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Efficient production of large deletion and gene fragment knock-in mice mediated by genome editing with Cas9-mouse Cdt1 in mouse zygotes

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ABSTRACT

Genetically modified mouse models are essential for *in vivo* investigation of gene function and human disease research. Targeted mutations can be introduced into mouse embryos using genome editing technology such as CRISPR-Cas. Although mice with small indel mutations can be produced, the production of mice carrying large deletions or gene fragment knock-in alleles remains inefficient. We introduced the nuclear localisation property of Cdt1 protein into the CRISPR-Cas system for efficient production of genetically engineered mice. Mouse Cdt1-connected Cas9 (Cas9-mC) was present in the nucleus of HEK293T cells and mouse embryos. Cas9-mC induced a bi-allelic full deletion of *Dmd*, GC-rich fragment knock-in, and floxed allele knock-in with high efficiency compared to standard Cas9. These results indicate that Cas9-mC is a useful tool for producing mouse models carrying targeted mutations.

1. Introduction

Targeted mutations have been induced by gene targeting methods using embryonic stem cells for over 20 years [1]. Recently, however, genome editing with CRISPR-Cas has enabled gene targeting in mouse embryos [2]. Embryo-based methods for producing genetically engineered mice are simple and are less time- and labour-consuming than traditional gene targeting methods. Knockout mice carrying small insertion or deletion mutations (indels) can be generated at a very high efficiency by simple microinjection or electroporation of CRISPR-Cas components [2,3].

CRISPR-Cas can be used to generate mice with large deletion mutations, which are useful for analysing promoter or enhancer regulation and the functions of untranslated regions [4,5]. Targeted knock-in mutations, e.g., genetic humanisation, fluorescence reporting, and Cre drivers, are also required to produce more advanced mouse strains for

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genetic and human disease research. However, mice with gene fragment knock-in or megabase-scale large deletion alleles are produced at low efficiency. Several methods have been established for enhancing knock-in efficiency in mouse embryo genome editing, including those using Cas9 fused with monomeric streptavidin and biotinylated repair templates by PCR amplification [6] or a long single-stranded oligodeoxynucleotide template [7]. The insert fragment is limited to approximately 2 kb [8] and there is a risk of unpredicted mutations because of the low polymerase fidelity when preparing single-stranded oligodeoxynucleotides. GC-rich cassettes such as the CAG promoter are difficult to amplify using DNA polymerase. Although some methods can be applied in zygote electroporation [9], pronuclear injection of plasmid DNA template is a reliable strategy for introducing a repair template encoding difficult-to-amplify and/or long cassettes into the pronucleus of mouse zygotes.

In this study, we focused on the nuclear localisation property of Cdt1 at the early stages of mouse embryos and examined the efficacy of mouse Cdt1-fused Cas9 (Cas9-mC) in mouse zygote genome editing.

2. Material and methods

2.1. Vector construction and mRNA in vitro transcription

The *pX330* vector was provided by the Zhang lab [10] through Addgene (plasmid #42230). cDNA fragments of mouse *Cdt1* were obtained from an adult C57BL/6J mouse heart. Total RNA from the heart was extracted with ISOGEN (Nippon Gene, Toyama, Japan). Reverse transcription was performed with oligo dT (Thermo Fisher Scientific, Waltham, MA, USA) and SuperScript III (Thermo Fisher Scientific). The Cdt1 (aa 31–132) coding sequence was inserted directly before SV40-NLS (downstream of h*Sp*Cas9) in *pX330* using In-fusion HD (Clontech, Mountain View, CA, USA). The sequences of *pX330-mC* are shown in Text S1. As there is no *Bbs*I recognition site in *Cdt1*, the entry site of *pX330-mC* were not changed from that in *pX330*. CRISPR target sequences were inserted into these vectors with annealed oligos (Table S1). Oligo annealing and insertion were performed as reported previously [11]. DNA plasmids for microinjection were collected from *Escherichia coli* with a FastGene Plasmid Mini Kit (Nippon Gene).

pT7-hCas9-mC-polyA (Text S2) was constructed from the T7-NLS hCas9-pA plasmid, which was kindly gifted from Tomoji Mashimo through RIKEN BioResource Research Center (BRC, RDB13130). Cas9 mRNAs were *in vitro*-transcribed from *pT7-NLS hCas9-pA* (*Nhe*I-digested) and *pT7-hCas9-mC-polyA* (*Nhe*I-digested) by using the mMESSAGE mMACHINE T7 ULTRA Transcription Kit (AM1345, Thermo Fisher Scientific).

All vectors were deposited to RIKEN BRC (Table S2).

2.2. Animals

Animal experiments were performed at the University of Tsukuba. C57BL/6J (egg donor) and ICR (recipient) mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). Animals were housed in plastic cages under specific pathogen-free conditions in a room maintained at 23.5 °C ± 2.5 °C and 52.5% ± 12.5% relative humidity under a 14:10-h light:dark cycle. Mice had free access to commercial chow (MF; Oriental Yeast, Tokyo, Japan) and filtered water. Breeding and experiments were performed humanely in accordance with the Regulations for Animal Experiments of the University of Tsukuba and Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology of Japan and with approval from the Institutional Animal Experiment Committee of the University of Tsukuba.

We performed intracytoplasmic sperm injection (ICSI) for immunostaining experiments as reported previously [12]. Each type of Cas9 mRNA was injected into the cytoplasm. Immuno-staining was performed as reported previously [13]. Briefly, embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min and permeabilised in 0.5% Triton X-100 in PBS for 10 min. Subsequently, blocking was performed with 1% bovine serum albumin (BSA) in PBS for 1 h followed by immunostaining with rabbit anti-FLAG antibody (1:1000; F7425, Sigma, St. Louis, MO, USA) overnight at 4 °C and Alexa Flour 555-conjugated donkey anti-rabbit IgG (1:400, #A-31572, Thermo Fisher Scientific) for 1 h. Antibodies were diluted in 1% BSA in PBS. Between each step, we washed the embryos two to three times with 1% BSA in PBS for 5 min each time. Fluorescent signals were observed using a laser-scanning confocal microscope (D-Eclipse C1; Nikon, Tokyo, Japan).

2.3. Transfection and immunostaining in HEK293T cells

The *pX330* and *pX330-mC* vectors were transfected into HEK293T cells using Lipofectamine LTX (Thermo Fisher Scientific) according to the manufacturer's protocol. After 24 h, the cells were fixed in 4% paraformaldehyde in PBS for 30 min and permeabilised with 0.2% Triton X-100 in PBS for 10 min. Subsequently, immunostaining was performed with FLAG antibody (1:300; Sigma, F1804) and Alexa Fluor 555 goat anti-mouse IgG (1:300; Thermo Fisher Scientific).

2.4. Microinjection

Female C57BL/6J mice (12 weeks old) were induced to superovulate by intraperitoneal administration of 5 units of pregnant mare serum gonadotropin (ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) and 5 units of human chorionic gonadotropin (ASKA Pharmaceutical) at a 48-h interval. Superovulated females were mated naturally with males, and mating was confirmed by the presence of a vaginal plug on the next day. We used fresh pronuclear-stage embryos from natural mating for microinjection; frozen-thawed and/or *in vitro* fertilised embryos were not used. DNA vectors (circular plasmids) were diluted to 5 ng/μL (CRISPR-Cas expression vector) and 10 ng/μL (knock-in donor plasmid) with deionised distilled water and mixed. Diluted and mixed DNA vectors were filtered using MILLEX-GV 0.22-μm filter units (Merck Millipore, Billerica, MA, USA). DNA vectors were microinjected into male pronuclei according to standard protocols [14]. Shortly thereafter (15 min to 2 h), living embryos were selected and transferred into the oviducts of pseudopregnant ICR mice. These ICR mice were anesthetised by a combination of the anaesthetics medetomidine, midazolam, and butorphanol [15]. Anesthetised animals were placed on a heating pad at 37 °C.

2.5. Genotyping PCR and sequencing

Genomic DNA was collected from <0.5-mm tail tips and purified by automated DNA extraction using the PI-200 (Kurabo, Osaka, Japan). To enhance sensitivity and specificity, we did not use crude samples but purified genomic DNA instead. Knock-in and long deletion alleles were detected by PCR with PrimeSTAR GXL DNA Polymerase (Takara, Shiga, Japan) and appropriate primers (Table S1). PCR for detection of random integration events was performed with AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific) and appropriate primers (Table S1). For DNA sequencing, PCR products and plasmid DNAs were purified with a FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan) and a FastGene Plasmid Mini Kit (Nippon Gene), respectively. Sequences were confirmed with a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific), FastGene Dye Terminator Removal Kit (Nippon Gene), and 3500xL Genetic Analyzer (Thermo Fisher Scientific).

3. Theory and calculation

We hypothesised that genome editing efficiency would be increased by using a modified Cas9 which is physically close to the genomic DNA.

Cdt1 is widely conserved among eukaryotic organisms. Cdt1 protein binds to the minichromosome maintenance proteins 2–7 (MCM2–7). The Cdt1-MCM2-7 complex binds to the Origin Recognition Complex (ORC)-Cdc6 complex that binds physically to genomic DNA [16,17]. Sakaue-Sawano *et al.* [18] developed a part of human Cdt1 (aa 30–120) fused to monomeric Kusabira-Orange2 fluorescence protein, which was detected in the nuclei *in vitro* and in vivo. Notably, Abe *et al.* [19] also showed that part of human Cdt1 (aa 30–120) fused to the mCherry fluorescence protein was detected in the nucleus of 2-cell stage embryos. The deletion mutant of human Cdt1 (aa 30–120) may not affect vital phenomena because it lacks the C-terminus of Cdt1 that includes the MCM complex binding domain, and the transgenic mice overexpressing the fluorescence protein did not show an abnormal phenotype.

To introduce the nuclear localisation property of Cdt1 into the CRISPR-Cas system to efficiently produce genetically modified mice, we fused mouse Cdt1 (aa 31–132) to Cas9 (Fig. 1A) and the resulting product was designated as Cas9-mC. Standard *Cas9* was replaced with *Cas9-mC* in *pX330* to yield the *pX330-mC* plasmid (Fig. 1B and Text S1). To confirm the localisation property of Cas9-mC proteins, we performed immunostaining with a FLAG antibody in *pX330*- and *pX330-mC*-transfected HEK293T cells. Signals were mostly observed in the cytoplasm of *pX330*-transfected cells in which standard Cas9 was expressed. In contrast, the signals were observed in the nucleus in *pX330-mC*-transfected cells (Fig. 2A).

Next, we replaced standard *Cas9* in the T7-NLS hCas9-pA plasmid with *Cas9-mC* (Text S2) to obtain Cas9-mC mRNA. Cas9-mC mRNA at a high concentration (2 μg/μL) was microinjected into the cytoplasm of mouse zygotes in the pronuclear stage and we performed immunostaining at the 2-cell stage. As expected, the FLAG signal was observed in the nucleus of mouse embryos (Fig. 2B). In standard Cas9 mRNA-injected embryos, FLAG dot signals were observed in the cytoplasm, although two SV40-NLS were originally fused to the standard Cas9 at both the N- and C-termini of the protein (Fig. 2B). We also microinjected the *pX330*- or *pX330-mC* plasmid DNA vector into the pronucleus of mouse zygotes, but no FLAG signals were observed in our experimental conditions (data not shown). This may be because the amount of Cas9 protein was below the detection threshold for immunostaining when using the plasmid DNA at the 2-cell stage.

These data indicate that Cas9-mC was superior to the standard Cas9 in the nuclear localisation of both HEK293T cells and mouse embryos.

4. Results and discussion

4.1. Producing large deletion mice with Cas9-mC

We previously reported that a set of tools, *pX330*, guide RNA (gRNA), and Cas9-expressing plasmid DNA, enabled induction of large deletion mutations and gene fragment knock-ins in mouse zygotes [20,21]. We hypothesised that genome editing with Cas9-mC could improve both large deletion and knock-in mouse production efficiencies because Cas9-mC was accumulated in the nucleus of mouse early embryos (Fig. 2B). We assessed the genome editing ability of Cas9-mC to induce a large deletion to excise the full-length gene. First, we targeted *Tyr*, for which null mutants showed an albino phenotype [11]. Two CRISPR targets located 1123 base pairs (bp) upstream and 2307 bp downstream of *Tyr* were designed. The distance between these two sites was 72.2 kb (Fig. 3A). We injected *pX330* or *pX330-mC* which carried these target sequences into mouse zygotes. Unexpectedly, offspring including five pups with albino and three with mosaic coat colour were found among the 34 pups in the Cas9-mC group. In contrast, only one mosaic offspring was found in the standard Cas9 group (Fig. 3B, C, and Table 1). Further genetic analysis revealed that the proportion of pups with a full-length gene deletion allele was higher in the Cas9-mC group (17/34, 50.0%) than in the standard Cas9 group (6/21, 28.6%) (Fig. S1 & Table 1).

To confirm the induction efficiency of large deletions of Cas9-mC in another gene, we excised full-length *Dmd* (Fig. 4A). *Dmd*, located on the X chromosome, is the largest protein coding gene (2.2 Mb) in the mouse genome. We generated full-length *Dmd* gene deletion embryos (Fig. 4A). The distance between the left and right gRNA target was 2.3 Mb. These two gRNA target sequences were inserted into *pX330* or *pX330-mC* and microinjected into mouse zygotes. The percentage of E18.5 embryos with the *Dmd* deletion allele in the Cas9-mC group (16.2%, 11/68) was approximately 1.7-fold larger than that in the standard Cas9 group (8.6%, 6/70) (Fig. 4B and Table 2). Because *Dmd* is an X-linked gene,

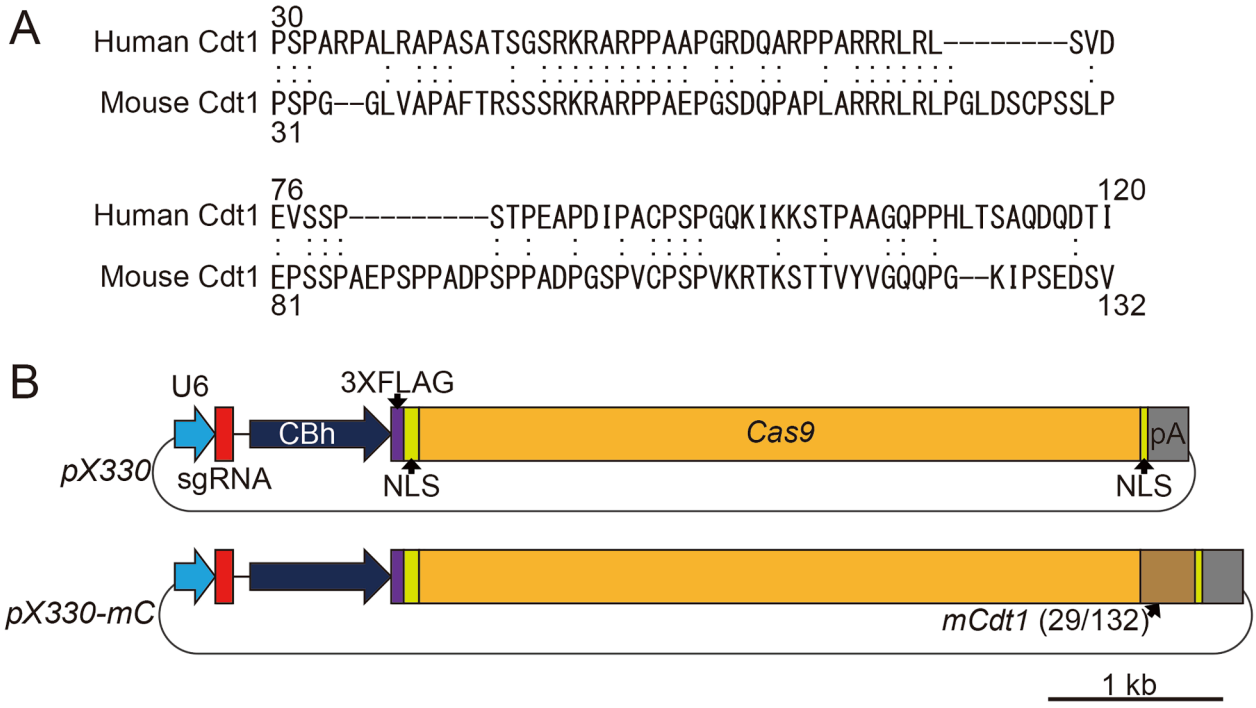

Fig. 1. Cas9-mouse Cdt1 (Cas9-mC). (A) Comparison of human Cdt1 (1/110) and mouse Cdt1 (1/107). (B) Vector map of *pX330* and *pX330-mouse Cdt1* (*mC*). Mouse *Cdt1* (aa 31–132) cDNA sequence was directly connected to the human-codon-optimised *Streptococcus pyogenes* Cas9 gene in *pX330*.

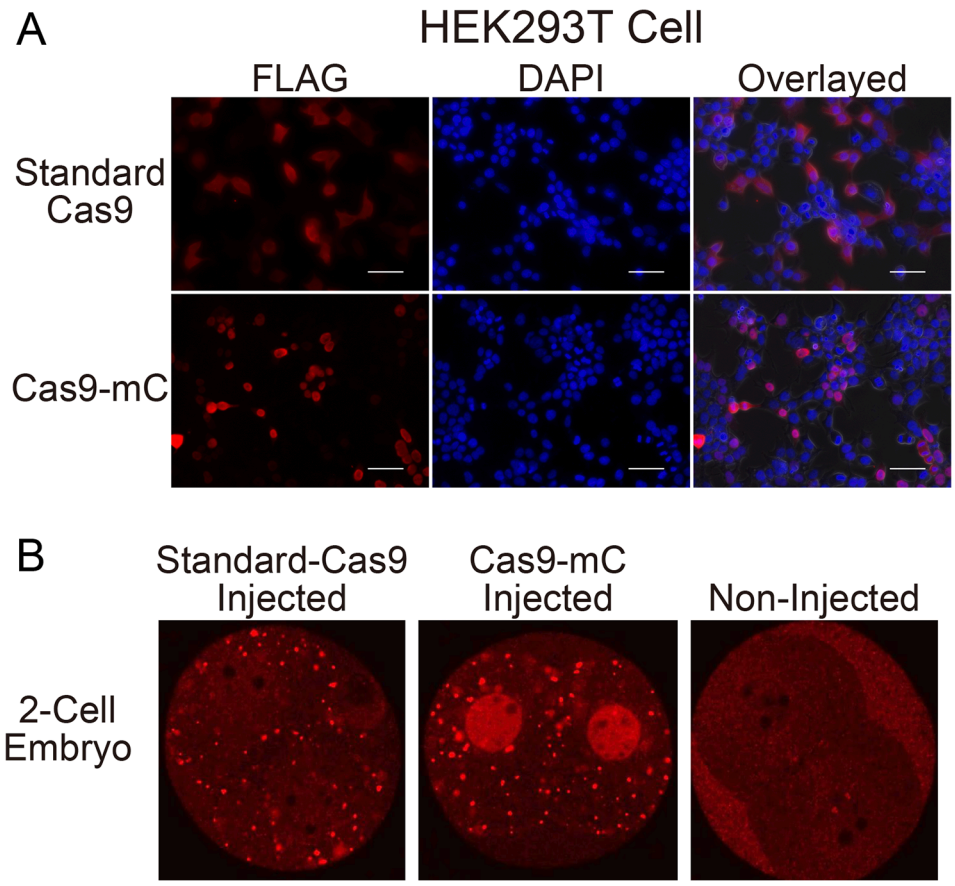

Fig. 2. Cellular localisation of Cas9-mC. (A) Vectors (*pX330* or *pX330-mC*) were transfected to HEK293T cells. We then immuno-stained these cells with an anti-FLAG antibody. Standard Cas9 from *pX330* were detected in the cytoplasm. In contrast, immunofluorescence signals of Cas9-mC were mostly observed in the nucleus. Scale bar: 50 μm. (B) Immunostaining of mouse embryos into which standard Cas9 or Cas9-mC mRNAs were injected. Red dot signals were detected in the cytoplasm for standard Cas9, whereas the signals of Cas9-mC were observed in the nucleus.

male embryos carrying both *Dmd* deleted and non-deleted alleles were predicted to be mosaics. In the standard Cas9 group, 3 of 4 male embryos carried both alleles. In contrast, 3 of 5 male embryos carried only the deleted allele in the Cas9-mC group. Moreover, one female (#D-26C) in the group carried a bi-allelic (compound heterogenic) deletion mutation without a non-deleted allele (Fig. 4B and C).

These results suggest that Cas9-mC improved the large deletion efficiency in the mouse embryo genome editing.

4.2. Producing Knock-in mice with Cas9-mC

We next evaluated using Cas9-mC for knock-in mouse production. As shown in Fig. 5, we attempted to knock-in a 5.7-kb gene fragment into the ROSA26 locus. The donor DNA, *pRosa-CAG-fEGFP-Cables1* (Text S3), contained a GC-rich region in the CAG promoter (67% GC), 5′-terminal 500-bp region of *Cables1* cDNA (75% GC), and ROSA 5′ homology arm (68% GC). This donor DNA was co-microinjected with *pX330-Rosa* or *pX330-mC-Rosa*. Knock-in alleles were confirmed by fluorescence observation and genotyping analyses (Fig. S2). Genotyping PCR analysis revealed that some knock-in mice had donor and/or randomly integrated *pX330* (data not shown); these mice were excluded when counting the number of founder mice. The knock-in frequency of the Cas9-mC group without random integration was still much higher than that of the standard Cas9 group (21.1% versus 0%), as expected (Table 3).

We further confirmed the Cas9-mC efficacy for knock-in at another locus by producing a *Prdm14* floxed mouse. We selected two guide RNA target sites located 558 bp upstream and 282 bp downstream of exon 6 of *Prdm14* (Fig. 6). These sequences were inserted into *pX330* and *pX330-mC*. Two CRISPR expression vectors and one donor vector *pflox-Prdm14* (Text S4) were co-microinjected. As shown in Table 4 and Fig. S3, a higher knock-in frequency was observed in the Cas9-mC group than in the Cas9 group (18.2% in Cas9-mC and 0% in standard Cas9). In this 2-cut strategy described in the previous section, deletion mutation may occur without templated repair. Cas9-mC also induced this deletion mutation at higher efficiency (Table 4).

Finally, we tested the ability of Cas9-mC to produce knock-in mice in seven different genome loci. All seven knock-in mouse strains were produced by pronuclear microinjection of *pX330-mC* and donor DNA vectors in the same manner as *ROSA-Cables1* knock-in. Knock-in alleles were confirmed by genotyping PCR at three weeks after birth. Consistent with *Rosa-Cables1* knock-in mouse production, knock-in mice without random integrations were obtained with high efficiency (10.3–31.8%) in every seven loci (Tables 5 and S3 and Fig. S4). Additionally, we produced seven floxed mouse strains with Cas9-mC using the same method as in *Prdm14* floxed mouse production. We obtained floxed mice with high frequencies (8.2–14.8%) in all seven loci (Tables 6 and S4 and Fig. S5). These results indicate that Cas9-mC is also useful for knock-in and floxed mouse production in various genomic loci.

4.3. Further considerations

Few studies have evaluated Cas9 cellular localisation in mouse embryos, although the nuclear-cytoplasmic ratio of Cas9 with different nuclear localization signal (NLS) peptides critically affects the genome editing efficiency in mammalian cells [22]. An increased concentration of Cas9-mC in the nucleus of mouse embryos resulted in the generation of large deletion and knock-in mice. Notably, our method using Cdt1-fused Cas9 in the form of a DNA plasmid may result in elongated expression of Cas9-mC as well as its accumulation in the nucleus. Although studies using mice and other laboratory animals revealed that the frequency of off-target mutation is minimal when using zygotes to generate genetically engineered animals [23–25], as specific gRNAs as possible should be used to reduce the risk of off-target mutagenesis.

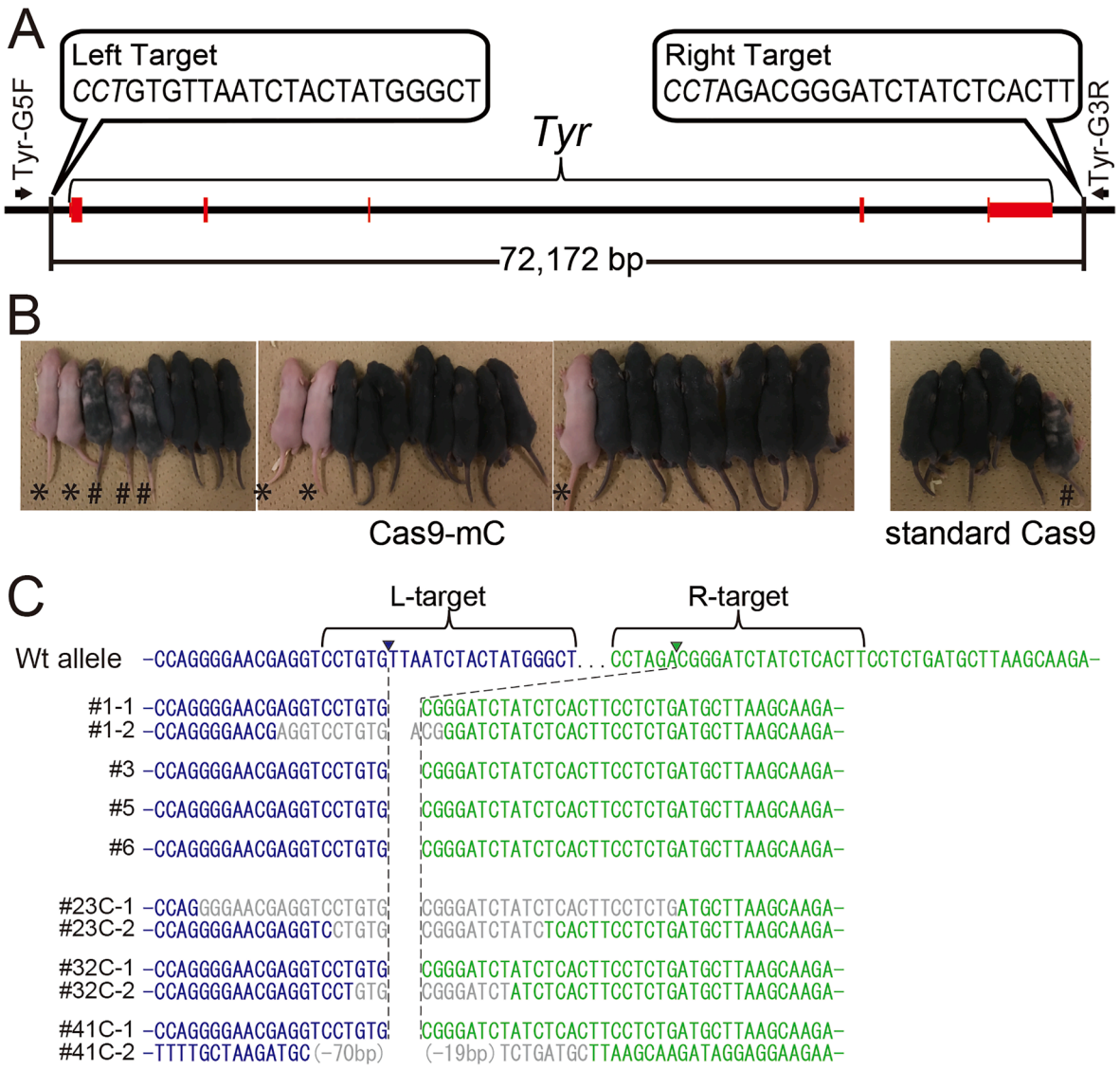

Fig. 3. Production of full-length *Tyr* deletion mice with *pX330-mC*. (A) Strategy for full-length *Tyr* deletion. The left target for Cas9 was 1123 bp upstream of *Tyr* and the right target was 2307 bp downstream of the gene. Red box: exons of *Tyr*, black arrows: primers (Tyr-G5F and R) for detecting the large deletion allele. (B) Founder mice from Cas9-mC or standard Cas9 injection groups. Albino (asterisk) and mosaic (sharp) founders were obtained. (C) Junction sequences in the large deletion alleles. Triangles indicate predicted cleavage sites at the left and right targets. Grey letters indicate the deleted region.

Table 1
Production of full-length *Tyr* gene deletion mice with *pX330-mC*.

Injected DNA	Number of Embryo		Newborn			
	Injected	Transferred	Total	Coat colour		Deletion
				Albino	Mosaic	
pX330-Tyr-L & pX330-Tyr-R	107	100	21	0	1	6 (28.6%)[a]
pX330-mC-Tyr-L & pX330-mC-Tyr-R	102	100	34	5	3	17 (50.0%)[a]

[a] Number of mice carrying large deletion/Number of mice examined.

Pronuclear injection of Cas9-mC protein or mRNA with donor DNA can be an alternative strategy in the future experiment.

On-target mutagenesis can also result in unintended small or large deletions and/or random integration and imprecise recombination of plasmids [26,27]. We observed random integration of donor plasmids or Cas9-mC plasmids in some weaned pups (Tables S3 and S4 and Figs. S4 and S5). Integrated genes or sequences can be easily detected by using specific PCR primers for commonly used drug-resistant genes or markers to select founder mating pairs to obtain the next generation [28]. For example, 71% of the pups (24 out of 34 pups) in which we detected the insert fragment were those with random integration of the Cas9-mC and/or donor plasmid in the *Cd153* knock-in mice production (Table S3). We can also segregate unwanted alleles by selecting pups in the N1 generation using the same genetic quality control strategy applied in the founders. Long read and classic short read sequencing and/or droplet digital PCR is considered another choice for confirming precise knock-in and copy numbers of the repair template, respectively [29,30].

The cell cycle is an important factor regulating the resection of double-strand breaks induced by Cas9 or other artificial nucleases. The classical non-homologous end joining pathway is active during G1, S, and G2 phases, whereas microhomology-mediated end joining and homology-directed repair are restricted to the S and G2 phases [31]. Our strategy using Cas9-mC can generate not only knock-in and floxed mice

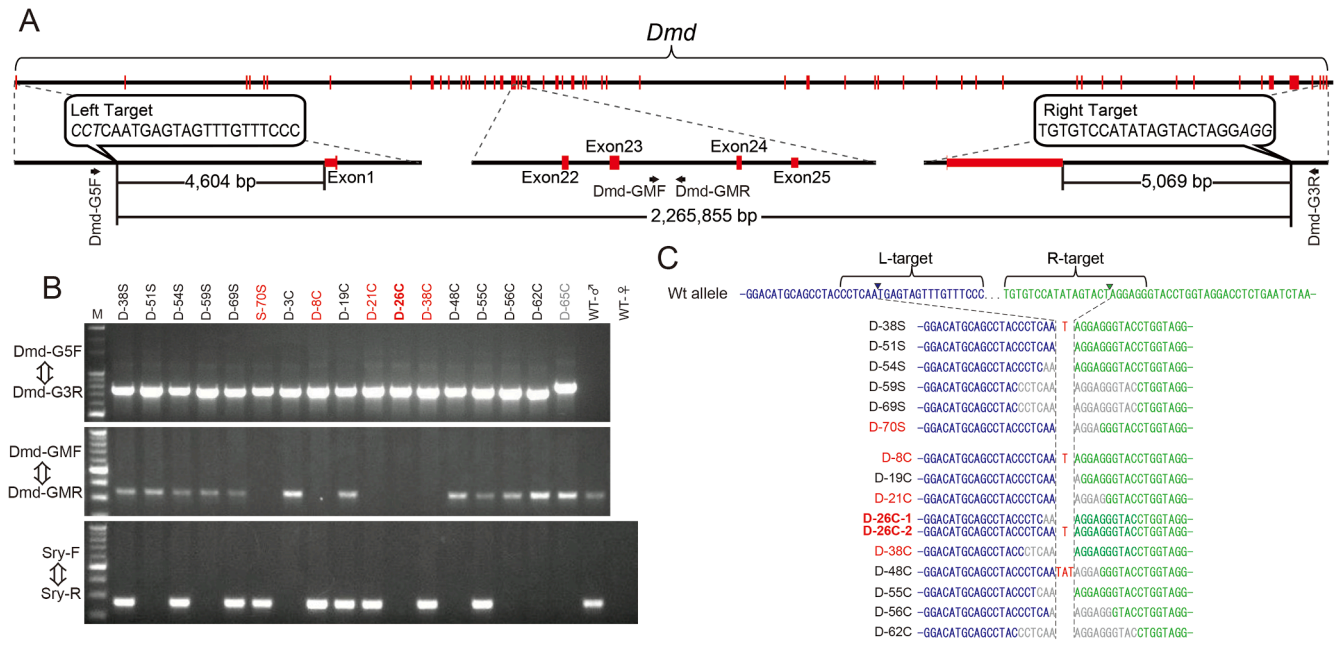

Fig. 4. Production of full-length *Dmd* deletion mice with *pX330-mC*. (A) Strategy for full-length *Dmd* deletion. Red box: exons of *Dmd*, black arrows: primers for detecting the large deletion allele. (B) Deletion and non-deletion alleles were detected by Dmd-G5F & Dmd-G3R primer pair and Dmd-GMF & Dmd-GMR primer pair, respectively. Genotyping was performed at embryonic day 18.5. Sex of founder candidate embryos was identified by PCR with *Sry* detection primers. M indicates 100-bp DNA ladder. (C) Junction sequences in the deletion alleles. Triangles indicate predicted cleavage sites at the left and right targets. Grey and red letters indicate deleted and inserted sequence, respectively.

Table 2
Production of full-length *Dmd* gene deletion mice with *pX330-mC*.

Injected DNA	Number of Embryo		Harvested E18.5 embryo		
	Injected	Transferred	Total	Deletion	
				Total	Male with deletion and non-deletion Female with deletion and non-deletion Male with only deletion Female with only deletion
pX330-Dmd-L & pX330-Dmd-R	166	154	70	6 (8.6%)[a]	4 2 0 0
pX330-mC-Dmd-L & pX330-mC-Dmd-R	165	149	68	11 (16.2%)[a]	2 3 5 1

[a] Number of embryos carrying large deletion/Number of embryos examined.

but also mice with megabase-scale large deletion with high efficiency, indicating that Cas9-mC exists in S and G2 phases in the mouse early embryo. We detected the FLAG signal at the 2-cell stage, 18 h after Cas9 or Cas9-mC mRNA microinjection (Fig. 2B), but not at earlier stages (data not shown). An earlier report using cultured cells showed that the DNA cleavage activity of Cas9 expressed from a plasmid can be detected 6 h after electroporation [32]. Therefore, we hypothesised that DNA cleavage and repair events may occur at or after the 2-cell stage.

In contrast to our strategy using Cas9-mC, signals from monomeric Kusabira-Orange2 fused to human Cdt1 (30–120) peptide degrade during the S and G2 phases [18]. There are two possible reasons why Cas9-mC was not completely degraded. The function of Skp1-Cul1-F-box-Skp2 complex that mediates degradation of Cdt1-fused proteins [18,19] may be weak in mouse early embryos. We used the CBh promoter to drive the strong expression of Cas9-mC, and this expression plasmid may exist in large quantities, albeit transiently, in mouse embryos. Additionally, the expression of Cas9-mC may have exceeded the degradation speed in the early stage embryos.

5. Conclusions

Cas9-mC was highly localised to the nucleus in both HEK293T cells and mouse embryos. As Cas9-mC can easily reach the target genome, knock-in and large deletion mutation efficiency were improved. Particularly, Cas9-mC induced bi-allelic deletion of *Dmd* and induced GC-rich fragment knock-in with high efficiency. These results suggest that Cas9-mC is a useful genome editing tool for creating various mouse models. Further studies of combination of Cas9-mC and other factors involved in DNA repair pathways may be important to increase the efficiency of model animal production. Cas9-mC can be used in multiple applications such as base editing, epigenome editing, and gene activation or inhibition using CRISPR-Cas.

CRediT authorship contribution statement

Saori Mizuno-Iijima: Conceptualization, Methodology, Writing - original draft, Visualization, Funding acquisition. **Shinya Ayabe:** Conceptualization, Methodology, Writing - original draft. **Kanako Kato:** Methodology, Investigation, Resources. **Shogo Matoba:** Methodology, Investigation, Resources. **Yoshihisa Ikeda:** Methodology, Investigation, Resources. **Tra Thi Huong Dinh:** Methodology, Investigation, Resources. **Hoai Thu Le:** Methodology, Investigation, Resources. **Hayate Suzuki:** Methodology, Investigation, Resources. **Kenichi Nakashima:** Methodology, Investigation, Resources. **Yoshikazu Hasegawa:** Methodology, Investigation, Resources. **Yuko Hamada:** Methodology, Investigation, Resources. **Yoko Tanimoto:** Methodology, Investigation, Resources. **Yoko Daitoku:** Methodology, Investigation, Resources. **Natsumi Iki:** Methodology, Investigation, Resources. **Miyuki Ishida:**

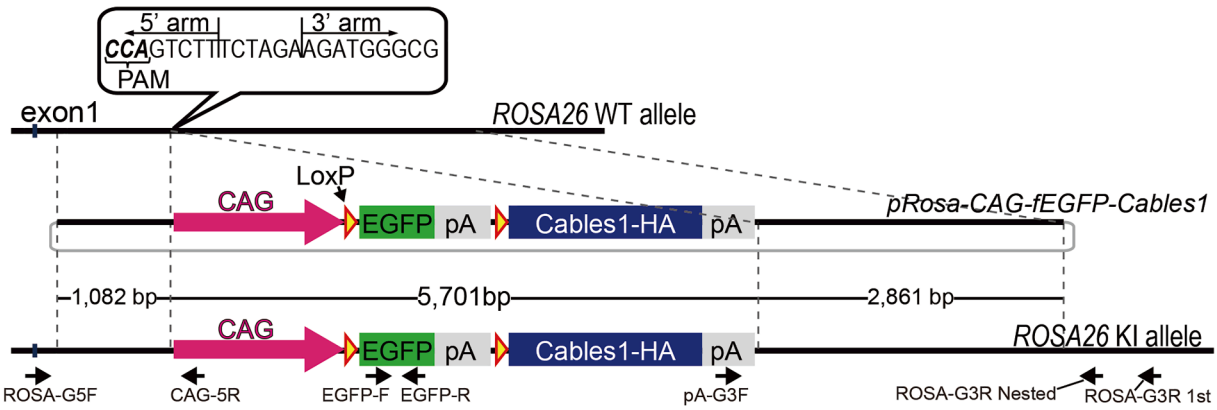

Fig. 5. Knock-in mouse production with Cas9-mC. Strategy for ROSA26 knock-in. Black arrows indicate primers for PCR screening. Blue box indicates HA tagged mouse *Cables1* cDNA. CAG: cytomegalovirus immediate early enhancer and a modified chicken beta-actin promoter, pA: rabbit globin polyadenylation signal.

Table 3
ROSA26 Knock-in mouse production with pX330-mC.

Injected DNA		Number of Embryo		Examined (weaning)	Knock-in
Donor	CRISPR-Cas	Injected	Transferred		
pRosa-CAG-fEGFP-Cables1	*pX330-ROSA*	122	106	44	0 (0.0%)[a]
	pX330-mC-ROSA	165	154	19	4 (21.1%)[a]

[a] Number of knock-in mice/Number of mice examined.

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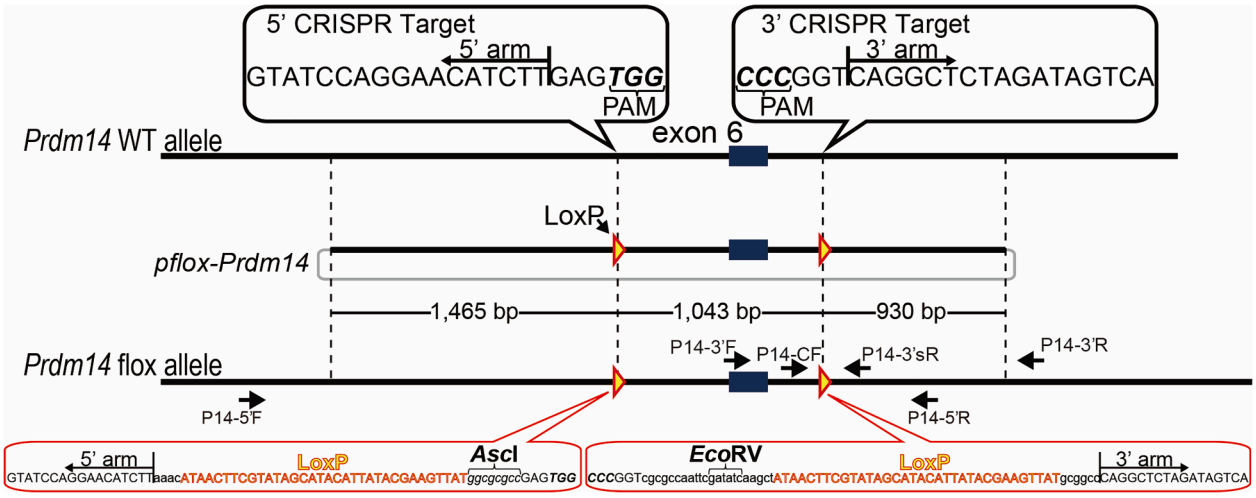

Fig. 6. Floxed mouse production with Cas9-mC. Strategy for *Prdm14* floxing. Upstream and downstream of exon 6 of *Prdm14* was cleaved at the 5′ and 3′ target sites. Black arrows indicate primers for PCR screening.

Table 4
Prdm14 floxed mouse production with *pX330-mC*.

Injected DNA		Number of Embryo		Newborn	Examined (weaning)	Carrying Exon6 floxed allele	Carrying Exon6 deletion allele
Donor	CRISPR-Cas	Injected	Transferred				
pflox-Prdm14-e6	*pX330-Prdm14-5′ pX330-Prdm14-3′*	104	85	26	25	0 (0.0%)[a]	12 (48.0%)[b]
	pX330-mC-Prdm14-5′ pX330-mC-Prdm14-3′	104	87	23	22	4 (18.2%)[a]	16 (72.7%)[b]

[a] Number of floxed mice/Number of mice examined.
[b] Number of mice carrying large deletion/Number of mice examined.

Table 5
Knock-in mouse production with *pX330-mC*.

Target Gene (Chromosomal Region)	Number of Embryo		Newborn	Examined (weaning)	Knock-in W/O random integration	Insertion length (kb)	Homology arm length (kb)	
	Injected	Transferred					5′ arm	3′ arm
Il-10 (1qE4)	153	140	41	39	4 (10.3%)[a]	1.7	2.3	1.6
Rubcn (16qB3)	229	208	71	66	21 (31.8%)[a]	1.8	1.8	1.9
Cd153 (4qC1)	247	217	56	55	10 (18.2%)[a]	2.8	1.9	2.3
Col5a1 (2qA3)	288	271	52	32	5 (15.6%)[a]	1.0	1.9	1.5
Camkk1 (11qB4)	168	157	53	48	7 (14.6%)[a]	0.6	1.7	1.9
Postn (3qC)	268	243	69	56	14 (25.0%)[a]	1.5	1.5	2.0
XXX (10qB4)	148	137	24	21	5 (23.8%)[a]	2.2	1.6	2.0

[a] Number of Knock-in mice/Number of mice examined.

Table 6
Floxed mouse production with *pX330-mC*.

Target Gene (Chromosomal Region)	Number of Embryo		Newborn	Examined (weaning)	Floxed W/O random integration	Floxed length (kb)	Homology arm length (kb)	
	Injected	Transferred					5′ arm	3′ arm
AA (2qC3)	320	273	31	25	3 (12.0%)[a]	2.1	2.1	1.9
BB (4qD2.2)	266	242	56	49	4 (8.2%)[a]	1.1	1.8	1.7
CC (13qD2.2)	312	287	81	81	8 (9.9%)[a]	1.4	1.6	2.3
DD (2qE5)	208	188	28	28	4 (14.3%)[a]	1.8	1.7	2.2
EE (2qH3)	224	203	43	28	4 (14.3%)[a]	1.2	2.2	0.7
FF (16qB3)	238	215	58	54	8 (14.8%)[a]	2.3	1.5	1.8
GG (19qD2)	234	225	65	65	7 (10.8%)[a]	2.6	1.8	1.3

[a] Number of floxed mice/Number of mice examined.

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Conflict of Interest Statement

YI is employed by Charles River Laboratories Japan, Inc. (CRJ) which provides the mice described in the manuscript. However, CRJ is not a publicly traded company and the author does not own any shares/equity in CRJ.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2020.04.007.

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