cell sorter do not greatly affect the ESCs’ morphology or their ability to convert to chimeric embryos (Fig. 2g,h). After trypsinization of ESC colonies, an aliquot of cells is lysed for screening, and the remaining cells are further cultured for expansion.

Screening for ESCs that underwent homologous recombination (Steps 34–68). Homologous recombination can be detected by PCR of the right (3’) homology arm region in cell lysates, using a pair of primers that anneal outside the homologous arms and within the inserted cassette³⁷ (P1 and P2 in Fig. 3a). It is important to carefully determine the PCR conditions for accurate and sensitive detection. For this purpose, we construct a control vector that contains the complementary sequence for the P2 primer on a longer right homology arm, which is not on the targeting vector (Fig. 3b). We then prepare a dilution series of the control vector in a solution containing the genomic DNA of the host ESCs. Using this dilution series as a template, we determine the PCR conditions capable of amplifying the desired single band from 0.5 fg of control vector contained in 10 ng of host ESC genomic DNA (Fig. 3c)⁵⁶. Under these optimized PCR conditions, we then use the above cell lysates of puromycin-resistant colonies as a template, and detect the homologously recombined right homology arm (Fig. 3d). We then expand the positive clones.

After expanding and storing the ESCs, ESC genome samples are prepared and used for further confirmation of genome integrity in the targeted region. For this test, genomic PCR is performed using a series of primers that anneal outside the homologous arms and within the inserted cassettes (P1 to P4 in Fig. 3a). Successful targeting in a single allele is confirmed by detecting all the PCR bands from the wild-type (WT) allele (P3 and P2), targeting the 5’- (P3 and P4) and 3’-homology arm regions (P1 and P2), and measuring the copy number of the puromycin-resistance gene³⁷ (Fig. 3e). By these steps, it is possible to quickly and accurately select clones that have recombined, without relying on the conventional Southern hybridization method.

Injection of KI ESCs into 8-cell-stage embryos to produce KI ES mice (Steps 69–89). This section provides the detailed procedure for the 8-cell injection used to produce KI ES mice (Fig. 4).

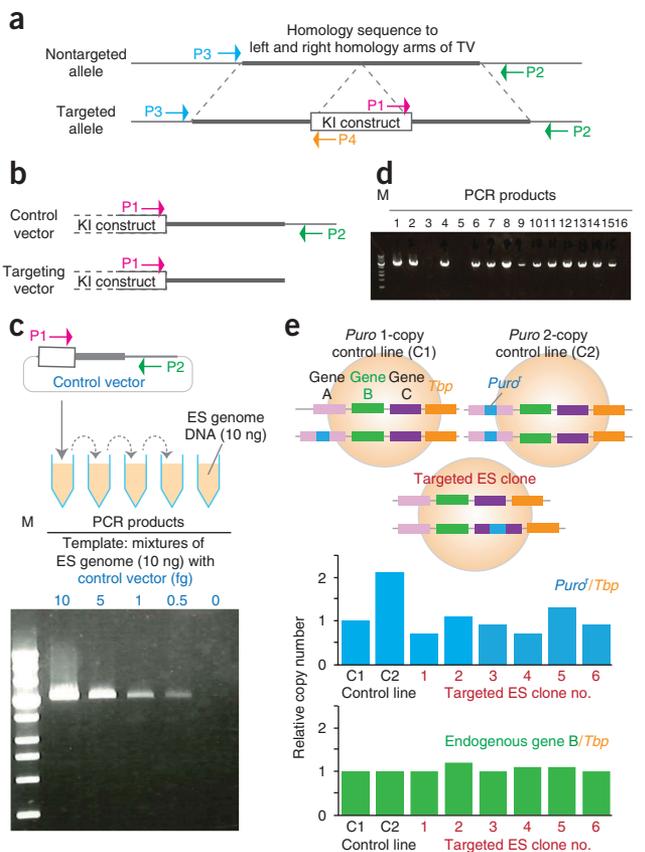


Figure 3 | Screening and genotyping procedures for KI ESCs, and typical results. **(a)** Illustration of the primer positions for screening and genotyping. The thin black lines indicate genomic DNA, and the thick black lines indicate the sequence corresponding to the homology arms of the targeting vector (TV). Arrows (P1 to P4) indicate the designed positions of the respective primers. The direction of the arrow indicates 5' to 3' direction. **(b)** Illustrations of the right homology arms of the targeting vector and control vector. The control vector contains a longer genomic DNA homologous sequence than the targeting vector. The P2 primer homologous sequence is contained only in the control-vector arm. **(c)** Illustration of the method for determining optimal screening PCR conditions. A dilution series of the control vector is prepared in a solution containing the genomic DNA of the host ESCs. Using this dilution series as a template, the PCR conditions capable of amplifying the desired single band from 0.5 fg of a control vector contained in 10 ng of host ESC genomic DNA using the P1 and P2 primers are determined. **(d)** Typical results of the PCR screening of targeted ESCs. PCR reactions using 16 puromycin-resistant ES colony suspensions were performed with the P1 and P2 primers, and the products were separated by electrophoresis. Normally, 80% or more of the clones are detected as positive clones. **(e)** Verification of the number of inserted puromycin genes. (Top) Schematic diagram of the genotype of control or a targeted ESC line containing one or two copies of the puromycin gene. (Bottom) Quantitative PCR was performed for the puromycin gene, the *Tbp* gene, or an endogenous gene (gene B in the diagram). The *Puro* or gene B value was normalized to the *Tbp* value. The results of these quantitative PCR assays are shown at the bottom. C1, control cell line containing one copy of the puromycin gene; C2, control cell line containing two copies of the puromycin gene; M, molecular-size marker of DNA; *Puro*, puromycin-resistant gene.

Limitations of KI ES mouse production and proposed future improvements

To establish a highly efficient method for the identification and analysis of molecular networks and/or cellular circuits in organisms,

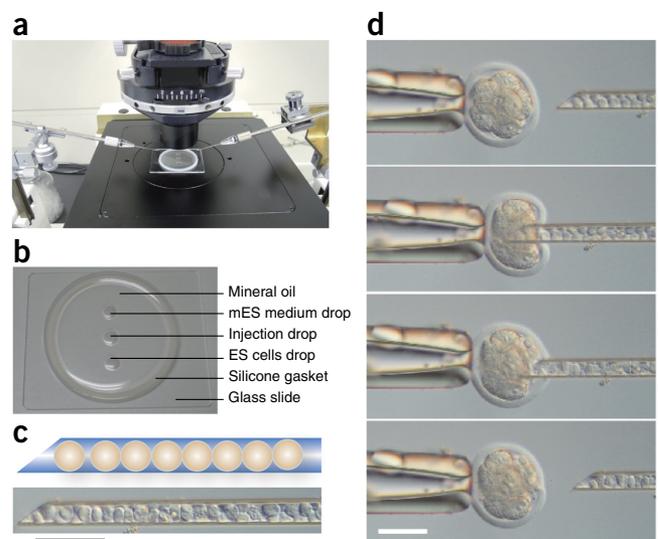


Figure 4 | Injection of KI ESCs into 8-cell-stage embryos to produce KI ES mice. **(a)** The micromanipulator into which the injection and holding pipettes are set. **(b)** Three cold medium-sized drops are made on the injection plate, which is prepared beforehand. The injection and holding pipettes are properly aligned within the mES-medium drop. ESCs are collected into the injection pipette in the ESC drop. The ESCs are then injected into 8-cell-stage embryos in the injection drop. **(c)** ESCs in the injection pipette. ESCs are collected into the injection pipette. An injection pipette with a suitable inner diameter eases the control of the ESCs. Scale bar, 40 μ m. **(d)** ESCs are injected into 8-cell-stage embryos so that they fill the perivitelline space. The injection should not cause the zona pellucida to expand. Scale bar, 40 μ m. All the experimental procedures and housing conditions were approved by the Institutional Animal Care and Use Committee of the RIKEN Kobe Branch, and all the animals were cared for and treated humanely in accordance with the institutional guidelines for experiments using animals.

we needed to improve both the throughput and the speed of the KI mouse production process. We improved the throughput of KI ES production by optimizing the transfection method, which is suitable for parallel procedures because it is simple and can be performed on a small scale. We also improved the throughput by simplifying the ESC culture using a feeder-free protocol. However, the throughput of the 8-cell injection procedure is still not very high in the present protocol, and it should be improved in the future. In particular, it will be necessary to automate the injection procedure or to develop a highly parallel cell-aggregation method that does not require exceptional skills or expensive and specialized equipment.

Regarding speed, by combining an efficient KI protocol for ESCs with an 8-cell injection protocol for 3i-cultured ESCs, we established a rapid procedure for producing mice that carry KI mutations throughout the body within ~3 months. A further increase in speed might be difficult with a two-stage method using genome editing of ESCs. A possible solution is editing of the zygotic genome, which enables one-step production of KI animals. The generation of transgenic mice, e.g., YACs/BACs/PACs-transgenic mice⁵⁷, by injection into the zygote is faster than the two-stage method, as there is no need for ES cell targeting and culture. One advantage of the transfer of these large fragments is position-independent transgene expression, so the use of YACs/BACs/PACs transgenics has been a fundamental approach to rescue of

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mutant phenotypes. However, the KI efficiency is low (5–20%⁵⁷), and transgenes are unstable (30–80% of transgenes are lost⁵⁷). In addition, random insertion of the transgene can also cause disruption of other genes, complicating phenotypic analysis. Usually, not all cells of the bodies of the produced KI mice carry the KI mutation, so further crossings of the offspring are needed to produce mice that carry KI mutations throughout the body for reliable phenotype analysis. Recent studies of KI into zygotes using efficient site-specific endonucleases, e.g., the CRISPR/Cas9 system, have enabled accurate insertion; however, the KI efficiency (20–45%) has still not been improved sufficiently^{10–14,16}. Thus, although one-stage methods require more substantial amounts of money, space, and effort to produce KI mice than do two-stage methods, the production of many types of KI mice is necessary for comprehensive identification and quantitative analysis of the molecular networks and/or cellular

circuits in an organism. Our two-stage method is more suitable than the one-step method for a comprehensive analysis, even if it takes time to target the ESCs using the current version of the protocol.

Another drawback of our protocol is that the ES mouse production rate can depend on the ‘quality’ of the cultured ESCs. Thus, genomic instability should be avoided during culture and passages because chromosomal aneuploidy can cause embryonic death. Although maintaining the ESCs in serum-free 3i/2i + LIF medium improved the efficiency of ES mouse production, better culture methods and ESC quality control should improve the efficiency further. A variety of treatments, including ERK/p38 inhibitor⁵⁸; PKC inhibitor^{59,60}; ROCK inhibitor^{61,62}; and HDAC inhibitors (e.g., trichostatin A, sodium butyrate, or valproic acid^{63–65}; or vitamin C⁶⁶) have been reported to improve the potency of ESCs, and inclusion of these or different reagents might further improve efficiency.

MATERIALS

REAGENTS

Animals

• We have used ICR female mice (CLEA Japan, cat. no. Jcl:ICR or Charles River Laboratories, cat. no. CrI:CD1 (ICR)) as pseudopregnant recipient mice and 8-cell-stage embryo donors. ICR mice are inexpensive, and in combination with B6-derived ES cells, the production efficiency of chimeric mice is also high. By combining ICR mouse embryos and B6 ES cells, the percentage contribution of ES cells can be readily recognized on the basis of simple coat-color markers. **! CAUTION** Animal experiments must be performed in accordance with governmental and institutional regulations regarding the use of animals for research purposes. All the experimental procedures and housing conditions were approved by the Institutional Animal Care and Use Committee of the RIKEN Kobe Branch, and all the animals used to produce the results shown in this protocol were cared for and treated humanely in accordance with the institutional guidelines for experiments using animals.

Vector construction

• Gateway system (Thermo Fisher Scientific, cat. no. 11791019)
• Golden Gate TALEN and TAL Effector Kit 2.0 (a gift from D. Voytas and A. Bogdanove, Addgene, kit no. 1000000024)

Cell culture and embryo manipulation

• 6-bromoindirubin-3'-oxime (BIO) for cell biology (Wako Pure Chemical Industries, cat. no. 029-16241) **! CAUTION** BIO is light sensitive.
• DMSO, Hybri-Max, sterile-filtered, BioReagent, suitable for hybridoma (Sigma-Aldrich, cat. no. D2650)
• KnockOut Serum Replacement (KSR) (Gibco, cat. no. 10828-028)
• 3i medium (iSTEM; Takara Bio, cat. no. Y40010) **! CAUTION** The attached inhibitors are light-sensitive.
• BSA (Sigma-Aldrich, cat. no. A8806)
• ESGRO leukemia inhibitory factor (LIF; Chemicon, cat. no. ESG1107)
▲ CRITICAL This solution can be stored at 4 °C. Freeze–thaw cycles will reduce its potency. Refer to the product vial label for the exact expiry information, and use it until the expiration date.
• DMEM, powder, high glucose (Gibco, cat. no. 12100-046)
• DMEM, high glucose, pyruvate (Gibco, cat. no. 11995-073)
• D-PBS (with CaCl₂ and MgCl₂; Sigma-Aldrich, cat. no. D8662)
• FBS (HyClone, cat. no. SH30910.03)
• Sodium pyruvate (100 mM; Gibco, cat. no. 11360-070)
• MEM non-essential amino acid solution, 100× (Gibco, cat. no. 11140-050)
• 2-Mercaptoethanol (Sigma-Aldrich, cat. no. M-3148)
• Sodium hydrogen carbonate (NaHCO₃; Nacalai Tesque, cat. no. 31213-15)
• Hydrochloric acid (HCl; Nacalai Tesque, cat. no. 18321-05)
! CAUTION HCl is toxic. Perform all procedures in a fume hood.
• Sodium chloride (NaCl; Nacalai Tesque, cat. no. 31320-05)
• Potassium chloride (KCl; Nacalai Tesque, cat. no. 28514-75)
• Disodium hydrogen phosphate 12-water (Na₂HPO₄·12H₂O; Nacalai Tesque, cat. no. 31723-35)
• Potassium dihydrogen phosphate (KH₂PO₄; Nacalai Tesque, cat. no. 28721-55)

• EDTA disodium salt dihydrate (EDTA-Na₂; Dojindo, cat. no. 345-01865)
• 2.5% (vol/vol) Trypsin (10×), no phenol red (Gibco, cat. no. 15090-046)
• Gelatin solution, type B, 2% (wt/vol) in H₂O, tissue-culture grade, sterile, BioReagent, suitable for cell culture (Sigma-Aldrich, cat. no. G1393)
• Xfect Transfection Reagent (Takara Bio, cat. no. 631321)
• Puromycin dihydrochloride from *Streptomyces alboniger*, powder, BioReagent, suitable for cell culture (Sigma-Aldrich, cat. no. P8833)
• PureYield Plasmid Miniprep System (Promega, cat. no. A1222)
• PureYield Plasmid Midiprep System (Promega, cat. no. A2496)
• Collagenase, Type 3, filtered (Worthington Biochemical, cat. no. CLS-3)
• Liquid hydrogen fluoride (Stella Chemifa, cat. no. 021049)
! CAUTION Liquid hydrogen fluoride is very toxic. Avoid its inhalation and contact with the skin and the eyes.
• Beeswax (Nacalai Tesque, cat. no. 03915)
• KSOM (ARK Resource, cat. no. KSOM(KSOMaa))
• Pregnant mare serum gonadotropin (PMSG; ASKA Pharmaceutical, cat. no. PMSG)
• Human chorionic gonadotropin (hCG; ASKA Pharmaceutical, cat. no. hCG)
• Milli-Q water (MQ; Merck, Integral 15 system)

Genotyping

• Proteinase K, recombinant, PCR grade (Sigma-Aldrich, cat. no. 03115836001)
• UltraPure DNase/RNase-free dH₂O (Thermo Fisher, cat. no. 10977-023)
• SYBR Premix Ex Taq GC (TaKaRa, cat. no. no. RR071A)
• KOD FX Neo (Toyobo, cat. no. KFX-201)
• Agarose S (Nippon Gene, cat. no. 318-01195)
• Tris-acetate EDTA buffer (10×; TAE buffer; Nacalai Tesque, cat. no. 35430-61)
• nexttec 1-step DNA Isolation Kit for Tissue & Cells (nexttec Biotechnologie, cat. no. 0635020)

Primers for PCR

• Primer P1: 5'-AGTCTAATGAGAACACAGGGCCAC-3'
• Primer P2: 5'-GGTGAAATGCTTGACTCCTAGACTT-3'
• Primer P3: 5'-GACAGGGTCTCTTACTAGCCTAAAC-3'
• Primer P4: 5'-TCCATCAGAAGCTGGTGCATC-3'
• Puro-F: 5'-CTCGACATCGGCAAGGTGTG-3'
• Puro-R: 5'-GGCCTTCCATCTGTTGCTGC-3'
• Tbp-F: 5'-CCCCCTCTGCACTGAAATCA-3'
• Tbp-R: 5'-GTAGCAGCACAGAGCAAAGCAA-3'

EQUIPMENT

Tool for designing TALEN

• TAL effector-nucleotide targeter (TALEN-NT) 2.0 (<https://tale-nt.cac.cornell.edu/>)

Tools for cell culture and embryo manipulation

• Nunc cell-culture treated multidishes (4-well plates; Thermo Fisher Scientific, cat. no. 176740)
• PureCoat Amine 6-well plate (Becton Dickinson Biosciences, cat. no. 356721)
• PureCoat Amine 24-well plate (Becton Dickinson Biosciences, cat. no. 356723)

- 96-well black with clear flat bottom plate (Corning, cat. no. 353948)
- 96-well clear round-bottom plate (Corning, cat. no. 353077)
- 15-ml conical tube (Corning, cat. no. 352096 or 188271)
- 50-ml conical tube (Corning, cat. no. 352070 or 227261)
- Bottle-top filter (Nalgene, cat. no. 595-3320)
- Millex-GP syringe filter unit, 0.22 μm (EMD Millipore, cat. no. SLGP033RB)
- Multipurpose refrigerated centrifuge (Tomy Seiko, cat. no. LX-130)
- Low-speed refrigerated centrifuge (Tomy Seiko, cat. no. AX310)
- High-speed refrigerated microcentrifuge (Tomy Seiko, cat. no. MX305)
- CO₂ multi-gas incubator (Astec, cat. no. SCA-325DRS or Panasonic, cat. no. MCO-170AICUVH)
- Phase-contrast microscope (Olympus, cat. no. CKX41SF or CKX31)
- Disposable hemocytometer (Funakoshi, cat. no. 521-10)
- pH meter (Horiba, cat. no. F-52)
- Electrode (Horiba, cat. no. 9611)
- Pre-separation filters (20 μm; Miltenyi Biotec, cat. no. 130-101-812)
- Flow cytometer (Furukawa Electric, Perflow Sort model)
- Bio-freezing vessel (Nihon Freezer, Bicell model)
- Glass capillary (Narishige, cat. no. G-1.2)
- Ampule cutter (Maruemu, cat. no. 0510-16)
- Microcaps (Drummond Scientific, cat. no. 1-000-0500)
- Terumo syringe (SS-20ESZ; Terumo, cat. no. 1-4908-06)
- Silicone tube (4 mm × 6 mm; Tigers Polymer, cat. no. SR1554)
- 50° Silicone gasket (2-mm thickness × 37-mm outer diameter × 32-mm inner diameter; Kokugo, custom order)
- Glass slide (no. 1; 40 × 50-mm; Matsunami Glass, cat. no. C040501)
- Microforge (Glassworks, cat. no. F-1200)
- Microgrinder (Narishige, cat. no. EG-401)
- Micropipette puller (Sutter Instrument, cat. no. P-1000 IVF)
- Micromanipulation system (Leica, system combining model nos. DMI6000B, MP-2, and CTR6000)
- CellTram vario (Eppendorf, cat. no. 5176000033)
- Microscope (Leica, system combining model nos. MZ95 and CLS150XD)

Tools for genotyping

- PCR Thermal Cycler Dice Gradient (TaKaRa, cat. no. TP600)
- MicroAmp Optical 384-well reaction plate (Thermo Fisher, cat. no. 4309849)
- MicroAmp Optical adhesive film (Thermo Fisher, cat. no. 4311971)
- QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher, model no. QuantStudio 7 Flex)
- 0.2-ml Hi-8-Dome Cap (TaKaRa, cat. no. NJ301)
- 0.2-ml Hi-8-Tube (TaKaRa, cat. no. NJ300)
- 1.5-ml microtubes, flat-bottom (Watson, cat. no. 131-415C)
- Electrophoresis system (Gel, model no. Mupid-2 Plus)

REAGENT SETUP

Eight-cell-stage embryos For efficient collection of embryos, ICR female mice are superovulated by injecting them with 5 IU PMSG, and then with 5 IU hCG 48 h later. Afterwards, they are mated with male mice immediately. Collect 8-cell-stage embryos from the uteri of 2.5-dpc (days post coitus) pregnant ICR female mice, and cryopreserve them in liquid nitrogen using the vitrification method for long-term storage⁶⁷.

PROCEDURE

Construction and preparation of the targeting (donor) vector and TALEN-expression vectors ● TIMING 1–4 weeks

1 | Construction of TALEN-expression vector. Design the target sequence of TALEN using TALEN-NT 2.0 (ref. 44; <https://tale-nt.cac.cornell.edu/>).

▲ **CRITICAL STEP** For targeting into the ROSA26 locus, we use TALENs that bind to the target sequence (5'-CTGCAACTC-CAGTCTTCTAGAAGATGGCGGGAGTCTTCTGGGCAGGCTTA-3'; TALEN-binding sequences are indicated with italics). The target sequence was designed using TALEN Targeter for C-terminal-truncated (+63) TALENs. As the architecture of TALENs affects the length of the spacer between the TALEN-binding sequences, it is necessary to design the target sequence according to the architecture of the TALEN used.

2 | Construct a pair of TALEN expression vectors (TALEN-L and TALEN-R) using construction kits⁶⁸ available from Addgene (<https://www.addgene.org/TALEN/>). Construct the vectors according to the detailed protocol provided by Addgene.

6-bromoindirubin-3'-oxime (BIO) medium To 425 ml of DMEM (high-glucose, pyruvate), add 75 ml of KSR, 0.1 mM MEM non-essential amino acid solution, 0.1 mM filtered 2-mercaptoethanol, 1,000 U/ml LIF, and 45 μl of 2 mM BIO.

▲ **CRITICAL** Store BIO medium at 4 °C and use it within 1 month.

BIO solution (2 mM) Dissolve 1 mg of BIO powder in 1,403 μl of DMSO.

▲ **CRITICAL** Prepare aliquots of the stock in tightly sealed vials; store them at –20 °C and use within 1 month.

BSA solution (1% or 10% (wt/vol)) Dissolve 0.1 mg or 1 mg BSA powder in 10 ml of PBS (without CaCl₂ or MgCl₂), and filter it using a Millex-GP syringe filter unit. Prepare aliquots of the stock solutions in vials; store them at –20 °C and use them within 1 year.

LIF solution (×1,000 stock solution) Dilute the LIF (10 million units/1 ml) in 9 ml of 1% (wt/vol) BSA. ▲ **CRITICAL** This solution can be stored at 4 °C and should be used within 1 year. Freeze–thaw cycles will reduce its potency.

Filtered 2-mercaptoethanol solution Add 14 μl of 2-mercaptoethanol to 20 ml of MQ, and filter the solution using a Millex-GP syringe filter unit.

▲ **CRITICAL** Store this solution at 4 °C, and use it within 1 week.

3i Medium After thawing the iSTEM, add the included inhibitors and 1,000 units/ml LIF. ▲ **CRITICAL** Store 3i medium at 4 °C and use it within 2 weeks.

mES medium Dissolve the DMEM powder in ~900 ml of MQ, and add 3.7 g of NaHCO₃. Adjust the pH (7.2–7.4) using HCl, and dilute the mixture to 1,000 ml with MQ. After filtering the mixture using a bottle-top filter, add 100 ml of FBS, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acid solution, 0.1 mM filtered 2-Mercaptoethanol, and 1,000 units/ml LIF. ▲ **CRITICAL** Store the mES medium at 4 °C and use it within 1 month.

PBS (without CaCl₂ or MgCl₂) Dissolve 8 g of NaCl, 0.2 g of KCl, 2.89 g of Na₂HPO₄·12H₂O, and 0.2 g of KH₂PO₄ in ~800 ml of MQ. After complete dissolution, adjust the pH (7.2–7.4) using NaOH, and dilute the solution to 1,000 ml with MQ. Sterilize the solution by autoclaving before use. Store the solution at room temperature (23 °C–26 °C) or at 4 °C, and use it within 1 year.

EDTA–PBS Dissolve 8 g of NaCl, 0.2 g of KCl, 2.89 g of Na₂HPO₄·12H₂O, 0.2 g of KH₂PO₄, and 2.2 g of EDTA-2Na in ~800 ml of MQ.

After complete dissolution, adjust the pH (7.2–7.4) using NaOH, and dilute the solution to 1,000 ml with MQ. Sterilize the solution by autoclaving before use. Store the solution at room temperature or at 4 °C, and use it within 1 year.

0.25% (vol/vol) Trypsin Dilute 2.5% (vol/vol) trypsin 10 times with EDTA–PBS. Store the solution at 4 °C for up to a week.

Puromycin solution (2 mg/ml) Dissolve 100 mg of puromycin powder in 50 ml of MQ, and filter the solution using a Millex-GP syringe filter unit. Prepare aliquots of the solution and store them at –20 °C for up to 1 year.

2 mg/ml Collagenase, type 3 Dilute 50 mg of collagenase, type 3 with D-PBS (with CaCl₂ and MgCl₂). After incubating the mixture for 1 h at room temperature, prepare aliquots and store them at –20 °C for up to 1 year.

Freezing medium (2×) Dilute DMSO to 20% (vol/vol) with 3i medium. The solution should be freshly prepared and kept on ice until use.

PROTOCOL

3| *Construction of the targeting (donor) vector.* Clone the regions of 4 kb or more in length on both sides of the TALEN-cleavage site of genomic DNA into arbitrary vectors as the 5'-homology arm and the 3'-homology arms, in the same direction and in the same order as the genomic sequence^{37,69}.

▲ **CRITICAL STEP** Both homology arms must be designed to not be digested by TALEN. The digestion of homology arms can be avoided by designing the inner termini of the homology arms (3-terminal of the 5' homology arm and 5'-terminal of the 3' homology arm) between the recognition sequences of the right and left TALEN.

4| Clone the gene of interest and drug-resistance gene into a position between the homology arms to complete the targeting vector construction. If it is necessary to change the insert genes, it is convenient to use a commercially available Gateway system^{37,70} (Thermo Fisher Scientific).

▲ **CRITICAL STEP** As the direction of cloning of the gene may affect the expression level, adjust the direction according to the purpose of the experiment.

5| *Preparation of vector DNA for transfection.* Prepare a highly purified targeting vector and TALEN-expression vectors by using a commercially available DNA purification kit according to the manufacturer's instructions.

▲ **CRITICAL STEP** For transfection, use highly purified endotoxin-free DNA. We use the PureYield Plasmid Mini/Midiprep System (Promega) to prepare the DNA.

Transfection of ESCs with the targeting vector and TALEN-expression vectors ● TIMING 5 d

6| Add 1 ml of BIO medium to each well of an amine-coated 6-well plate, and then incubate the plate overnight at 37 °C in 5% CO₂ (Fig. 2a).

▲ **CRITICAL STEP** Although pretreatment longer than one night should be avoided, as it tends to cause the colonies to float, it is essential to pretreat the wells' surface with BIO medium for more than 5 h at 37 °C in 5% CO₂.

7| Replace the BIO medium with 2 ml of 3i culture medium 30 min before starting the ESC culture, and then incubate the plates at 37 °C in 5% CO₂. Seed the ESCs at 1 × 10⁵ cells per well, and incubate the culture at 37 °C in 5% CO₂. Each day, replace the 3i medium of the culture with fresh 3i medium (preheated to 37 °C).

▲ **CRITICAL STEP** To avoid exposing ESCs to air during the daily medium-replacement work, exchange only ~90% of the culture medium, rather than all the culture medium.

8| 3 d after starting ES culture, add 1 ml of BIO medium to each well of a new amine-coated 6-well plate, to be used the next day for seeding the transfected ESCs. Then incubate the plate, which contains only BIO medium, overnight at 37 °C in 5% CO₂.

9| Replace the BIO medium (Step 8) with 2 ml of 3i culture medium before initiating transfection. Then incubate the plate at 37 °C in 5% CO₂.

10| *Preparation of suspended ESCs for transfection.* 4 d after starting ES culture, confirm that the cultured ES colonies have a good spherical morphology (Fig. 2b).

▲ **CRITICAL STEP** ES colonies that show a spherical form and have less contact with the dish should be used. ES colonies showing a flat shape should not be used.

11| Aspirate the medium. Add 300 µl of 0.25% trypsin-EDTA and wait until the cells start to detach. This usually takes ~1 min at room temperature.

12| Deactivate the trypsin by adding 700 µl of mES medium containing FBS. Gently pipette the medium up and down several times to generate a single-cell suspension.

13| Harvest the ESCs into a centrifuge tube containing 6 ml of DMEM without serum. Wash the wells with 1 ml of mES medium containing FBS, and collect the wash solution into the tube containing the ESCs.

14| Centrifuge the ESC suspension (190 g, 4 min, 22 °C), remove the medium, and resuspend the pellet in a small amount of 3i medium by gently pipetting several times.

15| Adjust the ESC concentration to 2×10^6 cells/ml, and place the cells on ice until use. We have no experience of the effects of placing the cells on ice for more than 30 min. Thirty minutes is sufficient time to prepare the transfection reagent–DNA complex.

▲ CRITICAL STEP There is no need to rinse the ESCs with PBS (without CaCl_2 or MgCl_2) before trypsinization, as the 3i medium does not include FBS, which inhibits the effect of trypsin. Do not overtrypsinize the cells. In addition, take care to avoid too much pipetting, which can damage the ESCs.

16| *Transfection with Xfect Transfection Reagent.* Prepare the DNA solution and polymer solution according to the manufacturer's instructions as follows: In a microcentrifuge tube, dilute 0.9 μg of a circular targeting vector and 1.2 μg each of TALEN-L and TALEN-R expression vectors with Xfect Reaction Buffer to a final volume of 25 μl . Mix the solution well by vortexing at high speed. In another microcentrifuge tube, add 1.65 μl of Xfect Polymer to 23.35 μl of reaction buffer. Mix the solution well by vortexing at high speed.

▲ CRITICAL STEP For transfection, use a high DNA concentration so that the concentration of the reaction buffer in the reaction solution does not decrease.

17| Add the polymer solution to the DNA solution, and mix well by vortexing at high speed. Incubate the polymer–DNA mixture for 10 min at room temperature.

18| While incubating the polymer–DNA mixture, dispense 150 μl of the cell suspension into a well of a 96-well plate.

19| After incubating the polymer–DNA mixture, add it to the cell suspension in the well. Immediately after this addition, pipette the suspension up and down three times gently to thoroughly mix the polymer–DNA mixture with the cell suspension (**Fig. 2c**).

20| Incubate the ESCs for 15 min at 37 °C in 5% CO_2 , then add 50 μl of 10% BSA, and mix the solution by pipetting. Incubate the ESCs for 5 min at 37 °C in 5% CO_2 .

21| Seed all the cell suspension into the wells prepared in Step 9. Incubate the ESCs for 3 d at 37 °C in 5% CO_2 . Each day, replace the 3i medium in the culture with fresh 3i medium (preheated to 37 °C).

Antibiotic selection of transfected ESCs ● **TIMING 5 d**

22| *2 d after transfection.* On the day before initiating antibiotic selection of the transfected ESCs by puromycin, add 1 ml of BIO medium to each new well of the same or a new amine-coated 6-well plate. Then incubate the plate overnight at 37 °C in 5% CO_2 .

▲ CRITICAL STEP When the automation protocol (Step 36B) is used for single-colony isolation after antibiotic selection, each new well of the same or a new 6-well plate should be pretreated with 1 ml of 1% gelatin (**Fig. 2f**) instead of BIO medium (**Fig. 2e**) in this step.

23| Replace the BIO medium with 1.5 ml of 3i culture medium before initiating the antibiotic-selection step (next step). Then incubate the plate at 37 °C in 5% CO_2 .

▲ CRITICAL STEP If the wells of a plate were pretreated with 1% gelatin instead of BIO medium in Step 22, replace the gelatin in this step.

24| *Antibiotic selection (first).* Aspirate the medium from the well containing transfected ESCs (from Step 21). Add 300 μl of 0.25% trypsin–EDTA to the well, and wait until the cells start to detach.

25| Add 700 μl of mES medium. Pipette the mixture up and down several times gently to generate a single-cell suspension. Filter the ESCs through pre-separation filters (20 μm , Miltenyi Biotec) and harvest them into a centrifuge tube. Wash the well with 1 ml of mES medium, filter the wash liquid, and add it to the ESC-containing tube.

▲ CRITICAL STEP Before initiating the antibiotic selection (Step 28), it is important to dissociate the culture to single cells to reduce the number of false-positive puromycin-resistant cells.

26| Centrifuge the ESC suspension (190 g , 4 min, 22 °C), remove the medium, and resuspend the pellet in 0.5 ml of 3i medium (preheated to 37 °C) by gently pipetting several times.

PROTOCOL

- 27|** Seed the entire ESC suspension into a well containing 1.5 ml of 3i medium, and incubate the cells for 5 h at 37 °C in 5% CO₂.
- 28|** Five hours after seeding the ESCs, replace all the culture medium with 2 ml of 3i medium containing puromycin (1.2 µg/ml). Then incubate the cells for 24 h at 37 °C in 5% CO₂.
- 29|** After 24 h (day 2) of puromycin treatment, replace all the culture medium with 2 ml of 3i medium without puromycin.
- 30|** Replace the medium with fresh 3i medium (preheated to 37 °C), and continue to incubate the cells for 24 h.
- 31|** *Antibiotic selection (second)*. Replace the medium with 2 ml of 3i medium containing puromycin (1.2 µg/ml). Then incubate the cells for 24 h at 37 °C in 5% CO₂.
- 32|** After 24 h of puromycin treatment, replace all the culture medium with 2 ml of 3i medium without puromycin.
- 33|** Each day, replace the medium with 2 ml of fresh 3i medium (preheated to 37 °C), until the ESC colonies are large enough to be picked (~7–8 d after the start of antibiotic selection).

? TROUBLESHOOTING

Single-colony isolation and screening for targeted ESCs ● TIMING 1 d

- 34|** The day before single-colony isolation of the ESC colonies, add 0.5 ml of BIO medium to each well of a new amine-coated 24-well plate, to be used for ESC expansion. Once you know your general targeting efficiency, you can determine the number of colonies to pick. We pick 12–16 colonies per construct.
- 35|** Replace the BIO medium with 1 ml of 3i culture medium before starting the colony picking. Then incubate the plate at 37 °C in 5% CO₂.
- 36|** Dispense single colonies into each well one by one by the conventional protocol (option A, **Fig. 2e**) or the automation protocol (option B, **Fig. 2f**).
- (A) Conventional protocol for single-colony isolation**
- Add 50 µl of trypsin per well to a 96-well round-bottom plate.
 - Replace the culture medium with 1 ml of PBS lacking CaCl₂ and MgCl₂.
▲ **CRITICAL STEP** The activity of trypsin is inhibited by divalent ions.
 - Place the plate under a microscope to select undifferentiated healthy colonies.
 - Using a P20 pipette set to 10 µl, carefully pick a colony without contamination from other colonies, and transfer it into trypsin in the 96-well plate. Continue picking colonies for 3 min, and then add 150 µl of mES medium to each well.
▲ **CRITICAL STEP** Do not use a multichannel pipette to add the mES medium; instead, use a single-channel pipette to add the medium at the same rate as the colonies had been picked into each well. Repeat Step 36A(iv) if the desired number of colonies was not picked within 3 min.
- (B) Automation protocol for single-colony isolation**
- Add 100 µl of collagenase (2 mg/ml) to the culture medium, and incubate the cells for 5 min at 37 °C in 5% CO₂.
▲ **CRITICAL STEP** To apply this method, the ESCs are cultured on gelatinized dishes.
 - Confirm that the cultured ESC colonies are floating and maintaining a spherical morphology (**Fig. 2g**).
 - Collect the entire culture into a conical tube (50 ml).
 - Dilute the culture to an appropriate concentration with PBS (without CaCl₂ or MgCl₂).
 - Using a cell sorter, automatically dispense each floating colony with a diameter of ~100 µm into separate wells of a 96-well round-bottom plate, one by one, using the procedure described in the instrument's instruction manual. We use a commercially available sorter, Perflow Sort (Furukawa Electric).
 - Centrifuge the 96-well plate (190g, 4 min, 22 °C), and then remove the medium using a multichannel pipette.
 - Add 50 µl of trypsin to each well using a multichannel pipette, and incubate the plate for 3 min at room temperature.
 - Add 150 µl of mES medium to each well using a multichannel pipette.
- 37|** Pipette the medium up and down several times using a multichannel pipette to break up the colonies.

38 | To create a sample for screening (Steps 41–45), prepare 100- μ l aliquots from each sample in 8-strip PCR tubes using a multichannel pipette, and place the tubes on ice until use. Then, transfer the remaining ESC suspension (~100 μ l) to each well of the 24-well plate prepared in Step 35, and continue culturing the cells for future use; e.g., stocking, genotyping, and 8-cell injection.

39 | Centrifuge the 8-strip PCR tubes containing the ESCs for screening (2,000*g*, 10 min, 4 °C), and remove the medium using a multichannel pipette set to 90 μ l.

! CAUTION To avoid losing pellets, leave a small amount (~10 μ l) of culture medium.

40 | Add 100 μ l of cold PBS to each tube using a multichannel pipette. Then centrifuge the tubes (2,000*g*, 10 min, 4 °C), and remove the PBS using a multichannel pipette set to 100 μ l.

! CAUTION To avoid losing pellets, leave a small amount (~10 μ l) of culture medium.

■ PAUSE POINT Unless the screening work is started within a few hours, these pellets should be stored in a freezer (–80 °C) until use. Under this condition, the pellet is stable, but it is better to finish the screening (Steps 41–45) before it is time to expand the cells (Step 46), which is usually within 3 d.

41 | Add 50 μ l of proteinase K solution (0.2 mg/ml) to each ESC-containing PCR tube. Then incubate the tube in a thermal cycler under the following conditions: 55 °C for 30 min, followed by 95 °C for 10 min.

42 | Pipette the mixture up and down several times to mix well, and add 5 μ l of the mixture to a new 8-strip PCR tube.

43 | Identify the targeted ESC clones by PCR and agarose gel electrophoresis according to preoptimized conditions (**Fig. 3c**). The conditions for detecting clones targeted at the ROSA26 region are shown below as an example. First, mix the following components in a PCR tube:

Component	Amount (μ l)	Final concentration
Colony lysate	5	
2 × PCR buffer for KOD FX Neo	12.5	1×
2 mM dNTPs	5	0.4 mM
10 μ M Primer P1	0.75	0.36 μ M
10 μ M Primer P2	0.75	0.36 μ M
KOD FX Neo (1 U/ μ l)	0.4	16 mU/ μ l
ddH ₂ O	0.6	
Total	25	

44 | Place the reaction tube in a thermal cycler, and run the following program:

Cycle no.	Denature	Anneal and extend
1	94 °C, 2 min	
2–33	98 °C, 10 s	68 °C, 3 min 50 s

45 | Run the PCR products on a 0.6% (wt/vol in TAE buffer) agarose gel, and confirm the presence of the intended amplicon (**Fig. 3d**).

? TROUBLESHOOTING

Expansion culture and genotyping of targeted clones ● TIMING 12–14 d

46 | Repeat the passaging of cells from Step 38 that were confirmed to have the intended amplicon, until the number of cells of each clone reaches the appropriate cell number for a 6-well cell culture plate.

PROTOCOL

- 47| Seed all the cells on a well of a BIO-treated 6-well plate and continue culturing them.
- 48| On the day before cell freezing (Step 51), add 1 ml of BIO medium to each new well of an amine-coated 6-well plate, into which the ESCs will be seeded (Step 55) to prepare the genomic DNA (Step 62). Then incubate the plate overnight at 37 °C in 5% CO₂. Next, on the day of cell freezing, replace the BIO medium with 2 ml of 3i culture medium before initiating the ESC harvest. Then incubate the plate at 37 °C in 5% CO₂ until use.
- 49| Confirm that the cultured ESCs have a good morphology and have reached a subconfluent state.
- 50| *Preparation of freezing medium.* Freshly prepare the freezing medium (2×) containing 20% DMSO immediately before use, and keep it on ice.
- 51| *Freezing of ESCs.* Aspirate the medium, then add 300 μl of 0.25% trypsin–EDTA, and keep the sample at room temperature until the cells start to detach.
- 52| Deactivate the trypsin by adding 700 μl of mES medium containing FBS. Pipette the medium up and down several times gently to generate a single-cell suspension.
- 53| Harvest the ESCs into a centrifuge tube. Wash the well with 1 ml of mES medium containing FBS, and collect the wash fluid into the tube containing the ESCs.
- 54| Centrifuge the ESC suspension (190g, 4 min, 22 °C), remove the medium, and resuspend the pellet in a small amount of 3i medium by gently pipetting it several times.
- 55| Adjust the concentration of ESCs to 2 × 10⁶/ml. Seed the ESCs (5 × 10⁵ cells/well) into the BIO-coated wells prepared in Step 48.
- 56| Gradually add an equal volume of cold 2× freezing medium to the remaining ESC suspension, and mix the suspension by pipetting it up and down several times.
- 57| Quickly prepare 1-ml aliquots of the cell suspension in freezing medium in labeled cryovials, and place them into a Bicell container (a freezing container provided by Nihon Freezer) precooled to 4 °C. Immediately place the Bicell container into a –80 °C freezer for 1 d, and then transfer the cryovials to a liquid-nitrogen tank for long-term storage.
- **PAUSE POINT** The ES cells can be stored until the start of the injection work; cells can be stored indefinitely in liquid nitrogen.
- 58| Confirm that the cultured ESCs for preparing genomic DNA have reached a subconfluent state.
- 59| *Preparation of genomic DNA.* Aspirate the medium, then add 300 μl of 0.25% trypsin–EDTA, and keep the samples at room temperature until the cells start to detach.
- 60| Deactivate the trypsin by adding 700 μl of mES medium containing FBS. Pipette the medium up and down several times gently to generate a single-cell suspension. Harvest the ESCs into a centrifuge tube. Wash the well with 1 ml of mES medium containing FBS, and collect the wash fluid into the tube containing the ESCs.
- 61| Centrifuge the ESC suspension (190g, 4 min, 4 °C), remove the medium, and wash the pellet with 300 μl of PBS. Centrifuge the ESC suspension (190g, 4 min, 4 °C), and remove the PBS.
- **PAUSE POINT** The ESC pellet can be stored in a –80 °C freezer for up to 1 year before starting the genome purification (Step 62).
- 62| Using a DNA isolation kit, purify the genomic DNA from the ESC pellet. We use the nexttec 1-step DNA Isolation Kit for Tissue & Cells (nexttec Biotechnologie) according to the manufacturer's instructions. Other commercially available products such as the Wizard Genomic DNA Purification Kit (Promega) can also be used.
- 63| *Confirmation of successful targeting into the correct locus.* Genomic PCR is performed using a series of primers that anneal outside the homologous recombination arms and within the inserted cassettes (**Fig. 3a**). The conditions for PCR depend on

the primer sets. Below, we show the conditions for detecting the arms targeting the ROSA26 region as an example. First, mix the following components in a PCR tube:

Component	Amount (μl)	Amount (ng)	Final concentration
Purified genomic DNA		100	4 ng/ μl
2 \times PCR buffer for KOD FX Neo	12.5		1 \times
2 mM dNTPs	5		0.4 mM
10 μM Primer Fw ^a	0.75		0.36 μM
10 μM Primer Rev ^a	0.75		0.36 μM
KOD FX Neo (1 U/ μl)	0.4		16 mU/ μl
ddH ₂ O	– ^b		
Total	25		

^aPrimer sets are as follows: for the left homology arm of the targeted allele—Primers P3 and P4; for the nontargeted allele—Primers P3 and P2. ^bAdjust the total volume of the reaction mixture to 25 μl .

64 | Place the reaction tube into a thermal cycler, and run the following program for the left homology arm of the targeted allele:

Cycle no.	Denature	Anneal	Extend
1	94 °C, 2 min		
2–31	98 °C, 10 s	60 °C, 30 s	68 °C, 4 min

Run the following program for the nontargeted (wild-type) allele:

Cycle no.	Denature	Anneal	Extend
1	94 °C, 2 min		
2–31	98 °C, 10 s	58 °C, 30 s	68 °C, 14 min

65 | Finally, run the PCR products on a 0.6% (wt/vol in TAE buffer) agarose gel, and confirm the presence of the intended amplicon.

66 | *Confirmation of successful targeting with a single copy.* Perform a quantitative PCR assay using the extracted ESC genomic DNA and primers that anneal to the coding sequence of the antibiotic-resistance gene. Below, we show the conditions for detecting the puromycin-resistance gene as an example.

First, mix the following components in a well:

Component	Amount (μl)	Final concentration
Sample genomic DNA ^a (0.2 ng/ μl) ^b	6	0.08 ng/ μl
SYBR Premix Ex Taq GC	7.5	1 \times
ddH ₂ O	1.02	0.4 mM
100 μM Primer-F ^c	0.09	0.36 μM
100 μM Primer-R ^c	0.09	0.36 μM
Reference dye	0.3	2%
Total	15	

^aAs a control, genomic DNA containing one or two copies of the *Puro* gene is used. ^bTo make a standard curve, a dilution series of genomic DNA containing one copy of the *Puro* gene (2, 0.4, 0.08, 0.016, and 0.0032 ng/ μl) is used. ^cPrimer sets are as follows: for the *Puro* gene—Primer Puro-F and Puro-R; for *Tbp*—Primer Tbp-F and Primer Tbp-R.

PROTOCOL

▲ **CRITICAL STEP** Although duplicate reactions are sufficient to create the standard curve, triplicate reactions for each sample are recommended.

67| Place the reaction plate into a QuantStudio 7 Flex Real-Time PCR System, and run the following program:

Cycle no.	Denature	Anneal	Extend
1	95 °C, 60 s		
2–46	95 °C, 10 s	60 °C, 50 s	
47	95 °C, 15 s	60 °C, 60 s	95 °C, 15 s

68| Normalize the *Puro* value to the *TATA box-binding protein (Tbp)* value.

▲ **CRITICAL STEP** The *Puro* value normalized to the *Tbp* value should be '1' if a single copy of the inserted cassette was harbored in the genome. Notably, cells that are confirmed to have an insertion with 1 or 2 gene copies can provide a useful sample for control experiments (Fig. 3e).

? TROUBLESHOOTING

Injection of ESCs into 8-cell-stage embryos ● TIMING 6 d

69| After genotyping is complete, add 1 ml of BIO medium to each of 2 wells of an amine-coated 6-well plate. Then incubate the plate overnight at 37 °C in 5% CO₂.

70| The next day, replace the BIO medium with 2 ml of 3i medium at least 30 min before starting to thaw the ESCs, and then incubate the plate at 37 °C in 5% CO₂.

71| *Thawing of ESCs.* Remove the vial from the liquid nitrogen, and warm it quickly in a 37 °C water bath.

72| When the ice crystals have almost disappeared, use a pipette to transfer the cell suspension aseptically to a 50- (or 15-)ml tube containing 10 ml of mES medium to dilute the DMSO.

73| Centrifuge the ESC suspension (190g, 4 min, 22 °C), remove the medium, and resuspend the pellet in 0.5 ml of 3i medium by gently pipetting it several times.

74| Seed 0.25 ml of the ESC suspension into each well containing 3i medium (Step 70). Incubate the ESCs for 3 d at 37 °C in 5% CO₂.

75| Each day, replace the 3i medium used for the culture with fresh 3i medium (preheated to 37 °C).

76| *Preparation of ESCs (day 3).* Replace the 3i medium of subconfluent ESCs with 2 ml of 3i medium (preheated to 37 °C), at least 2 h before injection.

▲ **CRITICAL STEP** Prepare at least 1,000 times more subconfluent ESCs than the number of embryos to be injected. The most important point is to prepare ES cells in a subconfluent state, as ESCs in the growing phase tend to contribute more to the chimeric mouse. In addition, if there are few cells, this can cause difficulty in manipulating the ESCs during preparation and injection, so we recommend that you prepare at least 1,000 times more ESCs than the number of embryos to be injected. These conditions can be achieved by following this protocol.

77| Aspirate the medium, add 300 µl of 0.25% trypsin–EDTA, and wait until the cells start to detach.

78| Deactivate the trypsin by adding 700 µl of mES medium containing FBS. Pipette the medium up and down several times gently to generate a single-cell suspension. Harvest the ESCs into a centrifuge tube. Wash the well with 1 ml of mES medium containing FBS, and collect the wash fluid into the tube containing the ESCs.

79| Centrifuge the ESC suspension (190*g*, 4 min, 22 °C), remove the medium, and resuspend the pellet with a small amount of 3i medium by gently pipetting it several times.

▲ CRITICAL STEP It is important to dissociate the ESCs to single cells for ease of injection, although care needs to be taken, as too much pipetting tends to damage the ESCs. Resuspending the cells in an excessive amount of medium may make it difficult to collect the ESCs for injection.

80| Place the injection and holding pipettes into the micromanipulator setup (**Fig. 4a**). Fill both pipettes with mineral oil by applying pressure with the CellTram vario (Eppendorf). Take extra care to avoid air bubbles.

▲ CRITICAL STEP Air bubbles in the tubing and pipettes make it difficult to control the handling of embryos and the movement of ESCs.

81| Prepare an injection plate in advance. Apply a 50° silicone gasket (Kokugo) to a 40 × 50-mm glass slide (Matsunami Glass) with beeswax (Nacalai Tesque) (**Fig. 4b**).

82| Place three drops on the injection plate (**Fig. 4b**). The first drop should contain only ES culture medium, the second is the injection drop (ES culture medium containing 8-cell-stage embryos), and the third is the ESC drop (ES culture medium containing ESCs). Cover these drops with mineral oil.

▲ CRITICAL STEP It is necessary to use precooled medium for all three drops. Otherwise, the ESCs will start aggregating and adhering to the bottom of the injection plate.

83| Move the tips of both pipettes into the mES medium drop, and collect a small amount of mES medium.

▲ CRITICAL STEP At this time, both pipettes should be properly aligned with each other and should be parallel to the plate surface.

84| Move the injection pipette into the ESC drop, and collect all the ESCs to inject them in one batch (**Supplementary Video 1**).

▲ CRITICAL STEP For easy injection, the ESCs should be collected into the pipette with as little space as possible between them (**Fig. 4c**). This can be achieved by using an injection pipette with a suitable inner diameter (17–20 μm). If the inner diameter is too wide, it is difficult to control the ESCs. If the inner diameter of the injection pipette is smaller than an ESC, the ESCs will be physically damaged, which will impair their effectiveness for chimera mouse production.

85| Move both the injection and holding pipettes into the injection drop. Hold an 8-cell-stage embryo with the holding pipette, and insert the injection pipette into the embryo.

▲ CRITICAL STEP Compaction-stage embryos can also be used for the injection. Although the details for the embryo-preparation methods are omitted here, the use of frozen embryos is extremely convenient for injection.

86| Inject 10–30 ESCs into each embryo to fill the perivitelline space (**Fig. 4d; Supplementary Video 2**).

▲ CRITICAL STEP The number of injected ESCs should be adjusted according to the size of the perivitelline space. Avoid injecting an excessive number of ESCs, which causes the zona pellucida to expand. Ideally, one set of injections should be finished in 30 min to 1 h. The number of embryos used for one set of injections should be decided upon depending on the skill of the operator. In our laboratory, 100 embryos are injected within 30 min by one operator.

87| Culture the injected embryos in KSOM-medium drops covered with mineral oil until the next day.

88| Move the (blastocyst) embryos to a KSOM-medium drop that is not covered with mineral oil before transfer.

▲ CRITICAL STEP It is necessary to remove the mineral oil from the surface of the glass capillary used for embryo transfer. Mineral oil in the uterus can disturb embryonic development.

89| Transfer the embryos into the uteri of 2.5-dpc pseudopregnant ICR female mice.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
33 (transfection)	Low number of colonies	Poor-quality DNA used for transfection Low concentration of DNA used for transfection Insufficient TALEN activity	Prepare new DNA for transfection. For transfection, use highly purified endotoxin-free DNA. We use the PureYield Plasmid Mini/Midiprep System (Promega) to prepare the DNA. Concentrate the DNA by ethanol precipitation, and suspend it in Xfect Reaction Buffer. At least half of the reaction solution used to prepare DNA-Xfect complexes must be occupied by Xfect Reaction Buffer. Validate the nuclease activity of TALEN by single-stranded-annealing assays with luciferase ⁸ . The TALEN for ROSA26 used in this report can be used as a positive control for comparing activities
45 (screening of KI ESCs)	Low percentage of correct KI ESCs	Inefficient transfection Insufficient activity of TALEN Insufficient selection by puromycin	Refer to comments for Step 33 Refer to comments for Step 33 Confirm the puromycin concentration and treatment duration. We used a puromycin-resistance gene that is expressed in a PGK promoter-dependent manner. If different promoters are used, optimization of the conditions may be necessary. Completely dissociate ESCs into single cells before starting antibiotic selection
68 (genotyping of KI ESCs)	High percentage of random integration	Contamination by linearized targeting vector Puromycin-selection pressure is too high	Confirm the form of the targeting vector by electrophoresis, and use a circular targeting vector. The linearization of targeting vectors increases the frequency of random integration. Confirm the puromycin concentration and treatment duration. We used a puromycin-resistance gene that is expressed in a PGK promoter-dependent manner. If different promoters are used, optimization of the conditions may be necessary
89 (transfer of the injected embryos)	Low numbers of 2.5-dpc pseudo-pregnant mice	Unknown causes	Transfer 8-cell-stage embryos to the oviducts of 0.5-dpc pseudo-pregnant mice. Note that the number of pups may decrease

● TIMING

Steps 1–5, construction and preparation of the targeting (donor) vector and TALEN-expression vectors: 1–4 weeks
 Steps 6–21, transfection of ESCs with the targeting vector and TALEN-expression vectors: 5 d
 Steps 22–33, antibiotic selection of transfected ESCs: 5 d
 Steps 34–45, single-colony isolation and screening for targeted ESCs: 1 d
 Steps 46–68, expansion culture and genotyping of targeted clones: 12–14 d
 Steps 69–89, injection of ESCs into 8-cell-stage embryos: 6 d

ANTICIPATED RESULTS

As a reference for the results of the KI experiment, we have provided typical data for inserting a targeting vector containing 8-kb and 4-kb arms into the ROSA26 region³⁷. Using 0.9 µg of the targeting vector under our transfection conditions, several hundred puromycin-resistant colonies were obtained, and >80% of the colonies bore the target gene inserted into the correct region (**Fig. 3d**).

The percentage of total pups that were 100% ES mice obtained from ESCs cultured in 3i medium on a BIO-pretreated amine-coated dish is usually 80% or more (**Fig. 2h**)³⁷. This percentage tends to be better than when ESCs are cultured on feeder cells³². Typically, pups are obtained from >20–30% of the embryos transferred into the uteri of pseudopregnant mice^{32,37}; so when 80 embryos are transferred, >16 100% ES mouse pups are usually obtained. After the KI work, some clones sometimes lose their ES mouse competency. As a reference for the ES mouse competency of ES clones after the KI procedures, we provide typical data for the ES-mouse competency of *Cry1* KI-rescue clones³⁷ in **Supplementary Table 1**. After the KI procedures, ~50% (29/59) of the ES clones maintained a pup rate (the number of pups per number of transferred embryos) of 10% or more. In addition, 100% ES mice could be obtained from ~50% (31/59) of the clones. These results show that 100% ES mice can be obtained by using 2 or 3 KI ESC clones for each targeting construct in the 8-cell injection.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS H.R.U., H.U. and H.K. designed the study and wrote the manuscript. H.U. developed, improved, and performed most of the protocols related to knock-in ESC establishment. H.K. developed most of the 8-cell injection protocol. All authors discussed the results and commented on the manuscript text.

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