

Generation of germ cell-deficient pigs by *NANOS3* knockout

Yuhei KOGASAKA¹), Sho MURAKAMI¹), Shiro YAMASHITA²), Daisuke KIMURA¹), Yoshinori FURUMOTO¹), Kana IGUCHI¹) and Yutaka SENDAI¹)

¹Biological Sciences Section, Central Research Institute for Feed and Livestock, Zen-noh, Ibaraki 300-4204, Japan

²Quality Control Research Section, Central Research Institute for Feed and Livestock, Zen-noh, Ibaraki 300-4204, Japan

Abstract. *NANOS3* is an evolutionarily conserved gene expressed in primordial germ cells that is important for germ cell development. Germ cell deletion by *NANOS3* knockout has been reported in several mammalian species, but its function in pigs is unclear. In the present study, we investigated the germline effects of *NANOS3* knockout in pigs using CRISPR/Cas9. Embryo transfer of CRISPR/Cas9-modified embryos produced ten offspring, of which one showed wild-type *NANOS3* alleles, eight had two mutant *NANOS3* alleles, and the other exhibited mosaicism (four mutant alleles). Histological analysis revealed no germ cells in the testes or ovaries of any of the nine mutant pigs. These results demonstrated that *NANOS3* is crucial for porcine germ cell production.

Key words: Germ cells, Knockout, *NANOS3*

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Germ cells are the only cells in mammals that produce the next generation. Unlike somatic lineages, germ cells are totipotent and experience specific phenomena such as genome-wide epigenome alterations and meiosis [1, 2]. The mechanisms underlying germ cell development and differentiation have been studied based on these unique characteristics.

Findings on mammalian germ cell development have been accumulated in mice [3, 4]. Primordial germ cells (PGCs), which are the founders of the germ cell lineage, initiate specification within the posterior epiblast cells in response to BMP signals derived from their proximal cell population at about E6 mouse embryos [3, 4]. During PGC specification, key transcription factors such as *BLIMP1*, *PRDM14*, and *TFAP2C* are expressed, and the transcription network formed by these factors facilitates PGC development by suppressing somatic gene expression, triggering genome-wide epigenetic reprogramming, initiating the expression of germ cell development genes, and reactivating pluripotent genes [3, 5–8]. Once the PGCs are specified, they migrate into the genital ridge, where they begin differentiation into either oocytes or spermatozoa [3]. During the migration process, several genes are expressed that are important for PGC survival, all of which encode RNA-binding proteins, such as *Nanos3*, *Tial1*, and *Dnd1* [9–12]. Disruption of these genes impairs PGC development (*Bmp*: reduction or loss of PGCs; *Blimp1*, *Prdm14*, and *Tfap2c*: impaired PGC specification; *Nanos3*, *Tial1*, and *Dnd1*: loss of PGCs) [3, 5–12].

In non-rodent mammals, the above molecular mechanisms are conserved, although certain aspects differ. In humans, an *in vitro* model showed that the induction of PGC-like cells (PGCLCs) from pluripotent stem cells required BMP supplementation [13]. Gene knockout (KO) studies have revealed that in addition to *TFAP2C* and *BLIMP1*, *SOX17*, *EOMES*, and *GATA* also served as key transcription factors for the specification of human PGCLCs, while *PRDM14* was

not expressed [14–17]. In pigs, it has been reported that the *SOX17*, *BLIMP1*, *TFAP2C*, *NANOG*, and *OCT4* proteins are expressed in PGCs, and *ex vivo* induction of PGCs requires BMP and WNT signals [18]. However, the precise molecular mechanisms involved in non-rodent PGC specification and migration remain unknown.

Pigs are among the main domestic animals used for meat production. They are also commonly used as experimental animals because their anatomical and physiological characteristics are similar to those of humans [19]. Thus, the accumulation of knowledge related to pig reproduction and germ cell development has both industrial and comparative biological significance.

Nanos3 is an evolutionarily conserved gene expressed in PGCs during specification and migration periods that has been implicated in the survival of migrating PGCs in mice [10, 20, 21]. *Nanos3*-KO showed complete germ cell loss in the gonads of both sexes of murines [10, 21] and in female cattle [22]. However, the function of *NANOS3* in pigs is unclear. In this study, we generated *NANOS3*-KO pigs using CRISPR/Cas9-modified embryos and analyzed their phenotypes.

Materials and Methods

Ethical statement

This study was conducted after approval by the Biosafety Committee for Recombinant DNA Research (Permit Number 20200603) and the Institutional Animal Care and Use Committee (Permit Number: 2021-2) of the Zen-Noh Feed and Livestock Central Research Institute. All *in vivo* experiments were performed using midazolam/medetomidine/butorphanol with a combination of isoflurane anesthesia, and suffering was minimized.

In vitro production of porcine embryos

In vitro production was conducted as described by Mito *et al.* [23] with slight modifications. Pig ovaries were collected from 5–6-month-old gilts (Landrace × Large White × Duroc) bred for meat production at a slaughterhouse at our institute. Cumulus oocyte complexes (COCs) were extracted from ovaries using a scalpel to cut the antral follicles in porcine oocyte/embryo collection medium (Research Institute for the Functional Peptides Co., Ltd.: RIFP, Yamagata, Japan) and washing multiple times in the medium. COCs with 2–3 layers of cumulus cells were cultured in HP-POM (RIFP) with 1 mM dibutyryl

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Correspondence: Y Kogasaka (e-mail: kogasaka-yuuhei@zennoh.or.jp)

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cyclic AMP (dbcAMP, RIFP) and 0.5 U/ml recombinant human follicle-stimulating hormone (Merck, Darmstadt, Germany). After 22 h of culture, COCs were transferred to HP-POM without dbcAMP and recombinant human follicle-stimulating hormone and further cultured for 24 h. Landrace semen was purchased from Zen-Noh Livestock Co., Ltd. (Tokyo, Japan). Highly active spermatozoa were separated using a 50%/80% Percoll (GE Healthcare, Chicago, IL, USA) gradient solution centrifuged at 700 g for 20 min, and washed twice with pig fertilization medium (RIFP) by centrifugation at 500 g for 5 min. Spermatozoa (1×10^6 /ml) were co-cultured with COCs in pig fertilization medium for 15 h. All incubations were performed at 39°C in an atmosphere of 5% CO₂, 5% O₂, and 90% N₂.

CRISPR/Cas9

The putative open reading frame of the porcine *NANOS3* gene was composed of two exons with no in-frame ATG sequences other than the initiation codon. The zinc finger domain (ZFD) sequence in exon 1 is essential for *NANOS3* gene function [20, 24]. Therefore, we designed two sets of three single-guide RNAs (sgRNAs) targeting the *NANOS3* gene initiation codon and ZFD, and performed the triple CRISPR method [25–27] (Fig. 1). The sgRNAs were designed using the CRISPR design tool GPP sgRNA Designer (<https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design>) [28], and the target sequences of each sgRNA are shown in Supplementary Table 1. The sgRNA synthesis template was obtained by PCR amplification using a template DNA sequence (5'-TTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCGTTCAACTTGAAAAAGTGGCACCCAGTTCGGTGCT-3'), top primer (5'-TAATACGACTCACTATAG-each sgRNA target sequence-GTTTTAGAGCTAGAAATAGCAAGTTAAA-3'), bottom primer (5'-AGCACCGACTCGGTGCCACT-3'), and the Phusion High-Fidelity Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). PCR was performed at 95°C for 3 min, followed by 45 cycles at 98°C for 10 sec, 68°C for 40 sec, and 68°C for 8 min. The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen, Venlo, Netherlands). The sgRNA synthesis and purification were performed using a Precision gRNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The concentration of sgRNA was measured using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and a Qubit RNA BR Assay Kit (Thermo Fisher Scientific). A mixture of 10 ng/μl sgRNA and 50 ng/μl Cas9 protein (Alt-R S.p. Cas9 Nuclease V3, Integrated DNA Technologies, Coralville, IA, USA) with nuclease-free water was used for microinjection.

Microinjections and embryo transfer

Fertilized oocytes that showed the second polar body 17–21 h after *in vitro* fertilization were microinjected using a piezo micro-manipulation system (Prime Tech, Ibaraki, Japan). The oocytes were fixed using a holding pipette (Prime Tech), and an injection pipette with a 3 μm outer diameter (Prime Tech, TPINS03-20T) was

inserted into the ooplasm after puncturing the zona pellucida with a piezo pulse. The injection volume was approximately 0.5–0.75 pl (1–1.5 embryo-lengths fluid volume in the pipette). Injected embryos were cultured in porcine zygote medium-5 (RIFP) for 24 h until embryo transfer, and some embryos that were not injected were cultured to blastocysts (5 days) for wild-type *NANOS3* genotyping. A multiparous sow was synchronized at estrus with 750 IU equine chorionic gonadotropin (i.m. injection; Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) on the day of weaning. On the day after estrus, the embryos were surgically transferred into the oviduct of the surrogate sow. Pregnancy was confirmed by ultrasound diagnosis, and delivery was induced on day 114 after embryo transfer using a prostaglandin F_{2α} analog (i.m. injection, MSD Animal Health K.K., Madison, NJ, USA) at day 113.

Genotyping

The offspring were euthanized after delivery (males at 1 day and females at 4 months of age), and testes and ovaries were collected. Ovaries from pigs numbered five and six (No. 5 and No. 6) were collected immediately after death. For each pig gonad, one tissue sample was subjected to genotyping and the other was subjected to histological analysis. Genomic DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions for the tissue sample, and 25 mM NaOH-200 μM EDTA solution, followed by heating at 95°C for 10 min was performed for the embryo sample. PCR was performed using the primers p*NANOS3*-8366 (T) (5'-TGGCACCTAATGACTCCTTG-3'), p*NANOS3*-12326 (B) (5'-CTGACTTCCCCAAAGGCAAT-3'), and PS-GXL (Takara Bio, Shiga, Japan) for the tissue sample, and Terra PCR Direct Polymerase (Takara Bio) for the embryo sample at 95°C for 3 min, followed by 35 cycles of 98°C for 10 sec, 60°C for 15 sec, 68°C for 1 min, and 68°C for 8 min (product size: 3961 bp). The PCR product was subcloned into a pCR-BluntII vector (Thermo Fisher Scientific) and Sanger sequencing was performed using the primers p*NANOS3*-8366(T), p*NANOS3*-9965(T) (5'-CCCCCTGTGACAATAAGGAA-3'), and p*NANOS3*-10749(B) (5'-ACCTTCTGAAGTGGGAAACC-3'), and a SeqStudio Genetic Analyzer (Thermo Fisher Scientific) in accordance with the manufacturer's protocols.

Histological analysis

Age-matched untreated wild-type pigs (Large White × Landrace) were used as controls. Tissues were fixed with a 10% formalin neutral buffer solution and the following procedures were conducted at KAC Co., Ltd. (Shiga, Japan). The fixed tissues were dehydrated and embedded in paraffin. Sections were prepared with a thickness of 2 μm. After deparaffinization and rehydration, hematoxylin and eosin staining and immunohistochemistry were performed. Antigen retrieval was performed using Tris-EDTA buffer at 110°C for 10 min. Endogenous peroxidase was removed with 3% hydrogen peroxide at room temperature (RT) for 20 min. Blocking was performed using

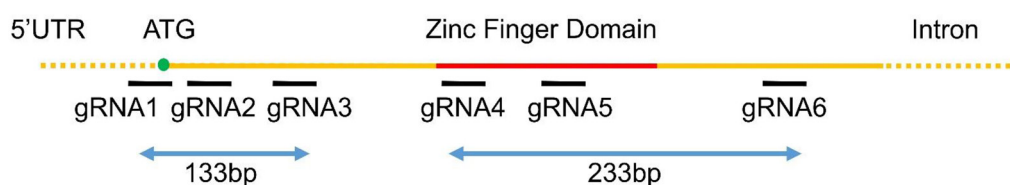


Fig. 1. Schematic view of genomic DNA around porcine *NANOS3* exon 1. gRNA, single-guide RNA.

Protein Block Serum-Free (Agilent Technologies, Santa Clara, CA, USA) at RT for 1 h. The tissues were then incubated with rabbit anti-human DDX4 antibody (Abcam, Cambridge, England, ab13840, diluted 1:1000) at RT for 1 h. A secondary HRP-conjugated anti-rabbit antibody (Agilent Technologies, K4003, ready-to-use) was incubated at RT for 1 h. DDX4 was stained with DAB chromogen, and nuclei were stained with hematoxylin.

Results

Embryo manipulation and piglet production

We injected three sgRNAs for the initiation codon (ATG set) into 100 fertilized oocytes and three sgRNAs for ZFD (ZFD set) into 98 fertilized eggs. The following day, 88 (88.0%) ATG set-injected embryos and 83 (84.7%) ZFD set-injected embryos showed cleavage (2–4 cells) with no abnormal morphology and were transferred to the foster mother, who conceived and delivered six female and four male piglets. Two females (No. 5 and No. 6) died two days after birth. The cause of death was presumed to be frailty because of the small birth weights (No. 5: 0.62 kg, No. 6: 0.51 kg, average of other offspring: 1.28 ± 0.47 kg). The other pigs showed no abnormalities in growth or behavior during breeding (data not shown).

Genotyping

As shown in Table 1 (the allele sequences are shown in Supplementary Fig. 1), pig No. 3 showed only the wild-type allele. Pigs No. 1, 2, and 6 were presumed to be derived from ATG set-induced embryos because they had alleles of the mutant ATG set target regions and intact ZFD set target regions. Pigs No. 4, 5, 7, 8, 9, and 10 had alleles of the intact ATG set target regions and mutant ZFD gRNA target regions, and were presumed to be derived from ZFD

set-induced embryos. Two alleles were detected in pigs No. 1, 2, 4, 5, 7, 8, and 10, and all were considered KO from the frameshift, ATG deletion, ZFD deletion, or loss of exon 1 coding sequence mutation. Pig No. 6 showed only one allele with a 113 bp deletion (frameshift, ATG deletion) in the ATG set. In some uninjected wild-type porcine embryos, direct sequencing of the *NANOS3* exon 1 region showed a double peak (A/G) 378 bp from the initiation codon, which is considered a polymorphism (synonymous substitution). Of the eight sequenced clones from pig No. 6, genotypes A and G were observed in six and two clones, respectively (Fig. 2). Therefore, No. 6 was considered to have a biallelic mutation. Pig No. 9 had a mosaic genotype because four mutant alleles were observed. Of these, one allele was not a frameshift mutation, but spanned a 61-bp deletion, a 55-bp insertion (total 6-bp deletion), and a 2-bp mutation, resulting in several amino acid deficiencies/substitutions in the ZFD.

Histological analysis of pig testes

There were no notable differences in the appearance of the male bodies (Figs. 3A, B, Supplementary Figs. 2A, E, I), and testes, or the size of testes (Figs. 3C, D, Supplementary Figs. 2B, F, J) between wild-type and pigs with only *NANOS3* mutant alleles (*NANOS3*^{mut/mut}; biallelic mutant No. 7, 8, and 10, and including mosaic mutant No. 9). Histological analysis showed gonocytes/prospermatogonia featuring large spherical nuclei [29, 30] and germ cell marker DDX4-positive cells [31, 32] in the seminiferous tubules of the wild-type (Figs. 3E, F), while the *NANOS3*^{mut/mut} tubules showed none of these cells (No. 7–10, Figs. 3G, H, Supplementary Figs. 2C, D, G, H, K, L).

Histological analysis of pig ovaries

There were no notable differences in the appearance of the female bodies (Figs. 4A, B, Supplementary Fig. 3A) and ovaries, or the

Table 1. Genotypes of *Nanos3*-modified pigs

| Pig | Sex | Mutations | | No. of clones * | Predicted target of gRNA set | Presumed null allele? | Mutation effect |
|-----|-----|--|--------------------------------|-----------------|------------------------------|-----------------------|---------------------|
| | | Around ATG set | Around ZFD set | | | | |
| 1 | ♀ | 50 bp del (ATG del), 2 bp mut | intact | 3/8 | ATG | Yes | ATG del, frameshift |
| | | 46 bp del (ATG del), 9 bp mut | intact | 5/8 | | Yes | ATG del, frameshift |
| 2 | ♀ | 88 bp del | intact | 8/8 | ATG | Yes | Frameshift |
| | | 967 bp del (exon 1 cds del), 1 bp ins, 21 bp mut | | 8/8 | | Yes | Exon 1 cds del |
| 3 | ♀ | intact | intact | 8/8 | ? | No | No mutation |
| 4 | ♀ | intact | 64 bp del | 8/8 | ZFD | Yes | ZFD mut, frameshift |
| | | 3531 bp del (exon 1 cds del) | | 8/8 | | Yes | Exon 1 cds del |
| 5 | ♀ | intact | 239 bp del, 6 bp mut | 8/8 | ZFD | Yes | ZFD mut, frameshift |
| | | 2358 bp del (exon 1 cds del), 3 bp mut | | 8/8 | | Yes | Exon 1 cds del |
| 6 | ♀ | 113 bp del (ATG del) | intact | 8/8 | ATG | Yes | ATG del, frameshift |
| 7 | ♂ | intact | 233 bp del | 8/8 | ZFD | Yes | ZFD mut, frameshift |
| | | 1706 bp del (ATG to ZFD del) | | 8/8 | | Yes | ATG to ZFD del |
| 8 | ♂ | intact | 174 bp del, 1 bp ins | 4/8 | ZFD | Yes | ZFD mut, frameshift |
| | | intact | 62 bp del, 5 bp ins | 4/8 | | Yes | ZFD mut, frameshift |
| 9 | ♂ | intact | 233 bp del | 10/16 | ZFD | Yes | ZFD mut, frameshift |
| | | intact | 26 bp del, 2 bp mut | 4/16 | | Yes | ZFD mut, frameshift |
| | | intact | 8 bp del, 1 bp mut | 1/16 | | Yes | ZFD mut, frameshift |
| | | intact | 61 bp del, 55 bp ins, 2 bp mut | 1/16 | | Yes | ZFD mut |
| 10 | ♂ | intact | 34 bp del, 9 bp ins, 2 bp mut | 5/8 | ZFD | Yes | ZFD mut, frameshift |
| | | intact | 178 bp del, 2 bp ins, 4 bp mut | 3/8 | | Yes | ZFD mut, frameshift |

* In animals described as 8/8 for mutations in both alleles, two bands of different sizes were observed after electrophoresis for PCR genotyping, and each band was gel-extracted and separately subcloned. ATG, initiation codon; cds, coding sequence; del, deletion; ins, insertion; mut, mutation; ZFD, zinc finger domain.

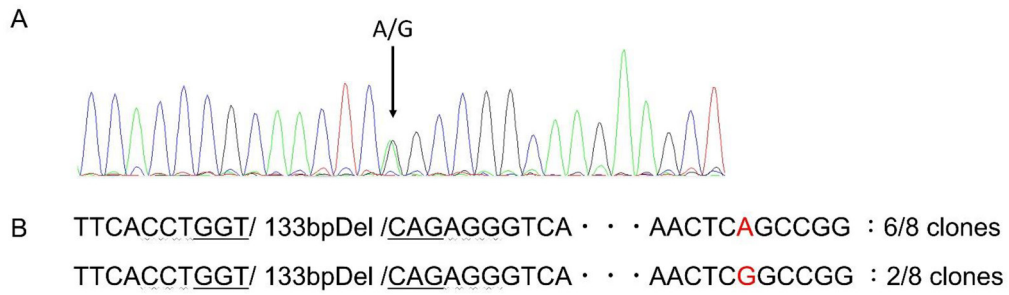


Fig. 2. Polymorphism in *NANOS3* gene exon 1. (A) Polymorphism (A/G) at 378 bp from the initiation codon in wild-type embryos. (B) Alleles of a pig with only one mutation allele (No. 6) detected by sub-cloning and sequencing. Del, deletion.

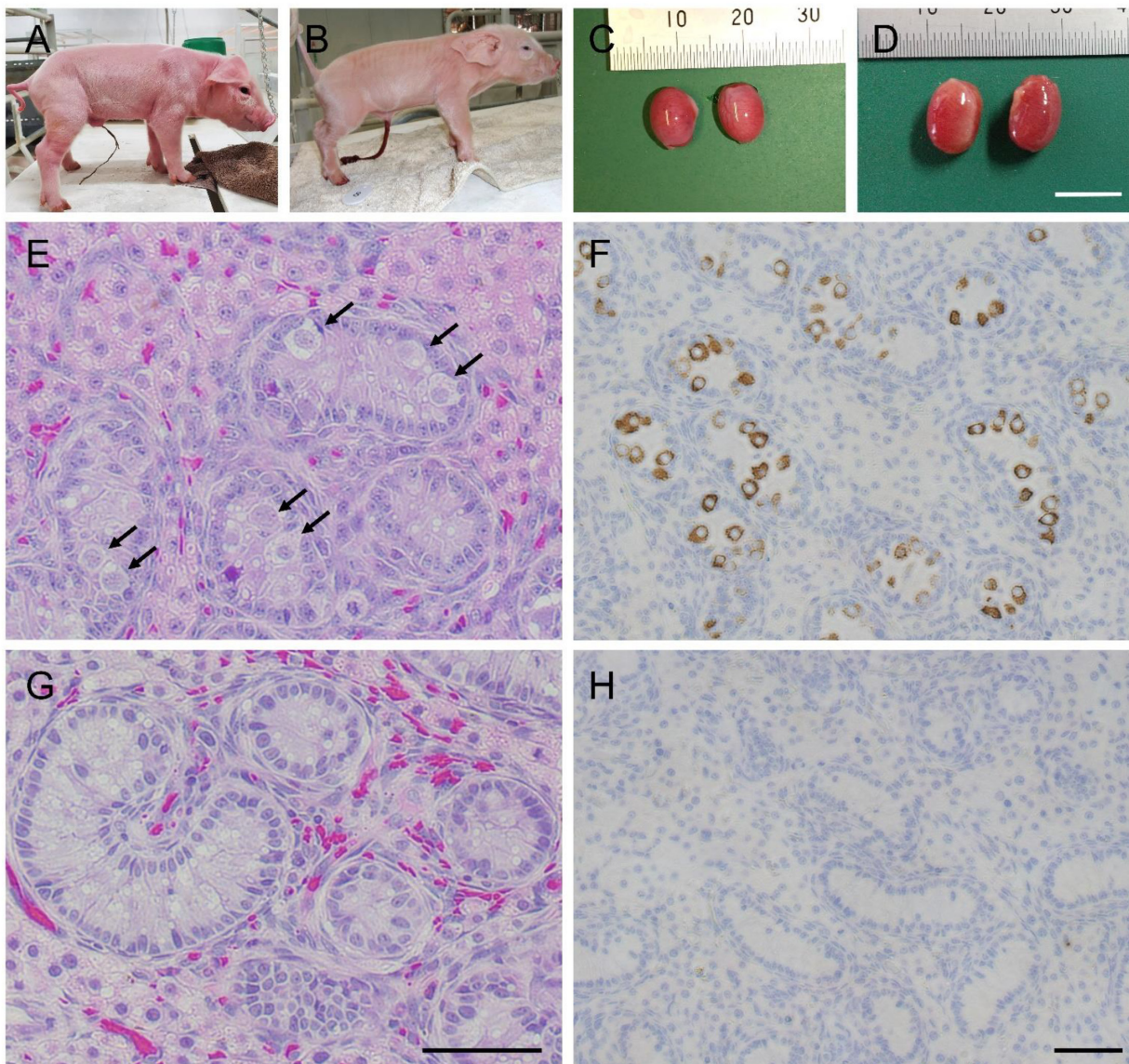


Fig. 3. Phenotype of *NANOS3* mutant male pigs. (A), (B) 1-day-old wildtype (WT) and *NANOS3* mutant pig (*NANOS3*^{mut/mut}, No. 8), respectively. (C), (D) Testes of WT and *NANOS3*^{mut/mut} pig (No. 8), respectively. (E), (G) Hematoxylin and eosin staining images of WT and *NANOS3*^{mut/mut} pig (No. 8) testes, respectively. (F), (H) DDX4 immunohistochemical images of WT and *NANOS3*^{mut/mut} pig (No. 8) testes, respectively. Arrows indicate gonocytes/prospermatogonia. Scale bars indicate 10 mm and 50 μ m in testes and histological images, respectively.

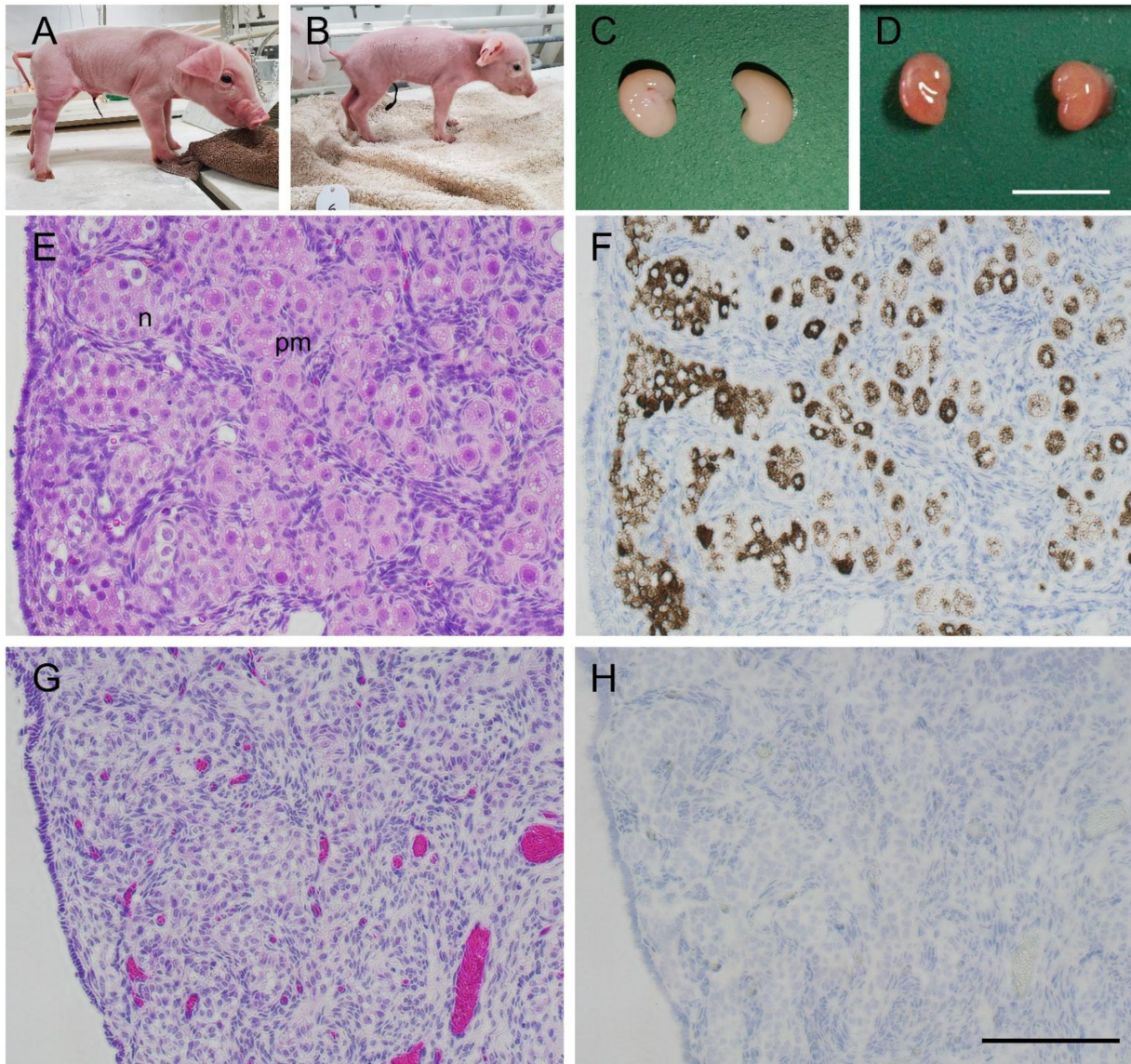


Fig. 4. Phenotype of 1-day-old *NANOS3* mutant female pigs. (A), (B) 1-day-old wildtype (WT) and *NANOS3* mutant pig (*NANOS3*^{mut/mut}, No. 6), respectively. (C), (D) Ovaries of WT and *NANOS3*^{mut/mut} pig (No. 6), respectively. (E), (G) Hematoxylin and eosin staining images of WT and *NANOS3*^{mut/mut} pig (No. 6) ovaries, respectively. (F), (H) DDX4 immunohistochemical images of WT and *NANOS3*^{mut/mut} pig (No. 6), respectively. (n), (pm) indicate egg nests and primordial follicles, respectively. Scale bars indicate 5 mm and 100 μ m in ovary and histological images, respectively.

size of ovaries (Figs. 4C, D; Supplementary Fig. 3B) between the wild-type and *NANOS3*^{mut/mut} pigs (No. 5 and No. 6). Histological analysis showed egg nests, primordial follicles, and DDX4-positive cells on the surface of the wild-type ovaries (Figs. 4E, F). No equivalent follicle structures or DDX4-positive cells were observed in ovaries from *NANOS3*^{mut/mut} pigs (No. 5 and No. 6, Figs. 4G, H, Supplementary Figs. 3C, D). There were no notable differences in the appearance of bodies among 4-month-old wild-type pigs (Fig. 5A), pigs with intact *NANOS3* alleles (*NANOS3*^{WT/WT}; No. 3, Fig. 5B), or *NANOS3*^{mut/mut} pigs (Nos. 1, 2, and 4, Figs. 5, C, Supplementary Figs. 4A, B). The ovary in the *NANOS3*^{WT/WT} pig (No. 3) contained many antral follicles (Fig. 5E), similar to the wild-type ovary (Fig. 5D), whereas ovaries from *NANOS3*^{mut/mut} pigs (Nos. 1, 2, and 4) were smaller and displayed no antral follicles (Fig. 5F, Supplementary Figs. 4C, D). Histological analysis showed antral follicles, preantral follicles, and DDX4-positive oocytes in wild-type and *NANOS3*^{WT/WT} (No.

3) ovaries (Figs. 5G–N). In contrast, follicle-like circular structures were observed, but no oocyte-like structures or DDX4-positive cells were found in ovaries from *NANOS3*^{mut/mut} pigs (Nos. 1, 2, and 4, Figs. 5O, P, Supplementary Figs. 4E–H).

Discussion

In the present study, CRISPR/Cas9 technology was used with porcine embryos to generate a *NANOS3*-KO model. The generation of a biallelic mutant was inefficient when only a single sgRNA was introduced, and a mutation was not necessarily a KO unless it introduced a frameshift change [33, 34]. We increased the efficiency of mutagenesis by using three sgRNAs [25–27]. By targeting the sgRNAs around the initiation codon or ZFD, which is essential for *NANOS3* function [20, 24], we expected that KO could be induced without a frameshift mutation. Mutant alleles were detected in 9/10

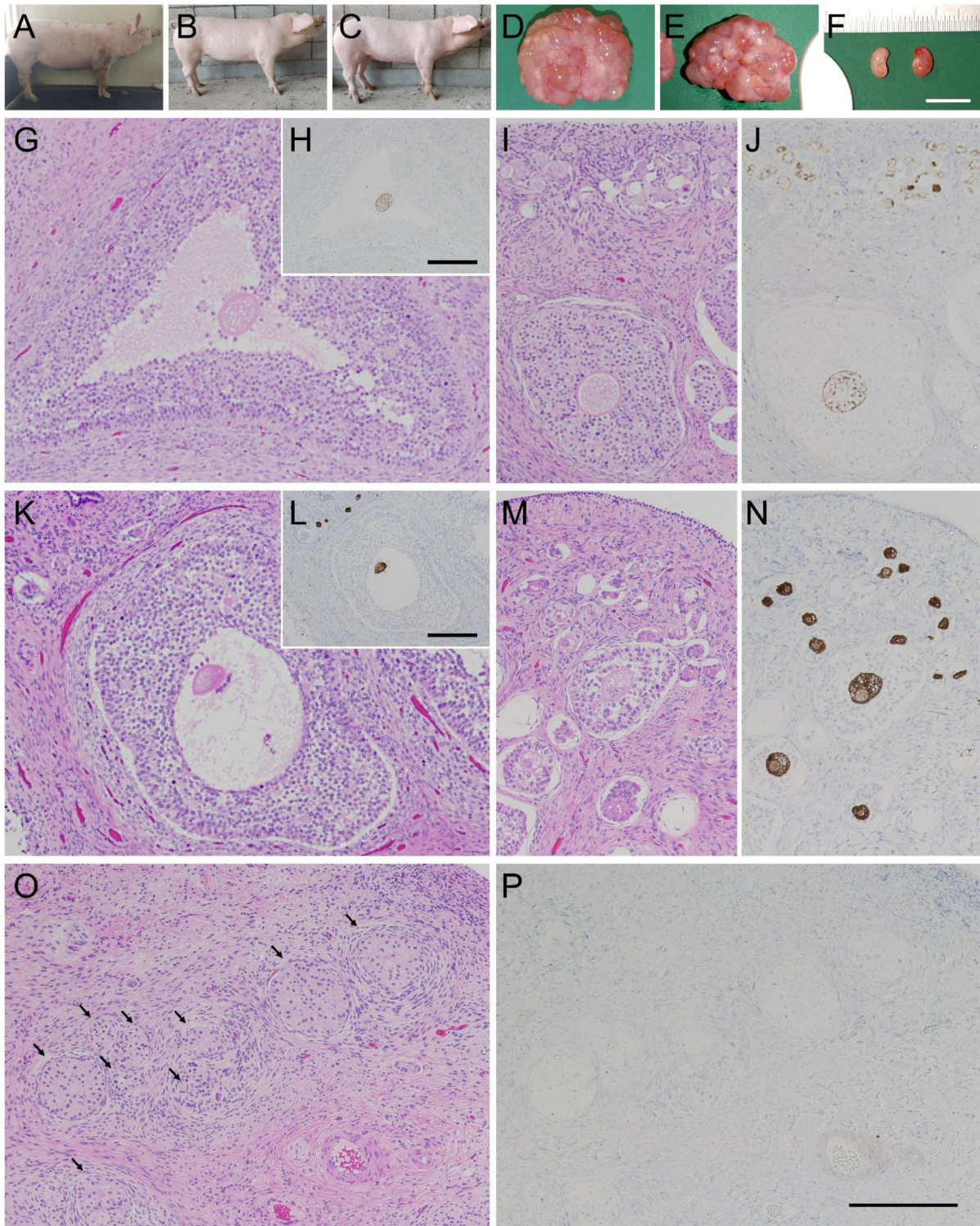


Fig. 5. Phenotype of 4-month-old *NANOS3* mutant female pigs. (A), (B), (C) Appearance of wildtype (WT), *NANOS3* intact (*NANOS3*^{WT/WT}, No. 3) and mutant (*NANOS3*^{mut/mut}, No. 1), respectively. (D), (E), (F) Ovaries of WT, *NANOS3*^{WT/WT} pig (No. 3) and *NANOS3*^{mut/mut} pig (No. 1), respectively. (G), (K) Hematoxylin and eosin (HE) staining images of antral follicles in WT and *NANOS3*^{WT/WT} pig (No. 3) ovaries, respectively, and (H), (L) show DDX4 immunohistochemical images of the same animals, respectively. (I), (M) HE staining images of preantral follicles in WT and *NANOS3*^{WT/WT} pig (No. 3) ovaries, respectively, and (J), (N) show corresponding DDX4 immunohistochemical images. (O), (P) HE staining and DDX4 immunohistochemical images, respectively, of *NANOS3*^{mut/mut} pig (No. 1) ovary. Arrows indicate follicle-like structures. Scale bars indicate 10 mm and 200 μ m in ovaries and histological images, respectively.

of the offspring, with the majority considered KO alleles because of a frameshift, ATG deficiency, or loss of exon 1 mutations. One mosaic mutant pig (No. 9) had mutations without a frameshift (total

6 bp deletion, 2 bp mutation). In humans, one amino acid substitution in the ZFD of *NANOS3* causes primary ovarian insufficiency and involves destabilization of *NANOS3*-RNA binding and the

disrupted prevention of apoptosis [24]. Therefore, we predicted that the non-frameshift mutation in the mosaic mutant pig (No. 9) would produce a non-functional protein because of the amino acid substitution and deficiency in ZFD.

It has been reported that mosaic embryonic development frequently occurs when CRISPR/Cas9 is introduced directly into pig embryos [33]. However, in the present study, only one mosaic animal was observed among the nine pigs with the detected mutations. Because there were large-scale deletions, such as 3531 bp, in the mutant alleles, and our analysis was restricted to the sequencing of 8–16 sub-clones, it is possible that a wider range of deletions and altered alleles with minor frequencies were not detected. However, the same germ cell-deficient phenotype was observed among all *NANOS3*^{mut/mut} pigs, which is consistent with the germ cell deficiency phenotypes observed in *Drosophila* (homolog *Nanos* gene [35]), mice [10, 21], and cattle [22] lacking the corresponding gene. Therefore, if a modified allele existed that was not detected in the current analysis, it was likely a *NANOS3* KO allele.

DDX4 is a germ cell marker expressed in gonocytes/prospermatogonia of immature pig testes [31] and oocytes at various stages of ovarian follicle development [32]. In the present study, no gonocyte-like or DDX4-positive cells were observed in the seminiferous tubules of *NANOS3*^{mut/mut} pigs (Nos. 7–10). At two months of age, pigs experience a dramatic increase in ovarian weight with the appearance of follicles ≥ 1 mm, and at four months of age, most porcine ovaries contain many antral follicles [36, 37]. In the *NANOS3*^{WT/WT} pig (No. 3), antral follicles, a normal follicle structure, and DDX4-positive oocytes were observed, similar to those of wild-type pigs. In *NANOS3*^{mut/mut} gilts (Nos. 1, 2, 4, 5, and 6), no oocytes or DDX4-positive cells were observed, and the ovaries were underdeveloped. These data show that *NANOS3* is involved in germ cell formation in porcine gonads. In both sexes of fetal mice, the *NANOS3* protein is expressed in PGCs and has been reported to be involved in germ cell formation by preventing the apoptosis of migrating PGCs [10, 21]. In pigs, *NANOS3* transcripts have been reported to be expressed in PGCs during expansion, migration, and gonadal colonization in the fetus during gestation days 14–31 [38]. These reports and the germ cell deficiency of *NANOS3*-KO neonatal pigs in the current study suggest that, similar to mice, *NANOS3* contributes to porcine germ cell formation during the fetal period. In postnatal mice, the *NANOS3* protein is expressed in germ cells during spermatogenesis and is involved in the maintenance of undifferentiated spermatogonia [21, 39]. In humans, this protein is expressed in germ cells during spermatogenesis and in oocytes and granulosa cells of various follicle stages [40]. Postnatal porcine effects of *NANOS3* were not analyzed in this study, but future investigations could compare its expression and function with that of other species. In addition, the *Nanos2* gene is expressed in male mouse germ cells, while in *Nanos2*-KO mice, germ cells are absent in males, but normal ovarian and oocyte development is observed in females [10, 21]. Similar to mice, *NANOS2*-KO pigs are specifically deficient in male germ cells [41]. These reports and our results support the conserved and key functions of the *NANOS* family of proteins among various species, including pigs. Interestingly, circular follicle-like structures were observed in *NANOS3*-KO ovaries in the current study; however, it was not determined whether these were actual follicles. These results may indicate that follicle organization is germ cell-independent, but further analysis is needed to evaluate this hypothesis.

Pigs are used as experimental animals because their anatomical and physiological characteristics are similar to those of humans [19]. Recently, genetic engineering technology has created various pig

models of human diseases; however, it is difficult to maintain and reproduce these animal models because of the associated symptoms of illness. Therefore, it is necessary to establish a stable production system for these models [42]. Blastocyst complementation has been proposed to overcome these issues [43], whereby genetically deficient organs or tissues are complemented by mixing organogenesis-disabled host embryos with donor embryos or stem cells [44]. Chimeric pigs derived from male and female embryos become male when germ cells are derived exclusively from male embryos, despite somatic cells with intersex chimerism [45]. Based on this finding, Matsunari *et al.* mixed genetically engineered male embryos with normal female embryos to generate healthy boars harboring sperm with mutated genomes of X-linked genetic disorders [43]. However, there have been no reports of using the blastocyst complementation method to generate healthy sows that can be bred to exclusively produce models of disease-impacting gametes. Chimeric animals derived from *NANOS3*-KO host embryos and donor blastomeres or pluripotent stem cells have been reported to possess germ cells exclusively derived from donor cells [22, 25]. By producing chimeric sows from *NANOS3*-KO host embryos and genetically engineered donor embryos or blastomeres, there is a new opportunity to generate sows that stably produce pig models of disease.

Conflicts of interests: The authors declare no conflict of interest.

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