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# An improved Red/ET recombineering system and mouse ES cells culture conditions for the generation of targeted mutant mice

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**Abstract:** Targeted mutant mice generated on a C57BL/6 background are powerful tools for analysis of the biological functions of genes, and gene targeting technologies using mouse embryonic stem (ES) cells have been used to generate such mice. Recently, a bacterial artificial chromosome (BAC) recombineering system was established for the construction of targeting vectors. However, gene retrieval from BACs for the generation of gene targeting vectors using this system remains difficult. Even when construction of a gene targeting vector is successful, the efficiency of production of targeted mutant mice from ES cells derived from C57BL/6 mice are poor. Therefore, in this study, we first improved the strategy for the retrieval of genes from BACs and their transfer into a DT-A plasmid, for the generation of gene targeting vectors using the BAC recombineering system. Then, we attempted to generate targeted mutant mice from ES cell lines derived from C57BL/6 mice, by culturing in serum-free medium. In conclusion, we established an improved strategy for the efficient generation of targeted mutant mice on a C57BL/6 background, which are useful for the *in vivo* analysis of gene functions and regulation.

**Key words:** C57BL/6, ES cells, gene retrieval, gene targeting, KSR

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## Introduction

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Targeted mutant mice are powerful tools for analysis of the biological functions of genes, and many investigators have generated knockout (KO), conditional knockout (cKO) and knockin (KI) mice by the gene targeting technology using mouse embryonic stem (ES) cells. Construction of a gene targeting vector is required for the generation of targeted mutant mice. Recently, several recombinase-based systems have been developed that have significantly simplified the construction of gene targeting vectors [33, 34]. A bacterial artificial chromo-

some (BAC) recombineering system involving DNA cloning and engineering has also been developed for the construction of such targeting vectors [33]. The first step in the construction of a gene targeting vector from a BAC clone is gene retrieval into a DT-A plasmid by Red/ET recombination. However, the efficiency of gene retrieval from BAC clones remains poor, and the toxicity of eukaryotic genomic sequences causes instability of the plasmid in *Escherichia coli* [5, 16, 31].

The C57BL/6 strain of mice is one of the best characterized for gene targeting, and was also the reference strain for establishing the mouse genome sequence [29,

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Supplementary Tables and Figures: refer to J-STAGE: <https://www.jstage.jst.go.jp/browse/exanim>

30]. This strain is widely used in a variety of biomedical fields, and in fact, the JAX Mice Database (<http://jax-mice.jax.org/>) includes many gene-targeted mouse lines with the C57BL/6 genetic background, including those expressing Cre recombinase, which is used for generating tissue-specific KO mice. Furthermore, large-scale mouse mutagenesis projects were organized by the International Knockout Mouse Consortium to select inbred C57BL/6 strain-derived ES cells for producing mutations of all the protein-coding genes in the mouse [3, 10]. Thus, the genetic background of the C57BL/6 strain is extremely useful for the generation of targeted mutant mice. However, for reasons that are not entirely clear, it remains difficult to acquire chimeric mice with confirmed germline competency on this background [2, 38, 42, 43]. A one of the technology that has been used to overcome this problem is the straightforward and efficient clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system for genome editing both *in vivo* and *in vitro* [6, 11, 13, 19, 27, 36]. This system has been demonstrated to cause double-strand breaks in mammalian cells, and to be useful for the generation of KO mice [41]. However, it was still difficult to produce cKO mice and KI mice of the inserted cDNA, as off-target mutagenesis frequently occurs in the genomic DNA when using the CRISPR/Cas9 system [12, 17, 35, 44].

We here describe an improved method to generate targeted mutant mice on a C57BL/6 background. The method involves improved gene retrieval from a BAC clone for the construction of a gene targeting vector, use of a lower voltage for electroporation, and use of a serum-free medium (KSR medium), which is widely available for culture. These improvements enabled more efficient generation of targeted mutant mice on a C57BL/6 genetic background and are expected to promote better use of the publicly available C57BL/6 strain ES cell resources by the biomedical research community.

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## Materials and Methods

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### *Generation of the gene retrieval vector*

A gene retrieval vector was constructed by isolating a genomic region of the *Wapl* gene from the BAC clone RP23-478G5 (Life Technologies Japan, Tokyo, Japan) and transferring it into the pMCS.DT-A plasmid (Addgene). Another gene retrieval vector was constructed with a genomic region of the *Pgr* gene that was isolated

from the BAC clone RP23-23L9 (Life Technologies Japan). The two end homologies (5' homology arm, *NotI/SacII* fragment; 3' homology arm, *SacII/SacI* fragment) were amplified by polymerase chain reaction (PCR) using the appropriate primer pairs (Supplementary Table S1) and each BAC clone as the template. The two amplified sequences were ligated into the pMCS.DT-A plasmid using Ligation High, ver.2 (Toyobo, Osaka, Japan). Ligation between the two end homologies and the pMCS.DT-A plasmid was confirmed by PCR using the primer pairs shown in Supplementary Table S1. Then, each plasmid DNA was linearized by digestion with *SacII*, and the phosphates were removed from the ends of the DNA to prevent religation of the linearized plasmid DNA using calf intestinal alkaline phosphatase (Takara Bio, Shiga, Japan). These plasmids were then used as the gene retrieval vectors.

### *Construction of the *Wapl* gene cKO targeting vector*

For construction of the *Wapl* gene cKO targeting vector, one loxP site was inserted upstream of exon 3 and another was inserted downstream of exon 4 to generate a frameshift by Cre-mediated deletion of exon 3 and 4 in the targeted *Wapl* gene region. The detailed protocol for the generation of this vector is explained in Supplementary methods.

### *Animals*

C57BL/6J and C57BL/6N mice were purchased from Charles River Laboratories, Japan (Kanagawa, Japan). ICR and pseudopregnant recipient mice were purchased from Sankyo Labo Service Corporation (Tokyo, Japan). The animals were housed in cages (five animals per cage) and provided access to water and food (FR-1; Funabashi Farm, Chiba, Japan) *ad libitum*. The animal rooms were maintained in a temperature-controlled (21°C–25°C) and light-controlled (12L:12D cycle, lights on at 0800 h) environment. All of the mice were bred and maintained under specific-pathogen-free conditions. All animal care and procedures that were performed in this study conformed to the guidelines for animal experiments of Tokyo Medical University, and they were approved by the Animal Research Committee of Tokyo Medical University.

### *Derivation and culture of mouse ES cells*

ES cells derived from C57BL/6J mice were established using standard methods [32], with some modifica-

tions in the duration of culture. Blastocysts collected at 3.5 days postcoitus (dpc) were cultured on 96-well tissue culture plates with a mouse embryonic fibroblast (MEF) feeder layer in KSR medium, which consisted of high-glucose DMEM (Gibco, Frederick, MD) supplemented with 15% Knockout-Serum-Replacement (Gibco), 2 mM glutamine (Gibco), 1 mM sodium pyruvate (Gibco), 0.1 mM non-essential amino acids (Gibco), 0.1 mM 2-mercaptoethanol (Nakalai Tesque, Kyoto, Japan) and 1,000 U/ml LIF (Merck Millipore, Darmstadt, Germany). After the emergence of primary ES cells, the cells were passaged three more times in KSR medium, and then frozen in liquid nitrogen for cryopreservation when they reached 70%–80% confluence (Supplementary Fig. S1A).

In the case of ES cells cultured on a MEF feeder layer in serum-containing medium (Figs. 2A and 3A), the medium consisted of high-glucose DMEM supplemented with 15% serum (Gibco), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, and 1,000 U/ml LIF. Mouse ES cells were passaged in serum-containing medium, when they reached 70%–80% confluence.

#### *Chromosome number analysis*

Chromosome number analysis of the ES cells was performed as described previously [39]. Well-spread metaphases were identified by microscopy, and the chromosome numbers were counted on the micrographs.

#### *Alkaline phosphatase staining and immunocytochemical analysis*

ES cells were fixed with 4% paraformaldehyde in phosphate buffer solution (Wako, Osaka, Japan). Alkaline phosphatase activity was detected using a commercial ES Cell Characterization Kit (Merck Millipore), as described previously [26]. Immunostainings for SSEA1 and Oct-4 were performed using the ES cell sample marker kit (Merck Millipore). The FITC-conjugated goat anti-mouse IgM antibody, AP128F (Merck Millipore) was used as the secondary antibody for the detection of SSEA1, and the Cy3-conjugated goat anti-mouse IgM antibody, AP128C (Merck Millipore) was used as the secondary antibody for the detection of Oct-4. Stained ES cells were observed under a fluorescence microscope (KEYENCE, Osaka, Japan).

#### *Isolation of ES clones with homologous recombination by electroporation*

ES cells ( $4 \times 10^7$ ) in HEPES-buffered salt buffer were transfected with 120  $\mu\text{g}$  of the linearized targeting vector in a 4-mm electrode gap cuvette ( $1 \times 10^7$  cells, 30  $\mu\text{g}$  of linear DNA, in a total volume of 700  $\mu\text{l}$ /cuvette) by electroporation using Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA). At 10 min after the transfection, the ES cells were plated onto a feeder layer. Selection with 300  $\mu\text{g}/\text{ml}$  of G418 (Gibco) was started 24 h after the electroporation. After 7–9 days of selection, ES cell colonies were picked up onto a 96-well tissue culture plate with a feeder layer. All of the colonies were replicated for cryopreservation and subjected to PCR and Southern blot analysis.

#### *Validation of homologous recombination*

PCR-based screening of the target *Wapl* gene ES cell clones was performed using the internal screening\_F4 primer (TGCCTGCTTGCCGAATATCA) and the external screening\_R4 primer (TGGACCACAATCACCACGTT) to detect the 2.6-kb PCR fragment. PCR-positive clones were confirmed by the presence of the 5' loxP and 3' loxP sites (Supplementary Table S2). Moreover, the PCR-positive clones were subjected to Southern blot analysis using the 3' external probe (500-bp) and to *ScaI* digestions for confirmation of 3' integration. The Neo insert was confirmed by *XhoI* digestion of genomic DNA (10  $\mu\text{g}$ ) and using Neo internal probe (804-bp). After digestion, the genomic DNA was separated by electrophoresis on a 0.8% agarose gel and blotted on to a nylon membrane (Pall corporation, Washington, NY) before hybridization with the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science, Mannheim, Germany). Blotting with the 3' external probe yielded a 5.0-kb fragment corresponding to the wild-type allele, and a 6.6-kb fragment corresponding to the mutant allele. Furthermore, blotting with the Neo internal probe yielded a 6.2-kb fragment corresponding to the mutant allele.

The 3' external probe was generated from a 500-bp PCR fragment using the ex3\_7\_F primer (TGATGTCA-CAGCCAAGAATT) and ex3\_7\_R primer (CAAAAC-TATTAGCTGCAAGT). The Neo probe was generated from an 804-bp PCR fragment using the Neo\_F1 primer (TCAGAAGAACTCGTCAAGAAG) and Neo\_R1 primer (ATGGGATCGGCCATTGAACA).

### Generation of chimeric mice

ES cell clones containing the target *Wapl* gene were aggregated for the generation of chimeric mice. The cell clones were placed on a MEF feeder layer 2 days before embryo manipulation. The propagated ES cells were dissociated with 0.05% trypsin just before the aggregation. Eight-cell-stage-embryos were collected at 2.5 dpc from female ICR mice that had been superovulated by intraperitoneal (i.p.) injection of 5.0 IU of equine chorionic gonadotropin (Serotropin; ASKA Pharmaceutical, Tokyo, Japan) at 1600 h, followed by injection of 5.0 IU of human chorionic gonadotropin (Gonadotropin 3000; ASKA Pharmaceutical) 48 h later and then mated naturally with male ICR mice. The zona pellucida was removed by treatment with acid Tyrode's solution (Sigma-Aldrich, St. Louis, MO). Fifteen to twenty ES cells were aggregated with each zona-free embryo and cultured overnight in micro-drops of KSOM medium (ARK Resource, Kumamoto, Japan) under mineral oil at 37°C, 5% CO<sub>2</sub>, and 95% humidity. The chimeric embryos were transferred at 2.5 dpc into the uterine horns of pseudopregnant female ICR mice (Sankyo Labo Service Corporation). Chimeric mice were identified 20 days after birth by their black eyes and their black coat color. Chimeric males showing germline transmission were crossed with C57BL/6 females.

### Statistics

Data are expressed as average values  $\pm$  SEM. The Student's *t*-test was used to statistically analyze the differences between two groups. A *P*-value of less than 0.05 was considered to indicate a significant difference between two groups.

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## Results

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### Gene retrieval from a BAC clone by Red/ET recombination

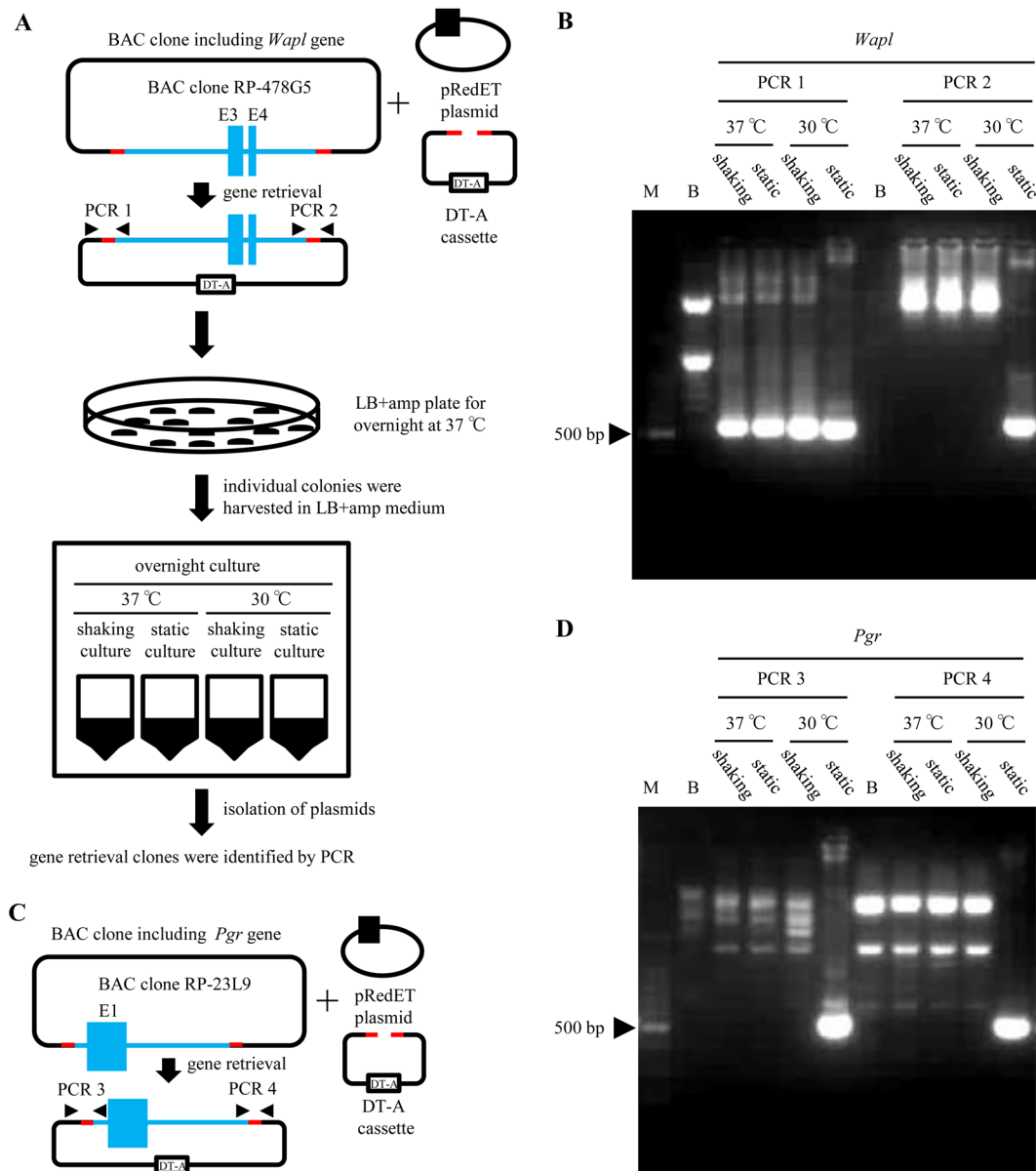
For the construction of a conditional *Wapl* gene targeting vector, we first retrieved a region of the *Wapl* gene from a BAC clone and inserted it into the DT-A cassette by Red/ET recombination (Fig. 1A). Gene retrieval from BAC clones are often unsuccessful, because of plasmid instability in *E. coli* owing to the presence of an unidentified promoter that produces a toxic peptide from eukaryotic genomic sequences [5, 16, 31]. Therefore, the challenge was how to maintain maximum plasmid stability in *E. coli* after gene retrieval. A previous study showed that the number of proteins synthesized is dra-

matically reduced in *E. coli* when cultured at low temperatures [20]. Moreover, static culture effectively suppressed the growth of ground beef microflora while allowing the healthy growth of *E. coli* [4]. Thus, we analyzed the culture conditions (shaking or static culture and high or low temperatures) that are the most suitable for obtaining optimal plasmid stability in *E. coli* after gene retrieval (Fig. 1A). Furthermore, to identify the gene retrieval clone, we performed PCR analysis. A 530-bp fragment including the targeted *Wapl* gene was detected upon both shaking and static culture at 37°C and 30°C by PCR 1. In contrast, a 588-bp fragment was detected in the static culture at 30°C by PCR 2 (Fig. 1B, Supplementary Table S1).

We next attempted to validate the gene retrieval of another gene, as a confirmatory experiment: genomic DNA from the *Pgr* gene was retrieved from a BAC clone and transferred into a DT-A cassette by Red/ET recombination (Fig. 1C). In the case of the *Pgr* gene, a 539-bp fragment was detected in static culture at 30°C by PCR 3. Furthermore, a 524-bp fragment was detected in static culture at 30°C by PCR 4 (Fig. 1D, Supplementary Table S1). These results showed that gene retrieval was accomplished for both genes by static culture at 30°C.

### Efficient gene retrieval from a BAC clone by static culture at a low temperature

To examine the efficiency of gene retrieval from a BAC clone by Red/ET recombination, we compared the rate of gene retrieval between shaking and static culture at high and low culture temperatures. For the case of the *Wapl* gene, following culture at 37°C after colony harvesting, the gene was not retrieved from any of the 500 colonies after either shaking or static culture (0.0%; Table 1). By contrast, following shaking culture at 30°C after colony harvesting, the gene was retrieved from two colonies out of the 50 colonies tested (4.0%; Table 1). Interestingly, the gene was retrieved from 40 colonies out of 50 colonies after static culture (80.0%; Table 1). Next, for the *Pgr* gene, following culture at 37°C after colony harvesting, the gene could not be retrieved from any of the 100 colonies after either shaking or static culture (0.0%; Table 1). In contrast, following static culture at 30°C after colony harvesting, the gene was retrieved from 44 colonies out of 50 colonies (88.0%; Table 1), although no positive colonies (0.0%; Table 1) were obtained with shaking culture. These results indi-



**Fig. 1.** Strategy for gene retrieval using the Red/ET recombining system. (A) Exon 3 and exon 4 of the *Wapl* gene were isolated from an RP-478G5 BAC clone and transferred to a DT-A cassette using the Red/ET recombining system; gene retrieval was attempted by shaking or static culture at 37°C or 30°C. (B) Genotype analysis by PCR. Subcloning by shaking culture at 37°C and 30°C yielded a 530-bp fragment amplified by PCR 1. On the other hand, subcloning by static culture at 30°C yielded a 588-bp fragment amplified by PCR 2. M, marker; B, BAC clone used as a negative control. (C) Exon 1 of the *Pgr* gene was isolated from an RP-23L9 BAC clone and transferred to the DT-A cassette using the Red/ET recombining system; gene retrieval was attempted by shaking and static culture at 37°C and 30°C. (D) PCR analysis of the genotype. Subcloning by static culture at 30°C yielded a 539-bp fragment amplified by PCR 3 and a 524-bp fragment amplified by PCR 4. M, marker; B, BAC clone used as a negative control.

cated that static culture at 30°C markedly improved the efficiency of gene retrieval from a BAC clone in *E. coli*.

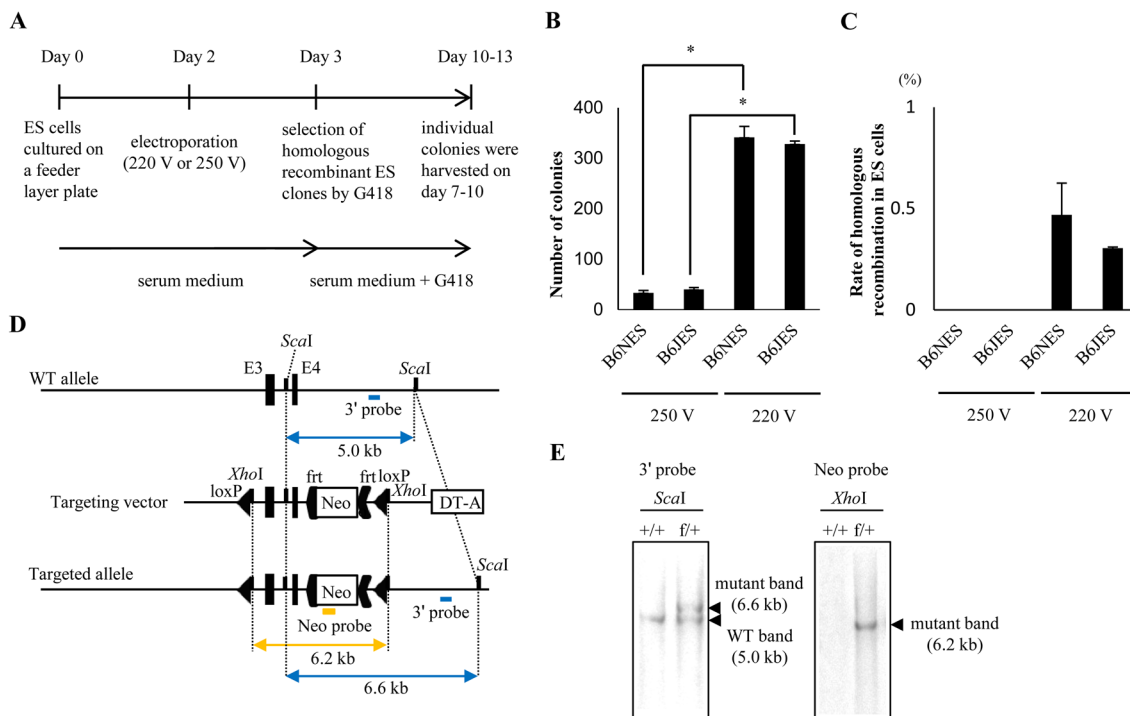
*Electroporation of constructs into C57BL/6 strain ES cells for the generation of conditional Wapl KO mice*

Using our newly established ES cell lines (Supplementary Fig. S1A-G), we next attempted to generate

**Table 1.** Efficiency of gene retrieval by Red/ET recombination

Target gene	Culture temperature	Culture method	No. of colonies tested	No. of colonies with the target gene (%) <sup>a</sup>
<i>Wapl</i>	37°C	shaking	500	0 (0.0) <sup>b,d</sup>
	37°C	static	500	0 (0.0) <sup>b,e</sup>
	30°C	shaking	50	2 (4.0) <sup>c,d</sup>
	30°C	static	50	40 (80.0) <sup>c,e</sup>
<i>Pgr</i>	37°C	shaking	100	0 (0.0) <sup>f,h</sup>
	37°C	static	100	0 (0.0) <sup>f,i</sup>
	30°C	shaking	50	0 (0.0) <sup>g,h</sup>
	30°C	static	50	44 (88.0) <sup>g,i</sup>

<sup>a</sup>No. of colonies with the target gene/no. of colonies tested. Fisher exact test was used for pairwise comparisons, as indicated by superscripts: <sup>b,f,h</sup> $P=1.0000$ ; <sup>c,e,g,i</sup> $P=0.0000$ ; <sup>d</sup> $P=0.0081$ .



**Fig. 2.** Generation of C57BL/6-strain ES cells containing a homologous recombination. (A) Experimental design to examine the optimal voltage for electroporation. (B) Number of ES colonies after electroporation. The average number was 33 for C57BL/6N ES colonies and 40 for C57BL/6J ES colonies after electroporation at 250 V, and 341 for C57BL/6N ES colonies and 328 for C57BL/6J ES colonies after electroporation at 220 V. \* $P < 0.01$ . (C) Rate of homologous recombination in the ES cells after electroporation. The average rate was 0.00% for C57BL/6N ES colonies and C57BL/6J ES colonies after electroporation at 250 V, 0.47% for C57BL/6N ES colonies and 0.31% for C57BL/6J ES colonies after electroporation at 220 V. \* $P < 0.01$ . (D) The structure of the murine *Wapl* gene and the targeted locus are shown. Genotyping by Southern blot analysis using the 3' external probe on *ScaI*-digested genomic DNA, and internal Neo probe on *XhoI*-digested genomic DNA. The blue arrowheads indicate band sizes of 5.0-kb (wild-type) and 6.6-kb (target) in the *ScaI*-digested genomic DNA using the external 3' probe. The orange arrowhead shows a fragment of 6.2-kb (target) in the *XhoI*-digested genomic DNA detected using the Neo internal probe. (E) Southern blot analysis of genomic DNA to confirm homologous recombination in the ES cells lines on a C57BL/6 strain background.

genetically modified mice on a C57BL/6J background. Specifically, we attempted to generate conditional *Wapl* gene KO mice on a C57BL/6J background, and towards that objective, we selected C57BL/6J ES-#2, which

yielded the largest number of 100% F0-chimeric mice, which confirms germline transmission, among the four newly established ES cell lines (Supplementary Table S3). For comparison, we also generated, in parallel, the

**Table 2.** Generation of germline-competent chimeric mice by induction of homologous recombination in ES cells

ES cell line	ES culture medium	No. of transferred chimeric embryos	No. of total pups (%) <sup>a</sup>	No. of colored F0-chimeric mice (%) <sup>b</sup>	No. of mice with a high contribution (> 70% chimerism) of ES cells <sup>c</sup> (%) <sup>b</sup>	Rate of GLT <sup>d</sup> in low contribution (< 70%) F0-chimeric mice	Rate of GLT <sup>d</sup> in high contribution F0-chimeric mice
B6NES	Serum medium	140	12 (8.6) <sup>e</sup>	3 (25.0) <sup>f</sup>	0 (0.0) <sup>g</sup>	0/3	0
	KSR medium	245	38 (15.5) <sup>e</sup>	30 (78.9) <sup>f</sup>	28 (73.7) <sup>g</sup>	0/2	25/28
B6JES	Serum medium	142	18 (12.7) <sup>h</sup>	5 (27.8) <sup>i</sup>	0 (0.0) <sup>j</sup>	0/5	0
	KSR medium	143	29 (20.3) <sup>h</sup>	22 (75.9) <sup>i</sup>	17 (58.6) <sup>j</sup>	0/5	16/17

<sup>a</sup>No. of total pups/no. of transferred chimeric embryos. <sup>b</sup>No. of F0 chimeras/no. of total pups. <sup>c</sup>mice that show coat color contribution of the aggregated ES cells in the host mouse. <sup>d</sup>GLT, germline transmission. Fisher exact test was used for pairwise comparisons, as indicated by superscripts: <sup>e</sup> $P=0.0588$ ; <sup>f</sup> $P=0.0012$ ; <sup>g,h</sup> $P=0.0000$ ; <sup>i</sup> $P=0.1099$ ; <sup>j</sup> $P=0.0021$ .

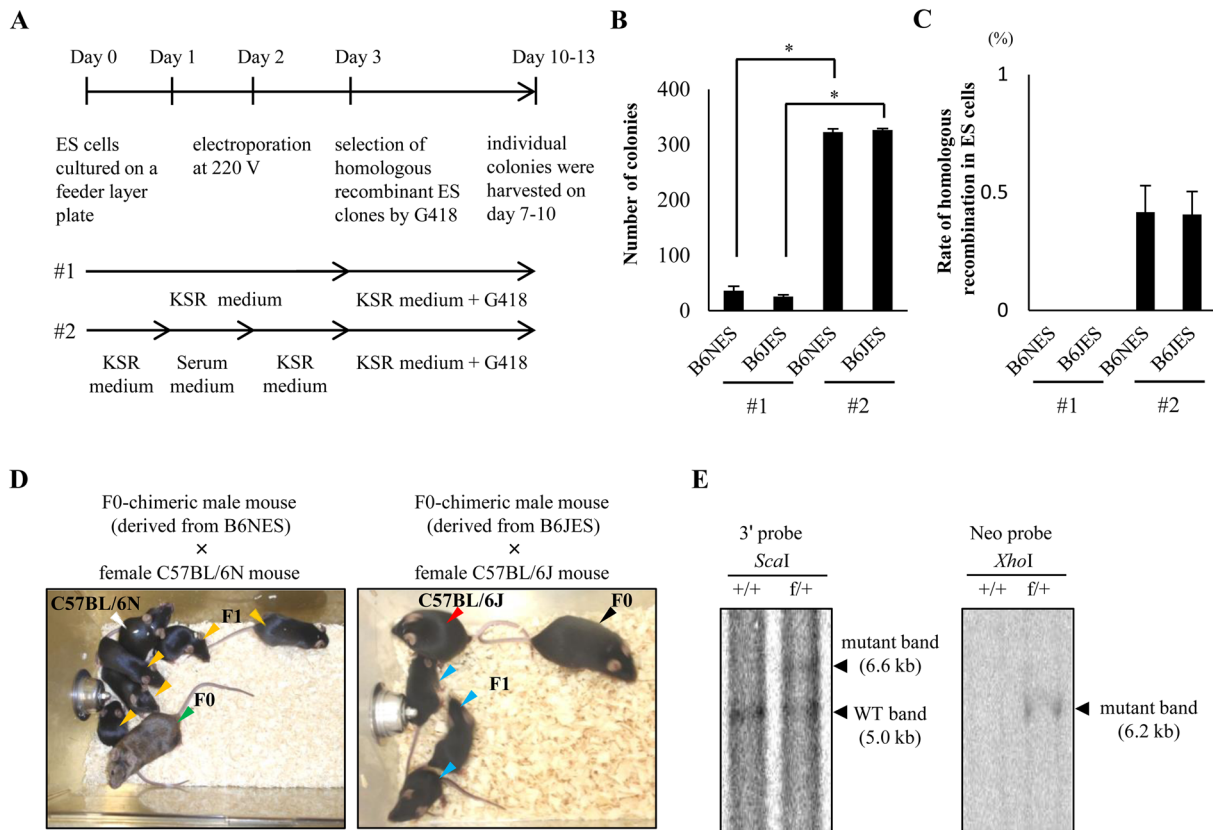
same mice using EGR-101, an ES cell line derived from C57BL/6N [14].

For the generation of cKO mice, electroporation of the construct is an important step, and the optimum electric voltage is known to vary among ES cell lines. We first tested 250 V, which is the voltage recommended for the TT2 ES cell line [45], as well as 220 V, which is recommended for the A2lox ES cell line [15] (Fig. 2A). After 7–10 days of selection with G418 following the electroporation, the number of C57BL/6 strain ES colonies obtained after electroporation at 250 V was found to be significantly lower than that after electroporation at 220 V (Fig. 2B). Moreover, although we could not confirm the occurrence of homologous in any of the C57BL/6 strain ES cells after electroporation at 250 V, successful homologous recombination was confirmed after electroporation at 220 V (Figs. 2C–2E). These results suggest that the number of C57BL/6 strain ES colonies obtained improves by electroporation at 220 V for obtaining homologous recombination in ES cells. We therefore set the voltage for electroporation to 220 V for the subsequent experiments.

#### *Efficient generation of targeted mutant mice*

For the generation of targeted mutant mice via homologous recombination in C57BL/6 strain cells, we attempted to generate targeted mutant mice with a high contribution of ES cells. However, mice with a high contribution of ES cells could not be obtained from homologous recombination in C57BL/6 strain cells (Table 2). Therefore, another challenge was to establish an optimal method for the maintenance of ES cells with homologous recombination, for generating targeted mutant mice. ES cells need to be maintained in both an undifferentiated and germline-transmitted state for the

generation of targeted mutant mice. Therefore, we examined various media to identify the most suitable for obtaining germline-transmitted chimeric mice. It has been reported that differentiation is more restrained in KSR medium than in fetal bovine serum-containing medium [8]. Therefore, the four newly established cell lines were cultured in KSR medium, which was found to be the suitable stem cell medium for stably maintaining the C57BL/6J ES cells in an undifferentiated (Supplementary Figs. S1B–E) and germline-transmitted (Supplementary Figs. S2F and 2G) state. First, we attempted homologous recombination in ES cells obtained in KSR medium before electroporation and cells cultured in KSR medium after electroporation (Fig. 3A, #1). However, only a small number of ES colonies were obtained and none of them had undergone homologous recombination (Figs. 3B and 3C). Thus, KSR medium before electroporation affects not only the survival rate of cells after electroporation. We suggest that the electroporation conditions affect the number of C57BL/6 strain ES colonies that are obtained, which is lower in serum-containing medium than in KSR medium (Fig. 2B). We therefore analyzed homologous recombination in ES cells obtained in serum-containing medium before electroporation and those obtained by culturing in KSR medium after electroporation (Fig. 3A, #2). Our results showed that the number of C57BL/6 strain ES colonies obtained after culturing in serum-containing medium was significantly higher than that obtained by culturing in KSR medium (Fig. 3B); furthermore, we confirmed that homologous recombination had occurred in these ES cells (Fig. 3C). Thus, the number of C57BL/6 strain ES colonies increased with the use of serum-containing medium before electroporation (Fig. 3B). Taken together, these results suggest that culturing the cells in serum-



**Fig. 3.** Generation of targeted mutant mice. (A) Experimental design for the culturing of ES cells, including the use of the KSR medium, before electroporation. (B) The number of ES colonies after electroporation. The average number was 36 for C57BL/6N ES colonies and 26 for C57BL/6J ES colonies by #1, and 323 for C57BL/6N ES colonies and 327 for C57BL/6J ES colonies by #2.  $*P < 0.01$ . (C) The rate of homologous recombination in the ES cells after electroporation. The average rate was 0.00% for C57BL/6N ES colonies and C57BL/6J ES colonies by #1, 0.42% for C57BL/6N ES colonies and 0.41% for C57BL/6J ES colonies by #2.  $*P < 0.01$ . (D) Photographs of an F0-chimeric mouse (green arrowhead) derived from C57BL/6N ES cells and an F0-chimeric mouse (black arrowhead) derived from C57BL/6J ES cells. Analysis of germline transmission by coat color analysis after natural mating between a male mouse with a high contribution C57BL/6N ES cells (green arrowhead) and a wild-type C57BL/6N female mouse (white arrowhead), and between a 100% F0-chimeric male mouse (black arrowhead) and a wild-type C57BL/6J female mouse (red arrowhead). Generation of F1 mice with a black coat color (orange arrowheads and blue arrowheads), confirming germline transmission in a male mouse with a high contribution of C57BL/6N ES cells and in 100% F0-chimeric male mice derived from each of the C57BL/6 ES cell lines. (E) Southern blot analysis of genomic DNA to confirm homologous recombination in the F1 mice.

containing medium before electroporation improves obtaining the number of C57BL/6 strain ES colonies for obtaining homologous recombination in ES cells.

Interestingly, not a single mouse with a high contribution of ES cells was generated from the transferred embryos aggregated with the ES cells cultured in serum medium, despite the generation of a certain number of colored F0-chimeric mice (Table 2). In contrast, in the case of embryos aggregated with the ES cells cultured in KSR medium, colored F0-chimeric mice were efficiently generated, most of which were mice with a high contribution of ES cells (Fig. 3D, Table 2). Furthermore,

germline transmission was confirmed in most of the mice with a high contribution ES cells (Figs. 3D, 3E, Table 2). In contrast, germline transmission was not confirmed in any of the F0 mice derived from C57BL/6J ES cells with less than 100% chimerism and C57BL/6N ES cells with less than 70% chimerism (Table 2).

Moreover, we investigated the generation of targeted mutant mice for the 4 targeted genes by using C57BL/6N ES cells (Supplementary Table S4, represented as A through D). Although the recombination frequencies to the homologous target loci of the surviving ES cells after selection with G418 were in the range of 0.6% to



5.5% in four independent experiments, homologous recombination in ES cells was obtained by culturing in serum-containing medium before electroporation and in KSR medium after electroporation in all experiments (Supplementary Table S4). Furthermore, in all the experiments performed by the aggregation method, the number of F0-chimeric mice obtained was between 7 and 12, mice with greater than 70% chimerism were obtained at a rate of 41.7% to 66.7%, and germline-transmitting-mice were successfully obtained at a rate of 16.6% to 55.6% (Supplementary Table S4). These results suggest that there is a high possibility that C57BL/6 strain ES cells can be germline-transmitted by culturing in serum-containing medium before electroporation and in KSR medium after electroporation.

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### Discussion

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The Red/ET recombineering system has been introduced as an efficient construction of a targeting vector for the generation of targeted mutant mice. However, as there is a loxP site in the BAC backbone, although a loxP-neo-loxP cassette is first inserted into the BAC, it is difficult to remove the genomic region of neo-loxP using the 706-Cre plasmid, when constructing conditional gene targeting vectors in a BAC. Therefore, we first attempted gene retrieval into a DT-A cassette from a BAC to remove the loxP site within the BAC backbone. However, gene retrieval was unsuccessful owing to the toxicity of eukaryotic genomic sequences that are unstable in *E. coli*. In a recent study, a toxic peptide from a eukaryotic genomic sequence was found to be produced by an unidentified promoter of a high-copy-number vector in *E. coli* [5, 16, 31]. Therefore, a low-copy-number vector was selected to reduce the possible toxic effects in *E. coli*. Moreover, it was reported that successful gene retrieval could be achieved by retransformation of the DNA isolated by mini-preparation; however, many points remain unclear regarding the details of this method [7]. On the other hand, we successfully achieved gene retrieval in an efficient manner, by static culture at 30°C. Therefore, our findings suggested that static culture at a low culture temperature prevents the production of toxic peptides from eukaryotic genomic sequences in *E. coli*, enabling efficient gene retrieval, and it may inhibit the expression of genes that promote self-ligation/end-joining of retrieval plasmid. Furthermore, previous studies showed that the loxP-oligo and loxP-neo cassette

can be simultaneously inserted into the BAC before retrieval, and hence it is possible to construct conditional gene targeting vectors in 7 days [33]. However, as the rate of loxP insertion into the BAC was 0%–6.2%, repeated trial and screening by PCR analysis would be required, resulting in high costs, which is unfavorable as a method for the construction of conditional gene targeting vectors. In this study, we demonstrated that our method takes 15 days, but requires fewer samples for screening by PCR analysis, as well as fewer trials and at a lower cost, and highly stable and hence useful for the construction of conditional gene targeting vectors. Therefore, our newly established method was able to overcome the problems of the previous methods.

KO mice generated by gene targeting technology are often used to analyze the biological functions of genes. Nearly two decades have passed since ES cell lines derived from C57BL/6 mice were first established and their usefulness has frequently been reported [21, 24], although developmental defects in these mice have been noted [18], and the cells often become unstable aneuploid cells and deteriorate under standard culture conditions [18]. Much fewer KO mice have been generated from C57BL/6 ES cell lines compared with from the widely used 129 ES cell lines [22]. In recent studies, modifications of the culture conditions of C57BL/6 ES cells have been proposed, one of which is the use of KSR medium for culturing of the ES cells [8]. Moreover, the use of two inhibitors, targeting ERK and GSK3 has been reported to prevent the differentiation of ES cells [46, 47]. Furthermore, the use of three inhibitors, targeting the FGF receptor, ERK, and GSK3, which were originally used to culture rat ES cells [25], has been reported to be effective for the maintenance of germline-competent C57BL/6 ES cells [23, 37]. However, the conditions of electroporation and of subsequent culture remained to be fully optimized for C57BL/6 ES cells. In this study, we first established four germline-competent C57BL/6J ES cell lines. Then, we attempted to generate germline chimeric mice in an efficient manner. However, electroporation at 250 V, which is the recommended voltage for TT2 cells, did not enable the satisfactory maintenance of ES cells, and homologous recombination was unsuccessful in these cells. On the other hand, electroporation at 220 V improved both the survival of the cells after electroporation and the homologous recombination efficiency. Furthermore, we used KSR medium for culturing the ES cells after electroporation. Inhibitors do no

need add to the medium in this strategy, and it is possible to prepare the medium at low cost, enabling the generation of a sufficient number of chimeric mice.

New technologies have recently been proposed for generating genetically engineered mice on a C57BL/6 background without the use of ES cells, including the use of zinc-finger nucleases (ZFNs) [28], transcription activator-like effector nucleases (TALENs) [40], and CRISPR/Cas9. Using these methods, targeted mutant mice can be generated by direct injection of the enzymes into the pronuclei and/or cytoplasm of the fertilized embryos [41]. However, the gene targeting region is restricted to the ZFNs and TALENs that can be constructed [28, 40]. Furthermore, off-target mutagenesis is an important critical issue when the CRISPR/Cas9 technology is used [12, 17, 27, 35, 44]. A recent study reported that although the frequency of off-target mutagenesis was reduced with the use of the CRISPR/Cas9 technology, the risk still exists, along with the risk of deficient gene disruption by off-target mutagenesis [9]. On the other hand, our improved conventional method can be used for artificial modification and for gene targeting of any genetic region, and allows complete genetic disruption while avoiding off-target mutagenesis, with the use of a minimum number of animals. A recent study has reported the construction of a targeting vector for the generation of KI mice using the CRISPR/Cas9 technology [1]. Therefore, our findings indicate that an improved method of gene retrieval from a BAC clone is required for the stable generation of targeted mutant mice.

In conclusion, we have established an improved strategy based on a conventional technique, to generate stable germline chimeric mice using C57BL/6-strain ES cell lines. First, efficient gene retrieval from a BAC clone into the DT-A cassette was accomplished using Red/ET recombination by static culture at 30°C. Then, a combination of electroporation at 220 V and culture in KSR medium after the electroporation enabled efficient generation of targeted mutant mice on a C57BL/6 background. This strategy is expected to improve the efficient generation of targeted mutant mice for the analysis of gene functions.

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### References

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1. Aida, T., Chiyo, K., Usami, T., Ishikubo, H., Imahashi, R., Wada, Y., Tanaka, K.F., Sakuma, T., Yamamoto, T., and Tanaka, K. 2015. Cloning-free CRISPR/Cas system facilitates functional cassette knock-in in mice. *Genome Biol.* 16: 87. [[Medline](#)] [[CrossRef](#)]
2. Auerbach, W., Dunmore, J.H., Fairchild-Huntress, V., Fang, Q., Auerbach, A.B., Huszar, D., and Joyner, A.L. 2000. Establishment and chimera analysis of 129/SvEv- and C57BL/6-derived mouse embryonic stem cell lines. *Biotechniques* 29: 1024–1028, 1030, 1032. [[Medline](#)]
3. Austin, C.P., Battey, J.F., Bradley, A., Bucan, M., Capecchi, M., Collins, F.S., Dove, W.F., Duyk, G., Dymecki, S., Eppig, J.T., Grieder, F.B., Heintz, N., Hicks, G., Insel, T.R., Joyner, A., Koller, B.H., Lloyd, K.C., Magnuson, T., Moore, M.W., Nagy, A., Pollock, J.D., Roses, A.D., Sands, A.T., Seed, B., Skarnes, W.C., Snoddy, J., Soriano, P., Stewart, D.J., Stewart, F., Stillman, B., Varmus, H., Varticovski, L., Verma, I.M., Vogt, T.F., von Melchner, H., Witkowski, J., Woychik, R.P., Wurst, W., Yancopoulos, G.D., Young, S.G., and Zambrowicz, B. 2004. The knockout mouse project. *Nat. Genet.* 36: 921–924. [[Medline](#)] [[CrossRef](#)]
4. Blais, B.W., Booth, R.A., Phillippe, L.M., and Yamazaki, H. 1997. Effect of temperature and agitation on enrichment of *Escherichia coli* O157:H7 in ground beef using modified EC broth with novobiocin. *Int. J. Food Microbiol.* 36: 221–225. [[Medline](#)] [[CrossRef](#)]
5. Boyd, A.C., Popp, F., Michaelis, U., Davidson, H., Davidson-Smith, H., Doherty, A., McLachlan, G., Porteous, D.J., and Seeber, S. 1999. Insertion of natural intron 6a-6b into a human cDNA-derived gene therapy vector for cystic fibrosis improves plasmid stability and permits facile RNA/DNA discrimination. *J. Gene Med.* 1: 312–321. [[Medline](#)] [[CrossRef](#)]
6. Carroll, D. 2014. Genome engineering with targetable nucleases. *Annu. Rev. Biochem.* 83: 409–439. [[Medline](#)] [[CrossRef](#)]
7. Chan, W., Costantino, N., Li, R., Lee, S.C., Su, Q., Melvin, D., Court, D.L., and Liu, P. 2007. A recombineering based approach for high-throughput conditional knockout targeting vector construction. *Nucleic Acids Res.* 35: e64. [[Medline](#)] [[CrossRef](#)]
8. Cheng, J., Dutra, A., Takesono, A., Garrett-Beal, L., and Schwartzberg, P.L. 2004. Improved generation of C57BL/6J mouse embryonic stem cells in a defined serum-free media. *Genesis* 39: 100–104. [[Medline](#)] [[CrossRef](#)]
9. Cho, S.W., Kim, S., Kim, Y., Kweon, J., Kim, H.S., Bae, S.,

- and Kim, J.S. 2014. Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Res.* 24: 132–141. [Medline] [CrossRef]
10. Collins, F.S., Finnell, R.H., Rossant, J., and Wurst, W. 2007. A new partner for the international knockout mouse consortium. *Cell* 129: 235. [Medline] [CrossRef]
  11. Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., and Zhang, F. 2013. Multiplex genome engineering using CRISPR/Cas systems. *Science* 339: 819–823. [Medline] [CrossRef]
  12. Cradick, T.J., Fine, E.J., Antico, C.J., and Bao, G. 2013. CRISPR/Cas9 systems targeting  $\beta$ -globin and CCR5 genes have substantial off-target activity. *Nucleic Acids Res.* 41: 9584–9592. [Medline] [CrossRef]
  13. Doudna, J.A. and Charpentier, E. 2014. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science* 346: 1258096. [Medline] [CrossRef]
  14. Fujihara, Y., Kaseda, K., Inoue, N., Ikawa, M., and Okabe, M. 2013. Production of mouse pups from germline transmission-failed knockout chimeras. *Transgenic Res.* 22: 195–200. [Medline] [CrossRef]
  15. Fujita, J., Crane, A.M., Souza, M.K., Dejosez, M., Kyba, M., Flavell, R.A., Thomson, J.A., and Zwaka, T.P. 2008. Caspase activity mediates the differentiation of embryonic stem cells. *Cell Stem Cell* 2: 595–601. [Medline] [CrossRef]
  16. Fütterer, J., Gordon, K., Pfeiffer, P., and Hohn, T. 1988. The instability of a recombinant plasmid, caused by a prokaryotic-like promoter within the eukaryotic insert, can be alleviated by expression of antisense RNA. *Gene* 67: 141–145. [Medline] [CrossRef]
  17. Fu, Y., Foden, J.A., Khayter, C., Maeder, M.L., Reyon, D., Joung, J.K., and Sander, J.D. 2013. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol.* 31: 822–826. [Medline] [CrossRef]
  18. Hughes, E.D., Qu, Y.Y., Genik, S.J., Lyons, R.H., Pacheco, C.D., Lieberman, A.P., Samuelson, L.C., Nasonkin, I.O., Camper, S.A., Van Keuren, M.L., and Saunders, T.L. 2007. Genetic variation in C57BL/6 ES cell lines and genetic instability in the Bruce4 C57BL/6 ES cell line. *Mamm. Genome* 18: 549–558. [Medline] [CrossRef]
  19. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., and Charpentier, E. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337: 816–821. [Medline] [CrossRef]
  20. Jones, P.G., VanBogelen, R.A., and Neidhardt, F.C. 1987. Induction of proteins in response to low temperature in *Escherichia coli*. *J. Bacteriol.* 169: 2092–2095. [Medline] [CrossRef]
  21. Kawase, E., Suemori, H., Takahashi, N., Okazaki, K., Hashimoto, K., and Nakatsuji, N. 1994. Strain difference in establishment of mouse embryonic stem (ES) cell lines. *Int. J. Dev. Biol.* 38: 385–390. [Medline]
  22. Keskinetepe, L., Norris, K., Pacholczyk, G., Dederscheck, S.M., and Eroglu, A. 2007. Derivation and comparison of C57BL/6 embryonic stem cells to a widely used 129 embryonic stem cell line. *Transgenic Res.* 16: 751–758. [Medline] [CrossRef]
  23. Kiyonari, H., Kaneko, M., Abe, S., and Aizawa, S. 2010. Three inhibitors of FGF receptor, ERK, and GSK3 establish germline-competent embryonic stem cells of C57BL/6N mouse strain with high efficiency and stability. *Genesis* 48: 317–327. [Medline]
  24. Ledermann, B. and Bürki, K. 1991. Establishment of a germline competent C57BL/6 embryonic stem cell line. *Exp. Cell Res.* 197: 254–258. [Medline] [CrossRef]
  25. Li, P., Tong, C., Mehrian-Shai, R., Jia, L., Wu, N., Yan, Y., Maxson, R.E., Schulze, E.N., Song, H., Hsieh, C.L., Pera, M.F., and Ying, Q.L. 2008. Germline competent embryonic stem cells derived from rat blastocysts. *Cell* 135: 1299–1310. [Medline] [CrossRef]
  26. Li, Z., Fei, T., Zhang, J., Zhu, G., Wang, L., Lu, D., Chi, X., Teng, Y., Hou, N., Yang, X., Zhang, H., Han, J.D., and Chen, Y.G. 2012. BMP4 Signaling Acts via dual-specificity phosphatase 9 to control ERK activity in mouse embryonic stem cells. *Cell Stem Cell* 10: 171–182. [Medline] [CrossRef]
  27. Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., and Church, G.M. 2013. RNA-guided human genome engineering via Cas9. *Science* 339: 823–826. [Medline] [CrossRef]
  28. Meyer, M., de Angelis, M.H., Wurst, W., and Kühn, R. 2010. Gene targeting by homologous recombination in mouse zygotes mediated by zinc-finger nucleases. *Proc. Natl. Acad. Sci. USA* 107: 15022–15026. [Medline] [CrossRef]
  29. Waterston, R.H., Lindblad-Toh, K., Birney, E., Rogers, J., Abril, J.F., Agarwal, P., Agarwala, R., Ainscough, R., Alexander, M., An, P., Antonarakis, S.E., Attwood, J., Baertsch, R., Bailey, J., Barlow, K., Beck, S., Berry, E., Birren, B., Bloom, T., Bork, P., Botcherby, M., Bray, N., Brent, M.R., Brown, D.G., Brown, S.D., Bult, C., Burton, J., Butler, J., Campbell, R.D., Carninci, P., Cawley, S., Chiaromonte, F., Chinwalla, A.T., Church, D.M., Clamp, M., Clee, C., Collins, F.S., Cook, L.L., Copley, R.R., Coulson, A., Couronne, O., Cuff, J., Curwen, V., Cutts, T., Daly, M., David, R., Davies, J., Delehaanty, K.D., Deri, J., Dermitzakis, E.T., Dewey, C., Dickens, N.J., Diekhans, M., Dodge, S., Dubchak, I., Dunn, D.M., Eddy, S.R., Elnitski, L., Emes, R.D., Eswara, P., Eyram, E., Felsenfeld, A., Fewell, G.A., Flicek, P., Foley, K., Frankel, W.N., Fulton, L.A., Fulton, R.S., Furey, T.S., Gage, D., Gibbs, R.A., Glusman, G., Gnerre, S., Goldman, N., Goodstadt, L., Grafham, D., Graves, T.A., Green, E.D., Gregory, S., Guigó, R., Guyer, M., Hardison, R.C., Haussler, D., Hayashizaki, Y., Hillier, L.W., Hinrichs, A., Hlavina, W., Holzer, T., Hsu, F., Hua, A., Hubbard, T., Hunt, A., Jackson, I., Jaffe, D.B., Johnson, L.S., Jones, M., Jones, T.A., Joy, A., Kamal, M., Karlsson, E.K., Karolchik, D., Kasprzyk, A., Kawai, J., Keibler, E., Kells, C., Kent, W.J., Kirby, A., Kolbe, D.L., Korf, I., Kucherlapati, R.S., Kulbokas, E.J., Kulp, D., Landers, T., Leger, J.P., Leonard, S., Letunic, I., Levine, R., Li, J., Li, M., Lloyd, C., Lucas, S., Ma, B., Maglott, D.R., Mardis, E.R., Matthews, L., Mauceli, E., Mayer, J.H., McCarthy, M., McCombie, W.R., McLaren, S., McLay, K., McPherson, J.D., Meldrim, J., Meredith, B., Mesirov, J.P., Miller, W., Miner, T.L., Mongin, E., Montgomery, K.T., Morgan, M., Mott, R., Mullikin, J.C., Muzny, D.M., Nash, W.E., Nelson, J.O., Nhan, M.N., Nicol, R., Ning, Z., Nusbaum, C.,

- O'Connor, M.J., Okazaki, Y., Oliver, K., Overton-Larty, E., Pachter, L., Parra, G., Pepin, K.H., Peterson, J., Pevzner, P., Plumb, R., Pohl, C.S., Poliakov, A., Ponce, T.C., Ponting, C.P., Potter, S., Quail, M., Reymond, A., Roe, B.A., Roskin, K.M., Rubin, E.M., Rust, A.G., Santos, R., Sapojnikov, V., Schultz, B., Schultz, J., Schwartz, M.S., Schwartz, S., Scott, C., Seaman, S., Searle, S., Sharpe, T., Sheridan, A., Shownkeen, R., Sims, S., Singer, J.B., Slater, G., Smit, A., Smith, D.R., Spencer, B., Stabenau, A., Stange-Thomann, N., Sugnet, C., Suyama, M., Tesler, G., Thompson, J., Torrents, D., Trevaskis, E., Tromp, J., Ucla, C., Ureta-Vidal, A., Vinson, J.P., Von Niederhausern, A.C., Wade, C.M., Wall, M., Weber, R.J., Weiss, R.B., Wendl, M.C., West, A.P., Wetterstrand, K., Wheeler, R., Whelan, S., Wierzbowski, J., Willey, D., Williams, S., Wilson, R.K., Winter, E., Worley, K.C., Wyman, D., Yang, S., Yang, S.P., Zdobnov, E.M., Zody, M.C., Lander, E.S., Mouse Genome Sequencing Consortium 2002. Initial sequencing and comparative analysis of the mouse genome. *Nature* 420: 520–562. [[Medline](#)] [[CrossRef](#)]
30. Nadeau, J.H., Balling, R., Barsh, G., Beier, D., Brown, S.D., Bucan, M., Camper, S., Carlson, G., Copeland, N., Eppig, J., Fletcher, C., Frankel, W.N., Ganten, D., Goldowitz, D., Goodnow, C., Guenet, J.L., Hicks, G., Hrade de Angelis, M., Jackson, I., Jacob, H.J., Jenkins, N., Johnson, D., Justice, M., Kay, S., Kingsley, D., Lehrach, H., Magnuson, T., Meisler, M., Poustka, A., Rinchik, E.M., Rossant, J., Russell, L.B., Schimenti, J., Shiroishi, T., Skarnes, W.C., Soriano, P., Stanford, W., Takahashi, J.S., Wurst, W., Zimmer, A., International Mouse Mutagenesis Consortium 2001. Sequence interpretation. Functional annotation of mouse genome sequences. *Science* 291: 1251–1255. [[Medline](#)] [[CrossRef](#)]
  31. Na, G., Wolfe, A., Ko, C., Youn, H., Lee, Y.M., Byun, S.J., Jeon, I., and Koo, Y. 2013. A low-copy-number plasmid for retrieval of toxic genes from BACs and generation of conditional targeting constructs. *Mol. Biotechnol.* 54: 504–514. [[Medline](#)] [[CrossRef](#)]
  32. Nagy, A. 2003. Manipulating the mouse embryo: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
  33. Newman, R.J., Roose-Girma, M., and Warming, S. 2015. Efficient conditional knockout targeting vector construction using co-selection BAC recombineering (CoSBR). *Nucleic Acids Res.* 43: e124. [[Medline](#)] [[CrossRef](#)]
  34. Nyabi, O., Naessens, M., Haigh, K., Gembarska, A., Goossens, S., Maetens, M., De Clercq, S., Drogat, B., Haenebalcke, L., Bartunkova, S., De Vos, I., De Craene, B., Karimi, M., Berx, G., Nagy, A., Hilson, P., Marine, J.C., and Haigh, J.J. 2009. Efficient mouse transgenesis using Gateway-compatible ROSA26 locus targeting vectors and F1 hybrid ES cells. *Nucleic Acids Res.* 37: e55. [[Medline](#)] [[CrossRef](#)]
  35. Pattanayak, V., Lin, S., Guilinger, J.P., Ma, E., Doudna, J.A., and Liu, D.R. 2013. High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nat. Biotechnol.* 31: 839–843. [[Medline](#)] [[CrossRef](#)]
  36. Sander, J.D. and Joung, J.K. 2014. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat. Biotechnol.* 32: 347–355. [[Medline](#)] [[CrossRef](#)]
  37. Sato, H., Amagai, K., Shimizukawa, R., and Tamai, Y. 2009. Stable generation of serum- and feeder-free embryonic stem cell-derived mice with full germline-competency by using a GSK3 specific inhibitor. *Genesis* 47: 414–422. [[Medline](#)] [[CrossRef](#)]
  38. Seong, E., Saunders, T.L., Stewart, C.L., and Burmeister, M. 2004. To knockout in 129 or in C57BL/6: that is the question. *Trends Genet.* 20: 59–62. [[Medline](#)] [[CrossRef](#)]
  39. Tanimoto, Y., Iijima, S., Hasegawa, Y., Suzuki, Y., Daitoku, Y., Mizuno, S., Ishige, T., Kudo, T., Takahashi, S., Kunita, S., Sugiyama, F., and Yagami, K. 2008. Embryonic stem cells derived from C57BL/6J and C57BL/6N mice. *Comp. Med.* 58: 347–352. [[Medline](#)]
  40. Tesson, L., Usal, C., Ménoret, S., Leung, E., Niles, B.J., Remy, S., Santiago, Y., Vincent, A.I., Meng, X., Zhang, L., Gregory, P.D., Anegon, I., and Cost, G.J. 2011. Knockout rats generated by embryo microinjection of TALENs. *Nat. Biotechnol.* 29: 695–696. [[Medline](#)] [[CrossRef](#)]
  41. Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M.M., Cheng, A.W., Zhang, F., and Jaenisch, R. 2013. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 153: 910–918. [[Medline](#)] [[CrossRef](#)]
  42. Ward, C.M., Barrow, K.M., and Stern, P.L. 2004. Significant variations in differentiation properties between independent mouse ES cell lines cultured under defined conditions. *Exp. Cell Res.* 293: 229–238. [[Medline](#)] [[CrossRef](#)]
  43. Ware, C.B., Siverts, L.A., Nelson, A.M., Morton, J.F., and Ladiges, W.C. 2003. Utility of a C57BL/6 ES line versus 129 ES lines for targeted mutations in mice. *Transgenic Res.* 12: 743–746. [[Medline](#)] [[CrossRef](#)]
  44. Wu, X., Scott, D.A., Kriz, A.J., Chiu, A.C., Hsu, P.D., Dadon, D.B., Cheng, A.W., Trevino, A.E., Konermann, S., Chen, S., Jaenisch, R., Zhang, F., and Sharp, P.A. 2014. Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells. *Nat. Biotechnol.* 32: 670–676. [[Medline](#)] [[CrossRef](#)]
  45. Yagi, T., Tokunaga, T., Furuta, Y., Nada, S., Yoshida, M., Tsukada, T., Saga, Y., Takeda, N., Ikawa, Y., and Aizawa, S. 1993. A novel ES cell line, TT2, with high germline-differentiating potency. *Anal. Biochem.* 214: 70–76. [[Medline](#)] [[CrossRef](#)]
  46. Yamagata, K., Ueda, J., Mizutani, E., Saitou, M., and Wakayama, T. 2010. Survival and death of epiblast cells during embryonic stem cell derivation revealed by long-term live-cell imaging with an Oct4 reporter system. *Dev. Biol.* 346: 90–101. [[Medline](#)] [[CrossRef](#)]
  47. Ying, Q.L., Wray, J., Nichols, J., Battle-Morera, L., Doble, B., Woodgett, J., Cohen, P., and Smith, A. 2008. The ground state of embryonic stem cell self-renewal. *Nature* 453: 519–523. [[Medline](#)] [[CrossRef](#)]