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CRISPR/Cas9-mediated gene targeting during embryogenesis in swine

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Abstract

Ability to disrupt genes is essential in elucidating gene function. Unlike rodents or amphibians, it has been difficult to generate gene targeted embryos in large animals. Therefore, studies of early embryo development have been hampered due to the limitation. A recent technology suggests that targeted mutations can be successfully introduced during embryogenesis, thus by-passing the need of breeding to produce gene-targeted embryos. This is particularly important in large animal models because of longer gestation period and higher animal cost. Here we describe a specific approach to disrupt up to two genes simultaneously during embryogenesis using the CRISPR/Cas9 technology in swine. The approach can help understand the mechanism of zygotic genome activation in large animals.

Keywords

Pig embryos; CRISPR/Cas9; Zygotic genome activation; Gene targeting; Early development

1. Introduction

Ability to generate gene-targeted embryos is essential in elucidating gene function. In mammals, the technology has been available mostly in the rodents, especially in the mouse. Development of embryonic stem (ES) cells and gene targeting using homologous recombination mechanism have made the mouse a leading species to study early development [1,2]. The mouse models have contributed greatly in biomedicine, and various signaling pathways have been elucidated using the models [1]. However, because of the differences in physiology, rodent models are not always the best model to recapitulate human biology [3].

The swine models, on the other hand, can closely represent human physiology due to their similarity in physiology. If the emphasis is in early development, swine embryos have similar development trajectory as human embryos. Both human and swine embryos have

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¹⁰Excess sperms, attached to zona pellucida, should be completely removed from the blastocyst to ensure accurate genotyping. Contamination of the sperm DNA can result in wild-type sequences in the genotyping results.

¹¹PCR amplification from an individual blastocyst is challenging because of extremely low amount of DNA. Number of PCR cycles used here should be at least 39 cycles. However, high cycle number may cause non-specific amplifications especially in the negative control. To prevent the non-specific amplifications, specificity and efficiency of primers should be tested. We use 40pg of standard wild-type genomic DNA to test the primers prior to using genomic DNA isolated from individual blastocyst.

major zygotic genome activation at the 4-cell stage [4,5]. In addition, changes in DNA methylation after fertilization is also observed in both species [6,7]. However, swine has not been considered to be a useful model to study early development because of difficulty in modifying its genome.

Conventionally, in swine, targeted modification was introduced into somatic cells then embryos/animals were generated through somatic cell nuclear transfer (SCNT) [8,9]. This approach allowed us to by-pass the need of ES cells in generating gene-targeted embryos/animals. However, because the process involves SCNT, i.e. cloning, animals generated through the method often presented developmental abnormalities associated with the cloning. Additional breeding should be the solution to this problem, but considering gestation period being close to four months (114 days), and housing and animal cost being high, the use of swine in biomedicine was limited.

A recent technology demonstrates that gene-targeted embryos can be generated without cloning or breeding by introducing Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system into early embryos [10,11]; reported efficiency of the targeting was as high as 100%. A recent report from our group also demonstrates that the technology can efficiently disrupt two genes simultaneously. Ability to introduce targeted modifications during embryogenesis will allow swine to be a model to study early embryo development. Here, we describe a specific approach to introduce targeted modifications during embryogenesis in swine.

2. Materials

2.1 Materials needed for *in vitro* maturation

2.1.1 Lab equipment

1. 5% CO₂ incubator.
2. Stereo microscope with warm plate.
3. Microneedles (18-gauge).
4. Syringes (10 ml).
5. Centrifuge tubes (50 ml).
6. Petri dishes (30 × 10 mm and 100 × 25 mm).
7. 4-well culture dishes.
8. Glass capillary tubes.
9. Captrol III[®] micropipet (Drummond Scientific).
10. Portable pipet-aid.
11. 10ml serological pipets.

2.1.2 Reagents

1. Saline: 0.9 % NaCl, supplemented with 100 U/ml penicillin-streptomycin.

2. Hepes-buffered Tyrode's Lactate (TL-Hepes) medium: 2.0 mM $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, 114.0 mM NaCl, 3.2 mM KCl, 2.0 mM NaHCO_3 , 0.4 mM NaH_2PO_4 , 10.0 mM Na Lactate (60 % syrup), 0.5 mM $\text{MgCl}_2 \times 6\text{H}_2\text{O}$, 10.0 mM Hepes, 12.0 mM sorbitol, 0.2 mM sodium pyruvate, 0.075 g/L penicillin, 0.05 g/L streptomycin, 1 ml phenol red (0.5%), 0.1 g/L polyvinyl alcohol (PVA); pH 7.4.
3. IVM medium: Medium 199 supplemented with 3.05 mM glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 10 ng/ml epidermal growth factor (EGF), 0.5 $\mu\text{g}/\text{ml}$ luteinizing hormone (LH), 0.5 $\mu\text{g}/\text{ml}$ follicle stimulating hormone (FSH), 10 ng/ml gentamicin, and 0.1% polyvinyl alcohol (PVA); pH 7.4.
4. IVM wash medium: IVM medium without LH and FSH; pH 7.4.
5. Embryo culture graded Mineral oil.

2.2 Materials needed for *in vitro* fertilization

2.2.1 Lab equipment

1. CO_2 incubator.
2. O_2/CO_2 incubator.
3. Stereo microscope with warm plate.
4. Benchtop centrifuge.
5. Microcentrifuge tubes (1.5 ml).
6. Vortex mixer.
7. Petri dishes (30 $\times$ 10 mm).
8. Glass capillary tubes.
9. Captrol III[®] micropipet (Drummond Scientific).
10. Portable pipet-aid.
11. 10ml serological pipets.

2.2.2 Reagents

1. IVF medium (mTBM): modified Tris-buffered medium with 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl_2 , 11 mM glucose, 20 mM Tris, 2 mM caffeine, 5 mM sodium pyruvate, and 2 mg/ml BSA; pH 7.4.
2. Manipulation medium: medium 199 supplemented with 0.6 mM NaHCO_3 , 2.9 mM Hepes, 30 mM NaCl, 10 ng/ml gentamicin, and 3 mg/ml BSA; pH 7.4.
3. Denuding medium: 0.3 M mannitol, 0.001% BSA, 0.03 % hyaluronidase, 5 % TL-Hepes medium in distilled water; pH 7.4.
4. AndroPRO[®] Plus semen extension medium (MOFA[®])
5. Sperm wash medium: DPBS with 0.1 % BSA and 10ng/ml gentamicin.
6. Embryo culture graded Mineral oil.

2.3 Materials needed for *in vitro* culture

2.3.1 Lap equipment

1. O₂/CO₂ incubator.
2. Stereo microscope with warm plate.
3. Petri dishes (30 × 10 mm).
4. Glass capillary tubes.
5. Captrol III[®] micropipet (Drummond Scientific).

2.3.2 Reagents

1. PZM3 culture medium [12].
2. Embryo culture graded Mineral oil.

2.4 Materials needed for microinjection of CRISPR/Cas9 system into presumable zygotes.

2.4.1 Lab equipment

1. Heat block (adjustable to 65 °C – 80 °C).
2. Thermocycler.
3. 37°C bacteria culture incubator.
4. 37°C bacteria culture shaker.
5. Water bath.
6. UV trans-illuminator.
7. FemtoJet (Eppendorf).
8. Micro manipulator.
9. O₂/CO₂ incubator.
10. Stereo microscope with warm plate.
11. Petri dishes (100 × 10 mm).
12. Glass capillary tubes.
13. Captrol III[®] micropipet (Drummond Scientific).
14. Nikon microscope with warm plate.
15. Microcapillary Puller (Shutter).
16. Benchtop centrifuge.
17. Microcentrifuge tubes (1.5 ml).
18. Electrophoresis.

2.4.2 Reagents

1. pX330 vector (Addgene).
2. BbsI enzyme (NEB).
3. T4 ligase and buffer.
4. mMESSAGE mACHINE® T7 Ultra Kit (Ambion).
5. MEGAshortscript™ Kit (Ambion).
6. Poly(A) Tailing Kit (Ambion).
7. mMESSAGE mACHINE® T7 Ultra Kit (Ambion).
8. Phusion High-Fidelity DNA Polymerase (ThermoFisher).
9. GeneJET PCR Purification Kit (ThermoFisher).
10. GeneJET Gel Extraction Kit (ThermoFisher).
11. GeneJET Plasmid Miniprep Kit (ThermoFisher).
12. PZM3 culture media.
13. Manipulation media.
14. Embryo graded mineral oil.
15. LB.
16. LB agar plate.
17. Ampicillin.
18. DNA ladder.
19. RNA ladder.
20. Agarose.
21. Chemical competent cells.

2.5 Genotyping of CRISPR/Cas9 injected embryos

2.5.1 Lab equipment

1. Thermocycler.
2. Stereo microscope with warm plate.
3. Petri dishes (100 × 10 mm).
4. Glass capillary tubes.
5. Captrol III® micropipet (Drummond Scientific).
6. PCR tube
7. Microcentrifuge tubes (1.5 ml)
8. Bioedit program

9. DNA electrophoresis units

2.5.2 Reagents

1. DPBS with 1% BSA media (pH 1.98).
2. DPBS with 1% BSA media
3. Embryo lysis buffer: 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl pH 8.5, 0.5% Nonidet P40, 0.5% Tween-20 and 200 µg ml⁻¹ proteinase K
4. Platinum taq
5. GeneJET PCR Purification Kit (ThermoFisher)
6. Agarose
7. 100bp DNA ladder

3. Methods

3.1 *In Vitro* Maturation (IVM)

1. In the morning, IVM and IVM washing dishes are prepared and incubated for 4–6 h at 38.5 °C/5% CO₂/100% humidity. The 4-well dishes containing 500 µl IVM medium covered with 450 µl mineral oil per well and two 30 mm IVM wash dishes with 3 ml IVM wash medium per dish
2. Porcine ovaries of pre-pubertal gilts or sows are obtained from slaughterhouse and transported to the laboratory.
3. The ovaries are washed with pre-warmed saline at 38.5 °C. Immature oocytes are aspirated from follicles (> 2 mm in diameter) using an 18-gauge microneedle attached to a 10-ml syringe, and harvested into 50ml centrifuge tube (See Note 1).
4. After 20 min incubation, the supernatant is removed then pre-warmed TL-Hepes medium is added for washing.
5. Washed aspirates are placed in the 100 mm petri dish.
6. Cumulus-oocyte complexes (COCs) with evenly granulated cytoplasm and intact surrounding cumulus cells are collected using a finely drawn glass pipet under a microscope (see Note 2).
7. The collected COCs are washed twice with pre-incubated IVM wash medium in the 30 mm dish.
8. Approximately 50 COCs are matured in a well (4-well dish) containing 500 µl IVM medium and incubated for 42–44 h at 38.5 °C, 5% CO₂, and 100% humidity.

¹-During aspiration, avoid blood contamination from the follicle. The blood may cause low maturation rate.

²-Good quality oocytes should consistently have two or more layers of cumulus cells. To minimally disturb the cumulus cells while transferring COCs, glass capillaries should be larger than 200 µm.

3.2 *In Vitro* Fertilization

In pigs, modified Tris-buffered medium (mTBM) is known to be proper to minimize polyspermy not affecting to the penetration rate [13,14]. For successful IVF, viability of sperm needs to be monitored prior to the IVF.

3.2.1 Preparation of oocytes

1. IVF medium (20 ml) is incubated at 38.5 °C/5% CO₂/100% humidity two days prior to the IVF.
2. IVF drops (each 50 µl) covered with mineral oil and two 3 ml mTBM washing medium are prepared in 30 mm petri dishes using the incubated IVF medium on the day before the IVF. These plates and the rest of media are incubated at the same condition until the IVF day.
3. After maturing COCs for 42–44 h, the COCs are transferred to 1.5 ml tube containing 1ml denuding medium pre-warmed at 38.5 °C, and the expanded cumulus cells surrounding oocytes are removed by vortexing for 3 min
4. Three manipulation dishes are prepared during the vortexing: A 30 mm dish with 2.5 ml and another two dishes with 3.5 ml manipulation medium pre-warmed at 38.5 °C.
5. Denuded oocytes are moved to the first manipulation dish (2.5 ml) and collection of matured oocytes with a visible polar body and washing are sequentially conducted in other two dishes (*see Note 3*).
6. After washing of the collected oocytes with the incubated IVF medium (two 30 mm dishes), 25 – 30 collected oocytes are placed in 50 µl droplets of IVF medium.

3.2.2 Sperm preparation

1. Fresh semen collected from a boar is diluted into an extender and stored at 17 °C. The semen can be sorted for up to 10 days.
2. For IVF 1 ml semen is washed in 9 ml sperm wash medium by centrifugation at 750 X g for 3 min.
3. The sperm pellet is resuspended and washed twice more with 10 ml wash medium at the same centrifugation condition. Washed sperm pellet is resuspended and diluted to 2.5×10^5 using the incubated IVF medium (*see Note 4*).

³.After freeing the oocytes from cumulus cells, searching for matured oocytes is an important step for successful IVF. The extrusion of first polar body is commonly used as an indicator of the maturation. However, the presence of the first polar body is not always apparent as the polar body can be located behind the cytoplasm. Use of manipulation medium can help because the medium has higher osmolality compared to other media. The diameter of the glass capillary used to transfer oocytes should be narrower (120 – 150 µm) now that cumulus cells are no longer attached to the oocytes.

⁴.The age and condition of boars can dramatically affect semen quality. We recommend that fresh semen from a boar aging from 1 to 4 years post-puberty. We generally incubate gametes 5 hours for IVF. However, the incubation time can be variable depending on the semen quality.

4. Then, 50 μ l sperm suspension is added to the prepared IVF droplets containing oocytes (final sperm concentration is 1.25×10^5) and incubated for 5 h at 38.5 °C/5% CO₂/100% humidity.

3.3 In Vitro Culture

1. PZM3 droplets, 20 μ l each placed on 30 mm dish, are covered with mineral oil and another two 30 mm wash dishes are filled with 3 ml PZM3, followed by incubation at 38.5 °C, 5% O₂, and 5% CO₂ in humidified air during the 5 h of IVF.
2. Oocytes fertilized for 5 h are transferred to the incubated wash dish containing PZM3 using a glass pipet.
3. Excess sperms attached to the oocyte surface (zona pellucida) are removed by repetitive passage of medium through glass pipet in the wash dish.
4. The oocytes are moved to the second wash dish.
5. 20 – 25 oocytes are placed in the pre-incubated PZM3 droplets and incubated at 38.5 °C/5% O₂/5% CO₂ in humidified air.

3.4 Microinjection of sgRNA and Cas9 mRNA into presumable zygotes

Introducing sgRNA and Cas9 mRNA into developing embryos can induce random insertion or deletion mutation (indel mutation) on a target sequence [15]. The random indel mutation can generate pre-mature stop codon thus disruption the gene function.

3.4.1 In vitro transcription of Cas9 mRNA

1. Using the pair of primers (Table 1) Cas9 mRNA sequence is amplified from pX330 vector using Phusion taq following the manufacture protocol. The PCR condition is as follows, initial denature at 98 °C for 2 min, denature at 98 °C for 30 sec, annealing at 62 °C for 30 sec and extension at 72 °C for 3min for 34 cycles, 72 °C for 5 min, and hold at 4 °C.
2. The PCR product is loaded on a 0.8% agarose gel for electrophoresis (see Note 6).
3. Once the size of the PCR product is confirmed, the rest of PCR products are purified using a PCR purification kit following the manufacture protocol.
4. In vitro transcription kit is used to generate Cas9 mRNA from the PCR product. Assembly of the transcription reaction are: 1 μ g of PCR product, 10 μ L of 2X NTP/CAP, 2 μ L of 10X buffer, 2 μ L of enzyme mix, and use nuclease-free water to bring the reaction volume to 20 μ L.
5. The reaction mixture is incubated at 37°C for 1 hour.

⁶.To generate Cas9 mRNA, template DNA used for in vitro transcription should be more than 1 μ g. And gel-extraction should be applied if you can't get homogeneous amplification of Cas9 gene. The Cas9 mRNA can be degraded easily thus must be stored at -80°C and kept on ice when it is used for microinjection.

6. After incubation, add 1 μ L of TURBO DNase into the reaction and incubate at 37°C for 15 min.
7. The in vitro transcribed product is placed into a fresh 1.5mL tube with 20 μ L of 5x E-PAP buffer, 10 μ L of ATP (10 mM), 10 μ L of MnCl₂ (25mM), 36 μ L of Nuclease-free water and 4 μ L of E-PAP enzyme to add additional poly A tail to the mRNA. The final volume should be 100 μ L. Incubate the reaction at 37°C for 1 hour.
8. To purify the mRNA, the entire reaction product (100 μ L) is mixed with 350 μ L of binding buffer from the RNA purification kit and add 250 μ L of 100% ethanol. The total of 700 μ L is then loaded into a filter cartridge. Centrifuge for 1 min at 15,000 x g.
9. Washing the filter cartridge 2 times with 500 μ L of washing buffer
10. To completely remove the traces of wash buffer, centrifuge for 1 min.
11. To elute the Cas9 mRNA, place the filter is to a new collection tube and add 50 μ L of elution buffer. Then, place the filter into a heating block at 65°C for 5min.
12. Centrifuge for 1min at 15,000 x g.
13. Load the purified Cas9 mRNA to a RNase-free gel for electrophoresis (see Figure .1)

3.4.2 sgRNA preparation

1. Digest 1 μ g of pX330 vector with BbsI enzyme. Reaction mixture contains 2 μ L of 10 x buffer, 1 μ L of BbsI enzyme, 1 μ g of pX330 vector and distilled water up to 20 μ L. Mixture is incubated at 37°C for 1 hour.
2. sgRNA sequences are designed using a web-based program (<http://crispr.mit.edu/>). Then, sequences with the highest score are selected. The target sequences are blasted against the entire pig genome to verify their specificity (see Note 5).
3. The selected sgRNA sequence is introduced into the linearized pX330 by following a standard protocol (<http://www.addgene.org/crispr/zhang/>) [16].
4. Using the plasmids carrying correct sgRNA as template, a pair of primers is used to amplify the sgRNA (Table. 1). Phusion taq is used for the PCR reaction following the manufacture protocol. The PCR condition is as follows, initial denature at 98 °C for 2 min, denature at 98 °C for 30 sec, annealing at 64 °C for 30 sec and extension at 72 °C for 30 sec for 34 cycles, 72 °C for 5 min, and hold at 4 °C.
5. The PCR product is loaded on a 2% agarose gel for electrophoresis.

⁵.sgRNA sequences obtained from web-based programs should be blasted against the whole pig genome to minimize potential off-targeting caused by the CRISPR/Cas9 system.

6. After verifying size of the amplicons, the rest of PCR products are purified using PCR purification kit following manufacture protocol.
7. For in vitro transcription, the following reaction mixture is assembled: 2 μ L of 10 x buffer, 2 μ L of ATP solution, 2 μ L of CTP solution, 2 μ L of GTP solution, 2 μ L of UTP solution, 2 μ L of enzyme, and 8 μ L of PCR product. Then, the mixture is incubated at 37°C for 3 hours.
8. After 3 hours, TURBO DNase (1 μ L) is added the reaction and incubation at 37°C for 15min.
9. The sgRNAs are then purified using RNA purification kit then used for microinjection.

3.4.3 Microinjection

1. After 2 hours post-IVF, zygotes are washed in manipulation medium. Then, the presumable zygotes are transferred to an injection dish (manipulation medium covered with mineral oil). Recommended concentrations of RNAs are 10 ng of sgRNA and 20 ng of Cas9 mRNA. The RNAs are injected into cytoplasm of the presumable zygotes using FemtoJet microinjector (see Note 7, 8). The microinjection is conducted on a heated plate at 37°C.
2. After microinjection, the zygotes are washed 2 times in PZM3 medium.
3. The zygotes are cultured in PZM3 at 38.5 °C, 5% CO₂, and 5% O₂ incubator for additional seven days.

3.5 Genotyping of CRISPR/Cas9 injected embryos

DNAs extracted from individual blastocyst are used to identify mutations on target sites, introduced by the CRISPR/Cas9 system.

3.5.1 DNA isolation and PCR

1. Seven days after IVF, blastocysts are collected for genotyping.
2. The blastocysts are placed in DPBS with 1% BSA adjusted pH 1.98 media drops and gently pipetted a few times to completely remove zona pellucida and sperms attached to the zona pellucida.
3. Genomic DNA from the blastocysts is extracted using embryo lysis buffer. An individual blastocyst is placed into a PCR tube with 12 μ L of embryo lysis buffer then incubated at 65 °C for 30 min followed by 95 °C for 10 min. (see Note 9)

⁷The amount of RNA introduced into each embryo is important. High volume can be detrimental to embryo development. Microinjection with water or PBS should be tested to identify if any adverse effect exist from the microinjection.

⁸The microinjection should be conducted at least two hours after IVF. Based on our experience, microinjection just after IVF can result in low embryo development. General concentration of sgRNA and Cas9 mRNA is 10ng and 20ng/ μ l respectively. However, the concentration depends on target location. According our experience, it is possible to completely disrupt a gene by using 2.5 ng/ μ l of sgRNA and 5ng/ μ l of Cas9 mRNA. However, some genes require higher concentration of CRISPR/Cas9 system. Therefore, it is important to conduct an optimization experiment to identify a working concentration.

⁹The lysis buffer should be freshly made each time. Based on our experience, old buffer leads to lower genomic DNA yield.

4. The genomic regions flanking CRISPR/Cas9 target region are amplified using Platinum Taq DNA polymerase. PCR conditions are as follows, initial denature at 95 °C for 2 min, denature at 95 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 30 sec for 39 cycles, 72 °C for 5 min and holding at 4 °C.
5. The PCR products are loaded on a 2% gel for electrophoresis.
6. Then the rest of the PCR products are purified using PCR purification kit and used for Sanger sequencing using the forward primer (Table 1).

3.5.2 Analysis of the genotyping

1. Results from the Sanger sequencing are opened with the Bioedit program (Chromas lite or other software can also be used). The sequencing results are blasted against wild type genomic sequence to verify modifications induced by the CRISPR/Cas9 system.
2. The blast results can indicate mutations introduced by the CRISPR/Cas9 system. To clearly distinguish the type of modifications (heterozygous, homozygous, biallelic, or mosaic), chromatogram from mutated embryos needs to be thoroughly compared with chromatogram peaks from wild-type control (see Note 12.).

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References

1. Capecchi MR (2005) Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. *Nature reviews Genetics* 6 (6):507–512. doi:10.1038/nrg1619
2. Thomas KR, Capecchi MR (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* 51 (3):503–512 [PubMed: 2822260]
3. Wilke M, Buijs-Offerman RM, Aarbiou J, Colledge WH, Sheppard DN, Touqui L, Bot A, Jorna H, de Jonge HR, Scholte BJ (2011) Mouse models of cystic fibrosis: phenotypic analysis and research applications. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society* 10 Suppl 2:S152–171. doi:10.1016/s1569-1993(11)60020-9 [PubMed: 21658634]
4. Braude P, Bolton V, Moore S (1988) Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* 332 (6163):459–461. doi: 10.1038/332459a0 [PubMed: 3352746]
5. Prather RS (1993) Nuclear control of early embryonic development in domestic pigs. *Journal of reproduction and fertility Supplement* 48:17–29 [PubMed: 7511691]
6. Santos F, Hyslop L, Stojkovic P, Leary C, Murdoch A, Reik W, Stojkovic M, Herbert M, Dean W (2010) Evaluation of epigenetic marks in human embryos derived from IVF and ICSI. *Human reproduction (Oxford, England)* 25 (9):2387–2395. doi:10.1093/humrep/deq151

¹²It is difficult to get clear genotyping results from sequencing only the PCR products. Biallelic mutation should have double peaks in the sequencing results but do not contain the wild-type sequence. Following each chromatogram peak will allow you to examine the presence of wild-type sequence. Mosaic mutation typically can have more than 3 peak in each nucleotide position. It is difficult to determine the presence of the wild type sequence in mosaic embryos. Alternatively, the PCR products can be cloned into a cloning vector and sequenced to identify modifications on each allele. Mosaic mutations will have more than two genotypes.

7. Deshmukh RS, Ostrup O, Ostrup E, Vejlsted M, Niemann H, Lucas-Hahn A, Petersen B, Li J, Callesen H, Hyttel P (2011) DNA methylation in porcine preimplantation embryos developed in vivo and produced by in vitro fertilization, parthenogenetic activation and somatic cell nuclear transfer. *Epigenetics : official journal of the DNA Methylation Society* 6 (2):177–187
8. Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, Samuel M, Bonk A, Rieke A, Day BN, Murphy CN, Carter DB, Hawley RJ, Prather RS (2002) Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science (New York, NY)* 295 (5557):1089–1092. doi:10.1126/science.1068228
9. Dai Y, Vaught TD, Boone J, Chen SH, Phelps CJ, Ball S, Monahan JA, Jobst PM, McCreath KJ, Lamborn AE, Cowell-Lucero JL, Wells KD, Colman A, Polejaeva IA, Ayares DL (2002) Targeted disruption of the alpha1,3-galactosyltransferase gene in cloned pigs. *Nature biotechnology* 20 (3): 251–255. doi:10.1038/nbt0302-251
10. Whitworth KM, Lee K, Benne JA, Beaton BP, Spate LD, Murphy SL, Samuel MS, Mao J, O’Gorman C, Walters EM, Murphy CN, Driver JP, Mileham A, McLaren D, Wells KD, Prather RS (2014) Use of the CRISPR/Cas9 System to Produce Genetically Engineered Pigs from In Vitro-Derived Oocytes and Embryos. *Biology of reproduction*. doi:10.1095/biolreprod.114.121723
11. Lei S, Ryu J, Wen K, Twitchell E, Bui T, Ramesh A, Weiss M, Li G, Samuel H, Clark-Deener S, Jiang X, Lee K, Yuan L (2016) Increased and prolonged human norovirus infection in RAG2/IL2RG deficient gnotobiotic pigs with severe combined immunodeficiency. *Scientific reports* 6:25222. doi:10.1038/srep25222 [PubMed: 27118081]
12. Yoshioka K, Suzuki C, Tanaka A, Anas IM, Iwamura S (2002) Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. *Biology of reproduction* 66 (1):112–119 [PubMed: 11751272]
13. Abeydeera LR, Wang WH, Cantley TC, Rieke A, Prather RS, Day BN (1998) Presence of epidermal growth factor during in vitro maturation of pig oocytes and embryo culture can modulate blastocyst development after in vitro fertilization. *Molecular reproduction and development* 51 (4):395–401. doi:10.1002/(sici)1098-2795(199812)51:4<395::aid-mrd6>3.0.co;2-y [PubMed: 9820198]
14. Lee K, Redel BK, Spate L, Teson J, Brown AN, Park KW, Walters E, Samuel M, Murphy CN, Prather RS (2013) Piglets produced from cloned blastocysts cultured in vitro with GM-CSF. *Molecular reproduction and development* 80 (2):145–154. doi:10.1002/mrd.22143 [PubMed: 23239239]
15. Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F, Jaenisch R (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 153 (4):910–918. doi:10.1016/j.cell.2013.04.025 [PubMed: 23643243]
16. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F (2013) Genome engineering using the CRISPR-Cas9 system. *Nature protocols* 8 (11):2281–2308. doi:10.1038/nprot.2013.143 [PubMed: 24157548]

Figure 1. Image of Cas9 mRNA with additional poly A tail. In vitro transcribed Cas9 mRNA was loaded on a 2% agarose gel. The mRNA was denatured prior to the loading. Only a single product is detected on the gel.

Figure 2.
Image of microinjection of CRISPR/Cas9 system into pig embryos. A: During microinjection, sgRNA and Cas9 mRNA are injected into the cytoplasm of presumable zygotes; B: After microinjection.

Figure 3. Sequencing results from *RAG2* and *IL2RG* double knock-out embryos. Sequencing readings from embryos carrying homozygous mutation have mutated single peaks. Biallelic mutations can be identified by having two polymorphic sequencing peaks but no wild type matching sequences are present. More than two sequencing peaks indicate the embryos have mosaic mutation; all embryos carrying mosaic genotypes do not have matching wild type sequence. The colors indicate each nucleotide; red – thymidine, black – guanine, green – adenine, and blue – cytosine.

Table 1.

Primers used to generate template DNAs for in vitro transcription and genotype CRISPR/Cas9 injected embryos for their modifications on *RAG2* and *IL2RG*.

To generate template DNA for in vitro transcription	
Cas9 mRNAF	TAA TAC GAC TCA CTA TAG GGA GAA TGG ACT ATA AGG ACC ACG AC
Cas9 mRNAR	GCG AGC TCT AGG AAT TCT TAC
T7 RAG2 F	TTA ATA CGA CTC ACT ATA GGT ATA GTC GAG GGA AAA GTA
T7 IL2RG F	TTA ATA CGA CTC ACT ATA GGG AAA CGG TTG AGA GTC CCA
T7 sgRNA R	AAA AGC ACC GAC TCG GTG CC
To genotype for <i>RAG2</i> and <i>IL2RG</i> mutations	
RAG2 F	AAG GAT TCC TGC TAC CTT CCT CCT
RAG2 R	AGA TAG CCC ATC TTG AAG TTC TGG
IL2RG F	CTG GAC TAT TAG AAG GAT GTG GGC
IL2RG R	ATA TAG TGG GAA GCC TGG GAT GCT