

# Somatic cell reprogramming-free generation of genetically modified pigs

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Genetically modified pigs for biomedical applications have been mainly generated using the somatic cell nuclear transfer technique; however, this approach requires complex micromanipulation techniques and sometimes increases the risks of both prenatal and postnatal death by faulty epigenetic reprogramming of a donor somatic cell nucleus. As a result, the production of genetically modified pigs has not been widely applied. We provide a simple method for CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 gene editing in pigs that involves the introduction of Cas9 protein and single-guide RNA into in vitro fertilized zygotes by electroporation. The use of gene editing by electroporation of Cas9 protein (GEEP) resulted in highly efficient targeted gene disruption and was validated by the efficient production of *Myostatin* mutant pigs. Because GEEP does not require the complex methods associated with micromanipulation for somatic reprogramming, it has the potential for facilitating the genetic modification of pigs.

## INTRODUCTION

Pigs are considered as one of the best animals for generating models of human diseases and for providing organs for xenotransplantation (1–3). For these purposes, the genetic modification of genes involving disease and immunorejection and of porcine endogenous retroviruses (4) with possible transmission after the implantation is essential (5). Currently, genetic modification in pigs has been mainly achieved by somatic cell nuclear transfer (SCNT) after the generation of engineered donor somatic cells (6–9). The SCNT technique is associated with technological limitations that prevent their widespread use in pigs (7, 10). In addition, the SCNT-dependent strategy sometimes results in incomplete epigenetic reprogramming (11–13), which can cause prenatal or postnatal death and a reduced efficiency of offspring production (14–17). Therefore, somatic cell reprogramming-free strategies are required for the practical use of genetically modified pigs in the medical field. If a micromanipulation-free method can be established for pig zygotes produced by in vitro maturation (IVM) and in vitro fertilization (IVF) techniques, which enable the preparation of large numbers of zygotes from the ovaries of slaughtered pigs, the rapid and large-scale production of mutant pigs may be feasible (Fig. 1). Here, we established a simple

GEEP (gene editing by electroporation of Cas9 protein) method for generating genetically modified pigs using IVF zygotes.

## RESULTS AND DISCUSSION

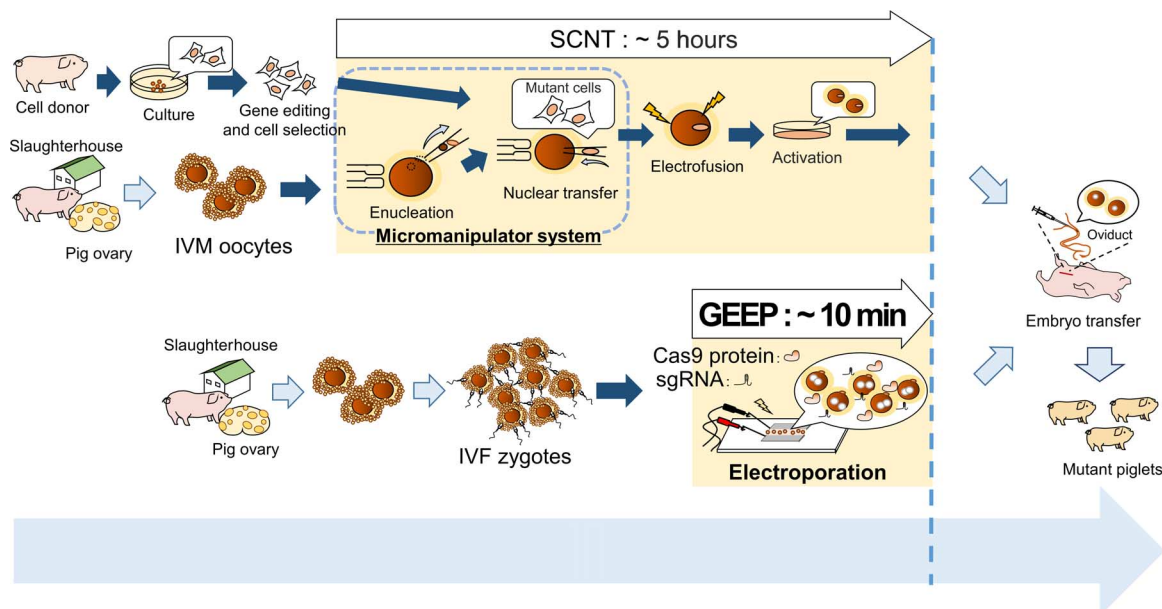
First, we evaluated the use of mouse zygote electroporation conditions (seven 3-ms pulses at a voltage of 30 V) (18) for introducing the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system into pig zygotes. Thirteen hours after IVF was initiated, the presumptive zygotes were electroporated with Cas9 mRNA (400 ng  $\mu\text{l}^{-1}$ ) and single-guide RNA (sgRNA; 200 ng  $\mu\text{l}^{-1}$ ) targeting the *FGF10* gene (18, 19). However, most of the electroporated IVF zygotes did not develop properly because of electroporation-induced damage (Fig. 2D). Therefore, we optimized the pig zygote electroporation conditions as follows. We electroporated the same concentrations of Cas9 mRNA and sgRNA into the presumptive zygotes, while varying the pulse duration (1 to 3 ms) and the number of pulses (three to seven) and keeping the voltage at 30 V (Fig. 2, A to D). The electroporated zygotes were cultured for 7 days until blastocyst formation, and then the frequency of base insertions or deletions (indels) in the *FGF10* gene was analyzed (Fig. 2, B and C). The frequency of indels increased with increasing pulse duration and number, whereas the blastocyst formation rates fell markedly when the pulse duration and number exceeded 1 ms and five, respectively (Fig. 2D). From these results, we concluded that five 1-ms pulses at a voltage of 30 V were the optimal electroporation conditions for introducing Cas9 mRNA and sgRNA into pig IVF zygotes. We also investigated the optimal timing of electroporation. We electroporated the presumptive zygotes at 11, 13, 19, 21, and 23 hours after the start of IVF. Although the efficiency of genome editing was the same among the groups, the rate of blastocyst formation was highest when the zygotes were electroporated 13 hours after the start of IVF (fig. S1). Therefore, electroporation was performed 13 hours after IVF initiation thereafter.

Although we succeeded in introducing indels into the pig genome using the optimized electroporation conditions, the mutation frequency

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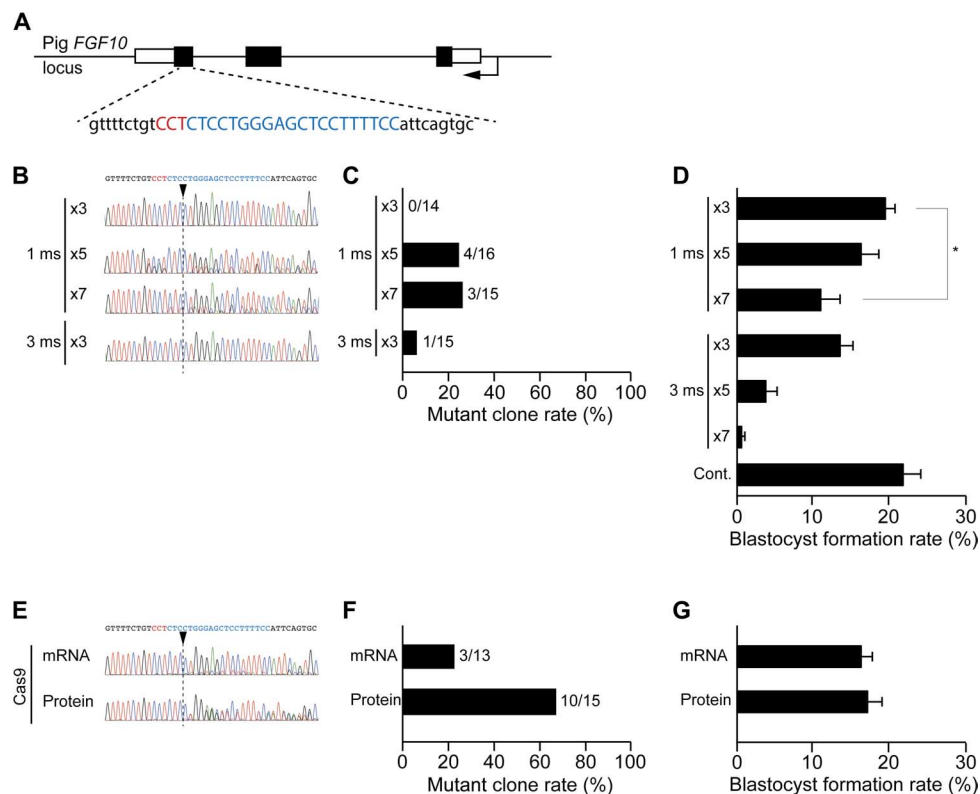
**Fig. 1. Comparison of the SCNT and the GEEP method.** In vitro matured pig oocytes are used for SCNT, and in vitro fertilized zygotes are used for GEEP. SCNT involves the removal of oocyte nuclei (enucleation), the transfer of mutant somatic cell nuclei (nuclear transfer), and the activation of the reconstructed embryos after electrofusion, all of which takes ~5 hours. SCNT also requires the generation of mutant donor cells, which takes ~2 weeks. On the other hand, GEEP takes ~10 min to transfer the genome editing system into the zygotes by electroporation. The total manipulation times represent the estimated time required to produce 100 mutant embryos by each method. Mutant embryos are transferred into recipient sows, resulting in the generation of mutant piglets.

was considerably lower than that observed in mice (18). Only 25% of the targeted genomic sequences in the pig contained mutations (Fig. 2C), whereas all of the targeted sequences carried mutations in mice. This outcome was not unexpected, because of the shorter pulses and the smaller number of pulses used during the electroporation of pig zygotes, presumably resulting in the introduction of reduced amounts of Cas9 mRNA into the zygotes. To circumvent this issue, we investigated whether Cas9 protein (160 kDa), which is much smaller than Cas9 mRNA (~1500 kDa), could be more efficiently introduced into pig zygotes, resulting in higher levels of genome targeting. We electroporated Cas9 protein (50 ng  $\mu\text{l}^{-1}$ ) with *FGF10* sgRNA (200 ng  $\mu\text{l}^{-1}$ ) into pig zygotes and found that the efficiency of genome editing was markedly improved, whereas the blastocyst formation rates were unaffected (Fig. 2, E to G).

We next examined whether our optimized conditions were applicable for the editing of other genes. We targeted the *Myostatin* (*MSTN*) gene, which encodes a negative regulator of myogenesis and whose disruption typically results in increased skeletal muscle mass in pigs (20, 21), cows (22, 23), and sheep (23, 24). We electroporated Cas9 protein and various sgRNAs targeting different sites in the first exon of *MSTN* (Fig. 3A) using the optimized conditions. We then evaluated the efficiency of genome editing in the resulting blastocysts. Of the seven sgRNAs tested, two (sgRNA6 and sgRNA7) induced high-efficiency genome editing and one (sgRNA1) induced moderate-efficiency genome editing (Fig. 3, B and C). The four remaining sgRNAs induced lower-efficiency genome editing (fig. S2, A and B). The rates of blastocyst formation were unaffected by the various sgRNAs (fig. S2C). We also analyzed the *MSTN* sequences present in each blastocyst (Fig. 3D). Some blastocysts carried two to four types of mutations and no wild-type sequence, whereas others carried both mutated and wild-type sequences at variable ratios.

These findings indicated that the GEEP method enabled genome editing in pigs and that its efficiency depended on the sgRNA target sequence.

We next investigated whether mutant pigs could be generated using the GEEP method. We introduced Cas9 protein and sgRNA1, sgRNA6, or sgRNA7 into zygotes by electroporation and then transferred them into the oviducts of two recipient gilts at ~12 hours after electroporation. Of the two recipients, one became pregnant and gave birth to 10 piglets 111 days after zygote transfer. One (10%) of the piglets died soon after birth. Genomic DNA was extracted from ear biopsies and analyzed to determine whether mutations were introduced into the *MSTN* gene. Sequencing of the *MSTN* genomic regions flanking the target sites revealed that 9 of the 10 piglets carried mutations in the *MSTN* gene (Fig. 4A and table S1). Among them, no wild-type sequences were detected in two piglets (#3 and #4), indicating that they carried biallelic mutations in the *MSTN* gene. The other piglets exhibited mosaic genotypes in which mutations were present in 7 to 79% in their genomes. We analyzed piglet #4 further. We detected no *MSTN* protein expression in muscle biopsies of piglet #4, whereas strong *MSTN* expression was detected in the biopsy of a wild-type piglet (Fig. 4B), indicating that the indels introduced into *MSTN* exon1 in piglet #4 caused a frameshift that prevented *MSTN* protein synthesis. The physical appearance of piglet #4 suggested that its muscle mass was greater than that of the wild-type piglet (Fig. 4C), and histological analysis of the longissimus thoracis muscle isolated from 40-day-old piglets also revealed that the muscle mass of piglet #4 was greater than that of wild-type piglets (Fig. 4D), which is commonly observed in *MSTN* mutant animals (20, 22, 25). We also investigated the distribution of skeletal muscle fiber type in the wild-type and mutant piglets (Fig. 4, E to G). Immunohistochemical analysis revealed that type I muscle fiber was reduced in the *MSTN*



**Fig. 2. Optimization of electroporation conditions for efficient genome editing in pig zygotes.** (A) Genomic structure of the *FGF10* locus and the sgRNA target sequence in the third *FGF10* exon. Nucleotides in blue represent the target sequence, and nucleotides in red represent the protospacer adjacent motif (PAM) sequence. (B) Genomic sequences of blastocysts formed after electroporation with Cas9 mRNA and *FGF10* sgRNA. Various pulsing conditions were tested (shown on the left) using a fixed voltage (30 V). The arrowhead indicates the Cas9 cleavage sites. (C) Frequency of mutations in the *FGF10* target region detected in PCR amplicons. (D) Blastocyst formation rates of the electroporated zygotes. (E to G) Comparison of the genome editing efficiency and blastocyst formation rates between Cas9 mRNA- and Cas9 protein-electroporated zygotes. (E) Direct sequencing of PCR amplicons from the blastocysts after electroporation. (F) Frequency of indels in the *FGF10* target region. (G) Blastocyst formation rates of the electroporated zygotes. \* $P < 0.05$  using one-way analysis of variance (ANOVA). Error bars, means  $\pm$  SEM.

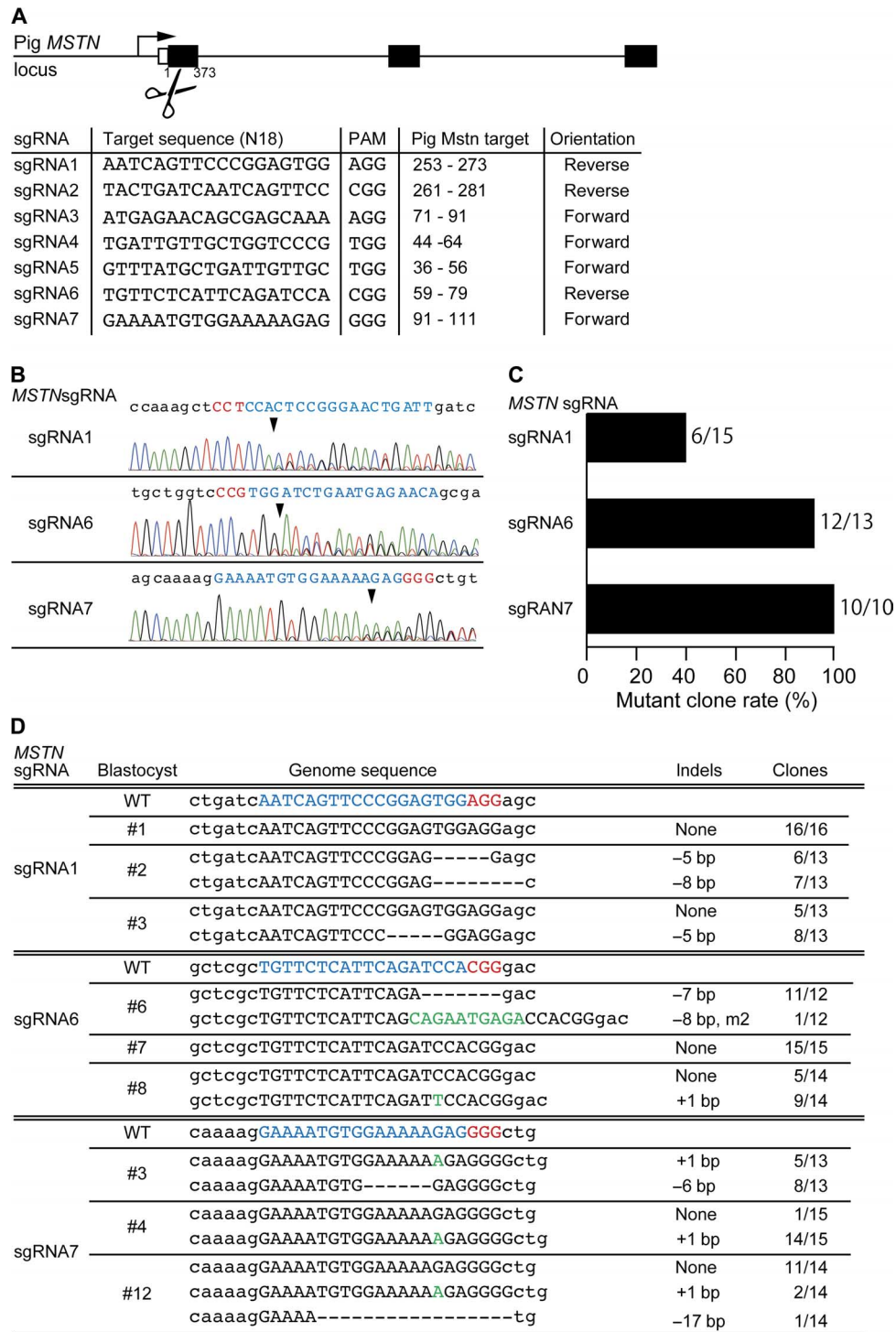
mutant pig, consistent with previous findings (25). Together, these results indicated that the electroporation of Cas9 protein and sgRNA into zygotes resulted in the efficient generation of genetically modified pigs.

Nonspecific cleavage of off-target sequences by Cas9 could be a major concern in the CRISPR/Cas9 system. To exclude this possibility, we analyzed the genome sequence of possible off-target sites. We searched the whole genome sequence of the pig [UCSC (University of California, Santa Cruz) Genome Browser on pig: SGSC Sscrofa10.2/susScr3] for potential off-target sites and found three sites each for sgRNA6 and sgRNA7 showing less than two mismatches (table S2). We amplified these sites and the *MSTN* target site from piglets #4 (sgRNA6) and #8 (sgRNA7) by polymerase chain reaction (PCR) and analyzed them using deep sequencing (next-generation sequencing). The PCR amplicons of each *MSTN* target site carried the same mutations as we detected in our cloning analysis (Fig. 4A and tables S1 and S3). In addition, a new mutation ( $-7$  base pairs) was detected in piglet #4 but with low frequency (2%) (table S3). On the other hand, more than 99% of the amplicons of possible off-target sites were made up of the wild-type sequence (table S2). The remaining 1% was composed of a small number of amplicons ( $<0.1\%$ ) carrying different sequences. These were also detected when we analyzed a wild-type piglet, indicating that these sequence changes

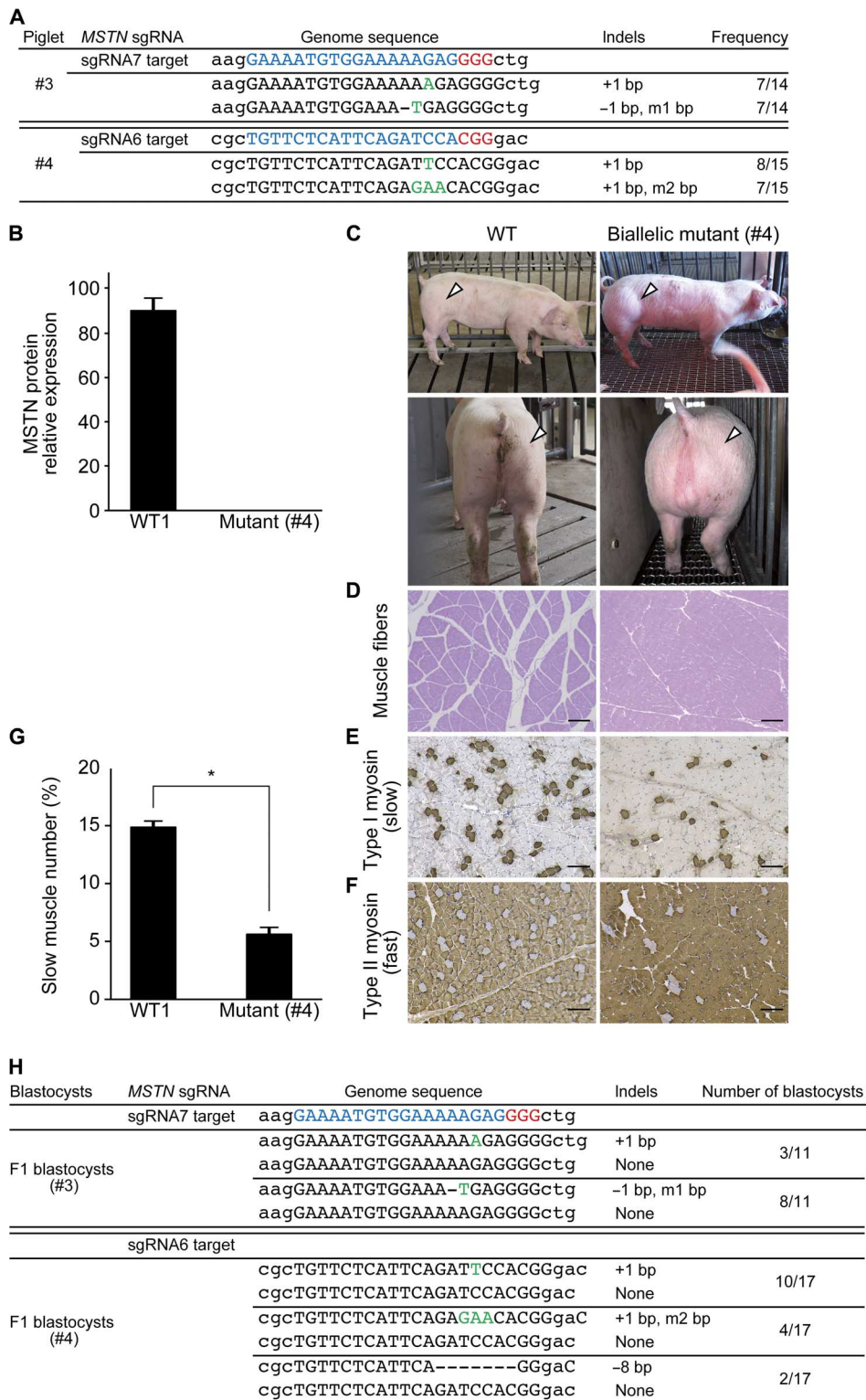
may be introduced by PCR errors or sequence errors. These results indicate that genome editing occurred only in the targeted region of the *MSTN* gene.

Finally, we investigated whether the mutation detected in the F0 pig was inherited by the next generation. The epididymal spermatozoa were collected from the F0 pig (#3 and #4) and were fertilized with in vitro matured oocytes from wild-type gilts. The zygotes were cultured in vitro for 7 days until blastocyst formation, and their genome was analyzed (Fig. 4H). All of the embryos from piglet #3 and 16 of 17 embryos from piglet #4 were heterozygotes carrying the mutations, which were detected in the F0 pig. The one remaining embryo carried only the wild-type sequence; presumably, it developed parthenogenetically. These results indicate that the mutation introduced by the GEEP method was inherited by the next generations.

In conclusion, we have established a new method for generating genetically modified pigs using the CRISPR/Cas9 system. We incorporated several elements that contributed to the increased efficiency of gene editing in pigs. First, we subjected oocytes from the ovaries of slaughtered pigs to IVM and IVF, to efficiently generate large numbers of zygotes. Second, we developed an optimized method for electroporating pig zygotes, which avoided the time-consuming method of mi-



**Fig. 3. Genome editing of the *MSTN* gene.** (A) Genomic structure of the *MSTN* locus and sgRNA sequences targeting the first *MSTN* exon. (B) Representative genomic sequences of blastocysts formed after zygote electroporation with Cas9 protein and *MSTN* sgRNAs (sgRNA1, sgRNA6, and sgRNA7; see also fig. S2). Arrowheads indicate the Cas9 cleavage sites. (C) Frequency of mutations in the PCR amplicons of each target region. (D) Alignment of sequences from each blastocyst after electroporation. Nucleotides in blue indicate target sequences, and nucleotides in red indicate PAM sequences. Nucleotides in green indicate inserted sequences. WT, wild type.



**Fig. 4. Generation of *MSTN* mutant piglets.** (A) Representative target region sequences in *MSTN* mutant piglets (see also table S1). Nucleotides in blue represent target sequences, and nucleotides in red represent PAM sequences. (B) Expression of *MSTN* protein in the longissimus thoracis muscle. (C) Phenotypic analysis of the biallelic mutant piglet showed enlarged muscle mass, compared with the WT control (arrowheads). (D) Hematoxylin and eosin staining of the longissimus thoracis muscle. Scale bars, 200  $\mu$ m. (E and F) Immunohistochemical analysis of fast and slow myosin expression in the muscle. Scale bars, 100  $\mu$ m. (G) Quantification of slow myofibers in the muscle. (H) Analysis of the genome sequence of F1 blastocysts of piglets #3 and #4. \* $P < 0.01$  using Student's *t* test. Error bars, means  $\pm$  SEM.

chromanipulation. Next, we incorporated the electroporation of Cas9 protein, which was predicted to be introduced more efficiently into zygotes than Cas9 mRNA. This change markedly improved the gene editing efficiency, resulting in high rates of mutant outcomes (90%). The GEEP method directly introduces genetic mutations into pig zygotes and does not require the epigenetic reprogramming of a donor somatic cell nucleus. Thus, GEEP may reduce the risks of both prenatal and postnatal death, which are sometimes observed in mutant pigs generated by the SCNT method (16, 17). Because GEEP does not require advanced skills and saves considerable time, it has the potential to facilitate the large-scale production of mutant pigs. Finally, the GEEP method will be applicable to the genetic modification of other mammals, in addition to pigs, and will undoubtedly contribute to the advancement of biomedical and agricultural research.

## MATERIALS AND METHODS

### Animals

All animal care and experiments were carried out in accordance with the Guidelines for Animal Experiments of Tokushima University and the National Institute of Agrobiological Sciences. This study was approved by the Ethics Committee of Tokushima University for Animal Research (approval number T28-21) and the Safety Management Section of the National Institute of Agrobiological Sciences (approval number H18-038).

### Oocyte collection, IVM, and IVF

Pig ovaries were obtained from prepubertal crossbred gilts (Landrace × Large White × Duroc breeds) at a local slaughterhouse. Cumulus-oocyte complexes (COCs) with a uniform ooplasm and compact cumulus cell mass were collected from follicles 2 to 6 mm in diameter. COCs were cultured in maturation medium at 39°C in a humidified incubator containing 5% CO<sub>2</sub> and 5% O<sub>2</sub> as described previously, with minor modifications (26). After 20 to 22 hours of maturation, the COCs were subsequently cultured for 24 hours in maturation medium without hormones.

The matured oocytes were subjected to IVF, as described previously (26). Briefly, frozen-thawed spermatozoa were transferred into 6 ml of porcine fertilization medium (PFM; Research Institute for the Functional Peptides Co.) and then washed by centrifugation at 500g for 5 min. The spermatozoa pellets were resuspended in PFM and adjusted to 5 × 10<sup>6</sup> cells ml<sup>-1</sup>. COCs were transferred to the PFM containing sperm co-incubated for 12 hours at 39°C under 5% CO<sub>2</sub> and 5% O<sub>2</sub>. After the co-incubation, the inseminated zygotes were denuded from the cumulus cells and the attached spermatozoa by mechanical pipetting.

### Preparation of sgRNA, Cas9 mRNA, and Cas9 protein

pDR274 plasmids carrying target sequences were constructed by the insertion of annealed oligos into the Bsa I site. The oligos shown in table S4 were purchased from Sigma-Aldrich. After Dra I digestion, sgRNAs were synthesized using the MEGAscript T7 Transcription Kit (Ambion) and then purified by phenol-chloroform-isoamyl alcohol extraction and isopropanol precipitation. The precipitated RNA was dissolved in Opti-MEM I. The RNAs were quantified by absorption spectroscopy and agarose gel electrophoresis and were stored at -30°C until use. Cas9 mRNA was prepared as described previously (18). The Cas9 protein in the Guide-it sgRNA Screening Kit (Takara Bio) was used for electroporation.

### Electroporation

Electroporation was performed as described previously with minor modifications (18). Briefly, the electrode (LF501PT1-20; BEX Co. Ltd.) was connected to a CUY21EDIT II electroporator (BEX Co. Ltd.) and set under a stereoscopic microscope. The inseminated zygotes were washed with Opti-MEM I solution (Life Technologies) and placed in a line in the electrode gap, in a chamber slide filled with 10 µl of Opti-MEM I solution containing sgRNA and Cas9 mRNA or protein (Takara Bio). Electroporation was then performed under various conditions. After electroporation, the zygotes were washed with pig zygote medium (Research Institute for the Functional Peptides Co.) and cultured either until embryo transfer (for 12 hours) or for 3 days. Embryos cultured for 3 days were subsequently incubated in porcine blastocyst medium (Research Institute for the Functional Peptides Co.) for 4 days.

### Analysis of the targeted genes after electroporation

Genomic DNAs were prepared from blastocysts or ear biopsies by boiling them in 50 mM NaOH solution. After neutralization, the genomic regions flanking the sgRNA target sequences were amplified by PCR using the following specific primers: FGF10, 5'-CCATCCCATT-TGATCTGCTT-3' (forward) and 5'-CTTCAACTGGCAGCA-CAATG-3' (reverse); MSTN, 5'-ATGCAAAAAGTCAAATCTATG-3' (forward) and 5'-TGTAGGCATGGTAATGATCG-3' (reverse). The PCR products were cloned into the pMD20 (Takara Bio) plasmid. More than 12 plasmids were isolated per blastocyst or biopsy, and the targeted genomic regions were sequenced. Sequencing was performed using the BigDye Terminator Cycle Sequencing Kit version 3.1 (Thermo Fisher Scientific) and the ABI 3500 Genetic Analyzer (Applied Biosystems).

### Embryo transfer

Two recipient gilts, whose estrous cycles had been synchronized, were prepared for embryo transfer as described previously (27). Approximately 12 hours after electroporation, the one- to two-cell stage embryos were transferred to the oviducts of a recipient gilt. Approximately 100 embryos were transferred to each oviduct, resulting in the transfer of ~200 embryos per gilt.

### Detection of MSTN protein

Muscle biopsies were obtained from the longissimus thoracis muscle of 40-day-old piglets with biallelic mutation (#4) or wild type piglets under anesthesia. Total protein was extracted using the M-PER Mammalian Protein Extraction Reagent (Life Technologies) and quantified using the bicinchoninic acid protein assay reagent (Thermo Fisher Scientific). All protein extracts were diluted to the same concentration (2.2 mg ml<sup>-1</sup>). The MSTN protein concentrations were determined with an enzyme-linked immunosorbent assay kit (R&D Systems), according to the manufacturer's protocol. We used MSTN propeptide (ProSpec) and MSTN protein (Wako) as negative and positive controls, respectively.

### Histochemical analysis of muscle samples

The muscle samples were fixed in 10% formalin-neutral buffer solution (Wako) and embedded in paraffin. Paraffin sections (10 µm) were prepared and stained with hematoxylin and eosin, using conventional techniques.

### Immunohistochemical analysis

Longissimus thoracis muscles obtained from 40-day-old piglets were frozen in dry-ice acetone (-78°C), and cryosections were generated

using a cryostat microtome (Sakura Finetek). To analyze the distribution of skeletal muscle fiber types, the sections were immunostained with anti-slow (clone M8421, 1:400; Sigma) and anti-fast (clone M4276, 1:200; Sigma) myosin heavy-chain monoclonal antibodies, which are specific markers of type I and type II myofibers, respectively. Histofine Simple Stain MAX PO (M) (Nichirei) was used as the secondary antibody. The proportion of each fiber type in each section of the longissimus thoracis muscle was determined using a digital microscope (VHX-5000; Keyence).

### Off-target analysis using deep sequencing

Genomic DNAs prepared from ear biopsies (piglet #4 and #8) were used as templates for PCR. The genomic regions flanking the sgRNA target sites or potential off-target sites were amplified by two-step PCRs using specific primers (table S5) and Index PCR Primers following the manufacturer's instruction (Illumina). After the gel purification, the amplicons were subjected to the MiSeq using the MiSeq Reagent Kit v3 (150 cycles) (Illumina).

### Inheritance analysis

Epididymal spermatozoa from piglets (#3 and #4) were used for the inheritance analysis. Spermatozoa were collected from the epididymides of piglets (7 months old) and were frozen, as described previously (28). At the time of insemination, the frozen spermatozoa were thawed at 37°C and then fertilized with in vitro matured oocytes from wild-type gilts as described above. After IVF, the zygotes were cultured for 7 days and the targeted genomic regions in blastocysts that were developed were sequenced.

### Statistical analysis

Five replicates per treatment group were performed and analyzed. Percentages of embryos developed to the blastocyst stage were subjected to arcsine transformation before ANOVA. The transformed data were tested by ANOVA, followed by protected Fisher's least significant difference test, using the StatView software (Abacus Concepts). Differences with a probability value of  $P < 0.05$  were considered statistically significant.

## SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/2/9/e1600803/DC1>

Supplementary Text

fig. S1. Optimization of the time period between IVF initiation and electroporation.

fig. S2. Genome editing of the *MSTN* gene.

table S1. Sequence of the *MSTN* target region in piglets.

table S2. Off-target analysis of the piglets by deep sequencing.

table S3. Sequence analysis of the *MSTN* gene in piglets #4 and #8 by deep sequencing.

table S4. Oligonucleotide sequences used to generate sgRNA.

table S5. Oligonucleotide sequences used for off-target analysis.

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method for generating mutant pigs. F.T., T.T., M.H., and T.O. are listed as inventors of the method and will receive part of any reward it may generate. All other authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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