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Research Article

Generation of chimeric mice with spermatozoa fully derived from embryonic stem cells using a triple-target CRISPR method for *Nanos3*[†]

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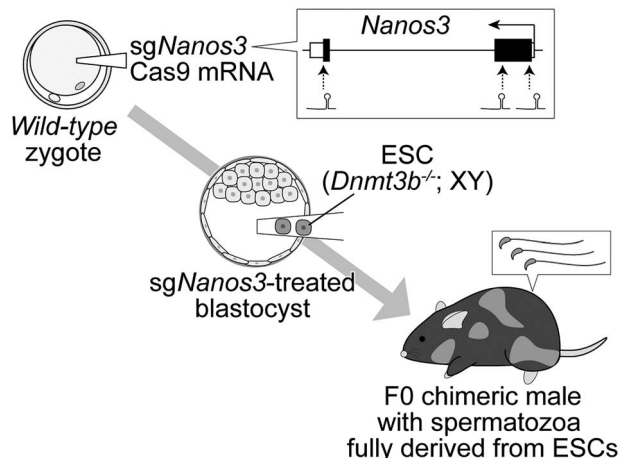
Abstract

Conditional knockout (cKO) mice have contributed greatly to understanding the tissue- or stage-specific functions of genes in vivo. However, the current cKO method requires considerable time and effort because of the need to generate two gene-modified mouse strains (Cre transgenic and loxP knockin) for crossing. Here, we examined whether we could analyze the germ cell-related functions of embryonic lethal genes in F0 chimeric mice by restricting the origin of germ cells to mutant embryonic stem cells (ESCs). We confirmed that the full ESC origin of spermatozoa in fertile chimeric mice was achieved by the CRISPR/Cas9 system using three guide RNAs targeting *Nanos3*, which induced germ cell depletion in the host blastocyst-derived tissues. Among these fertile chimeric mice, those from male ESCs with a *Dnmt3b* mutation, which normally causes embryo death, also produced F1 mice derived exclusively from the mutant ESCs. Thus, our new chimeric strategy readily revealed that *Dnmt3b* is dispensable for male germ cell development, in agreement with a previous cKO study. Our new approach enables us to analyze the germ cell functions of embryonic lethal genes in the F0 generation without using the current cKO method.

Summary Sentence

A CRISPR/Cas9 system using three guide RNAs targeting *Nanos3* enabled the production of chimeric mice with spermatozoa fully derived from ESCs and confirmed that *Dnmt3b* is not essential for male germ cell development.

Graphical Abstract



Key words: chimeric mouse, germ cell, spermatozoon, CRISPR/Cas9, *Nanos3*, embryonic stem cell, *Dnmt3b*, blastocyst complementation.

Introduction

Gene knockout (KO) is one of the most powerful tools available to analyze the functions of genes of interest *in vivo* in model organisms such as the mouse. However, it is known that homozygous mutations of about 30% of genes in mice are lethal at the embryonic or neonatal stages, which makes analysis of their functions in adults impossible [1]. To circumvent this problem, the so-called conditional KO method (cKO) of lethal genes has been employed widely, using systems such as Cre-loxP-induced recombination [2]. In this system, cKO can be induced *in vivo* by mating gene knockin (KI) mice carrying a target gene flanked by two loxP sites (flox mice) with a tissue/cell-specific promoter-driven Cre-transgenic mouse strain (Cre mice). In germ cell studies (e.g., germ cell-specific Cre mice), mice expressing tissue nonspecific alkaline phosphatase-Cre and *Vasa*-Cre are often used [3, 4]. However, homozygous cKO mice—Cre mice carrying the loxP-containing targeting allele (double heterozygous mice)—must be prepared. Furthermore, the generation of loxP mice is often technically troublesome, even though the CRISPR/Cas9 method has now been improved for generating KI mice [5]. The proper maintenance of these two genetically modified lines can also be a burden for laboratories. Thus, the current cKO system is time-consuming and laborious and, sometimes, is not applicable because of the unavailability of appropriate KI strains.

To circumvent these drawbacks of the current cKO system, we sought to design a new system that could avoid the combined use of tissue/cell-specific transgenic and flox mice. The blastocyst complementation method has been applied in the fields of developmental biology and regenerative medicine [6–8]. With this method, a target organ exclusively derived from embryonic stem cells (ESCs) or induced pluripotent cells (iPSCs) can be achieved in chimeric animals by injecting these cells into blastocysts that have been modified genetically to prevent the development of the targeted organ [7–9]. We expected that this strategy could be applied to the substitution of the cKO system for analyzing the tissue-specific functions of embryonic lethal genes. For this purpose, while the resultant animals are chimeras of the donor ESCs/iPSCs and host blastocysts, the target organ should be derived exclusively from the donor stem cells.

To ensure this, we applied a modified CRISPR/Cas9 system using three guide RNAs (triple-target CRISPR), which has been shown to enable immediate production of biallelic mutant mice at nearly 100% efficiency in the F0 generation [10, 11].

Here, we aimed to establish a new chimera-based method substituting for the conventional cKO system by combining blastocyst complementation and the triple-target CRISPR methods. To examine the feasibility of this strategy, we performed functional assays of male germ cells derived from ESCs with *Dnmt3b* deficiency, which causes embryonic death at embryonic day 15.5 (E15.5) [12]. Germ cell loss in the blastocyst-derived tissues was induced by CRISPR using three single-guide (sg) RNAs for *Nanos3* (sg*Nanos3*) whose mutation is known to cause complete germ cell loss at the primordial germ cell (PGC) stage [13].

Materials and methods

Mice

ICR strain mice were obtained from CLEA Japan Inc. (Tokyo), and B6D2F1 (or BDF1; C57BL/6 N × DBA/2) and C57BL/6 N (B6N) strain mice were purchased from Japan SLC Inc. (Shizuoka, Japan) at >10 weeks of age. Enhanced green fluorescent protein (GFP)-expressing transgenic C57BL/6-Tg (CAG-EGFP) C14-Y01-FM131Os strain mice (B6-GFP) [14] were gifts from Dr Masaru Okabe, and *Oct4*-ΔPE-GFP strain mice [15] were donated by Dr Yasuhisa Matsui. Mice were housed in specific-pathogen-free conditions with food and water *ad libitum* under controlled conditions of temperature (24 ± 1 °C), humidity (55 ± 2%), and daily lighting (0700–2100 h). All animal experiments were approved by the Institutional Animal Care and Use Committee of RIKEN Tsukuba Branch. All methods described were performed in compliance with the relevant guidelines and regulations for good laboratory practice of RIKEN.

In vitro fertilization

Adult mice (>10 weeks of age) were used for *in vitro* fertilization (IVF). Briefly, spermatozoa were collected from the epididymides of adult males and incubated in droplets of human tubal fluid (HTF)

medium for 1 h at 37 °C under 5% CO₂ in humidified air. Cumulus-oocyte complexes (COCs) were collected from the oviducts of female mice that had been superovulated by injecting 7.5 IU of pregnant mare serum gonadotropin (ZENOAG, Tokyo, Japan) and/or anti-inhibin serum [16] (a gift from Dr Gen Watanabe) followed by 7.5 IU of human chorionic gonadotropin (hCG; ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) at a 48-h interval. Then, 15–17 h after the hCG injection, the COCs were isolated and incubated in HTF medium containing 0.125–1.25 mM glutathione (L-glutathione reduced, #G6013; Merck, Darmstadt, Germany) for 1 h before insemination. Next, preincubated spermatozoa were introduced to the COC-containing HTF droplets. At 5–6 h after insemination, fertilized zygotes were washed, transferred to droplets of potassium-enriched simplex optimized medium (KSOM) and cultured as above.

Triple-target CRISPR

Triple-target CRISPR was performed as described [10, 11]. Briefly, three sgRNAs targeting distinct positions of coding exons of *Nanos3* were designed using the Massachusetts Institute of Technology sgRNA design tool (currently closed). Sequences complementary to the sgRNAs were cloned into the px330 vector by oligo annealing (Supplementary Table S1). The T7 promoter was added to sgRNA templates by polymerase chain reaction (PCR) using the primers listed in Supplementary Table S1; then, sgRNAs were synthesized by in vitro transcription (IVT) from the templates using MEGAscript T7 transcription kits (#AM1354; Thermo Fisher Scientific, Waltham, MA, USA). Synthesized sgRNAs were purified using MEGAclear Transcription Clean-Up kits (#AM1908; Thermo Fisher Scientific). Cas9 mRNA was synthesized from the px330 vector by addition of T7 followed by IVT using mMACHINE mMACHINE T7 Ultra transcription kits (#1345; Thermo Fisher Scientific). The sgRNAs and Cas9 mRNA were adjusted to 500 ng/μl with ultrapure water, and aliquots were frozen at –80 °C until use. A mixture of the three sgRNAs (50 ng/μl each) targeting *Nanos3* and Cas9 mRNA (100 ng/μl) was injected into zygotes using a piezo-driven micromanipulator (Prime Tech Ltd., Ibaraki, Japan) at 5–6 h after insemination. The injected embryos were cultured as above in KSOM for 24 h until they reached the 2-cell stage or for 96 h until they reached the blastocyst stage.

Generation of chimeric mice and embryo transfer

Male B6-GFP or *Dnmt3b*^{-/-} ESCs were injected respectively into ICR × ICR or BDF1 × B6-GFP blastocysts, which had been treated with sg*Nanos3* and Cas9 mRNA at the zygote stage. About 10–15 ESCs were injected into each blastocyst. In the experiments shown in Figures 1 and 2, 2-cell stage embryos were transferred to the oviducts of E0.5 pseudopregnant ICR female mice. In the experiments shown in Figures 3 and 4 (the generation of chimeric mice), blastocysts were transferred to the uteri at E2.5 or, in a few cases, to the oviducts at E0.5 in pseudopregnant ICR female mice. For the embryonic analysis, the recipient females were euthanized at E13.5–14.5. The sex of embryos was determined from morphological differences between their gonads [17]. For the recovery of full-term pups, recipients were euthanized and subjected to Caesarean section at E19.5. Living pups were nursed by lactating ICR females. The systemic contribution of ESCs in chimeric mice was estimated from their coat color.

Embryonic stem cells

We established a male B6-GFP-ESC line from blastocysts generated by IVF using oocytes from B6N female mice and spermatozoa

from B6-GFP (C57BL/6 J and C57BL/6 N mixed background) male mice. To establish ESCs, developing blastocysts were transferred to Dulbecco's modified Eagle's medium (DMEM, #D6429; Merck) supplemented with 15% KnockOut™ Serum Replacement (#10828028; Thermo Fisher Scientific), 1% MEM non-essential amino acid (NEAA) solution (#11140050; Thermo Fisher Scientific), 0.1% GlutaMax (#35050061; Thermo Fisher Scientific), 0.1 mM 2-mercaptoethanol (#M7522; Merck), and 1000 U/ml mouse leukemia inhibitory factor (LIF; #ESG1107; Merck). They were cultured at 37 °C under 5% CO₂ in humidified air. Mouse embryonic fibroblasts (MEFs) were treated with 10 μg/ml mitomycin C (#M0503; Merck) and used as feeder cells. ESCs were established by plating blastocysts on a feeder layer in gelatin-coated dishes. Several days after plating, expanded colonies derived from the inner cell mass were obtained, dissociated with 0.25% trypsin (#15090046; Thermo Fisher Scientific) and cultured in the same medium on MEF feeder cells. After several passages, cell lines with typical ESC colonies were used for generating chimeric mice. A male 129-*Dnmt3b*^{-/-} ESC line (AES0165) was obtained from RIKEN BioResource Research Center and cultured in Glasgow's MEM (#11710035; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate (#11360070; Thermo Fisher Scientific), 0.1% GlutaMax, 1% NEAA, 0.1 mM 2-mercaptoethanol, and 1000 U/ml mouse LIF.

Natural mating

Adult mice (>10 weeks of age) were used for natural mating. Each F0 generation founder male was mated with one or two wild-type females in one cage. We defined the male as infertile if no pups were obtained by 2 months after the onset of mating trials.

Germline transmission test

To examine the germline transmission potential of ESCs, F1 progenies were generated by natural mating or IVF using wild-type female mice. ICR female mice were used for generating F0 male mice from B6-GFP ESCs and sg*Nanos3*-treated ICR blastocysts (Figure 3). B6N (a/a) females were used for generating F0 male mice from 129 (A/A)-*Dnmt3b*^{-/-} ESCs and sg*Nanos3*-treated BDF1 (a/a) × B6-GFP (a/a) blastocysts (Figure 4). Using this mating strategy, ESC-derived F1 (A/a) pups show agouti coat color (Figure 4B) and the blastocyst-derived F1 (a/a) pups show black coat color (Supplementary Figure S2).

Histology

After recording weights, testes and ovaries from euthanized mice were fixed in 4% paraformaldehyde at 4 °C overnight and embedded in paraffin wax. Serial sections (4-μm thick) were subjected to hematoxylin and eosin (HE) staining and immunohistochemistry. For immunohistochemistry, the sections were treated with Antigen Unmasking Solution (#H-3300; Vector Laboratories, Burlingame, CA, USA) at sub-boiling temperature (<100 °C) for 10 min to unmask antigen epitopes followed by 0.3% hydrogen peroxide treatment for 20 min to inactivate endogenous peroxidase. After blocking with 1% bovine serum albumin in phosphate-buffered saline for 1 h at room temperature, they were incubated with a mouse anti-protamine 1 (PRM1) primary antibody (1/500 dilution, #HUP1N; Briar Patch Biosciences, Livermore, CA, USA; RRID:AB_2651186), a chicken anti-GFP primary antibody (1/5000 dilution, #GFP-1020; Aves Labs, Inc., Davis, CA, USA; RRID:AB_10000240), or a rabbit anti-DEAD-box helicase 4 (DDX4) primary antibody (1/5000 dilution, #ab13840; Abcam, Cambridge, UK; RRID:AB_

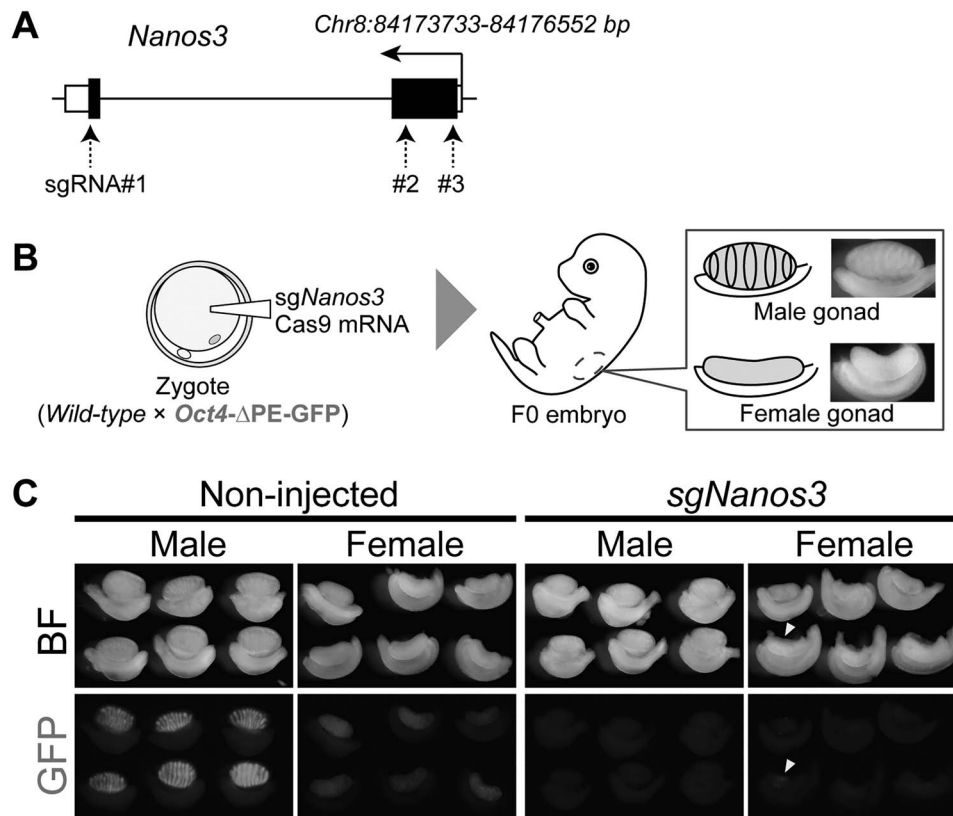


Figure 1. Loss of germ cell-specific GFP in the gonads of E13.5–E14.5 embryos derived from *sgNanos3*-treated zygotes. (A) Schematic illustration of the triple-target CRISPR approach. Three sgRNAs (#1–3) targeting protein-coding exons were designed for *Nanos3*. Black and white boxes represent protein-coding and nonprotein-coding exons, respectively. (B) Schematic representation of the triple-target CRISPR method targeting *Nanos3* in zygotes, and analysis of embryonic gonads. The *sgNanos3* and Cas9 mRNA sequences were microinjected into zygotes derived from wild-type oocytes (ICR, B6N, or BDF1) and *Oct4-ΔPE-GFP* spermatozoa. The *sgNanos3*-treated zygotes were transferred into the oviducts of recipient pseudopregnant female mice, and fetal gonads were isolated from the embryos at E13.5–E14.5. (C) Bright field (BF) and GFP fluorescence images of fetal gonads derived from noninjected or *sgNanos3*-injected BDF1 × *Oct4-ΔPE-GFP* embryos. Most *sgNanos3*-injected embryos showed the loss of GFP signals in the gonads. The arrowhead (rightmost panels) indicates a slight GFP signal in a gonad (see Table 1).

443012). Following three washes, the sections were incubated with biotinylated secondary antibodies (Dako, Glostrup, Denmark) for 1 h at room temperature. The biotin signals were then amplified using Vectastain Elite ABC kits (#PK-6100; Vector Laboratories) and detected using 3,3'-diaminobenzidine. For double staining, the signals were detected with the following secondary antibodies: anti-chicken Alexa Fluor 488 or anti-rabbit Alexa Fluor 546 antibody (Thermo Fisher Scientific). The fluorescence-labeled sections were mounted using Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, # H-1200) and were observed using confocal microscopy (Nikon C2, Tokyo, Japan).

Genomic DNA isolation and genotyping PCR with spermatozoa

Genomic DNA isolation from spermatozoa was performed as described [18]. Briefly, spermatozoa were collected from the epididymides of adult males after euthanasia and suspended in a lysis buffer (0.14 mM β -mercaptoethanol, 0.24 mg/ml proteinase K, 150 mM NaCl, 10 mM Tris-HCl [pH 8.0], 10 mM EDTA [pH 8.0], and 0.1% SDS) and incubated at 55 °C overnight. Then, sperm genomic DNA was isolated using phenol/chloroform extraction and ethanol precipitation. Three primers were used for

genotyping PCR for GFP using spermatozoa of fertile chimeric mice (Supplementary Figure S3) according to the instructions of RIKEN BioResource Research Center (https://mus.list.brc.riken.jp/ja/wp-content/pdf/00267_PCR.pdf). Genotyping PCR was performed using Tks Gflex DNA Polymerase (#R060A; Takara Bio, Shiga, Japan).

Statistical analysis

Data are presented as the mean \pm standard error of mean (SEM; Tables 2 and 4) as appropriate. Mean testis weights were evaluated between treatments using Student's unpaired two-tailed *t*-test as a parametric test or the Mann–Whitney nonparametric *U* test after checking for normal distribution using the Shapiro–Willk test (Table 2). Statistically significant differences between variables were determined at $P < 0.05$.

Results

Mice derived from zygotes treated with triple-target *sgNanos3* produced no spermatozoa

We designed three single-guide RNAs targeting distinct protein-coding exons for *Nanos3* (Figure 1A; Supplementary Table S1). To

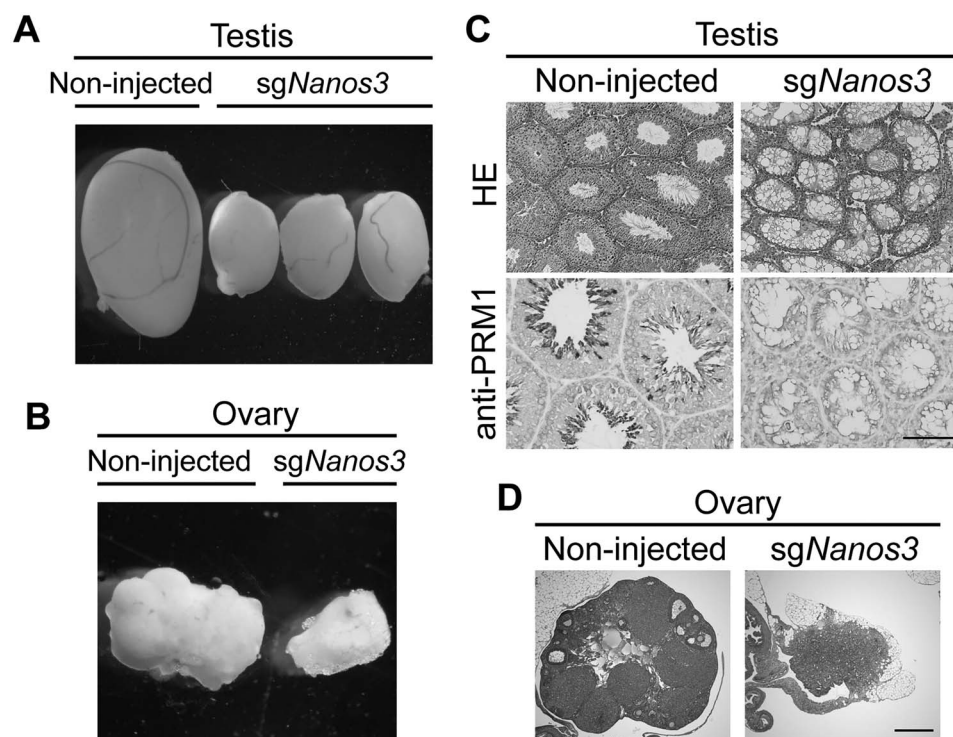


Figure 2. Hypogonadal phenotypes with severe loss of germ cells in adult mice derived from *sgNanos3*-treated zygotes. (A, B) Testes and ovaries of F0 generation adult mice (age > 10 weeks) derived from *sgNanos3*-treated zygotes (BDF1 × BDF1). (C, D) HE and anti-PRM1 immunostained images of the testis (C) and HE-stained images of the ovary (D). These mice showed hypogonadal phenotypes with severe loss of spermatozoa or oocytes. None of the *sgNanos3*-treated F0 male mice ($n = 13$) showed PRM1-positive spermatozoa in their testes. Scale bars = 100 μm (C) and 500 μm (D).

confirm the efficiency of the triple-target *sgNanos3*, we microinjected them together with Cas9 mRNA into zygotes derived by IVF (Figure 1B). IVF was performed using wild-type oocytes from one of three mouse strains—ICR, B6N, or BDF1—and spermatozoa from *Oct4*- Δ PE-GFP transgenic mice expressing germ cell-specific GFP fluorescence [15]. After transfer of these embryos into the oviducts of pseudopregnant females, we examined their phenotypes at E13.5–14.5. The embryonic gonads derived from control (noninjected) zygotes showed GFP signals (Figure 1C; Table 1) as reported [15]. In contrast, most (80–100%) of the embryonic gonads derived from the zygotes treated with *sgNanos3* showed no GFP signals, suggesting complete loss of PGCs in their gonads (Figure 1C, Table 1). We also checked the testes and ovaries of adult founder mice (age > 10 weeks) derived from zygotes treated with *sgNanos3*. Their testes and ovaries were smaller than those of control mice, showing a hypogonadal phenotype as reported [13] (Figure 2A and B, Table 2). Histology revealed that *sgNanos3*-treated testes showed no germ cells in most seminiferous tubules (Figure 2C). Although residual germ cells were observed in a few seminiferous tubules in some *sgNanos3*-treated testes, no PRM1-positive mature spermatozoa were detected (Figure 2C; Supplementary Figure S1), indicating that the residual germ cells were composed of spermatogenic cells preceding the elongating spermatid stage. We confirmed that the cauda epididymidis in all these mice contained no spermatozoa. In *sgNanos3* females, no follicles were found in most ovaries, but a few developed occasionally (Figure 2D; Table 2).

F0 chimeric male mice from B6-GFP ESCs and *sgNanos3*-treated blastocysts produced F1 mice all derived from ESCs

Next, we generated chimeric mice by injecting donor B6-GFP male ESCs into *sgNanos3*-treated ICR strain blastocysts (Figure 3A). This combination enabled us to distinguish ESC-derived tissues from blastocyst-derived tissues by their GFP signals and black coat color. Of 10 F0 chimeras obtained, seven were male in appearance (Table 3). Only one of these mice was fertile and produced 55 pups by natural mating or IVF using ICR females (Table 3). All the offspring had black coat color, indicating that only ESC-derived spermatozoa contributed to their production (Figure 3B; Table 3). The remaining six male mice were sterile, as confirmed by repeated natural mating over 2 months. IVF was not performed with them because of the lack of spermatozoa in their epididymides. This was also the case with non-ESC-injected male mice ($n = 8$; Table 3). The fertile male mouse had larger testes than did the infertile ones (Figure 3C and D; Table 4). Histology of the testes revealed that normal spermatogenesis occurred in the fertile chimeric male, while only early germ cells, if any, were found in the infertile ones (Figure 3D). Immunostaining for GFP and DDX4 confirmed the ESC origin of the spermatogenic cells in the fertile male's testes (Figure 3D). These data suggest that we had successfully generated chimeric mice with mature spermatozoa fully derived from ESCs by injecting them into *sgNanos3*-treated blastocysts.

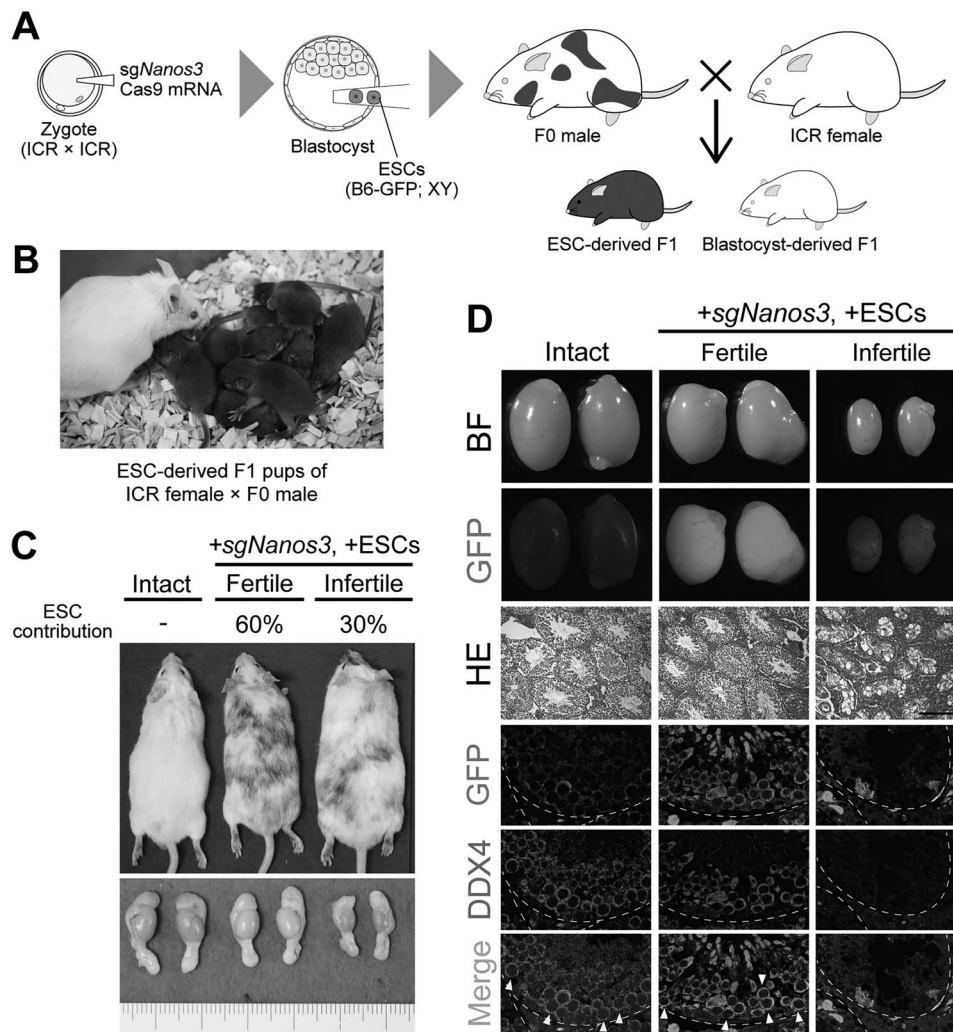


Figure 3. Generation of chimeric mice derived from *sgNanos3*-treated ICR × ICR zygotes and male B6-GFP ESCs. (A) Schematic representation of the generation of chimeric mice derived from *sgNanos3*-treated ICR × ICR zygotes and XY B6-GFP ESCs and the analysis of their germline transmission by natural mating and/or IVF with ICR strain female mice. We confirmed successful germline transmission of the ESC genome by the black coat color of F1 generation mice. (B) One-week-old F1 pups born after mating of a chimeric F0 male with a female ICR mouse. All F1 pups had black coat color, indicating their donor ESC origin (see Table 3). (C) Chimeric male mice generated by injecting B6-GFP ESCs into ICR × ICR blastocysts treated with *sgNanos3* and their testes. An intact male mouse was used as a control without *sgNanos3* treatment or ESC injection. The ESC contribution rate of each chimeric mouse was estimated from the coat color (Table 4). (D) BF, GFP fluorescence, HE staining, and anti-GFP and DDX4 staining images of the testes from an intact control male and F0 chimeric males. The testes from the fertile chimeric male generated from a *sgNanos3*-treated zygote were GFP positive and larger than those from an infertile chimeric male. They showed normal spermatogenesis with GFP-positive/DDX4-positive male germ cells (white arrowheads) in the seminiferous tubules. The intact mouse testis contained GFP-negative/DDX4-positive male germ cells (yellow arrowheads), while the infertile chimeric mouse testis contained no germ cells. The dotted lines denote the boundaries between the seminiferous tubule and interstitium. Scale bar = 200 μ m.

F0 chimeras produced with *sgNanos3*-treated blastocysts revealed dispensability of the embryonic lethal gene *Dnmt3b* for spermatogenesis

Based on the above results, we next attempted to generate chimeric mice with spermatozoa fully derived from ESCs carrying a lethal gene mutation. For this purpose, we used ESCs with a *Dnmt3b* mutation because this mutation causes embryonic death at E15.5 [12], but plays no essential roles in germ cell development, as revealed by a germ cell-specific cKO study [19]. We injected male *Dnmt3b*^{-/-} ESCs with the 129Sv/Jae genetic background (129-*Dnmt3b*^{-/-} ESCs) (*non-agouti* gene: *A/A*) [12, 20] into *sgNanos3*-treated BDF1 × B6-GFP blastocysts (*a/a* × *a/a*) and examined germline transmission of the mutant genome to F1 mice by natural mating or IVF using

B6N (*a/a*) females (Figure 4A). Although five of seven F0 chimeric male mice were infertile, the other two were fertile and produced F1 pups with agouti coat color (*A/a*), indicating that they were derived from the donor ESCs (Figure 4B; Table 3). If they had been derived from the host blastocysts, they should have had black coat color (*a/a*) (Supplementary Figure S2). Consistent with this, genomic PCR amplification for the gene encoding GFP did not detect the GFP gene (host blastocyst origin) in spermatozoa from these two fertile F0 males (Supplementary Figure S3). There seemed to be no correlation between the coat color pattern and germline transmission in this case (Table 4). Although the testes of the fertile males were smaller than those of intact controls (no *sgNanos3* treatment, no ESC injection), they were larger than those of the infertile ones (Figure 4C, D;

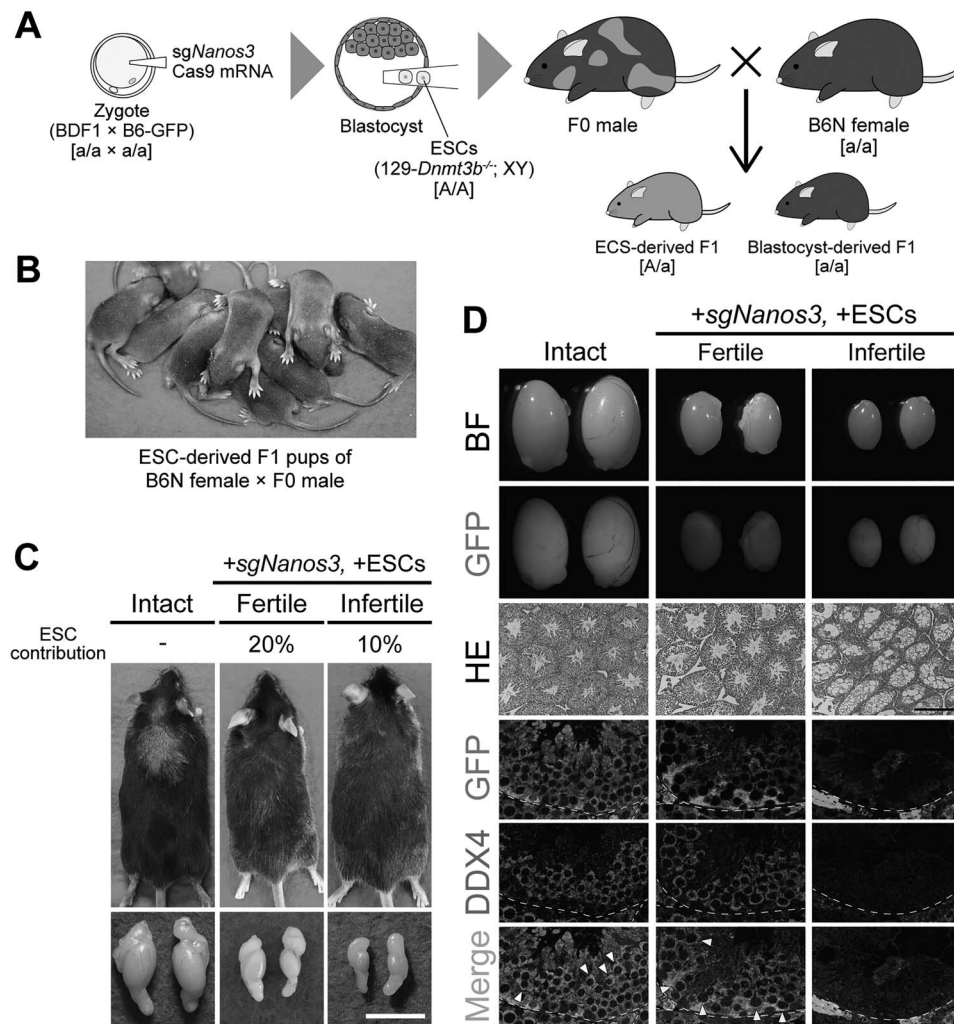


Figure 4. Generation of chimeric mice from *sgNanos3*-treated BDF1 × B6-GFP zygotes and male 129-*Dnmt3b*^{-/-} ESCs. (A) Schematic representation of the generation of chimeric mice derived from *sgNanos3*-treated BDF1 × B6-GFP zygotes and 129-*Dnmt3b*^{-/-} ESCs and analysis of their germline transmission by natural mating and/or IVF with B6N females. We confirmed germline transmission of the ESC genome by the agouti coat color of F1 mice (A/a). The black coat color (a/a) of F1 mice indicates the blastocyst origin of their spermatozoa. (B) One-week-old F1 pups obtained by IVF using a F0 chimeric male and a B6N female. All these pups had agouti coat color, indicating their donor ESC origin (see Table 3). (C) An intact control (no *sgNanos3* treatment and no ESC injection) BDF1 × B6-GFP male and F0 chimeric males generated by injecting male 129-*Dnmt3b*^{-/-} ESCs into BDF1 × B6-GFP blastocysts treated with *sgNanos3*, and their testes. The ESC contribution rate of each chimeric mouse was estimated from the coat color (Table 4). (D) BF, GFP fluorescence, and HE-, anti-GFP-, and DDX4-staining images of the testes from an intact control male and F0 chimeric males. The testes from the fertile chimeric male generated from a *sgNanos3*-treated zygote were larger than those from an infertile chimeric male. They show normal spermatogenesis with GFP-negative/DDX4-positive germ cells derived from ESCs (yellow arrowheads). The intact testis contains GFP-positive/DDX4-positive germ cells (white arrowheads). The dotted lines denote the boundaries between the seminiferous tubule and interstitium. Scale bars = 10 mm (C) and 200 μm (D).

Table 4) and showed normal spermatogenesis (DDX4 positive) in many seminiferous tubules, as did the control mice (Figure 4D). Thus, we have confirmed that the embryonic lethal *Dnmt3b* gene is dispensable for normal spermatogenesis in the mouse. Together with the results shown in Figure 3, we obtained three fertile chimeric male mice, all of which exclusively produced ESC-derived pups ($n = 92$ from 10 l) (Table 3).

Discussion

Here, we aimed to establish a method for producing chimeric mice with germ cells fully derived from ESCs carrying a lethal gene mutation. Previously, chimeric mice have been produced with the aim

of rescuing male germ cells carrying an embryonic lethal mutation and the effects of the mutation on sperm function were analyzed [21–23]. However, in those methods, as male germ cells could be derived from either ESCs or blastocysts, it is difficult to distinguish them, unless the mutant spermatozoa carry specific markers such as GFP-tagged acrosin [21–24]. We overcame this problem by employing the triple-target CRISPR method targeting *Nanos3*, which abolishes germ cells at the PGC stage. This method is easy and highly reproducible, as shown previously [10, 11]. Using this experimental system, we obtained F0 chimera mice that produced spermatozoa derived only from the embryonic lethal *Dnmt3b*^{-/-} ESCs (Figure 4). This result suggests that *Dnmt3b* is dispensable for spermatogenesis and spermiogenesis, consistent with a previous report showing that no apparent change in phenotype was observed

Table 1. Loss of GFP in the gonads of E13.5–14.5 embryos derived from sgNanos3-treated zygotes carrying the *Oct4-ΔPE-GFP* allele.

Female	Male	Injection	No. of transferred embryos (no. of recipients)	No. (%) of embryos	No. of embryos by sex		No. (%) of GFP levels in gonads			
					Male	Female	Intact	Faint	Undetectable	
ICR	<i>Oct4-ΔPE-GFP</i>	Non-injected	52 (2)	35 (67)	Male	22	22 (100)	0 (0)	0 (0)	
					Female	13	13 (100)	0 (0)	0 (0)	
					sgNanos3/Cas9	7	0 (0)	1 (14)	6 (86)	
BDF1	Non-injected	63 (3)	46 (73)	Male	30	30 (100)	0 (0)	0 (0)		
				Female	16	16 (100)	0 (0)	0 (0)		
				sgNanos3/Cas9	13	0 (0)	0 (0)	13 (100)		
C57BL/6 N	Non-injected	42 (2)	24 (57)	Male	18	18 (100)	0 (0)	0 (0)		
				Female	6	6 (100)	0 (0)	0 (0)		
				sgNanos3/Cas9	9	0 (0)	0 (0)	9 (100)		
					9	0 (0)	0 (0)	0 (0)	9 (100)	

in germ cell-specific *Dnmt3b* mutant mice [19]. Thus, our method substitutes for the current cKO strategy, at least for functional assays of male germ cells carrying an embryonic lethal mutation. We expect that the same strategy will be broadly applicable for the assessment of the tissue-specific functions of embryonic lethal genes without the need for complicated cKO procedures.

The triple-target CRISPR method is a powerful tool, but it is not always perfect. Although we could largely reproduce the phenotype of *Nanos3* KO mice with the loss of germ cells as reported previously [13], there were a few exceptional cases with residual PGCs in embryonic gonads or active seminiferous tubules in adult testes (Figure 1; Table 1; Supplementary Figure S1). The original study of the triple-target CRISPR method also mentioned its efficiency was “almost” perfect (~96–100% whole-body KO) [10]. Nevertheless, in our study, the sgNanos3-treated adult male mice showed no mature spermatozoa (Figure 2C; Supplementary Figure S1) and were infertile (Table 3). In addition, the F0 chimeric male mouse generated from sgNanos3-treated blastocysts injected with ESCs produced only F1 pups of donor ESC origin (Figures 3B and 4B; Table 3). We further confirmed the absence of host-derived spermatozoa in the epididymides of the fertile F0 chimeras by genomic PCR (Supplementary Figure S3). However, the presence of residual germ cells in the testes derived from sgNanos3-treated blastocysts suggests that the sgNanos3 treatment might not be appropriate for the functional analysis of the genes in earlier stages of spermatogenesis. Alternative targets of sgRNA for this purpose could include *Prdm14*, which is essential for the development of early PGCs at around E6.5 in mice [25]. We confirmed that treatment with *Prdm14* sgRNA resulted in the complete depletion of germ cells in most gonads at E12.5 (Supplementary Figure S4; Supplementary Table S2). It is possible that sgPrdm14 can be more broadly used than sgNanos3 for the analysis of male fertility-related functions of embryonic lethal genes.

In our study, many of the F0 chimeric males were infertile and unable to be used for germline transmission tests (Figures 3C and D and 4C and D; Table 3). Consequently, the rates of generation of fertile F0 chimeric males were rather low: 14% (1/7) in ICR × ICR blastocysts with B6-GFP ESCs and 29% (2/7) in BDF1 × B6-GFP blastocysts with 129-*Dnmt3b*^{-/-} ESCs. It is generally known that the efficiency of germline transmission in chimeric animals can be affected by multiple factors: the quality of ESCs [26–29]; the method used to generate chimeric embryos [30–32]; and the combination of mouse strains used for the host embryos and ESCs [33, 34]. Specifically, concerning the stage of host embryos, it was reported that 8-cell embryos were better than blastocysts for generating F1 mice from B6-background ESCs [31]. Thorough optimization of these factors might have improved the germline transmission in our experimental system. The broad interstrain applicability of this triple-target CRISPR should be a significant advantage for this purpose (Table 1). Moreover, the fact that fertile chimeric males ($n = 3$) exclusively produced ESC-derived pups ($n = 92$ from 10 litters) (Table 3) verifies the robustness of our strategy.

This study has important implications for developments in stem cell research and transplantation medicine. Previously, intra- or interspecies chimeric animals with organs derived exclusively from ESCs or iPSCs have been generated using blastocysts carrying mutations inducing loss of specific organs/tissues (e.g., *Pdx1* for the pancreas, *Sall1* for the kidney, and *VEGFR2* for vascular endothelial and hematopoietic cells) [7, 35, 36]. In those studies, the host blastocysts carrying organ-specific mutations were prepared by fertilization using mutant animals. As shown in this and other studies, the triple-

Table 2. Testis or ovary weights of F0 founder adult mice derived from sgNanos3-treated zygotes.

Female	Male	Injection	No. of transferred embryos (no. of recipients)	No. (%) of F0 pups	Mean weight [mg] (n)	
ICR	ICR	Non-injected	N/A ^c		Testis	307 ± 10 (6)
					Ovary	33 ± 1 (7)
		sgNanos3/Cas9	103 (4)	54 (52)	Testis	55 ± 3 (8) ^a
					Ovary	9 ± 6 (8) ^{a,b}
BDF1	B6-GFP	Non-injected	65 (4)	38 (58)	Testis	224 ± 6 (9)
					Ovary	22 ± 2 (6)
		sgNanos3/Cas9	62 (3)	18 (29)	Testis	37 ± 1 (5) ^a
					Ovary	2 ± 1 (7) ^a

^a*P* < 0.05 vs. "non-injected" control group.^bA few follicles developed in three females.^cDerived from natural mating.**Table 3.** The fertility and germline transmission of F0 founder male chimeras derived from sgNanos3 blastocysts and ESCs.

Female	Male	Injection	ESC strain	No. of F0 male/offspring	No. (%) of F0 chimeric males ^b	No. (%) of fertile F0 males	No. (%) of F0 males with GLT ^c	No. of F1 offspring		No. (%) of ESC-derived F1 offspring
ICR	ICR	sgNanos3/ Cas9	Non-injected	8 ^a	N/A	0 (0) ^c		N/A		
			B6-GFP	7/10	2/7 (29)	1 (50) ^c	1 (100)	Mating	31	31 (100)
				IVF	24	24 (100)				
BDF1	B6-GFP	sgNanos3/ Cas9	Non-injected	5/13	N/A	0 (0) ^d		N/A		
			129- <i>Dnmt3b</i> ^{-/-}	7/29	2/7 (29)	1 (50) ^d	2 ^f (100)	Mating	11	11 (100)
				IVF	26	26 (100)				
Sum of ESC chimeras				14/39	4/14 (29)	2 (50)	3 (100)	92	92 (100)	

^aOf 54 offspring obtained, eight male mice were subjected to IVF trials, but no epididymal spermatozoa were found.^bDetermined by the coat color.^cMated with ICR females.^dMated with B6N females.^eGermline transmission.^fOne from a black (nonchimeric coat color) male.**Table 4.** The testis weights of F0 generation founder male chimeras derived from sgNanos3 blastocysts and ESCs.

Female	Male	Injection	ESC strain	Fertility (n)	ESC contribution in % ^a (n)	Mean weight of testes [mg] (n)
ICR	ICR	sgNanos3/Cas9	B6-GFP	Fertile (1)	60 (1)	212 (1)
				Infertile (6)	0 (5), 30 (1)	59 ± 3 (6)
BDF1	B6-GFP	sgNanos3/Cas9	129- <i>Dnmt3b</i> ^{-/-}	Fertile (2)	0 (1), 20 (1)	73 ± 2 (2)
				Infertile (5)	0 (4), 10 (1)	36 ± 3 (5)

^aJudged from the coat color.

target CRISPR method is highly effective in ablating a single gene, or even multiple genes at one time, using zygotes [10, 37]. Therefore, in vivo generation of organs from ESCs/iPSCs in chimeric animals for research and clinical uses will become easier technically by using this triple-target CRISPR system.

In conclusion, we have established a novel strategy to produce chimeric mice that have spermatozoa fully derived from ESCs using triple-target sg*Nanos3* blastocysts. Based on our system, it would become more feasible to identify germ cell-related functions of genes whose mutations cause embryonic death. It could also be applied for other combinations of organs and embryonic lethal genes by using appropriate triple-target sgRNAs.

Supplementary data

Supplementary data are available at *BIOLRE* online.

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Conflict of interest

The authors declare no conflict of interest.

Authors' contributions

K.M., S.M., and A.O. conceived the project, designed the experiments, and wrote the manuscript. K.M. performed most of the experiments. M.H. established the B6-GFP ESC line.

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