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Efficient bi-allelic gene knockout and site-specific knock-in mediated by TALENs in pigs

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Pigs are ideal organ donors for xenotransplantation and an excellent model for studying human diseases, such as neurodegenerative disease. Transcription activator-like effector nucleases (TALENs) are used widely for gene targeting in various model animals. Here, we developed a strategy using TALENs to target the *GGTA1*, *Parkin* and *DJ-1* genes in the porcine genome using Large White porcine fibroblast cells without any foreign gene integration. In total, 5% (2/40), 2.5% (2/80), and 22% (11/50) of the obtained colonies of fibroblast cells were mutated for *GGTA1*, *Parkin*, and *DJ-1*, respectively. Among these mutant colonies, over 1/3 were bi-allelic knockouts (KO), and no off-target cleavage was detected. We also successfully used single-strand oligodeoxynucleotides to introduce a short sequence into the *DJ-1* locus. Mixed *DJ-1* mutant colonies were used as donor cells for somatic cell nuclear transfer (SCNT), and three female piglets were obtained (two were bi-allelically mutated, and one was mono-allelically mutated). Western blot analysis showed that the expression of the DJ-1 protein was disrupted in KO piglets. These results imply that a combination of TALENs technology with SCNT can efficiently generate bi-allelic KO pigs without the integration of exogenous DNA. These *DJ-1* KO pigs will provide valuable information for studying Parkinson's disease.

Pigs have been extensively used in biomedical research and are an excellent models for human diseases, especially for cardiovascular disease, atherosclerosis, cutaneous pharmacology, wound repair, cancer, diabetes, ophthalmological studies, toxicology research, lipoprotein metabolism, lung disease, and neurodegenerative diseases, and being considered as a potential source of organs for xenotransplantation¹. The similarities of organ size, anatomic and physiologic characteristics as well as genome sequence between pigs and humans have stimulated a wide range of application of pigs in biomedical research. While pigs offer many similarities to human anatomy, metabolism, neurobiology and physiology^{2–5}, furthermore, pigs have a longer life span, are easily bred, reach puberty at 6–8 months, and generate less ethical concerns when compared with non-human primates.

The ability to make genetic modifications in pigs facilitates the establishment of a human genetic disease model for the exploration and development of new therapies. Because no characterized embryonic stem cells (ESCs) have been generated in pigs that can be used for gene targeting, homologous recombination (HR) in somatic cells followed by somatic cell nuclear transfer (SCNT) is the primary strategy for creating knockout (KO) animals⁶. However, the very low rate of HR in somatic cells has hindered the establishment of KO animals. Therefore, highly efficient targeting in somatic cells is imperative and will facilitate the ability to genetically modify pigs. The emergence of zinc finger nucleases (ZFNs) has improved the efficiency of gene targeting greatly⁷, and ZFNs have been used to make various KO animals, such as pigs^{8–10}, human cells¹¹, and rats¹². Although ZFN-mediated gene targeting has progressed in large animals, some disadvantages of ZFNs, such as off-target cleavage, cytotoxicity, and limited target sites^{13–15}, have limited their applications.

Transcription activator-like effector nucleases (TALENs) were first reported in 2010¹⁶. Similar to ZFNs, TALENs are composed of a TALE DNA binding domain and a *FokI* endonuclease. TALENs have been shown to possess comparable or higher efficiency for gene targeting, are more straightforward, and can theoretically target any sequence. TALEN-mediated targeting has been used successfully in different organisms, such as zebrafish^{17,18}, rat¹⁹, and pig⁶.



Table 1 | Summary of the targeting efficiency of the 3 TALEN pairs in pig FFCs

TALEN pair	Cell type	Mutant colonies (%)	Bi-allelic mutant colonies (%)
<i>GGTA1</i>	Male	2/40 (5%)	2/2 (100%)
<i>Parkin</i>	Male	2/80 (2.5%)	0
<i>DJ-1</i>	Female	11/50 (22%)	4/11 (36.4%)

used in the current study have high specificity for their target sequences.

Site-specific insertion via TALENs. We next investigated the possibility of TALEN-mediated site-specific insertion by co-transfection of TALENs with single-strand oligodeoxynucleotides (ssODN). ssODN was first used in homology-directed repair (HDR) in ZFN-induced gene editing, and at least 20–25 bp homology arms on each side are recommended³⁴. Considering the high targeting efficiency of the *DJ-1* TALENs, it was chosen for subsequent investigation in female FFCs. An ssODN was designed to contain a *HindIII* restriction enzyme site (Fig. 5A) that would facilitate the subsequent detection of an insertion. The ssODN was co-transfected into fibroblasts with the *DJ-1* TALENs and the pCAG-GFP plasmid, and single colonies were screened as described above. Ten of 49 colonies were mutated in the *DJ-1* gene, and one (the DS21 colony) had the *HindIII* site insertion (Fig. 5B). Sequencing results confirmed the successful site-specific insertion of ssODN that was mediated by the *DJ-1* TALENs (Fig. 5C). However, results of the restriction digest map and sequencing showed that the DS21 colony was not truly mono-colony. It may be mixed with wild type cells, which would be caused by the method we used for cell plating. The insertion rate was 10% among edited.

Production of TALEN-modified piglets by somatic cloning. The *DJ-1* mutant female FFCs (a mix of mono-allelic and bi-allelic mutant colonies) were used as donor cells for SCNT and embryo

transfer to examine TALEN-mediated KO cells for producing KO animals. A total of 687 embryos were delivered to 5 surrogates, and one pregnancy was established. Three live-born piglets and one stillborn piglet were obtained, and the piglets were genotyped as 1 mono-allelic mutation and 3 bi-allelic mutations (Fig. 6A). The sequencing results showed that the mono-allelic KO piglet was derived from the D9 colony (Fig. 6B), which harbored an insertion-induced frame-shift in one allele, resulting in a prematurely truncated DJ-1 ORF. The two bi-allelic KO live-born piglets and the one stillborn piglet were derived from the D27 colony (Fig. 6B), which harbored an 8-bp deletion in one allele and a 10-bp deletion in another allele, also resulting in a prematurely truncated DJ-1 ORF. Unfortunately, the 2 bi-allelic mutant live-born piglets died within two days after birth, and the mono-allelic mutant piglet died at day 8. No obvious defect in the pig organs was observed by autopsy, and the deaths may have been caused by cloning defects. Tissues were dissected from the dead piglets, and Western blot analysis was performed to confirm the disruption of DJ-1 in these pigs. Compared with wild-type piglets, DJ-1 was partially repressed in the mono-allelic mutant piglet and completely absent in the bi-allelic mutant piglet (Fig. 6C), suggesting the successful generation of *DJ-1* KO piglets.

Discussion

In the current study, three pairs of TALEN expression plasmids were designed to target the *GGTA1*, *DJ-1* and *Parkin* genes in pigs. First, the efficiency of TALEN-mediated gene targeting was detected for the *GGTA1* gene. Exon 6 was chosen for gene targeting because there is a catalytic domain of the $\alpha 1$, 3-galactosyltransferase enzyme. The targeting efficiency was 5% (2/40), and both colonies were bi-allelically modified, demonstrating the establishment of TALEN-mediated gene targeting in the lab. Then, two more TALEN pairs were designed to target the *Parkin* and *DJ-1* genes, respectively. The efficiency of TALEN pairs designed against the *DJ-1* gene was 22% (11/50). Various mutation patterns at the target site were observed, including small deletion (-1 base), large deletions (-226 bases), and small insertions (+1 base), which are characteristic of non-homologous end joining (NHEJ)-induced DNA damage response. The vast

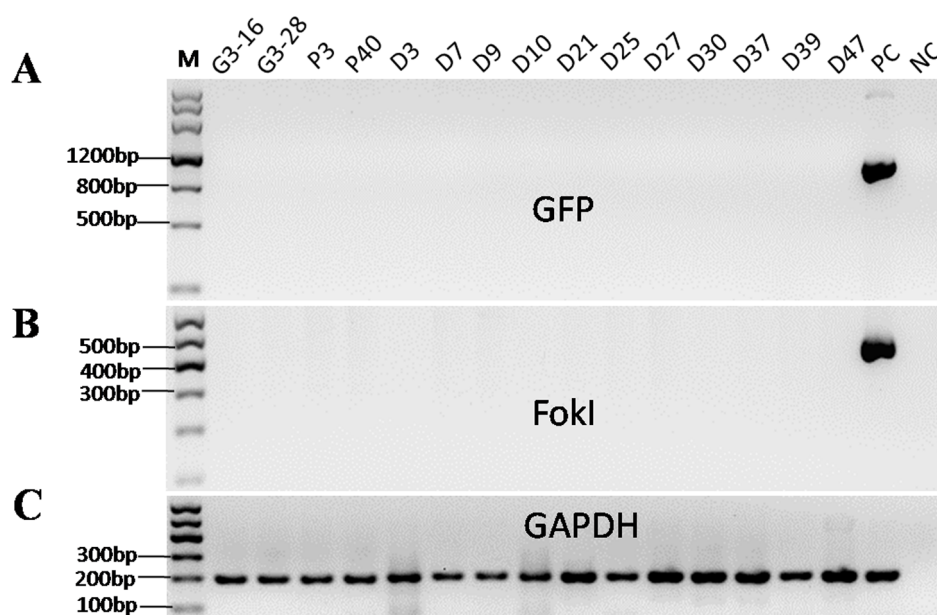


Figure 4 | PCR amplification of GFP and *FokI* confirmed the loss of GFP and no TALEN plasmid integration in mutant colonies. M represents the DNA marker, PC represents the positive control, and NC represents the negative control. GAPDH was used to confirm the DNA quality of all the samples.

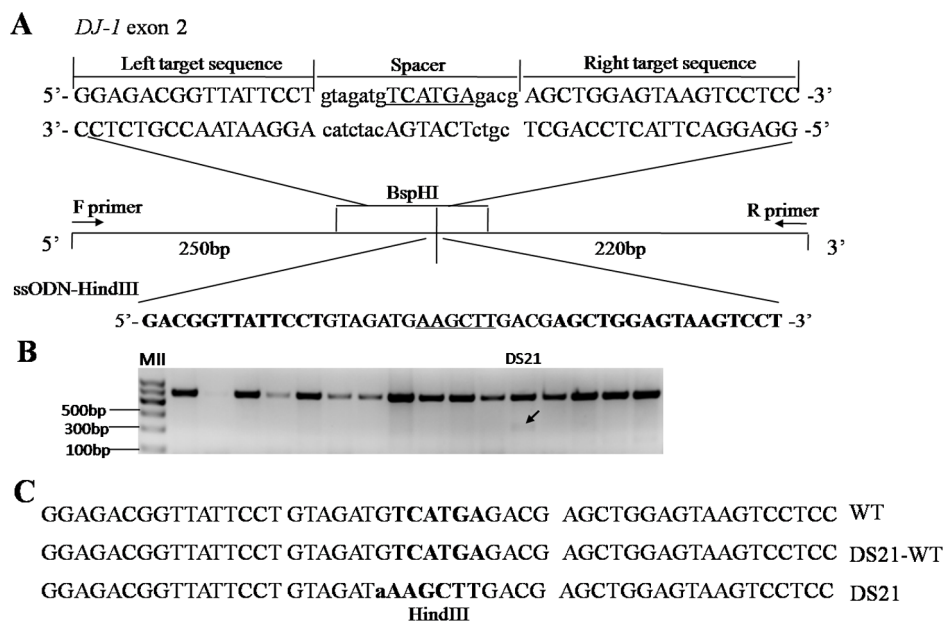


Figure 5 | Site-specific insertion of *HindIII* site mediated by TALENs. (A) Schematic of the *DJ-1* locus with the ssODN sequence used to introduce the *HindIII* sequence (underlined) into the genome in vivo. (B) One of the colonies (DS21) could be cut by the *HindIII* restriction enzyme. The arrow shows the resulting fragments cut by *HindIII*. (C) DNA sequences of the DS21 colony at the target site. The *HindIII* site is shown by bold uppercase letters. There is a different base before the *HindIII* site, as shown in lowercase letters. DS21-WT represents sequence of the mixed wild type cells in DS21 colony.

majority of mutations were deletions, which were also reported previously in mice and pigs^{32,35}. Among all the targeted cell colonies, more than 1/3 were bi-allelic mutations, and this amount was consistent with previous reports in pigs^{6,32}.

To induce mutations into the pig genome without the integration of exogenous DNA, circular pCAG-GFP plasmid was co-transfected with each TALEN pairs into pig FFCs and then sorted based on GFP expression by FACS to screen the mutant cell

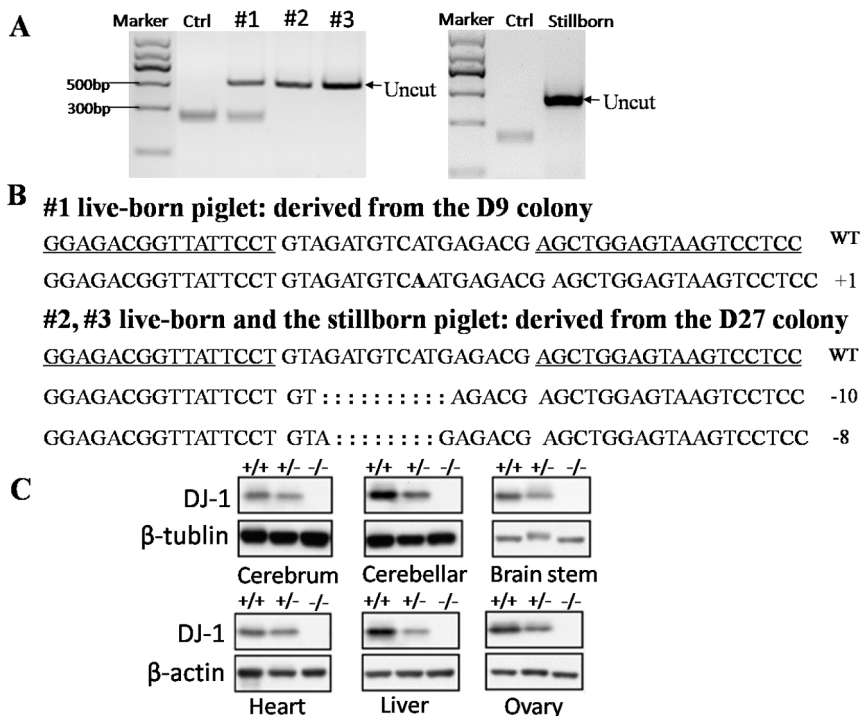


Figure 6 | Female piglets obtained from SCNT and embryo transfer using mixed KO colonies. (A) *BspHI* digestion of PCR products from piglets #1, #2, #3 and the stillborn piglet. The genotyping result of piglets #1, #2, #3 was cropped from full-length gels indicated in supplementary files by discarding lanes of irrelevant samples. (B) DNA sequence of the *DJ-1* gene at the target site in wild-type cells (wt) and KO piglets. The inserted base is shown by bold uppercase letters, and the deleted bases are indicated by colons. (C) Western blot results in six tissues of wild-type and KO piglets. +/+ represents mono-allelic KO piglet (8-day-old), -/- represents bi-allelic KO piglet (2-day-old), and +/+ represents wild type pigs (10-day-old, female). β-tubulin and β-actin served as housekeeping gene controls.



colonies instead of the usage of antibiotic markers to screen positive colonies. PCR results confirmed that there was no integration of the GFP or TALEN plasmids in the targeted cell colonies.

Off-target activity is another concern regarding nucleases mediated genome editing. In the current study, no off-target cleavages were identified for all the 3 pairs of TALENs. This lack of off-target cleavage is an advantage of TALENs over ZFNs and CRISPR/Cas9 system, which was reported to cause high off-target mutagenesis^{13,14,36}. Take these data described above, TALENs are a reliable gene targeting tool for high efficient genetic modification in porcine somatic cells.

Site-specific knock-in is another advantage of nuclease-mediated gene targeting in large animals. TALEN-mediated site-specific insertion using ssODN with a *HindIII* site was successfully introduced to the somatic cell genome, however, a base substitution just before the *HindIII* site was identified. This substitution may be the result of re-cleavage of TALEN pairs after insertion of *HindIII*³⁷ because the length of the spacer region between the TALEN pairs was the same as the wild-type sequence, which could be easily re-cleaved in cells cultured for extended periods. ssODN combined with ZFNs^{34,38,39}, TALENs^{18,40} and Cripsr/Cas9⁴¹ was used widely for introducing small indels, point mutation, and even deletion of large fragment (up to 100 kb) because of its easy design, rapid synthesis, and comparable efficiency as classical targeting vector^{34,39}. However, since ssODN is very short (tens of nucleotides), it's difficult to detect the random integration of ssODN into genome. Deep sequencing might be necessary to evaluate the random integration of ssDNA. Now, we are trying to obtain longer donor template (>2 kb) to mediate site-specific insertion of long fragments, which will complement the future advances of gene therapy for repairing disease-related genes in humans.

Previous studies have constructed many mouse models with different fragments deleted from the *Parkin* and *DJ-1* genes^{21–29}, but few of them recapitulate human PD very well. One of the reasons may be that mice have a short life span, and PD is age-dependent, often occurring in aged people. Considering that pigs have a longer life span than mice and the other similarities between pigs and humans, pigs are ideal for constructing models of PD. Therefore, we used the *DJ-1* KO cell colonies combined with SCNT and embryo transfer to generate KO piglets. One *DJ-1*^{+/-} and two *DJ-1*^{-/-} female piglets were obtained. Western blot analysis showed that the DJ-1 protein was repressed in all the detected tissues of *DJ-1* KO piglets, indicating the successful construction of a *DJ-1* KO pig model. Unfortunately, the piglets all died due to cloning defect.

In conclusion, TALEN mediated efficiently generation of bi-allelic *DJ-1* KO pigs without the integration of exogenous DNA was successfully developed. The highly efficient gene targeting was also verified in the targeting of *GGTA1* and *Parkin*. The results in the current study further confirmed that TALENs can be used conveniently and efficiently for site-specific knock-in for precise gene modification in large animals. The bi-allelic *DJ-1* KO pigs could provide valuable information for understanding the mechanisms underlying PD.

Methods

Animals. Pigs were raised at the Beijing Farm Animal Research Center and had *ad libitum* access to a commercial pig diet (nutrient levels according to the NRC) and water during the entire experimental period. All experiments involving animals were performed according to procedures approved by the Institutional Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences, China.

Generation of TALENs. TALENs expression constructs were assembled by Viewsolid Biotech Company (Beijing, China). Three pairs of TALENs were designed to target exon 6, exon 1, and exon 2 in the porcine *GGTA1*, *Parkin*, and *DJ-1* genes (ensembl ID: ENSSSCG00000005518, ENSSSCG00000004032 and ENSSSCG00000003385), respectively. The *GGTA1* TALENs recognition sequences were as follows: left TALEN 5'-CTTATCCCCAGAATACT-3' and right TALEN 5'-ATCCACAGACATGCCT-3'. The *Parkin* TALENs recognition sequences were as follows: left TALEN 5'-GTTTGTCAAGGTTCAACT-3' and right TALEN 5'-

GTCAGAATCGACCTCCACT-3'. Between the two binding sites was a 16-bp spacer with a *NcoI* site (CCAGCCATGGTTTCCC, *NcoI* underlined). The *DJ-1* TALENs recognition sequences were as follows: left TALEN 5'-GGAGACGGTTATTCCT-3' and right TALEN 5'-GGAGACTTACTCCAGCT-3'. Between the two binding sites was a 17-bp spacer with a *BspHI* sequence (GTAGATGTCATGAGACG, *BspHI* underlined). The TALEN pairs were transfected into porcine fetal fibroblast cells to detect the targeting efficiency.

Cell culture and transfection. Primary fetal fibroblast cells (FFCs) were isolated from 42-day-old Large White porcine fetuses (male and female) and digested by collagenase-DNaseI in cell culture medium (DMEM high glucose (Gibco BRL, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS, Hyclone, Logan, UT), 0.032% collagenase (Sigma, St. Louis, MO), 25 Kunitz units/ml DNaseI (Sigma, St. Louis, MO) and 40 µg/ml gentamicin (Sigma, St. Louis, MO) for 4–6 h at 37°C. Digested cells were washed and plated in 25-cm² flasks in cell culture medium (DMEM high glucose supplemented with 15% FBS). FFCs were frozen when they became confluent. Two days before transfection, FFCs were thawed and cultured in 75-cm² flasks. When the cells become 80% confluent, transfection was performed by electroporation as described previously³². To avoid antibiotic gene insertion, the pCAG-GFP plasmid was co-transfected with TALENs to be used as an indicator for FACS sorting and was eliminated after several passages. Forty-eight to seventy-two hours after transfection, cells were subjected to FACS sorting based on the expression of GFP fluorescence. Single cells were plated in each well of 96-well plates and cultured for approximately 10 days in cell culture medium supplemented with 2.5 ng/mL basic fibroblast growth factor^{32–44} (Sigma, St. Louis, MO). The medium was replaced every 4 days. Confluent cell colonies were propagated and genotyped by PCR, restriction enzyme digestion and sequencing. Cell colonies harboring mutations were cryopreserved for SCNT.

For TALEN/ssODN-induced insertion, 0.5 µg of each of the TALEN pairs was co-transfected into female FFCs with 2 nmol ssDNA (a 20-bp 5' homologous arm and a 20-bp 3' homologous arm with a *HindIII* recognition site in the center, synthesized by Invitrogen). The pCAG-GFP plasmid was also included in the transfection system to provide an indicator for FACS sorting. A similar strategy was used to screen single-cell colonies and for genotyping.

Detection of gene mutations. The genomic DNA of each cell colony was extracted using the TIANamp genomic DNA kit (Tiangen, Beijing, China). Genotyping was conducted using PCR followed by restriction enzyme digestion. For *GGTA1*, the genotyping primers were as follows: 5'-AGCACGAGGTGGACTTC-3' and 5'-CAGATGTTATTTCTAACCAAAT-3'. The primers for *Parkin* genotyping were as follows: 5'-AAGGGCAGTCCCACAGGAAACTC-3' and 5'-TGGAGTAAAGTTCAAGGAATCCCC-3'. The primers for *DJ-1* genotyping were as follows: 5'-GTGCTTCTTCTATTCATTGTGC-3' and 5'-CAGGTCTGTAAAGAAATATGGGATG-3'. Mutations in *GGTA1* were assessed by the T7 endonuclease I (T7EI) assay. Briefly, the purified PCR products from colonies and wild-type cells (200 ng each) were denatured at 95°C for 5 min and re-annealed at room temperature for 10 min, then digested by T7EI (New England Biolabs) at 37°C for 0.5 h. Different bands were expected for mutant colonies. Mutations in *Parkin* and *DJ-1* were assessed by the loss of restriction enzyme sites harbored in the spacer region. PCR products of colonies were cut by *NcoI* or *BspHI*. Uncut bands were expected for mutant colonies. To verify the mutations, the gel-purified PCR products were cloned into the pEASY-T3 vector using the pEASY-T3 cloning kit (Transgen, Beijing, China) and then sequenced. To detect whether there was pCAG-GFP or TALEN plasmid integration in the positive colonies, PCR was conducted using the primers 5'-CAAGCTTCAAGTTCGAGTCG-3' and 5'-TGATGAGTTGGACAAACCACAAC-3' for GFP detection and the primers 5'-ATTTGGTGGATCAAGGA-3' and 5'-GTTTATCTCGCGTTATT-3' for *FokI* detection. One hundred and fifty ng of genomic DNA was used for PCR analysis. Conditions for PCR amplification were one cycle of pre-denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 62.7°C (for GFP primers) or 52°C (for *FokI* primers) for 30 seconds, and elongation at 72°C for 30 seconds, and one cycle of post-elongation at 72°C for 7 min. To assess the *HindIII* insertion, the PCR products of each cell colony were digested by the *HindIII* restriction enzyme, and colonies that could be cut by *HindIII* were then confirmed by sequencing.

Oocyte maturation. Pig ovaries were collected from a local slaughterhouse and transported to the laboratory within 1 h in 0.9% saline maintained at 37°C. Oocytes were aspirated from antral follicles (3–6 mm in diameter) with an 18-gauge needle fixed to a vacuum pump. Compact cumulus oocyte complexes (COCs) were selected under a stereomicroscope, washed and cultured in maturation medium at 39°C under 5% CO₂. The maturation medium was composed of tissue culture medium (TCM) 199 (Hyclone, Logan, UT) supplemented with 0.1% (w/v) polyvinyl alcohol, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 0.5 µg/ml of luteinizing hormone, 0.5 µg/ml of follicle-stimulating hormone, 10 ng/ml of epidermal growth factor, 10% (v:v) of porcine follicular fluid, and 10 µg/ml of gentamicin. After 42–44 h of *in vitro* maturation culture, COCs were denuded by vigorous vortexing for 5 min in TL-HEPES supplemented with 0.1% polyvinyl alcohol and 0.1% hyaluronidase. Oocytes with polar bodies were selected under a stereomicroscope and used for somatic cell nuclear transfer (SCNT).



Somatic cell nuclear transfer and embryo transfer. Mature MII oocytes were enucleated by aspirating the first polar body and adjacent cytoplasm with a glass pipette (20 µm in diameter) in manipulation medium supplemented with cytochalasin B (5 mg/ml stock, 4 µl stock per 3 ml manipulation medium). A single *DJ-1* KO fibroblast cell (donor cell) was injected into the perivitelline space of an enucleated oocyte. The donor cell membrane should be in contact with the oocyte cytoplasmic membrane. Oocyte cytoplasmic complexes were then fused and activated by electric pulse. Reconstructed embryos were cultured in porcine zygote medium (PZM3) in 5% CO₂ at 39°C for 14–16 h until embryo transfer. Embryos in good condition (120–200 embryos) were surgically transferred into the oviduct of a surrogate the day after observed estrus. Gestation was detected by ultrasound at day 28 after embryo transfer. If the surrogate was pregnant, the gestation was monitored every 2 weeks. On the day of birth, an ear skin biopsy was isolated from each piglet for genomic DNA extraction and genotyping.

Western blot analysis. Different tissues were dissected from dead piglets, frozen immediately in liquid nitrogen and stored at –80°C until use. Twenty to thirty milligrams of different tissues was lysed in a buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 100 mM NaF, 0.05% SDS, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 g/ml aprotinin and leupeptin, 2 mM benzamide, 1 mM sodium vanadate, 1 mM EGTA and 0.1% deoxycholate for 15 min on ice, and 50 µg of protein was subjected to Western blot analysis. The *DJ-1* antibody (DJ-1 (D29E5) XP® Rabbit mAb #5933) was purchased from Cell Signaling Technology (Danvers, MA, USA), and a concentration of 1 : 1000 was used to detect the expression of DJ-1 in different tissues. β-actin and β-tubulin were used as housekeeping genes to confirm equal sample loading.

Off-target analysis. To examine whether these three pairs of TALENs have off-target effects, we explored two approaches to predict potential off-target sites in the porcine genome, namely E-PCR^{31,32} and TALENoffer³³. For E-PCR (<http://www.ncbi.nlm.nih.gov/tools/epcr/>), we used the same criteria as Yong Lei and Xiaogang Guo (2012) described. Briefly, up to three mismatches and a 1-bp gap for each binding site was allowed, and the spacer between the two TALEN binding sites was <100 bp because longer spacers could interfere with *FokI* dimerization⁴⁵. For TALENoffer, the default parameters were used to perform the predictions. The regions around potential off-target sites were amplified in mutant colonies and sequenced to confirm off-target cleavage.

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Author contributions

Z.Y. and J.Z. conceived and designed the study. J.Y., J.H., T.H., X.W., G.Q., H.Z. and R.W. performed the study. C.C. predicted the off-target sites. J.X. designed the TALEN plasmid pairs. J.Y. and J.Z. analyzed the data and wrote the manuscript. All authors reviewed the manuscript.

Additional information

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