Supplementary kphqto cvkqp.'F cvc'U3'O cvgtkcnu'cpf 'Methods

Animals

All animal work was handled according to the Guidelines for the Care and Use of Laboratory Animals established by the Beijing Association for Laboratory Animal Science, and approved under Animal Ethics Committee of Institute of Zoology, Chinese Academy of Sciences.

Construction of Cas9 and sgRNA vector

To construct the Cas9 encoding plasmid, the Cas9 coding sequence was synthesis by Taihe Biotechnology Company (Beijing) as we previously reported, and then cloned into the pEASY-T1 vector, which harbored a T7 promoter for the *in vitro* transcription of Cas9. A SV40 polyadenylation signal was at the 3' end of the Cas9 cassette, and a unique HindIII restriction site was outside of SV40 signal for linearization.

The T7 promoter containing sgRNA scaffold was also ordered from Taihe Biotechnology Company (Beijing), and it was cloned into a promoterless pUC19 vector. Two BsaI restriction sites was used for the spacer insertion and the plasmid can be linearized by PsiI for *in vitro* transcription. For targeting vector construction, a site-specific 20 nt spacer was synthesis and cloned into T7-sgRNA scaffold between BsaI restriction sites.

***In vitro* synthesis of Cas9 mRNA and sgRNA**

To prepare Cas9 mRNA, T7-Cas9 expression plasmid was linearized by HindIII, and purified using DNA Clean & Concentrator^{TM}-5 (ZYMO Research).

To prepare sgRNA, the sgRNA vector was linearized using PsiI and purified by using DNA Clean & Concentrator^{TM}-5 (ZYMO Research).

All the linearized plasmid were *in vitro* transcribed by T7 High Yield RNA Synthesis Kit (NEB) following the manufacturer's instruction. To synthesize Cas9 mRNA, the $m^7G(5')G$ RNA Cap Structure Analog (NEB) was additionally added to stabilized the transcribed mRNA. Prepared RNAs were purified using MicroElute RNA Clean-Up Kit (Omega) and recovered in DEPC water.

Cytoplasmic microinjection of RNAs

The cytoplasmic RNA microinjection was performed according to previous report[1] and developed from our previous work[2]. Simply, the zygotes from Bama minipig were collected on the next of insemination, transferred to manipulation medium and subjected to a single cytoplasmic

microinjection of 2-10 pl of 125 ng/ul Cas9 mRNA and 12.5 ng/ul sgRNA. To test the viability of pig embryos after RNA injection, *in vitro* produced parthenogenetic embryos were used for preliminary experiment. For parthenogenetic embryos preparation, pig ovaries were collected, washed with pre-warmed saline and follicles aspirated. Oocytes were washed in TL-HEPES before culturing in maturation medium for 44 hours. Matured MII oocytes were depleted off surrounding cumulus cells by gentle pipetting, followed by electrical activation by two direct current pulses (1-sec interval) of 1.2 kV/cm for 30 microseconds. Activated oocytes were transferred to TL-HEPES medium and subjected to a single 2–10 pl cytoplasmic injection of 125 ng/ul Cas9 mRNA and 12.5 ng/ul sgRNA.

Zygotes and activated oocytes were cultured to blastocyst stage in PZM3 medium for 144 hours under 5% CO2, 39℃.

Embryo transfer

The survived embryos were transferred into the oviduct of recipient gilts on the day or 1 day after the onset of estrus, following mid-line laparotomy under general anesthesia. Pregnancy was diagnosed after 28 days, and then was checked regularly at 2-week intervals by ultrasound examination. All of the microinjected piglets were delivered by natural birth.

T7 endonuclease I assay and indel rate analysis

Genomic DNA was extracted from the ear tissue of the assayed pups. The target fragment was amplified using the followed primers: *vWF*-F: GCTTGTGAGATGCTGCCTGAAG, *vWF*-R: TGGTGGCTACAGCTCCGATTC. And the product is 477 bp. After finishing the amplification, PCR products were reannealed to generate heteroduplexed DNA in the following program: 95 ℃ 10 min; 95 ℃ to 85 ℃ (-2.0 ℃ /s), 85 ℃ 1 min; 85 ℃ to 75 ℃ (-0.3 ℃ /s), 75 ℃ 1 min; 75 ℃ to 65 ℃ (-0.3 ℃ /s), 65 ℃ 1 min; 65 ℃ to 55 ℃ (-0.3 ℃ /s), 55 ℃ 1 min; 55 ℃ to 45 ℃ (-0.3 ℃/s), 45 ℃ 1 min; 45 ℃ to 35 ℃ (-0.3 ℃/s), 35 ℃ 1 min; 35 ℃ to 25 ℃ (-0.3 ℃/s), 25 ℃ 1 min; 4 ℃ Hold. 0.4 ul T7 endonuclease I (NEB) and 1/10 volume Buffer 2 were added and incubated at 37 ℃ for 2 hours. The 2% agarose gel (Takara) are used for cleavage analysis according to previous describe[3].

Sanger sequencing of mutated sites

The targeted fragment was amplified using the primers mentioned above (*vWF*-F and *vWF*-R). PCR products from T7 endonuclease I (T7EI) assayed founders were cloned into pMD18-T vector (Takara) and transformed into competent *E. coli* strain Trans1-T1 (Transgen). After overnight culture at 37 ℃, at least ten valid colonies were picked and sequenced with M13F primer. Mutations were identified by alignment of sequenced alleles to wild-type allele.

Off-target sites screening and detection

The potential off-target sites were identified based on more than 14 bp identity to the sequence at the 3' end of the 20 bp spacer as reported previously[4]. The higher homology at the 3' end means the higher potential of off-target. We screened the potential off-target site in the whole genome of pig. Then T7EI assay were carried out to confirm whether off-targeting mutation existed.

Bleeding time measurement

The bleeding time was measured after severing a 3mm deep incision on tail at 1cm far from the tail tip. The time required for stopping bleeding was defined as the bleeding time. If no cessation of bleeding occurred after 10 hours, the tail was cauterized and the bleeding time was recorded as 600 min.

Hematological Analysis

Blood was sampled from precaval vein using a syringe and quickly removed to a BDTM vacutainer tubes containing plastic citrate and BD™ vacutainer tubes containing dipotassium EDTA. Blood samples were delivered to the laboratory within 3 h of collection and promptly assayed. Complete blood counts, hematocrit and other normal hematological values were determined by using an automatic cell counter (Coulter). Plasma was prepared by centrifugation of the blood at 2,500g for 10 min at room temperature for further Disseminated intravascular coagulation（DIC）test, vWF antigen, and FVIII activity determination. DIC parameters including activated partial thromboplastin time (aPTT) and prothrombin time (PT) was determined using reagent from Instrumentation Laboratory on ACL top 700 (Instrumentation Laboratory). FVIII activity and vWF antigen was determined by similar method using FVIII activity kit (0020011800, Instrumentation Laboratory) and vWF antigen kit (0020002300, Instrumentation Laboratory).

Statistics

Data were presented as mean ± standard deviation (SD). Student's t test was used to compare the means among different groups and calculate the statistical significance.

1. Hauschild, J. et al. Efficient generation of a biallelic knockout in pigs using zinc-finger nucleases. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 12013-12017 (2011).
2. Li, W., Teng, F., Li, T. & Zhou, Q. Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems. *Nat Biotechnol* **31**, 684-686 (2013).
3. Cong, L. et al. Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819-823 (2013).
4. Jinek, M. et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816-821 (2012).